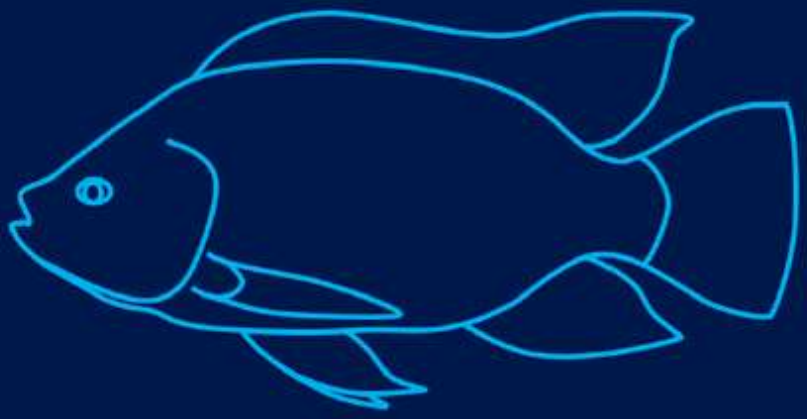
Breeding for salinity tolerant tilapia



Priadi Setyawan

Propositions

1. A salinity-tolerant tilapia strain is needed to empower small-scale brackish water farmers.
   (this thesis)

2. Selection is better than hybridization to produce salinity-tolerant tilapia.
   (this thesis)

3. Failure to disseminate scientific results is a waste of energy and budget.

4. Research in life sciences should be driven by societal goals.

5. Poverty is the worst enemy of personal development.

6. Increasing farmers income requires growing more species than just the economically most important ones.

Propositions belonging to the thesis, entitled

Breeding for salinity tolerant tilapia

Priadi Setyawan

Wageningen, 2 October 2023

Breeding for salinity tolerant tilapia

Priadi Setyawan

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Breeding for salinity tolerant tilapia

Priadi Setyawan

Thesis

Submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
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Priadi Setyawan

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Contents

Chapter 1

General introduction

1.1. Overview of Indonesian aquaculture

Indonesia is an archipelago state that consists of thousand islands, and around 70% of the area is covered with water. With this advantage, Indonesia has become the third major world aquaculture producer (FAO, 2022). Shrimp, tilapia and seaweeds are the main products contributing to the increase of gross domestic product (GDP), gross regional domestic product (GRDP) and the volume of national aquaculture production from 2010 to 2018 (Oktopura et al., 2020). The Coastal environment offers a huge potential area for aquaculture, nationally involving more than 800.000 brackish water farmers. Total shrimp production was 0.88 million ton in 2022 of which 27.1% was exported, only second to tilapia (1.17 million ton), representing an export value of around 2.04 billion US dollars (MMAF, 2022). With strong demand and high prices, shrimp farming has become the most valuable aquaculture sector, and this encouraged shrimp farmers to increase their production. However, a combination of poor water management, increasing density and high levels of commercial feed during intensification has caused serious land and environmental degradation. Disease outbreaks have caused repeated farming failures resulting in many shrimp farmers suffering from financial problems and quitting shrimp farming altogether.

1.2. Alternative species for shrimp farming

Shifting to other species has been a commonly used strategy to minimize economic losses since shrimp farming has become a high-risk industry. Shrimp and milkfish have traditionally dominated brackish water pond production (MMAF, 2018). Grouper and seabass are also produced in brackish water, but in intensive, tank based, systems because these species need specific requirements to grow normally. Smallholder farmers cannot access the financial and technical resources required to farm these two species.

Farming tilapia in brackish water is becoming more preferred in the coastal areas. To date, the majority of tilapia are produced in fresh water, and brackish water farming contributes only 3.3% of total production. Fisheries extension services in Central Java have recently reported that the number of tilapia farmers in brackish water ponds is increasing. The rapid growth of tilapia in brackish water has also encouraged many milkfish farmers to shift to tilapia farming, particularly in East and Central Java. The selling price for tilapia in many

areas is comparable to milkfish at the same size, while the growth rate of tilapia is much higher.

Tilapia has the highest salinity tolerance range among freshwater fish and grows faster than most other freshwater species (Cnaani & Hulata, 2011; Popma & Masser, 1999). However, salinity tolerance varies enormously between tilapia species. *Oreochromis mossambicus* is well known as the most salinity tolerant of tilapia species (Cnaani & Hulata, 2011; Fiess et al., 2007; Kamal & Mair, 2005; Popma & Masser, 1999; Suresh & Lin, 1992). It can be directly transferred from fresh water to sea water, can reproduce in 49 ppt and can grow at 69 ppt (El-Sayed, 2006), far beyond the typical salinity of around 35 ppt found in seawater. Nevertheless, *O. mossambicus* has a lower growth rate than Nile tilapia (*Oreochromis niloticus*) (Cnaani & Hulata, 2011; Popma & Masser, 1999). Because of this, *O. mossambicus* is not a common species for fish farming activities (Suresh & Lin, 1992) but hybridization of this species with Nile tilapia is commonly applied for brackish water production. The average heterosis of this hybrid for weight gain is 1.24 and for biomass is 1.33 at salinities above 10 ppt (Kamal & Mair, 2005). However, the survival rate of the hybrid was significantly lower than *O. mossambicus*. Blue tilapia (*Oreochromis aureus*) is moderately salinity tolerant, less than *O. mossambicus* but more than *O. niloticus* (Cnaani & Hulata, 2011; El-Sayed, 2006; Popma & Masser, 1999; Watanabe et al., 1985). A selection line of this species has been developed for years for increasing salinity tolerance (Cnaani & Hulata, 2011; El-Sayed, 2006). Despite this knowledge, until now, most of brackish water farmers in Indonesia are using freshwater tilapia strains because of lacking availability of a specific tilapia strain for brackish water environment that meet the quality and quantity for commercial production.

1.3. A Breeding program for salinity-tolerant tilapia in Indonesia

1.3.1. Tilapia breeding programs

Developing salinity tolerant tilapia for brackish water ponds in Indonesia is crucial to meet the farmers need. A few studies have investigated GIFT1) tilapia performance in brackish water ponds. Vietnam has developed a breeding program for brackish water tilapia, which started in 2007 using a synthetic population from three Nile tilapia strains (GIFT, Taiwan strain and NOVIT4 as GIFT-derived strain) this for four generations from 2008-2011 (Ninh et al., 2014). Research Institute for Aquaculture No.1 (RIA.1) in Vietnam investigated the GIFT tilapia performance in brackish water using 261 full-sib families over three generations (Luan et al., 2008). Results from these breeding programs indicate that GIFT has good growth rate but low to moderate tolerance to salinity (<20 ppt).

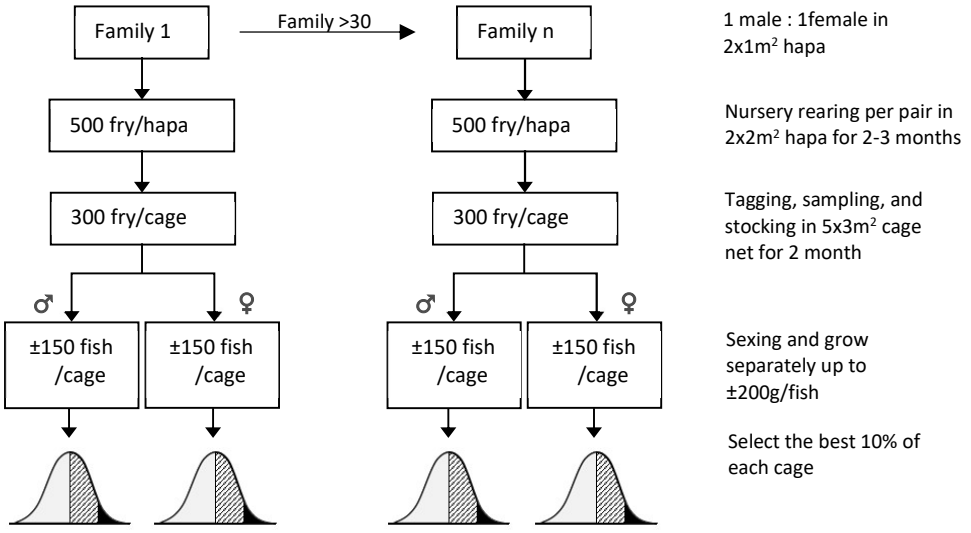
Hybridisation has been investigated to combine good growth of GIFT with salinity tolerance of other species. Comparing hybrids of *O. spilurus*, *O. aureus*, *O. mossambicus* with three *O. niloticus* strains in brackish water ponds resulted in the best growth performance of a hybrid between *O. mossambicus* and *O. niloticus* (Tayamen et al., 2002).

A breeding program for brackish water ponds has been developed in the Philippines using an F1 of GIFT strain x feral *O. mossambicus* and repeated backcrossing to *O. mossambicus*, resulting in the Molobicus strain (de Verdal et al., 2014). Two lines were selected in the Molobicus program for intensive and extensive farming conditions. In the seventh generation of the Molobicus program a significant excess of *O. Niloticus* alleles was shown for genes that are responsible for higher growth rate in intensive farming conditions (Bartie et al., 2020).

In Indonesia, I used a hybridization strategy to produce salinity tolerant tilapia by crossing Nirwana (a local strain of *O. niloticus*) with a Blue tilapia strain called Sukamandi strain. The resulting hybrid is called Srikandi, and shows high growth rate in brackish water ponds (Setyawan et al., 2015). I improved the Sukamandi strain since 2011 following a national protocol for fish breeding programs developed by the Ministry of Marine Affairs and

1) GIFT (Genetic Improvement of Farmed Tilapia) is a tilapia strain improved for growth performance that has been developed since 1988 under International Center for Living Aquatic Resources Management (ICLARM) and its partners in Philippines. Eknath, A. E., Dey, M. M., Rye, M., Gjerde, B., Abella, T. A., Sevilleja, R., Tayamen, M. M., Reyes, R. A., & Bentsen, H. B. (1998). Selective breeding of Nile tilapia from Asia. In (Vol. 27: Reproduction; fish breeding; genetics and the environment; genetics in agricultural systems; disease resistance; animal welf, pp. 89–96).

Fisheries in Indonesia (Fig. 1). The protocol is based on phenotypic selection of harvest weight where pedigree information was neglected in the selection and mating process. Tag information was used but only to identify harvest weight. With this protocol, I produced the first to fourth generation of Sukamandi strain selecting the top 10% of harvest weights from each generation of fish grown in commercial farms in brackish water areas. The selected parents were used for multiplication, and the top 50% of their male offspring were used to produce Srikandi strain by hybridization with local females of Nile tilapia. A cross between *O. niloticus* and Sukamandi strain theoretically should produce all male populations as we assumed the Sukamandi strain to be of *O. aureus* origin (El-Sayed, 2006) but we found both males and females equally in the Srikandi offspring. This indicates that our Sukamandi strain is not a pure *O. aureus*.



1.4. Motivation and objectives of the study

Aquaculture needs to increase its production in a sustainable way to address food security issues for a growing population in Indonesia. Coastal area with a high potential for aquaculture can be the future for fish production. A specific salinity-tolerant tilapia could be farmed in unused shrimp ponds which account for around 60% of all the ponds in brackish water area (Aliah, 2017). The lack of stability in growth and survival rates of tilapia

currently impedes aquaculture development in coastal areas. A novel breeding program to improve growth and survival of tilapia in the dynamic environments in coastal areas is highly needed. For this, an optimised breeding strategy is required. Definition of the production system is crucial in optimization of the breeding strategy. Breeding program should be conducted as much as possible in the same conditions as in the commercial production environment. However, information on how the farmers have adopted tilapia and the economic benefit of their developed farming systems are not well recorded.

In the first to fourth generation of the selection program, there were repeated issues with high mortality of selection candidates during grow out and transportation from the testing sites to the broodstock facilities in freshwater. Field trials with the Srikandi strain also showed wide variation in growth and mortality in shrimp farm environments.

A nucleus breeding program with a safe environment for the selection candidates could be a solution for this problem. However, this strategy requires information on genotype by environment interaction between nucleus and production system. Furthermore, a breeding program can be expensive, especially for a developing country. For this, we need to evaluate strategies that can be applied to reduce breeding cost and complexity of maintaining the breeding program.

The aim of this thesis is to investigate tilapia farming in shrimp ponds and to provide knowledge to improve the salinity-tolerant tilapia breeding program for brackish water ponds that has been developed in Indonesia. The specific objectives may be summarised as follows:

1. To investigate the farming characteristics and the adopted farming systems for shrimp and tilapia in brackish water ponds and their economic contribution to shrimp-tilapia farmers,
2. To investigate the genotype by environment interaction between freshwater ponds and commercial production environment in brackish water ponds,
3. To investigate physiological responses of selected tilapia for growth in brackish water and freshwater ponds,
4. To evaluate the long-term genetic response of the improved breeding strategy, and to

use this information to develop a low cost and sustainable design for a breeding program in Indonesia.

1.5. Outline of the thesis

This thesis consists of six chapters. In this **Chapter 1**, we provide a general introduction on the importance of aquaculture in Indonesia, the problems and the need for solutions. In **Chapter 2**, we show the results of a questionnaire-based survey to investigate the characteristics of shrimp-tilapia farms and their developed farming systems to grow tilapia in shrimp farm environments. In this chapter, we show how farmers have adopted various combinations of monoculture and polyculture of shrimp with tilapia and we present the economic profitability of each farming system. In **Chapter 3**, we investigate the genetic parameters and the genotype by environment (GxE) interaction between brackish and freshwater ponds using BLUP selection on the fifth generation of Sukamandi strain. In **chapter 4**, we investigate physiological differences between groups contrasted on their EBVs for growth in brackish and freshwater to evaluate the traits underlying the interaction of genetics with the environment. In **chapter 5**, we compare genetic parameters from monoculture of tilapia with polyculture of shrimp with tilapia and investigate the genetic trend, bias and accuracy of selection from G5 to G6. Finally, in the general discussion (**Chapter 6**), I discuss my findings and provide directions for a feasible, low cost and sustainable breeding program for salinity-tolerant tilapia in Indonesia and its dissemination.

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Chapter 2

Current status, trends, and future prospects for combining salinity tolerant tilapia and shrimp farming in Indonesia

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Abstract

Aquaculture plays a key role in Indonesian seafood production and food security. The most valuable species in Indonesian aquaculture is shrimp, which has been widely farmed since the early 1980s. Beginning in the 1990s, recurrent farm failures and increasing production losses caused by disease outbreaks have hampered the growth of shrimp production. Many small-scale shrimp farmers face severe financial challenges and are looking for alternative crops to supplement their livelihoods. In recent years, many farmers have adopted rotational cropping and polyculture systems as a sanitary practice to reduce disease outbreaks. One promising strategy is to add tilapia as a second species on existing shrimp farms. Few studies have investigated the economic potential for using tilapia in rotational cropping or co-culture systems with shrimp, especially in the context of high spatial and temporal variability that can reduce tilapia growth rate in saline coastal shrimp ponds. We conducted a survey to acquire information on current tilapia production covering 17 sites in four provinces of Java (Banten, West Java, Central Java and East Java). This paper presents an analysis on the extent to which and how tilapia has been incorporated into traditional shrimp farming systems, and its economic implications based on the survey data. We selected a total of 224 representative shrimp-tilapia farmers in consultation with the Extension and Community Empowerment Centre of Marine and Fisheries, Ministry of Marine Affairs and Fisheries (MMAF), and interviewed them in small focus groups from every site. Local fisheries extension officers guided the interviews. Our results indicate that a majority of small-scale farmers in all four provinces have low income status. To sustain their production levels, farmers have developed novel rotational and co-culture farming systems. The farmers' income status influenced their decisions to adopt specific farming systems. The poly-rotational farming system, which is farming shrimp and tilapia in the same ponds during the rainy season followed by farming only shrimp in dry season provided the highest farm gross profit. Higher income status farmers have more access to high quality feed, pond lining materials, and aeration systems, which resulted a higher farm gross profit across all four farming systems.

Key words: small-scale farmers, rotational farming, polyculture, farming system, income status

2.1. Introduction

Indonesia is the world's third largest aquaculture producer, contributing significantly to global fish production at ~4.9 million tonnes/year (FAO, 2020). The aquaculture sector accounts for 69.5% of Indonesian fish production, more than double that of capture fisheries, and aquaculture production grew at a rate 11.47% per year from 2012 to 2017 (MMAF, 2018). Indonesian aquaculture occupies around 1.32 million ha consisting of 54.15% brackish water ponds managed by 857,151 farmers, whereas freshwater and marine culture make up only 24.25% and 21.62% of the aquaculture area respectively (MMAF, 2018). Approximately 2.96 million ha of undeveloped and/or marginal land alongside the coastline are available for the potential expansion of brackish water pond culture.

Shrimp is the most valuable aquaculture product in Indonesia and dominates aquaculture production in brackish water areas. Shrimp farming activity began in 1962 in South Sulawesi using wild-collected larvae of Tiger shrimp (*Penaeus monodon*) from estuarine and coastal waters (Sianipar & Genisa, 1987). Hatchery production of Tiger shrimp seed started in 1970 (Sianipar & Genisa, 1987). By the 1990s, intensification was a common strategy for Indonesian shrimp farmers to boost their yields. In 2001, white leg shrimp (*Litopenaeus vannamei*) was officially introduced by Indonesian Ministry of Marine Affairs and Fisheries (Paena et al., 2009) as a superior variety. White leg shrimp are smaller than Tiger shrimp, but more resilient in brackish water (7-34 ppt), performing best under isosmotic conditions (10-15 ppt). In addition, white leg shrimp can be reared at densities of up to 100 seed/m^2, more than double that of Tiger shrimp in these intensive farming systems. Consequently, white leg shrimp rapidly became more popular than Tiger shrimp and is widely farmed in many areas in Indonesia.

Despite some variation, shrimp production increased between 2008 and 2019 (Fig.1). Total shrimp production was 409,590 tons in 2008, declined to 338,060 tons in 2009 and gradually increased to 932,698 tons in 2018. Disease outbreaks were the main cause of reduced shrimp production (Juarno et al., 2011). The number of farmers in brackish water areas showed a similar pattern, starting from 469,201 in 2008 to 857,151 farmers in 2018 (Fig.1).

Increasing shrimp density, establishing new ponds and the conversion of agricultural land to shrimp ponds contributed to this overall growth (Rimmer et al., 2013), and the high profitability of shrimp farming incentivized farmers to maximize production despite the environmental impacts and emerging disease issues (Widiatmaka et al., 2015).

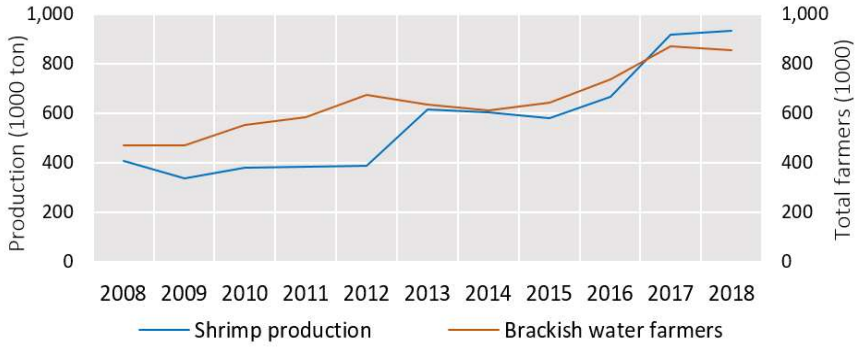

Figure 1. Shrimp production and number of brackish water farmers in Indonesia from 2008 to 2018 (MMAF, 2021)

This growth has, however, fallen short of expectations considering the large potential area available for shrimp farming. The Ministry of Marine Affairs and Fisheries projected total shrimp production of 1,134,700 tons in 2018, but only 82.2% of that target was realized, mainly due to recurrent farming failures. Many publications have suggested that poor water management and disease outbreaks are the main causes of recurring shrimp farming failures (Crab et al., 2012; Juarno et al., 2011; Liao & Chien, 2011; Rimmer et al., 2012). Early-on, white leg shrimp suffered from abdominal segment deformity disease (ASDD), an organ deformity triggered by an unknown retrovirus-like agent that can cause high mortality (Flegel et al., 2008). In addition, viral diseases, especially monodon-type baculovirus (*Penaeus monodon or MBV*) and white spot syndrome virus (WSSV), a member of the Whispoviridae, are serious problems (Flegel et al., 2008; Liao & Chien, 2011; Rimmer et al., 2012; Yuvaraj et al., 2015).

Anecdotal accounts suggest that disease outbreaks also lead to financial problems for Indonesian shrimp farmers. Currently, shrimp farming is considered a high-risk industry due to instability in shrimp prices and high mortality caused by diseases (Lestariadi & Yamao,

2018). Consequently, it is increasingly difficult for shrimp farmers to obtain financing, and many have abandoned land and ponds in traditional shrimp farming areas.

Switching to alternative species and rotational cropping are common strategies to minimize such economic losses. Milkfish and tilapia are the most popular fish species in brackish water ponds. According to MMAF (2018), total milkfish and tilapia production in brackish water ponds in 2017 were 632,885.6 tons and 42,225.6 tons, respectively. Grouper and seabass are also produced in brackish water, but the farming systems for these species require intensive pond management and high-quality water. Smallholder farmers, the majority in brackish water areas, cannot access the requisite financial and technical resources. Tilapia is the most attractive fin fish species in brackish water environments because it has the highest salinity tolerance and range, grows faster than most other freshwater fish species, including milkfish (Cnaani & Hulata, 2011; El-sayed, 2006; Popma & Masser, 1999), and in many areas, the selling price for tilapia is slightly higher than milkfish at the same size. In recent years, the rapid growth of tilapia in brackish water has encouraged many milkfish farmers to shift to tilapia, particularly in East and Central Java.

Rotational cropping or polyculture between shrimp and tilapia are increasingly practiced in brackish water ponds. Rotational cropping aims to reduce disease incidence or predation problems and is widely used in terrestrial agriculture as a sanitary practice and an integral part of plant health management (Paclibare et al., 1998; Yuvaraj et al., 2015). Polyculture is growing two or more species together in the same pond, and shrimp farmers could earn extra income from tilapia as a second crop. Polyculture between shrimp and tilapia has been applied in many countries, including Brazil (Junior et al., 2012), Philippines (Cruz et al., 2008), Thailand (Ferreira et al., 2015), and Mexico (Hernández-Barraza et al., 2012; Rosales et al., 2019). Several publications have demonstrated that this combination results in a positive interaction during grow out, and that adding tilapia to shrimp ponds can significantly reduce shrimp disease and mortality (Erwiantono et al., 2020; Jaspe & Caipang, 2011; Lio-Po, 2017; Paclibare et al., 1998).

Our hypothesis was that adopting tilapia in shrimp farming environments has contributed to farm profit. However, there is little information available on current practices,

particularly on the economics of shrimp-tilapia rotational and polyculture farming systems in Indonesia, the farmers themselves, the characteristics of their farms, and how this varies with farmers' income status. This paper addresses these knowledge gaps to provide a foundation for data-driven decisions on the prospects for rotational-and polyculture to enhance the economic performance of Indonesian shrimp farms. Specifically, we address the following questions:

- What are the characteristics of current Indonesian shrimp-tilapia farmers and their farms?
- To what extent are various farming systems practised by the farmers on Java Island, and what factors underly their decisions to adopt specific systems?
- What is the relative profitability of different farming systems and how does the farmers' income status influence the gross profit of these systems?

With this information available, farmers, breeders, extension agents, and government officials will be able to make more informed decisions to increase farm productivity and food security in the coastal areas. This information will be also useful to demonstrate the potential benefits of a breeding program to improve the performance and profitability of tilapia in brackish water aquaculture areas of Indonesia.

2.2. Material and methods

2.2.1. Developing the questionnaire

We developed an Indonesian-language questionnaire to facilitate discussions between farmers and extension agents in Java Island. It consisted of a set of structured questions intended to collect general information about farmers and the characteristic of their ponds, the farming systems they use, and their income status. We conducted a workshop in 2019 to adjust an existing questionnaire based on the farmers and farm characteristics. The workshop involved 23 participants from the three Fisheries Training and Extension Centres in Java (Central Java, West Java, and East Java), and resulted a final questionnaire that covered all needed information.

The background information section of the questionnaire covered basic information related to the respondents themselves. The socio-economic characteristics of the farmers were adapted from Omasaki et al. (2016a). We categorised their family income status based on local income standards as low income (US$<210/month), medium (US$ 210-490/month), and high (>US$ 490/month). We defined the salinity of their ponds as the average salinity during farming activities (i.e., excluding periods when the ponds were empty). We defined "farm distance" as the distance of the farm from the coastline, categorised as close (<1 km), medium (1-3 km) and far (>3 km). "Productive ponds" was defined as the percentage of total of pond area used for shrimp or tilapia production excluding ponds used for water filtration or recirculation. "Water source difficulty" was the percentage of ponds in which salinity levels cannot be maintained or controlled during the production cycle because of a lack of water to make the adjustments.

Table 1. The patterns of farming cycles per year for the four farming systems

Farming system	Farming cycles per year (times)		
	Tilapia	Shrimp	Tilapia + Shrimp
Monoculture (T, T)	1-3	-	-
Polyculture (T+S, T+S)	-	-	1-3
Mono-rotation (T, S)	1-2*	1-2**	-
Poly-rotation (T+S, S)	-	1-2**	1-2*

* during the wet season, ** during the dry season.

S refers to shrimp and T refers to tilapia. Monoculture (T, T) is farming tilapia in dry and rainy season. Mono-rotation (T, S) is a combination of farming tilapia in the rainy season and shrimp in the dry season. Polyculture $(T + S, T + S)$ is farming tilapia and shrimp at the same time in the same pond year round. Poly-rotation $(T + S, S)$ is farming tilapia and shrimp at the same time in rainy season and shrimp in dry season.

We categorized the farming "methods" as single crop or co-culture, single crop being farming only tilapia or shrimp, and co-culture being farming tilapia and shrimp simultaneously in the same pond. The majority of farmers practice a semi-intensive method particularly for single crop farming using commercial feed in combination with low density. We defined the farming "system" based on the pattern of farming cycles of shrimp and/or tilapia over a year and identified four distinct systems (Table 1). Monoculture is farming

only tilapia in every production cycle, and these farmers normally do not operate their ponds when salinity increases in the dry season. Polyculture is simultaneously farming shrimp and tilapia in every cycle. Mono-rotational cropping is farming only tilapia when salinity is low and only shrimp when salinity is higher, typically during the dry season. Poly-rotational cropping is defined as farming tilapia and shrimp together in the same pond during the rainy season, and only shrimp in dry season. Using these categories, we could account for the number of farming cycles per year for every individual farm in our gross profit calculations.

2.2.2. Sampling and interviews

We administered the questionnaire only in Java, the most densely populated island in Indonesia for both logistical and economic reasons. Java has the largest brackish water area among the five largest islands, consisting of 167,827 Ha or 31.52% of the total brackish water area available for aquaculture in Indonesia. Brackish water aquaculture in Java also supports the largest number of workers relative to other islands at 94,980 households or 36.04% of the total households in brackish water areas (MMAF, 2018). Furthermore, Java contributed 32.82% of national shrimp production or around 283,230 tons in 2019 (MMAF, 2021).

We conducted personal interviews based on the final version of the questionnaire in four provinces in Java. The survey participants were predominantly small-holder brackish water farmers who have incorporated tilapia to some extent into their farming system. The target areas and participants were also selected in consultation with the Extension and Community Empowerment Centre of Marine and Fisheries, Ministry of Marine Affairs and Fisheries. A small group of shrimp-tilapia farmers that represent each particular area was chosen by the extension agents who also coordinated and guided the interview process. In total, the study involved 224 shrimp-tilapia farmers, 17 from Banten Province, 84 from West Java, 69 from Central Java, and 54 from East Java. We conducted interviews at 17 sites, spending one day at each site. There were 5 sites in Central Java, 7 in West Java, 4 in East Java, and 1 in Banten.

2.2.3. Data analysis

2.2.3.1. Descriptive analyses of shrimp-tilapia farmers and farming systems

To describe the patterns and trends in combined shrimp-tilapia farming, we summarized the number of farmers in low, medium, and high income status categories as percentages for each of the provinces. We further summarized the farm characteristics based for each income status category. The distances of shrimp-tilapia ponds from the coast, salinity levels of the ponds, productive ponds, difficulty of accessing water, and shrimp farming failures in the last five years are expressed as percentages of farms for each level of income status. We also summarized the farm inputs - types of water aeration, pond lining systems, the use of commercial feed, the feeding rate and type of fertilizer - as percentages of farms that invest in these inputs. We categorised three types of feed used by the farmers as commercial feed, self-formulated feed, and naturally-occurring food from the pond. For the total feed used, we grouped the respondents based on daily feeding rates of <3%, 3-5% and >5% of fish biomass.

2.2.3.2. Comparing the profitability of alternative farming systems

We compared the four farming systems by calculating farm-level gross profits separately for every farm, taking into account their reported costs and revenues for a single production cycle and the number and types of production cycles achieved in a year. The annual gross profit for each farm is, therefore the combination of the gross profit per hectare per cycle of each type within a year and the number of cycles achieved. To calculate the per hectare, per cycle gross profit, we assumed a simplified production system for which we developed an approximate gross profit equation.

The general form of the gross profit equation per hectare per cycle is:

$$Gross\ profit\ margin\ per\ ha = GR - (C_{TF} + C_{TS} + C_{TO})$$

where GR (gross revenue) is defined as the total selling price of fish and/or shrimp at harvest time per cycle per ha. We defined C_{TF} (total feed cost) as total feed used for production, and C_{TS} (total seed cost) as a number of seed stocked at the pond times price per seed. We defined C_{TO} (total operation cost) as the total operational costs for electricity, fuel, lining, fertiliser and labour per cycle per ha which was discussed with fishery agents.

Because the ponds are generally inherited by the farmers rather than purchased (personal communication during focus group discussions in 2020), the costs of acquiring land were not taken into account in total cost. Other fixed costs are not affected by the farming system, and therefore are omitted in this calculation. The costs of rent, insurance and machinery which are common in large-scale, intensive shrimp farming do not apply to small scale farmers. Small-holder farmer are family-operated and thus do not incur permanent labour costs. Small-scale farmers recruit workers when they have extra work on daily basis for occasional tasks such as maintaining ponds, water quality and harvesting which were included as a variable cost of temporary labour.

We calculated GR per hectare per cycle as:

$$GR = (SD_{tilapia} * SR_{tilapia} * 10{,}000\ (ha) * \frac{harvest\ size}{1000\ g} * tilapia\ price/kg) +$$

$$(SD_{shrim} * SR_{shrim} * 10{,}000\ (ha) * \frac{harve\ \ size}{1000\ g} * shrimp\ price/kg)$$

where SD is defined as stocking density (fish/m^2) and SR is defined as survival rate. We estimated the number of seed stocked per hectare based on farmers' answers to the questionnaire. We also used shrimp and tilapia price per kg, harvest weight, and survival rate information provided by individual farmers to calculate farm-specific GR per cycle.

We used a tilapia growth model of Daily Growth Coefficient (DGC) to calculate C_{TF} for tilapia in monoculture and polyculture systems. First, we calculated the DGC following Omasaki et al. (2016b) for every farm using the formula:

$$DGC = \frac{\sqrt[3]{HW} - \sqrt[3]{SW}}{growing\ days} * 100$$

where HW is harvest weight and SW is stocking weight. Then we calculated the average of monthly body weight ($\overline{BW}$) for each month as:

$$\overline{BW} = \left(\frac{DGC * days\ in\ a\ month}{100} + \sqrt[3]{SW}\right)^3$$

Based on body weight information, we calculated total feed provided per fish per cycle as an accumulation of monthly feed. We calculated monthly feed using the formula:

$$Monthly\ feed\ = \overline{BW} * FR * n\ days\ in\ a\ month$$

where FR is feeding rate. We used 2% and 4% as the average feeding rate approximation for the <3% and 3-5% categories, respectively. The total feed consumption per fish is an accumulation of monthly feed consumption. Finally, we estimated C_{TF} with total feed per fish information using the formula:

$$C_{TF} = \frac{(1 - (0.5 * (1 - SR) * total\ feed\ per\ fish * SD * 10.000\ (ha)}{1000\ g} * feed\ price/kg$$

We assumed that fish that die during the growing period die, on average, half-way through the rearing period. Total feed was used to calculate realized feed conversion ratio ($rFCR$), which is a ratio between estimated total feed provided and fish production per cycle (Omasaki et al., 2017), accounting for mortality.

We calculated C_{TF} for shrimp in rotational farming as:

$$C_{TF} = FCR * SD * SR * 10{,}000\ (ha) * \frac{harvest\ size}{1000\ g} * feed\ price\ per\ kg$$

where FCR used in this calculation is assumed to be 1.5, which is generally applicable to small scale shrimp farmers in Java based on the information from fisheries extension agents in Gresik and Cirebon regencies (pers. comm. to PS, 2021). The farmers do not directly feed shrimp in polyculture systems and stock shrimp at a lower density than in single crop farming.

We estimated total seed cost (C_{TS}) as :

$$C_{TS} = \left(SD * 10{,}000 * Seed\ price_{tilapia}\right) + (SD\ x\ 10.000\ (ha) * Seed\ price_{shrimp}$$

We defined Total operation cost (C_{TO}) as:

$$C_{TO} = Lining\ cost + fertilizer\ cost + ((labour\ cost\ + aeration\ cost\ ) * n\ months)$$

Output from the pond per ha per cycle model above becomes the input for the yearly farm model which consists of four farming systems. The types and number of production cycles

per year (n) for each farm is known from the questionnaire. Therefore, the average of yearly gross profit for `each farming system per ha can be defined as:

$$Farm\ gross\ profit = Profit\ of\ pond\ per\ per\ ha\ per\ cycle * n$$

where n is a number of production cycle per year. The formulas were used to calculate farm gross profit of every farmer in the questionnaire. These gross profits were then averaged by farming system and farmers income status.

2.3. Results

2.3.1. Shrimp-tilapia farmers and farm characteristics

Table 2. The percentage of shrimp-tilapia farmers for each level of family income status in all provinces

Provinces	Income status (%)		
	Low	Medium	High
Central Java	46.4	36.2	17.4
West Java	48.8	29.8	21.4
East Java	61.1	27.8	11.1
Banten	52.9	47.1	0

The family income status of shrimp-tilapia farmers are presented in Table 2. The majority of shrimp farmers on Java Island (46.4-61.1%, depending on the province) have low income status. Medium income status account for around a third, and less than a quarter of the farmers surveyed are high income status. All respondents in Banten have medium and low income status.

Farmers with higher income status have a higher proportion of farms located more than three kilometres from the coastline. It also seems that farms inland have better access to a water source, as the higher income status category has a lower percentage of farms with water source difficulty (Table 3). Interestingly, while all income status categories have variation in farm location and water source difficulty, a majority of shrimp-tilapia farmers (67.12-77.78%) are able to limit their ponds salinity to less than 20 ppt, which is suitable for

some tilapia species. High income status farmers, on average, inherited a larger farm at around triple the average size of low income status farmers. High income status farmers also have a higher percentage of productive ponds for farming shrimp and tilapia.

Table 3. The characteristics of the shrimp-tilapia farms across three income status

Variables	Distribution based on income status		
	Low	Medium	High
Farm distance to coastline > 3 km (%)	36.52	38.46	44.45
Farm have water source difficulty (%)	78.26	76.71	61.11
Pond salinity levels <20 ppt (%)	77.39	67.12	77.78
The average of farm size (ha)	1.26	1.96	3.30
Total ponds used for production (%)	64.35	71.23	88.89

Turning to previous shrimp farming failures, all small scale farmers have experienced recurrent farming failures. Table 4 shows that more than half of the farmers in Banten and East Java suffered a total loss of shrimp production more than 10 times in the last five years, and all farmers in Banten experienced farming failures more than five times. The farmers in West Java and Central Java encountered fewer problems with shrimp farming with a lower percentage having experienced >10 failures than other two provinces. Additionally, about one third of farmers in these provinces have experienced shrimp farming failures less than five times.

Table 4. The percentage of farmers that experienced shrimp farming failures across the four provinces

Farming failures	Provinces			
	Banten	West Java	Central Java	East Java
<5 times (%)	0	38.24	37.68	11.54
5-10 times (%)	43.75	20.59	21.74	25
>10 times (%)	56.25	41.18	40.58	63.46

2.3.2. Shrimp-tilapia farming systems

The farming systems adopted by shrimp farmers vary across the four provinces. East Java has the highest proportion of farmers using polyculture systems (Figure 2), indicating that a majority of shrimp farmers in this area produce shrimp in combination with tilapia. Moving to the west, the percentage of farmers implementing polyculture decreases, and the fraction using poly-rotation increases. In contrast, a majority of shrimp farmers in Banten shifted entirely to tilapia monoculture over the whole year, with the remainder (~6%) adopting a mono-rotational system.

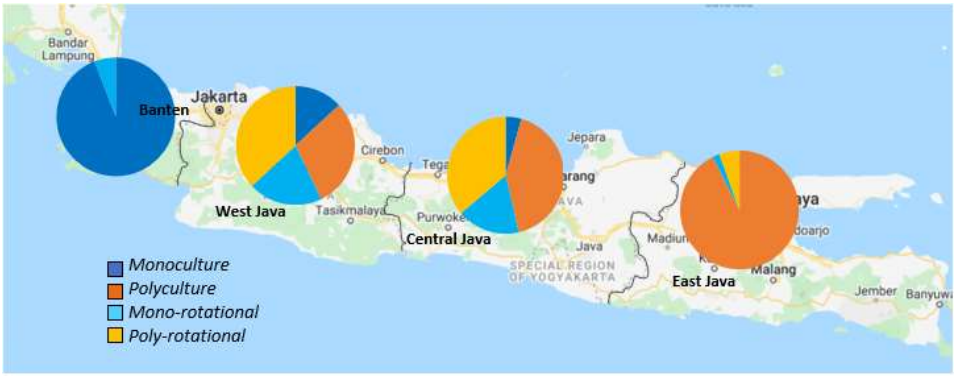

Figure 2. The proportion of practiced shrimp-tilapia farming system across the four provinces in Banten, West Java, Central Java and East Java

Apparently, income status influences farmers' decisions of which system to adopt; there is a trend to shift to poly-rotational farming with increasing income status (Fig. 3). The highest income status category has the lowest percentage of farmers producing tilapia in a monoculture system (8.3%). In contrast, the percentage of farmers using the polyculture system (farming shrimp and tilapia in the same pond), declines with increasing income status. In general, the polyculture system is the most commonly practised by farmers in all income status levels.

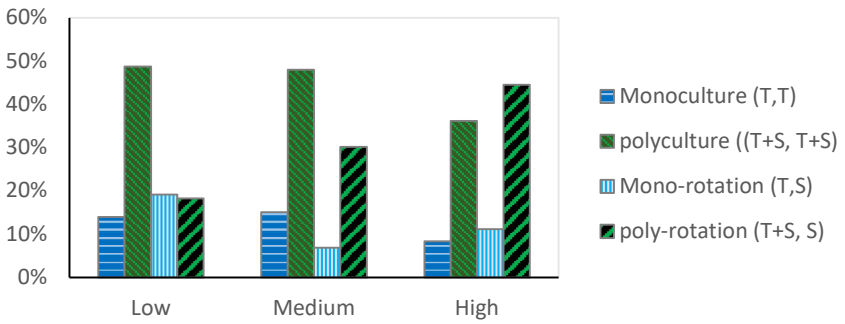

Figure 3. The percentage of farmers practicing the four shrimp-tilapia farming systems across the three income status categories.

Table 5. The distribution of farmers who use farming inputs based on farming system

Farming input	Distribution of farmers based on farming system			
	Monoculture	Polyculture	Mono-rotational	Poly-rotational
Pond aeration (%)	3.33	13.46	48.39	57.63
Pond lining (%)	0.00	0.96	22.58	30.51
Use tilapia feed (%):				
Commercial feed	66.67	79.92	74.19	76.27
Own formulated	26.67	3.85	16.13	13.56
Feeding rate (%):				
<3 %	71.43	61.90	53.57	69.81
3-5 %	28.57	38.10	46.43	30.19
Pond fertilizer (%):				
Biological	56.67	60.58	54.84	71.19
Chemical	40	34.62	35.48	25.42

Our results show that farmers practicing different farming systems input different levels of aeration, pond lining, feed, fertilizer and seed. A higher percentage of farmers who practise the two rotational systems invest in aeration and lining (Table 5). In contrast, none of the farmers applying monoculture tilapia line their ponds and a very small percentage use aeration in their production systems. Regarding feeding, on average three quarters of the farmers use commercial feed, another 11% use own formulated feed. The remaining farmers rely entirely on natural food in their brackish water ponds.

Our results also show that farm inputs are different in each income status group. The higher income status group has a higher percentage of farmers using aeration and lining their ponds with polyethylene or plastic as well as a higher percentage using commercial feed (Table 6). Interestingly, the proportion of farmers applying the lower feeding rate is also higher for higher income status farmers because a higher percentage of farmers use nutritionally denser commercial feed. A higher percentage of farmers in the higher income status category use chemical pond fertiliser.

Table 6. The distribution of farmers who use farming inputs based on income status

Farming input	The percentage of farmers based on income status		
	Low	Medium	High
Pond aeration (%)	19.13	30.14	55.56
Pond lining (%)	6.96	9.59	30.56
Type of tilapia feed (%)			
- Commercial feed	63.48	84.93	91.67
- Own formulated	14.78	8.22	5.56
- no feed (%)	21.74	6.85	2.78
Feeding rate (%)			
- <3%	48.70	58.90	69.44
- 3-5%	29.57	34.25	27.78
Pond fertilizer (%):			
- Biological	58.26	68.49	61.11
- Chemical	33.91	28.77	38.89

Different farming systems also stock at different seed densities resulting in different realized FCR (rFRC; Table 7). The farmers applying the mono-rotational system stock a higher density of tilapia and the polyculture system stocks both tilapia and shrimp at lower single-species densities than the rotational system. In the polyculture system, the rFCR was also lower than other systems. Furthermore, the monoculture system uses the lowest density at around four tilapia per square meter. Stocking density and rFCR are also different in each income status group. The higher income status group has a higher percentage of farmers using higher average stocking densities, which results in a lower rFCR for tilapia

feed in a monoculture system. However, the medium income status has the highest rFCR in a polyculture system.

Table 7. The average of stocking density and realized FCR (rFCR) in different farming systems and farmers' income status categories

Categories	Group	Stocking density (seed/m^2)			rFCR tilapia (%)
		Tilapia	Shrimp	Tilapia:Shrimp	
Farming system	Monoculture	4	-	-	1.26
	Polyculture	-	-	5:5	1.11
	Mono-rotation	10	15	-	1.34
	Poly-rotation	-	17	6:11	1.21
Income status	Low	7	14	4:6	1.34 (Monoculture), 1.01 (Polyculture
	Medium	7	16	6:8	1.25 (Monoculture), 1.33 (Polyculture)
	High	10	21	7:12	1.16 (Monoculture), 1.15 (Polyculture)

2.3.3. Economic comparison of the different farming systems

Farm inputs have an important impact on farm gross profit estimation. Pond aeration and lining add significant extra costs for the farm. In addition, there were notably different prices between commercial and own formulated feed as well as different pond fertilisers. The average labour cost per hectare per month is approximately USD 140. The price for tilapia fingerlings and shrimp seed vary as a consequence of the different sizes used by farmers. The farmers' use of different size of seed is mainly due to seed availability in the market. The table size of shrimp and tilapia also vary due to harvest size and the seasonal price (Table 8). All these reported variations in prices between farms were used to produce farm-specific gross profit calculations.

Regarding the farm gross profit, our equations indicate that monoculture tilapia, which is farming only tilapia in every production cycle generates the lowest gross profit in all income status categories (Table 9). Furthermore, the higher income status group produces a better gross profit in all farming systems. Adding shrimp significantly increases farm gross profit. The farm gross profit of monoculture is even higher if they combine tilapia and shrimp in a

polyculture system with an increase of about 340% whereas gross profit in poly-rotation increases by about 178% over mono-rotation. In general, the highest farm gross profit is achieved when farmers apply a poly-rotational system.

Table 8. Farming inputs and prices from farmers' answer to the questionnaire used for farm gross profit estimation

Farming input	Price	
	value	unit
Pond aeration:		
- Gas engine paddle wheels	744.9	USD/ha/month
- Electric paddle wheels	550.6	USD/ha/month
Pond lining:		
- Polyethylene pond lining	2,348.7	USD/ha/cycle
- Plastic pond lining	851.4	USD/ha/cycle
Tilapia feed:		
- Commercial feed	0.70	USD/kg
- Own formulated feed	0.42	USD/kg
Pond fertiliser:		
- Biological pond fertilizer	209.35	USD/ha
- Chemical pond fertilizer	348.92	USD/ha
Labour cost	140	USD/ha/month
Tilapia fingerling	4.19-13.96*	USD/1000 seed
Shrimp seed	1.40-4.19*	USD/1000 seed
Shrimp feed	1.05	USD/kg
Table size tilapia	1.12-1.74*	USD/kg
Table size shrimp	3.14-5.58*	USD/kg

(1 US$ = Rp. 14,330 in March 2022).
* The range values are the value used in the calculation based on the seed and table size of shrimp and tilapia on each farm.

Table 9. The average of farming systems gross profit per year per ha in USD across three income status

Income status	Farming system			
	Monoculture	Mono-rotation	Polyculture	Poly-rotation
Low	1,739	3,630	5,524	7,492
Medium	2,965	7,186	8,222	10,579
High	3,659	11,036	15,897	19,850

2.4. Discussion

Many smallholder shrimp farmers in Indonesia have either added tilapia as a second crop or shifted entirely to tilapia farming in abandoned and under-producing brackish water shrimp ponds as the best alternative to address recurrent shrimp farming failures (Rimmer et al., 2012). However, prior to our study, we lacked systematic data on the extent to which farmers have modified their farming systems and the economic factors driving their decisions. In the next three sections, we discuss the characteristics of shrimp-tilapia farmers who are adopting tilapia, the economic aspects of different farming systems, and the future prospects and directions for producing tilapia in systems initially developed for shrimp production.

2.4.1. Shrimp tilapia farmers characteristics and the adopted farming systems

Our data show that majority of shrimp-tilapia production consists of small-scale farmers with low income status in all provinces of Java (Table 2). This result was as expected based on existing data (MMAF, 2018) indicating that small-scale farmers constitute around 65% of brackish water farmers in Indonesia. A majority of these farmers operate their ponds using a traditional low input, low productivity system and are highly dependent on natural conditions and inputs. Quach et al. (2017) showed that traditional shrimp farmers are more vulnerable to shrimp farming failures related to climate change because they are more subject to natural variation in salinity and have limited options to stabilize it (Hukom et al., 2020). This group of farmers also has difficulty with access to water, and as a result, cannot optimize the conditions in their ponds for production (Table 3). Consequently, they cannot produce shrimp year-round. In line with this, a recent study by Hukom et al. (2020) also showed that the majority of shrimp farmers in Sidoarjo, East Java, are small scale farmers. Nevertheless, the small-scale farmers in this area improve their farms' technical efficiency through a community based group that monitors water and coastal resources to prevent unwanted problems such as pollutant and disease outbreaks. It seems reasonable, therefore, to conclude that their low income status is associated with low input production systems and low farm productivity since low input results in low production, a situation that Yi et al. (2018) described as a low productivity "trap" in aquaculture. As farmers improve

their income status, they can have a better access to water sources and use a larger percentage of their ponds for production as well as increasing investments in farm inputs such as higher quality feed, aeration, and linings.

It also appears that farmers' income status drives or constrains their decisions regarding which farming system to adopt. In contrast to large companies and intensive shrimp farmers, a majority of small-scale farmers inherit farms that have been family-owned for generations rather than purchasing them. Therefore, their income status depends largely on farm size and location. In all provinces, the small percentage of farmers with high income status have an average pond area that's approximately triple that of low income status farmers (Table 3). With a higher income, the farmers in this group are more able to invest in their farm, and are more likely to use aeration, lining, and high-quality feed (Table 6), making it possible to stock fish at higher density (Table 7). As a consequence, this group also has a higher percentage of farmers who have adopted a poly-rotational system (Figure 3). These inputs are particularly important for farming only shrimp in a rotational system or farming tilapia and shrimp in high density polyculture systems (Table 7). Ruiz-Velazco et al. (2010) reported that the most important determinant of dissolved oxygen in shrimp production is stocking density, and Martinez-Cordova et al. (1998) showed that aerating ponds for 6 hour per day produced better shrimp growth at a stocking density 10 shrimp per m^2.

Farmers' prior experience with farming failures also seems to be an important determinant of farming system preferences. Farmers in Banten and East Java, where more than half have experienced multiple, serious shrimp farming failures (Table 4), grow fewer shrimp in their ponds. The most extreme example is that the vast majority of farmers in Banten have totally shifted to tilapia due to disease outbreaks on their farms. Our data support the conclusion of Rimmer et al. (2012) that shrimp farming failures are mainly caused by disease problems which led to the abandonment of many shrimp ponds. In line with this, Liao and Chien (2011) showed that disease related problems caused by viruses such as MBV and WSSV are responsible for slow annual growth in cultured shrimp production in Indonesia. Farmers in West Java and Central Java, where approximately one third of farmers have experienced total loss fewer than five times (Table 4) are still farming shrimp using a rotational system

during the dry season (Figure 2). Polyculture and poly-rotational systems have been widely adopted across all provinces except Banten.

Despite these constraints and issues, small scale farmers are managing to operate their farms and have been developing innovative farming systems that seem to be more sustainable and profitable than shrimp monoculture. Rotational farming reduces shrimp diseases, suggesting that tilapia can be used as a form of biological control for pathogens in shrimp ponds, increases shrimp survival and productivity (Yuvaraj et al., 2015), and improves nutrient usage, which reduces the environmental impact of shrimp production (Yuan et al., 2010). Our results also agree with previous research in Aceh, Sumatra (Rimmer et al., 2012), demonstrating that rotational cropping is an economically promising alternative to avoid shrimp farming failures.

Our data indicate that polyculture is the most common farming system for small scale shrimp-tilapia farmers (Figure 2). In the last two decades, adopting this system has significantly reduced some shrimp farmers' problems (Martínez-Porchas et al., 2010). Furthermore, probably because these two species have distinct trophic niches which reduces competition for the same resources (Junior et al., 2012), polyculture of shrimp and tilapia results in a positive interaction during grow out (Cadiz et al., 2016; Ferreira et al., 2015; Hernández-Barraza et al., 2012), increasing growth and yield compared to monoculture. In addition, polyculture reduces shrimp disease and mortality, including reductions in *Vibrio parahaemolyticus*, green colony Vibrios (Cadiz et al., 2016), and eutrophication (Ferreira et al., 2015). A study by Tendencia et al. (2006) showed that tilapia and grouper hampered significantly luminous bacteria growth in shrimp ponds and increased shrimp survival rate.

Reducing eutrophication has a positive environmental effect, decreasing organic outputs from farming activity by recycling wastes and residues through a second cultivated species rather than releasing them to the surroundings (Junior et al., 2012). A study by Rosales et al. (2019) showed that the polyculture system at low stocking density using the combination of ten shrimp and four tilapia per m^2 optimized nutrient utilization and pond water quality in both dry and rainy season. In agreement with this, the polyculture system at a low

stocking density adopted by small-scale farmers in Java at five shrimp and five tilapia per m^2 (Table 7) seems to be a beneficial combination on their farms.

2.4.2. Economic aspects of tilapia farming in shrimp farming environment

Our data show that, if successful, shrimp farming is generally more profitable than tilapia on a per hectare, per cycle basis. However, shrimp farming produces only one or two cycles per year (Table 1) during the dry season when pond salinity level is high enough. This agrees with a previous study by Hukom et al. (2020) that traditional shrimp farmers in Sidoarjo, East Java prefer higher salinity, which is more efficient for their farms. Since around two thirds of small-scale farmers have difficulties with access to water sources (Table 3), they are not able to operate their farms when salinity gets too high for shrimp due to evaporation or too low due to high rainfall. Furthermore, the high probability of total crop losses make shrimp farming risky. This is most apparent in Banten province where pond productivity is low and a majority of farmers grow only tilapia. A few respondents in Banten applied monoculture shrimp farming at low density, but experienced low survival rates, resulting in lower farm gross profit than farming tilapia.

A mono-rotational system is a viable alternative strategy to optimize farm gross profit. This practice provides a better gross profit than farming tilapia in an entirely monoculture system (Table 9). Rotational farming is categorized as a sanitation practice. Previous studies have demonstrated that rotational cropping between shrimp and tilapia results in lower disease occurrences in shrimp farming (Paclibare et al., 1998; Paena et al., 2009), and Jaspe and Caipang (2011) found that combining shrimp farming with tilapia reduces the incidence of fibrosis disease in shrimp ponds. Consequently, using tilapia in a rotational cropping system is a potentially more sustainable strategy to reduce shrimp mortality than farming shrimp in monoculture system continuously. Rotational cropping between fish and shrimp has been demonstrated to significantly increase shrimp survival by approximately 25% and to increase shrimp productivity (Yuvaraj et al., 2015), improve nutrient usage and reduce the environmental impact of shrimp production (Yuan et al., 2010).

Farm-level gross profit is higher for polyculture systems than monoculture (Table 9). This practice is most suitable during the rainy season when disease outbreaks are common.

Small scale farmers adopting this system can produce between one and three cycles per year (Table 1). The best farm gross profit is achieved when the farmers apply a poly-rotation system. However, this system requires more farm inputs (Table 5). As a consequence, a high income status farmer has more opportunities to adopt this system as shown in the higher percentage of farmers in this group adopted poly-rotation system.

2.4.3. Implication and future directions

The Indonesian shrimp industry is struggling with disease issues and environmental impact, and co-culturing tilapia and shrimp can potentially mitigate some of the issues. Adding tilapia to traditional shrimp monoculture systems has potential for growth and generates higher gross profits for farmers who adopt novel, modern rotational and polyculture systems. Similarly, Erwiantono et al. (2020) showed that this combination increased farm gross profit significantly and recommended it to decrease poverty in the coastal areas. By reducing the risks of crop failures, these recently-developed farming systems can improve access to capital and encourage investment in feed, aeration, and other forms of modernization. Furthermore, the government, through its network of fisheries extension agents, can assist the farmers to develop the most profitable farming systems for their specific locations. Poly-rotational and polyculture, the most profitable farming systems in many areas, should be actively promoted and supported to encourage implementation by farmers.

Furthermore, farming tilapia in brackish water ponds could be a potential solution to the shortage of freshwater ponds. Tilapia production contributed significantly to the national fish production at around 1.27 million ton in 2017 with an annual improvement of 13.1% from 2012 to 2017 (MMAF, 2018). Most of tilapia production occurs in freshwater ponds, and around a quarter is produced in floating cages in reservoirs and lakes. Since 2016, the Indonesian government tightened regulations on fish production in reservoirs due to increasing water pollution and carrying capacity issues. Another concern is that escaping tilapia could endanger many endemic species due to its invasive behaviour. As a result, many floating cage activities have been closed or restricted. The potential area for aquaculture in brackish water is larger than for freshwater, and only one fifth of this area is

currently used for fish production. The fact that tilapia has the highest salinity tolerance among freshwater species could be a major reason that its development in this area is increasing. The faster growth, feed efficiency, and simplicity of tilapia farming are comparative advantages of tilapia farming in brackish water. In addition, the farmers and fisheries extension agents mentioned that the stability of selling price (Table 8) influenced their decision to adopt tilapia.

The trend of increasing tilapia farming in brackish water ponds is also economically feasible, and farmers themselves have developed farming systems that seem to be more sustainable and profitable than traditional methods. Our preliminary economic analysis indicates that farming tilapia increases and stabilizes farm productivity and profitability. Furthermore, given that farmers are already making this transition, a more salinity tolerant tilapia strain adapted to brackish water systems would also enhance production and reduce the risks of tilapia crop failures. A majority of surveyed farmers use tilapia strains that are poorly adapted to brackish conditions resulting in low farm gross profit, particularly in the monoculture system. The growth rate of tilapia is decreased and more susceptible to bacterial disease at salinity higher than 20 ppt (Rimmer et al., 2012). Consequently, production is constrained by the limited salinity tolerance of these freshwater tilapia strains, and, developing salinity tolerant tilapia for brackish water ponds in Indonesia could expand production. A genetically rigorous and disciplined breeding program that evaluates performance in real-world farming systems developed and implemented by farmers themselves and aims to produce strains adapted to these specific conditions could help to achieve this potential. This requires focusing selection pressure on traits that most strongly influence performance in these emerging systems. Desired gains, profit equations, and bio-economic models can all be used to evaluate the economic potential of desired traits for future development.

There are three key strategies to increase national shrimp and tilapia production. The first strategy is to involve fisheries extension agents in actively promoting and supporting polyculture or poly-rotational systems based on specific regional and local farm conditions. Our data suggest that poly-rotation is the most profitable overall, but additional feasibility studies are required to identify and adapt the best farming system for specific farms. The

second strategy is to enhance investment and financial support for shrimp and tilapia farmers to modernize their farms and adopt more profitable farming systems. Finally selective breeding to produce a more salinity tolerant tilapia strain for brackish water ponds through a rigorous, accurate, and disciplined breeding program would further enhance the profitability of both polyculture and monocultures systems in brackish water environments.

2.5. Conclusion

Farmers' experiences of farming failures in the past drove decision to farming system they use. A majority of shrimp-tilapia farmers in all provinces are in low-income status who traditionally used low input production systems that create a low productivity trap. The farmers are currently exploring four different farming systems, but for the most part, their income status drives farm inputs and farming systems applied. The higher income status group has access to better farm inputs. This group has a higher ratio of farmers applying polyculture and poly-rotation system. In general the poly-rotational system is the most profitable farming system even though requires a higher investment and farm inputs. This system should be systematically promoted in brackish water pond through government policy, involving fisheries extension agents to actively assist the implementation of this system in every regency.

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Chapter 3

Genetic parameters and genotype by environment interaction in a unique Indonesian hybrid tilapia strain selected for production in brackish water pond culture

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Abstract

Recurrent farming failures due to disease outbreaks have driven Indonesian shrimp farmers to develop co-culture between shrimp and tilapia. For this reason the Research Institute for Fish Breeding (RIFB) Indonesia has started to develop a fast-growing tilapia with good growth over a range of fluctuating salinities in brackish water ponds. A freshwater nucleus and evaluation breeding program is the simplest strategy to implement, but requires knowledge on the extent of genotype by environment (GxE) interaction between fresh and brackish water environments. The objectives of this study were: 1) to investigate the presence of GxE between brackish water and freshwater ponds, 2) to investigate the impact of salinity on genetic parameters, and 3) to investigate gonadal development of tilapia in both environments. We produced 91 fish families and for each family, randomly choose 2 groups of 20 fingerlings for communal grow-out in brackish water at salinity 6 to 25 ppt and freshwater for 120-147 days. We recorded harvest weight (HW) and standard length (L) and calculated daily growth coefficient (DGC), growth rate in length (GR(L)) and condition factor (K) for each fish. Gonadosomatic index and maturation score (0/1) was recorded on 6 fish from each family per environment. We estimated genetic parameters using bivariate animal models in ASReml version 4.1. Results: HW, L, DGC and GR (L) in brackish water were significantly higher than in freshwater. Heritability was moderate for all traits in both environments (0.35-0.50). Genetic correlations between brackish water and freshwater for HW, SL, DGC and GR(L) were 0.65-0.74. Gonad weight for males and females, and gonadosomatic index for females in brackish water were significantly higher than in freshwater ($P<0.05$). Gonad maturity for both sexes had low heritability in brackish water than in freshwater, (0.12 and 0.04 respectively) with a genetic correlation of 0.47. We conclude that there is substantial GxE interaction for growth between brackish water and freshwater. However, the higher mean growth in brackish water suggests that this is not due to salinity per se, but more likely to other differences between the pond environments. We recommend that a breeding program for salinity tolerant tilapia with a safe, stable, low-risk, and bio-secure fresh water nucleus should incorporate sib information on growth performance in brackish water.

Keywords: breeding program, brackish water, salinity, heritability, Genotype by environment interaction

3.1. Introduction

The Indonesian shrimp industry consists of approximately 65% small-scale farmers who have been abandoning their ponds in many areas due to repeated crop failures, and these reductions in production will likely accelerate as climate change drives significant changes in salinity and sea level rises (Dabbadie et al., 2019; Kalikoski et al., 2018; Maulu et al., 2021). Because shrimp production is the most important aquaculture industry in Indonesia with the highest contribution to the national income (MMAF, 2018), this has important economic and societal repercussions.

To address this situation, many shrimp farmers have shifted to a shrimp and tilapia co-culture farming system in brackish water ponds. Most tilapia grow quickly in freshwater, but some species and strains can also be cultivated in brackish water (Cnaani & Hulata, 2011; Stickney, 1986; Suresh & Lin, 1992). In addition, several studies (Ath-thar & Gustiano, 2010; Putra et al., 2013) demonstrated that hybrids and improved strains have higher growth than Nile tilapia in brackish water ponds (Aliah, 2017; Setyawan et al., 2015).

To facilitate this transition, the Research Institute for Fish Breeding (RIFB) has been conducting a small-scale breeding program for salinity tolerant tilapia for four generations. The institute acquired a tilapia strain from a private feed company in 2007 that was thought to be blue tilapia (*Oreochromis aureus*). The founders were spawned for multiplication in early 2008 before the start of the breeding program and named the Sukamandi strain. However, Yu et al. (2022) recently compared the whole genome sequence of this strain to both Nile tilapia (*Oreochromis niloticus*) and blue tilapia and discovered that it is actually a hybrid that is genetically closer to Nile tilapia than to blue tilapia. Signatures of introgression suggest that specific genes related to salinity adaptation (*slc25a24* and *cdhl*) have been introgressed from blue tilapia. We assume that inadvertent mixing between blue tilapia and Nile tilapia occurred between 2008 and 2011 because in subsequent communication with the feed company, they explained that they kept blue tilapia in a separate closed facility. Although this was unintentional, it has apparently contributed to improving salinity tolerance in the Sukamandi strain by introducing favourable genetic variants to the population.

To date, the Research Institute for Fish Breeding's breeding program for salinity tolerant tilapia has been based entirely on mass selection using own performance records of candidates from the Sukamandi strain evaluated in a range of brackish water environments. The average selection response after four generations of phenotypic selection for increased harvest weight after ~120 days of growth is 10.29% for males and 9.29% for females (unpublished results).

The current strategy exposes selection candidates to conditions with poor biosecurity, high mortality losses and associated risks. Many practical challenges such as limited control over environmental conditions and transportation from test sites to the hatchery have also resulted in high risk and costs. An alternative strategy is a nucleus-based breeding program similar (Bentsen & Gjerde, 1994; Olesen et al., 2015), to those previously implemented for other tilapia strains (Omasaki et al., 2017), salmonids (Yáñez et al., 2014), and gilthead seabream (Janssen et al., 2018). This approach maintains all selection candidates in a safe and bio-secure environment that does not represent commercial growing conditions and selects among them using performance information from relatives grown in a production environment using mixed-model BLUP to estimate their breeding values (Trong, 2013).

In this case, the population of selection candidates can be kept in safer and more stable freshwater conditions at the research institute and their progeny and/or sibs can be grown and evaluated in brackish water test locations. Because genotypes may perform differently in the holding vs. testing environments due to genotype by environment interaction (Falconer & Mackay, 1996) the performance of candidates in freshwater may not predict their performance in brackish water. Depending on the strength of GxE, this approach may require predicting the breeding values of candidates based on the performance of their relatives rather than own records. GxE interaction between freshwater and brackish water has been studied previously by Luan et al. (2008), Thodesen et al. (2011) and Thoa et al. (2016) based on the genetic correlation between final weight in different environments using models that treat them as separate traits. Thoa et al. (2016), for example, estimated the genetic correlation between harvest weight in freshwater and brackish water (15-20 ppt salinity) as 0.92 ± 0.04 which suggests that selection based on freshwater performance can transfer 92% of the genetic gain achieved to brackish water performance without evaluating

sibs or other relatives in the brackish water environment. If this is also true for the Sukamandi strain, it may not be worthwhile to evaluate sib or progeny performance in brackish water.

The previous focused on final weight, but growth rate during on-growing period is the main priority for fish farmers. Selecting on fast-growth is preferable to selecting on harvest weight because it increases feed efficiency, which further contributes to profitability and sustainability of production (Aubin et al., 2009; Besson et al., 2016; de Verdal et al., 2018). However, the genetic correlations between final weight and growth rate were greater than 0.97 (de Oliveira et al., 2016). Consequently, selection for harvest weight within a batch of selection candidates will improve growth rate as a correlated response. In this study, we directly quantify growth rate, which can be expressed as absolute or specific growth rate (Hopkins, 1992), thermal growth coefficient (Jobling, 2003) or daily growth coefficient (Cho, 1992). We expected that higher salinity would reduce growth rates and the expression of genetic variation resulting in lower genetic variance as well as re-ranking of candidates (GxE) between brackish water and freshwater. We also address the implications of GxE for breeding program to improve growth of tilapia in brackish water.

Selection for improved performance in brackish water also raises concerns about the potential for increased reproduction and higher probability that escapees may become invasive in estuarine ecosystems via correlated responses to selection. Therefore, we would ideally select for fast-growth, but lower reproduction performance in brackish water without compromising reproductive performance in the freshwater nucleus. This requires further knowledge on the correlation structure between growth and reproduction in both fresh and brackish water.

3.2. Materials and methods

3.2.1. Selected parents

We produced our experimental fish using the 4th generation of the Sukamandi strain as selected parents at the Research Institute for Fish Breeding, Indonesia. We maintained the parents in separate 15 m^2 hapas (5 x 3m), cage-like, rectangular nets with a mesh size of 5

mm suspended in 2000 m^2 freshwater ponds in single sex groups. They were fed twice a day on a commercial pelleted feed with approximately 30% crude protein and 5% fat, at a daily feeding rate of 3% of biomass for four weeks.

3.2.2. Family production

We produced full- and half-sib families in 65 smaller breeding hapas (4 m^2; 2 × 2m), suspended in three 200 m^2 earthen ponds at the Research Institute for Fish Breeding. Each of these hapas was stocked with one male and three females by introducing the males to the hapas 1 day before the 3 females. Because tilapia are mouth-breeders in which the female keeps fertilized eggs in her mouth until hatching, and there was only one male in each breeding hapa, this method produces full-sib families from each mated female and paternal half-sib families if a male mates with multiple females.

We conducted this mating process in 7 day cycles, and if none of the females produced eggs during a cycle, we replaced the male. We replaced the male in every hapa after 2 cycles, and replaced spawned females with new females. At the end of each cycle, we collected the fertilized eggs or hatchling/swim-up fry from the females' mouths, recorded her unique identification tag number, and subsequently incubated the eggs from each female in a single cone-shaped hatching jar (25 cm diameter and 40 cm height) with a constant flow of water until they hatched and grew into functional hatchling/ swim-up fry. We labelled the cone based on the female ID and recorded the collection date of eggs or larva and the male parent for each female's progeny. During this incubation period, we removed dead eggs and fry daily. We also stabilised the water temperature during incubation between 28°C and 30°C with aquarium water heaters.

In total, we produced 91 families over a period of 105 days (from 21 May to 22 August 2019) consisted of 53 full sib families and 38 paternal half-sib families. In order to facilitate the next steps of the experiment, we divided the resulting families into three batches based on the spawning date. We labelled the first 35 full-sib families from the first four weeks of the reproduction period as batch 1, the next 27 families as batch 2, and the last 29 families as batch 3.

3.2.3. Fingerling Nursery Rearing and Tagging

Fry hatched after about 5–7 days. After yolk-sac absorption, we transferred swim-up fry from each family into 4 m^2 nursery hapas (2 x 2, mesh size 1 mm) suspended in a 2,000 m^2 earthen pond. For this, we randomly sub-sampled 200 fry and stocked them into a single nursery hapa, equivalent to a nursing density of 50 fish per m^2. During this period, we fed them twice daily using a commercial powder feed with a dietary protein level of 30%, at the rate of 10-15% of their body weight during the first 3 to 4 weeks. The second nursery period continued until tagging at an average bodyweight of 16g during which we fed the fingerlings a commercial pelleted feed consisting of 30% protein twice daily at the rate of 10% of total body weight. This nursery period of separate family rearing in nursery hapas ranged from 120 to 161 days. At the end of this period, we randomly chose and tagged 40 individuals from each family using PIT (Passive Integrated Transponder) tags and recorded their identification number, stocking weight (SW) and standard length (L0).

Table 1. Total number of families, range (mean) of stocking age and harvesting age and rearing period in days, stocked number and harvested number of fish in brackish water (B) and freshwater (F)

Batch	Environ-ment	Number of families	Stocking age	Harvest age	Number stocked	Number Harvested	Rearing period
1	B	35	127-155 (142.6)	253-281 (268.6)	640	511	126
	F	35	133-161 (148.6)	258-286 (273.6)	649	482	125
2	B	27	123-154 (134.9)	243-274 (254.9)	489	348	120
	F	27	130-161 (141.9)	270-301 (281.9)	488	425	140
3	B	29	128-148 (139.4)	250-270 (261.4)	568	449	122
	F	29	131-151 (142.4)	278-298 (289.4)	574	484	147
All	B	91	123-155	243-274	1697	1308	120-126
	F	91	130-161	258-298	1711	1391	125-147

A total 20 fingerlings per family were grown in brackish water and another 20 in freshwater. For logistical reasons, we tagged the fish for brackish water grow out first. The first batch of 35 families were tagged at an average age of 142 days for brackish water grow out and 148 days for freshwater grow out (127 to 161 days of age post-hatching interval) as summarized in Table 1. We tagged the second batch at the average age 134 and 141 days for brackish water and freshwater respectively (123 to 161 days of age post-hatching interval), and the third batch at the average age of 139 for brackish water and 142 for freshwater (128 to 151 days of age post-hatching interval). We then pooled all tagged fingerlings within a batch and water treatment after 3 days of conditioning in fiberglass tanks with minimum feeding rate of 1-3% body weight.

3.2.4. Testing environments

The test location for brackish water was at the Technical Implementation Unit for Brackish water Culture Karawang (-6.106192, 107.428710), at salinity around 20 ppt, and the location for freshwater/nucleus was at Research Institute for Fish Breeding (-6.371860, 107.623815). Both locations are in the West Java area close to the North Java Sea.

We stocked the tagged and mixed fingerlings from each batch of families in separate ponds on each site at an initial stocking density of ~5 fish per m^2. To minimize stress and mortality during the stocking process, prior to stocking the brackish water ponds we temporarily reduced their salinity level from ~20ppt to 10ppt s by reducing the water level and re-filling the pond with freshwater from the irrigation waterway. During the grow out period, we fed the fish twice daily between 07:00 and 09:00 in the morning and between 15:00 and 17:00 in the afternoon with a commercial pellet diet containing 28% protein at a rate of 3-5% bodyweight. We also recorded water parameters such as dissolved oxygen, pH, temperature, and salinity daily using digital water quality tester.

3.2.5. Trait measurements

Following a grow-out period of 150 to 210 days, we harvested the fish, initially using three drags of a seine net, after which we drained the pond to catch all the remaining fish. We transferred all caught fish directly into a plastic container with diameter around 80 cm

containing clove oil (~0.4 ml per litre of water) as an anaesthetic agent. This process was performed to avoid fish mortality due to handling stress during catching and measuring the phenotypic traits. The number of surviving fish at harvest ranged from 3 to 19 fish/family in brackish water and from 1 to 20 fish/family in freshwater (72.9±16.6% in brackish water and 77.1±19.6% in freshwater). During measurements, we weighed each fish for harvest weight (HW) using a digital scale to the nearest to 0.1 g. We also measured the standard length (L) with a ruler to the nearest 1 mm.

From the individual stocking and harvest weights, we calculated daily growth coefficients (DGC, (Bureau et al., 2000)) as:

$$DGC = \frac{HW^{\frac{1}{3}} - SW^{\frac{1}{3}}}{growing\ days}\ x\ 100$$

where SW is body weight at stocking, HW is harvest weight, and growing days is the growing time between stocking and harvest.

Similarly, we calculated individual growth rate for length, GR(L), as:

$$GR(L) = \frac{SL_f - SL_0}{growing\ days}$$

where SL_0 is standard length at stocking, SL_f is standard length at harvest, and growing days is the growing time between stocking and harvest.

We calculated the condition factor (K) according to Weatherley et al. (1987):

$$K = \frac{HW}{L^3} \times 10^5$$

With HW in grams and L in mm

For reproductive performance, we measured gonad weight and maturation stage for 6 fish per family in each environment. We measured gonad weight with digital scale (0.01g), and macroscopically determined the maturation score (MS) based on Legendre and Ecoutin (1989) with three stages for males and five stages for females. Gonadosomatic index (GSI) was determined as:

$$GSI = \frac{Gonad\ weight}{Body\ weight} \times 100$$

We analysed gonad weight and gonadosomatic index separately for each sex because the differences of the scores between sexes was significant.

3.2.6. Data analysis

3.2.6.1. Descriptive statistics

We prepared and checked the raw data recorded in Microsoft Excel using R version 4.1 (R-Core-Team, 2021). In total, we obtained data for 1308 fish in brackish water and 1391 in freshwater ponds from 91 families (Table 1). We estimated descriptive statistics and checked for data anomalies using R version 4.1. We performed student t-test to evaluate whether HW, L, DGC, GR (L), K, and GW differed between environments.

3.2.6.2. Phenotypic and genetic parameters

We estimated genetic parameters for performance traits using a total of 2699 individual fish for HW, L, DGC, GR (L) and K. The animal model included fixed effects for pond, sex within pond and harvest age within pond for HW and L. Sex was coded as male (m) and female (f) and harvest age was calculated as the number of days between the stocking date and harvest date. We nested sex within ponds to take into account differences in age and sexual maturity of the different groups of families by estimating different effects of sex in each of the ponds.

We estimated the genetic correlations between environments using a bivariate animal model in ASReml version 4.1 (Gilmour et al., 2015) that treats growth traits in different environments as different traits:

$$y_{ijkl} = \mu + \mathrm{POND}_i + \mathrm{SEX\ (POND)}_{i,j} + \mathrm{AGE\ (POND)}_{i,k} + \mathrm{INIT} + \mathrm{a}_l + \mathrm{e}_{ijkl}$$

where: y_{ijkl} is vector of single growth trait in fresh and brackish water; μ is overall mean; POND_i is fixed effect that accounts for both pond and batch effects (i=1– 3 for BW, and 4-6 for FW) ; $\mathrm{SEX\ (POND)}_{i,j}$ is the fixed effect of sex nested within pond (j =m, f); $\mathrm{AGE\ (POND)}_{i,k}$ is harvest age nested within pond; INIT is initial weight for DGC, initial

length for GR and the ratio of initial weight and initial length for K which were standardized by scaling it to a standard normal distribution; a_l is the random additive genetic effect of the l-th individual; e_{ijkl} is random residual effect associated with an individual.

Common environmental effects (c^2) were expected in this study because families were reared separately in family-specific hatching jars and nursing hapas until tagging. However, solutions for c^2 could not be obtained because family effects are confounded with dam effects because our breeding strategy produced very few half-sib families and we had only shallow pedigree information. We tried to fit models that included c^2, but they did not converge. Without the common environmental effect in the model, the solutions converged and the genetic correlations could be estimated. The full model was used to analyse the DGC, GR (L) and K. A simplified model that excluded the fixed effect for initial value (INIT) was used for HW and L.

We calculated the heritability as the ratio between additive genetic variance (σ_A^2) and phenotypic variance (σ_P^2), $\frac{\sigma_A^2}{\sigma_P^2}$. Genetic and phenotypic correlations between different traits in the same environment were also obtained from bivariate analyses. The animal effects were distributed as N(0,A⊗G) with the additive genetic variance covariance matrix (G) is $\begin{bmatrix} \sigma_{A,1}^2 & r_{A,12}\sigma_{A,1}\sigma_{A,2} \\ r_{A,12}\sigma_{A,1}\sigma_{A,2} & \sigma_{A,2}^2 \end{bmatrix}$ where $\sigma_{A,1}^2$ ($\sigma_{A,2}^2$) is the additive genetic variance of trait 1 (trait 2), and $r_{A,12}\sigma_{A,1}\sigma_{A,2}$ is the additive genetic covariance between trait 1 and trait 2. The residuals were distributed as N(0, I⊗R) with residual variance-covariance matrix (R) is $\begin{bmatrix} \sigma_{e,1}^2 & r_{e,12}\sigma_{e,1}\sigma_{e,2} \\ r_{e,12}\sigma_{e,1}\sigma_{e,2} & \sigma_{e,2}^2 \end{bmatrix}$ where $\sigma_{e,1}^2$ ($\sigma_{e,2}^2$) is the residual variance of trait 1 (trait 2), and $r_{e,12}\sigma_{e,1}\sigma_{e,2}$ is the residual covariance between trait 1 and trait 2. Genetic and phenotypic correlations among traits were calculated as the covariance divided by the product of the standard deviations of the two traits in the bivariate model.

For reproductive performance, we also estimated the genetic parameters with bivariate animal models that take into account the fixed effects of pond and harvest age. For genetic analysis of gonad maturity, we reclassified the maturity score as mature (1) and immature (0) according to Legendre and Ecoutin (1989). We classified females as immature when they

were in stage 1 to 3, and as mature when they were in stage 4 and 5. Whereas for males, they were classified as mature when they were in stage 2 to 3. Then we analysed males and females together with sex nested within pond as a fixed effect.

We estimated the genetic correlation between the same traits measured on different (related) individuals in the brackish and freshwater ponds with the bivariate model above. For this model, the additive genetic variance-covariance matrix is $\begin{bmatrix} \sigma^2_{A,B} & r_{A,BF}\sigma_{A,B}\sigma_{A,F} \\ r_{A,BF}\sigma_{A,B}\sigma_{A,F} & \sigma^2_{A,F} \end{bmatrix}$ where $\sigma^2_{A,B}$ is the additive genetic variance for the traits in brackish water, $\sigma^2_{A,F}$ is the additive genetic variance for the traits in freshwater and $r_{A,BF}$ is the additive genetic correlation between brackish water and freshwater.

The covariances of residuals between environments was set to zero, as individual fish were evaluated in only one environment. Consequently, the residual variance-covariance matrix is $\begin{bmatrix} \sigma^2_{e,B} & 0 \\ 0 & \sigma^2_{e,F} \end{bmatrix}$ where $\sigma^2_{e,B}$ is the residual variance for the trait in brackish water and $\sigma^2_{e,F}$ is the residual variance for the trait in freshwater.

3.3. Results

3.3.1. Descriptive statistics

The average salinity, morning and afternoon water temperature in the brackish water pond were 16.21 ppt, 29.57°C and 33.71°C, respectively. The salinity in the brackish water was highly variable, fluctuating over time between 6 and 25 ppt as shown in Figure 1. The lowest salinity was 6 ppt which occurred in raining period. The temperature profiles for the brackish and freshwater ponds are very similar (Figure 1).

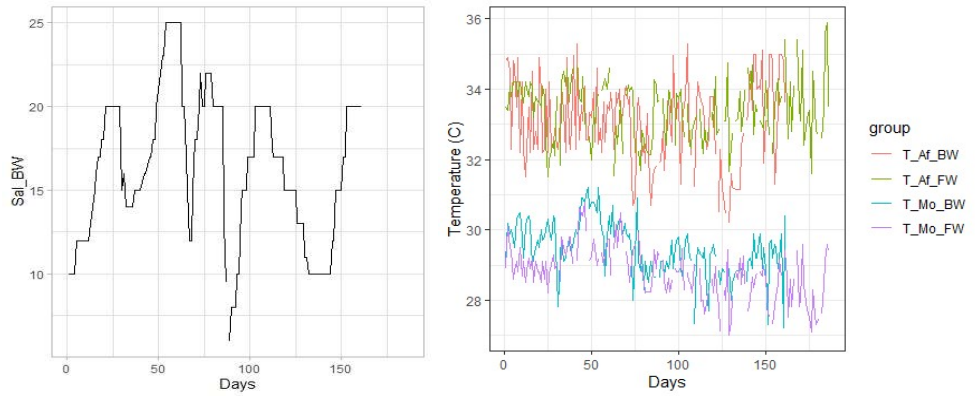

Figure 1. Daily salinity fluctuation in the brackish pond (left) and temperature (right) in the freshwater pond in the morning (T_Mo_FW) and afternoon (T_Af_FW), and in the brackish water pond in the morning (T_Mo_BW) and afternoon (T_Af_BW) during the experimental rearing period.

Grow out in brackish water pond resulted in 77.08% survival, and we recovered 1308 out of 1697 fish at harvest time after 120-126 days rearing period. In the freshwater pond, we observed higher survival of 81.82% or 1391 out of 1700 fish after 125-147 days rearing period. Descriptive statistics of SW, HW, L, DGC, GR, K and survival are shown in Table 2. The average stocking weight is similar between brackish water (16.11±7.79 g) and freshwater (15.65±7.75 g). HW, L, DGC and GR were higher for males compared to females in both brackish water and freshwater, but K was similar. However, the coefficient of variation for females was higher than males for all growth traits in both brackish water and freshwater. At harvest time, HW and L were higher in brackish water and significantly different ($P<0.05$) compared to freshwater. DGC in brackish water was higher (3.38±0.43) and significantly different ($P<0.05$) compared to freshwater (2.72±0.44). In brackish water, GR(L) during grow-out period was significantly higher compared to freshwater ($P<0.05$). The difference between K in brackish water (4.02±0.37) and in fresh water (3.98±0.36) was not significant. The regression coefficients and intercepts of log(HW) against log(L), were similar in brackish water and freshwater (Figure. 2). Overall, brackish water leads to higher HW, L, DGC, GR (L) compared to freshwater.

Table 2. Number of observations (n), mean and coefficient of variation (CV in %) for stocking weight (SW) and harvest weight (HW), standard length (L), daily growth coefficient (DGC), growth rate (GR (L)), condition factor (K) and survival rate (S) of male and female *Oreochromis niloticus* in brackish water and freshwater

Trait	Brackish water					Freshwater				
	n	Male		Female		n	Male		Female	
		Mean	CV	Mean	CV		Mean	CV	Mean	CV
SW (g)	1697	16.8±7.49	44.58	16.1±8.60	53.27	1711	15.8± 7.28	46.07	15.1 ± 8.06	53.40
HW (g)	1308	324.7± 61.43*	18.92	255.0± 53.85*	21.14	1391	261.5 ±49.72*	19.01	201.7 ±46.60*	23.10
L (cm)	1308	19.9±1.32*	6.63	18.5±1.32*	7.12	1391	18.7±1.20*	6.45	17.1±1.30*	7.62
DGC ($g^{1/3}$ /day)	1308	3.5± 0.38*	11.02	3.12± 0.40*	12.42	1391	2.84±0.39*	13.81	2.5±0.45*	18.01
GR(L) (cm/day)	1308	0.10±0.011*	10.75	0.09±0.011*	11.74	1391	0.08±0.017*	21.68	0.07±0.012*	17.39
K	1308	3.95±0.38		4.05±0.36		1391	3.98±0.35		3.98±0.36	
S (%)		(77.07)					(81.29)			

*p< 0.05 Student-T test comparing brackish and freshwater ponds

Table 3. Means ($\bar{x}$), standard deviations (σ), coefficients of variation (CV in %) of gonad weight and GSI male and female from brackish water and freshwater

Traits	Brackish water				Freshwater			
	n	$\bar{x}$	σ	CV	n	$\bar{x}$	σ	CV
Gonad weight male (g)	299	1.03*	1.50	145.1	280	0.70*	0.76	109.7
Gonad weight female (g)	177	4.52*	3.56	78.6	198	2.42*	2.16	89.2
GSI male	299	0.31	0.50	159.7	280	0.28	0.31	111.1
GSI female	177	1.82*	1.40	77.2	198	1.26*	1.54	91.2

*p< 0.05 Student-T test comparing brackish and freshwater ponds

We evaluated the reproductive performance of males and females in both environments. Macroscopic analysis of gonad weight (Table 3) showed that gonad weight for both males and females and gonadosomatic index for females in brackish water was higher than in freshwater (P<0.05), but for males the difference in gonadosomatic index between brackish water and freshwater was not significant (P>0.05). Gonad maturity score varies between sexes and environments (Table 4).

Table 4. Gonad maturity score (MS) for males and females in brackish water and freshwater

Male			Female		
Stage	Brackish water	Freshwater	Stage	Brackish water	Freshwater
1	13 (4%)	8 (3%)	1	3 (2%)	2 (1%)
2	39 (13%)	57 (21%)	2	37 (20%)	47 (23%)
3	248 (83%)	212 (77%)	3	56 (30%)	67 (33%)
			4	72 (38%)	66 (33%)
			5	19 (10%)	20 (10%)

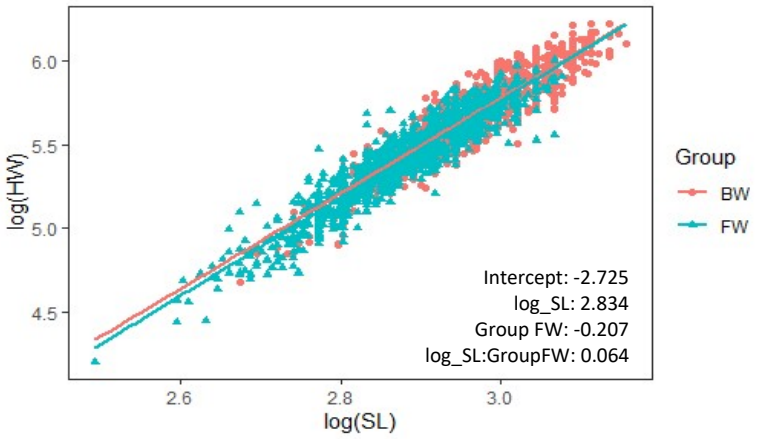

Figure 2. Log10(HW) plotted against Log10(L) in brackish water and freshwater ponds. HW=harvest weight, L=length.

We evaluated the regression coefficient between log(HW) and log(SL) in fresh and brackish water using a separate slopes analysis of covariance (Figure 2). The estimated regression coefficients in brackish water and freshwater are 2.834 and 2.898, respectively, and are not significantly different (Interaction $p>0.05$, Table 5). However, the intercept in brackish water was significantly higher than in freshwater (Group effect $p<0.05$, Table 5).

Table 5. Separate slopes analysis of covariance for the relationship between standard length (SL) and harvest weight (HW) brackish and freshwater ponds

Source	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Log_SL	1	179.36	179.36	21903.095	2e-16
Group	1	0.20	0.20	24.108	9.66e-07
Interaction	1	0.02	0.02	2.129	0.145
Residuals	2686	22.00	0.01		

3.3.2. Genetic parameters of traits within environments

Genetic and phenotyping variances estimates for all traits in freshwater were lower compared to brackish water, except standard length (Table 6). The h^2 estimates for HW, L, DGC, GR (L) and K were moderate, from 0.35 to 0.50 with small standard error ranging from

0.06 – 0.09. All h^2 estimates for HW and L were higher in freshwater ponds compared to brackish water pond while for the ratio traits (DGC, GR (L) and K), h^2 estimates were higher in the brackish water pond compared to the freshwater pond. We estimated the genetic parameters for reproduction traits separately for the sexes in both environment as shown in Table 7. The h^2 estimates varies from low to moderate (0.03-0.54). The genetic correlation of GW in both environments was higher in male (0.85) than in female (0.50). Genetic correlations for reproductive traits had higher standard errors than growth traits due to the smaller sample size (6 fish/family rather than ~ 20).

Table 6. Additive genetic variance (σ^2_A), total phenotypic variance (σ^2_P), heritability (h^2), genetic correlation (r_g), phenotypic correlation (r_p), and their standard errors (se) and genetic coefficient of variation (GCV) from bivariate analysis for harvest weight (HW), standard length (L), daily growth coefficient (DGC), growth rate (GR (L)) and condition factor (K) in brackish water and freshwater ponds

	Brackish water				Freshwater					
Trait	σ^2_A	σ^2_P	h^2 (se)	GCV*	σ^2_A	σ^2_P	h^2 (se)	GCV*	r_g	r_p
HW	1048.47	2958.8	0.35	10.2	790.57	2104.7	0.38	5.2	0.66	0.24
	(222.13)	(143.58)	(0.06)		(162.67)	(102.22)	(0.06)		(0.10)	(0.05)
L	0.48	1.24	0.39	3.2	0.53	1.30	0.41	2.7	0.73	0.29
	(0.09)	(0.06)	(0.07)		(0.10)	(0.06)	(0.07)		(0.09)	(0.06)
DGC	0.47 x 10-01	0.11	0.43	6.5	0.37 x10-01	0.09	0.42	3.7	0.65	0.28
	(0.97 x 10-02)	(0.58 x 10-02)	(0.07)		(0.74 x 10-02)	(0.45 x 10-02)	(0.06)		(0.09)	(0.06)
GR (L)	0.30 x 10-04	0.73 x10-04	0.42	4.5	0.26 x10-04	0.65 x10-04	0.39	2.8	0.74	0.30
	(0.84 x 10-05)	(0.47 x 10-05)	(0.09)		(0.67 x 10-05)	(0.39 x 10-05)	(0.08)		(0.31)	(0.09)
K	0.56 x 10-01	0.11	0.50	5.3	0.54 x10-01	0.122	0.44	5.2	0.83	0.34
	(0.11 x 10-01)	(0.62 x 10-02)	(0.07)		(0.11 x 10-01)	(0.63 x 10-02)	(0.07)		(0.06)	(0.06)

* GCV was calculated as: $\left(\frac{\sigma_A}{\mu}\right) \text{x } 100$

Table 7. Heritability (h^2) and genetic correlation (r_g) for gonad weight (GW), gonadosomatic index (GSI) and Maturity score (MS) in brackish and freshwater and their standard errors (se)

Trait	Sexes	h^2 (se) Brackish water	h^2 (se) Freshwater	r_g (se)
GW	Male	0.13 (0.13)	0.38 (0.14)	0.85 (0.45)
	Female	0.30 (0.17)	0.21 (0.17)	0.50 (0.46)
GSI	Male	0.06 (0.11)	0.54 (0.14)	0.75 (0.74)
	Female	0.30 (0.16)	0.03 (0.15)	-
MS	Both sexes	0.12 (0.07)	0.04(0.07)	0.47 (0.74)

The genetic correlations (r_g) between HW, L, DGC, and GR(L) within an environment (brackish or freshwater) were high (Supplementary table S1), varying from 0.81 to 0.99 and from 0.79 to 0.99, respectively. Similar trends appear in the phenotypic correlations (r_p). The estimated r_p were high in both environment from 0.86 to 0.99, except for r_p between GR(L) and HW in freshwater which was very high, and the software generated an estimated value >1 (Supplementary table S2). The r_p and r_g between GR(L) and HW in freshwater could not be estimated due to model convergence problems.

3.3.3. Genotype by environment interactions

The genetic correlations between brackish water and freshwater for HW, L, DGC and GR (L) were moderate ranging from 0.65 to 0.74. Figure 3 shows the patterns of the re-ranking of the parents of all families between brackish water and freshwater for DGC based on their estimated breeding values. The DGC interaction plot has many crossings and more families switch rank between environments resulting in lower genetic correlation than other traits. There are crossovers of high-ranking parents between the two environments, indicating that these families will perform differently in both environments. Genetic correlations for

reproductive traits (GW, GSI and MS) show high variation between 0.47 and 0.85 with very high standard error (Table 7).

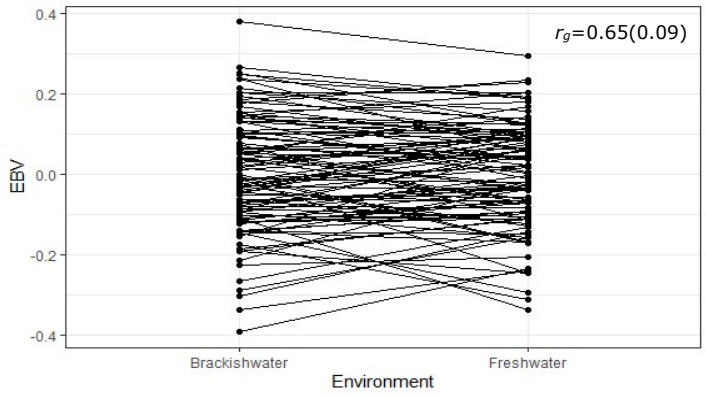

Figure 3. Reranking of parental estimated breeding valued (EBV) for daily growth coefficient (DGC) between brackish water and freshwater ponds. The genetic correlations (r_g) with standard error are included inside the plot.

3.4. Discussion

The objectives of our study were to investigate the extent of GxE interactions for growth and reproductive traits between brackish and freshwater ponds in the Indonesian Sukamandi tilapia strain being selected for improved salinity tolerance. This information will be important for redesigning and refining the breeding program. In the next three sections, we discuss the performance of the Sukamandi strain in brackish water, potential for further improvement of this strain and the implications for the (re)design of the selective breeding program.

3.4.1. The Sukamandi strain performance in brackish water

The Sukamandi strain grew better in brackish water ponds than in freshwater ponds. At ~16.2 ppt HW, DGC, L and GR (L) were significantly higher in brackish water than in freshwater. Because physiological adaptation to elevated salinity requires energy to maintain osmotic homeostasis (Kültz, 2015) and the diverted energy to osmoregulation should reduce growth (Boeuf & Payan, 2001; Tseng & Hwang, 2008), we expected the opposite result based on previous studies of Nile tilapia, which has lower performance in

brackish water than in freshwater ponds (Cnaani & Hulata, 2011; Fineman-Kalio, 2008; Kamal & Mair, 2005). The energetic requirements for osmoregulation to depend on the environment, and both hypo- hyper-osmotic conditions require energy to maintain internal homeostasis. Consequently, the best growth performance of tilapia is achieved when they are in isosmotic conditions. Blue tilapia (*O. aureus*) and Mozambique tilapia (*O. mossambicus*) have higher salinity tolerance than Nile tilapia and grow well in brackish water ponds up to 20 ppt for blue tilapia and close to full-strength seawater for Mozambique tilapia (Popma & Masser, 1999). Blue tilapia is in isosmotic conditions at salinities of 8 to 12 ppt based on blood chemistry (Semra et al., 2013). A Molobicus hybrid tilapia strain has a salinity tolerance close to *O. mossambicus* (Mateo et al., 2004) and can reproduce in brackish water (Cnaani & Hulata, 2011). Our test condition were at salinity range around 16 ppt, which is closer to the isosmotic condition than freshwater. Our results indicate that the salinity tolerance of the Sukamandi strain (*Oreochromis spp*.) is closer to that of blue tilapia, than of Nile tilapia, most likely because this strain is a unique composite strain of Nile tilapia with introgressed salinity tolerance genes from blue tilapia (Yu et al., 2022). In addition, the strain has been selected for growth and survival in brackish water ponds for 4 generations, prior to the current experiment.

Our expectation was that fish would grow less in brackish water and have lower fecundity. However, we observed higher mean growth in brackish water, compared to freshwater, while gonadal development and maturation was comparable in both environments. The survival rate in brackish water (77%) was close to survival rate in freshwater (81%) which also indicates that the Sukamandi strain has a good salinity tolerance. The regression coefficient between HW and L in Figure 2 indicate whether fish grow thicker or thinner at the same length. When the slope below 3.0 indicates that fish become leaner and when the slope exceeds 3.0 indicates fish become fatter (Silva et al., 2015). In our study, the regression coefficients are 2.834 for brackish water and 2.898 for freshwater. They are statistically equal as indicated by the non-significant interaction effect ($p>0.05$) and close to 3, indicating that the fish were in a good condition in both environments. However, brackish water ponds provided a better environment for fish growth than freshwater ponds as

indicated by the significant main effect of group ($p<0.05$). This suggests that salinity itself had no negative effects but that other aspects of the brackish water environment were biologically different and more beneficial for growth than the freshwater pond. A study by Dewi et al. (2012), found abundant phytoplankton and zooplankton, particularly *Calanus sp* and *Acartia sp* in brackish water ponds at 95,570 ind./L and 17,120 ind./L far higher than in freshwater ponds at RIFB at 604 ind/L for *Fillinia sp*. This additional natural food could boost fish growth and all related traits. Taken together our results show that the combination of inadvertent hybridization and mass selection have already enhanced the salinity tolerance of Sukamandi strain, making it a unique and valuable genetic resource for Indonesian tilapia breeding to produce superior strain for tilapia culture.

3.4.2. Potential for further improvement of the Sukamandi tilapia strain

The moderate heritabilities for all production-related traits indicate the presence of sufficient additive genetic variance for future selection on these traits to produce significant responses. Our estimate of h^2 for HW in the brackish water (0.35) is higher compared to what has been estimated for growth in intensive (0.19±0.07) and extensive systems (0.17±0.06) in Molobicus hybrid tilapia strain (de Verdal et al., 2014) but is lower compared to what has been reported in previous studies for Nile tilapia grown in saline environments (0.53-0.57; Thoa et al. (2016) and Ninh et al. (2014)). It is possible that the large fluctuation in salinity in this study (6-25ppt) inhibited the Sukamandi strain from expressing its full genetic potential for growth. Alternatively, the difference in heritability could be due to strain differences. The Sukamandi strain is of hybrid origin and has been selected for 4 generations in brackish water.

We encountered problems with including common environmental effect (c^2) in our models, most likely due to shallow pedigree information and limited pedigree connections between families. Our dataset consisted mostly of full-sibs families and very few half-sib families. Consequently, genetic correlations between observed traits within and between environments were obtained from models without the common environmental effect and this can influence estimates of genetic variance. Maluwa et al. (2006), Trọng et al. (2013) and Omasaki et al. (2016) also reported that a multivariate model to estimate genetic

correlation including a common environmental effect did not converge. Not including c^2 usually leads to over-estimated heritability's, as common environmental effects are absorbed in the additive genetic variance component. Expressing growth as DGC makes it less dependent of initial (i.e. pre-tagging) body weight which is the stage most affected by common environmental effects (Bureau et al., 2000; Cho, 1992; Trọng et al., 2013). This trait represents grow out period from stocking to harvest, while harvest weight is a cumulative growth from spawning to harvest. The estimated heritability for DGC in our study agrees with (Trọng et al., 2013) who also omitted c^2 from the model.

Our estimates for all growth parameters showed substantial GxE between brackish and freshwater ponds. The between-environment genetic correlation for DGC was 0.65 (0.09), which suggests substantial re-ranking of genotypes between the two environments. Significant GxE was also reported for HW of Nile tilapia tested in brackish water and freshwater ponds by Luan et al. (2008) at 0.45±0.09. The design of our experiment followed the recommendation of Sae-Lim et al. (2010) with ~1000 fish/environment with equal representation of families, so we assume that our estimates are unbiased. However, the number of fish/environment did not solve the structure problem in our estimation when the number of half-sib families is low, resulted in not converge in the model. GR(L), K, GSI, GW and MS were also indicated substantial GxE between brackish and freshwater. However, small sample size at 6 fish per family for all reproductive traits due to logistical reason resulted in very high standard error, and not estimable GxE of GSI in females as shown in Table 7.

3.4.3. Implications for future breeding program

To date, the breeding program has been based on selection for own performance (mass selection) for harvest weight, conducted in various shrimp farm environments. The advantage of this breeding scheme is the high accuracy of selection due to selection on own growth performance in brackish water. However, this breeding scheme has several drawbacks related to high mortality of selection candidates during the grow out period in unpredictable salinity condition, security issues, and mortality during the transportation

and adaptation from the testing site to the selection site in freshwater. There is also a potential risk in disease transfer from the test pond in brackish water to the brood stock facility in freshwater. Another issue is related to escapees during the grow-out of selection candidates that potentially spread into the natural brackish water environments. To avoid this, closed and restricted testing facilities should be implemented to prevent this threat. Furthermore, reducing the reproductive performance of tilapia in brackish water is desired and could be included in the selection criteria. However, this has positive impact to the environmental and negative consequences to breeding program. The negative consequence could be related to the mating problem to produce sufficient number of families in the next generation.

A shift from brackish water to freshwater pond for fish selection could minimize some of these downsides. However, when G×E interactions are strong, it could result in a reduction in genetic gain due to inaccurate selection of breeding candidates in freshwater (Mulder & Bijma, 2005). Re-ranking of genotypes is not substantial if the genetic correlation between environments is above 0.8 (Robertson, 1959). In this study, however, the genetic correlation was 0.65, which means that it is essential to incorporate information from full-sibs in brackish water. Further, combining own performance in freshwater with sib records in brackish water could increase the accuracy of selection and maximise the genetic gain. With own-performance records, we can exploit within-family variation to increase accuracy compared to using only sib information. In practical terms, a sib selection program has several advantages: eliminating transportation costs of testing fish and selection candidate from brackish water to freshwater, and reducing chance of disease transfer from the test pond in brackish water to the nucleus in freshwater.

3.5. Conclusion

Our results show that brackish water ponds provided a positive environment for the Sukamandi strain. However, there was substantial re-ranking shown by genetic correlations of 0.65-0.74 in all observed growth traits. Based on this, we suggest to perform a nucleus breeding program in freshwater and incorporate sib information from brackish water ponds to increase the accuracy of breeding value estimation and to optimize genetic gain.

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Supplementary Information

Supplementary table S1. Estimated genetic correlations (above diagonal) and phenotypic correlations (below diagonal) between harvest weight (HW), standard length (L), daily growth coefficient (DGC), growth rate (GR(L)) and condition factor (K) in brackish water ponds. Standard errors are in brackets.

Trait	HW	L	DGC	GR (L)	K
HW	x	0.84 (0.04)	0.93 (0.02)	0.83 (0.04)	0.36 (0.12)
L	0.87 (0.01)	x	0.81 (0.05)	0.99 (0.00)	-0.15 (0.14)
DGC	0.97 (0.003)	0.87 (0.01)	x	0.89 (0.03)	0.35 (0.12)
GR (L)	0.87 (0.01)	0.99 (0.00)	0.89 (0.01)	x	-0.07 (0.14)
K	0.42 (0.03)	0.02 (0.04)	0.45 (0.03)	0.03 (0.04)	x

Supplementary table S2. Estimated genetic correlations (above diagonal) and phenotypic correlations (below diagonal). between harvest weight (HW), standard length (L), daily growth coefficient (DGC), growth rate (GR(L)) and condition factor (K) in freshwater ponds. Standard errors are in brackets.

Trait	HW	L	DGC	GR(L)	K
HW	x	0.87 (0.03)	0.95 (0.01)	Not estimable	0.25 (0.13)
L	0.88 (0.01)	x	0.79 (0.05)	0.99 (0.00)	-0.19 (0.14)
DGC	0.97 (0.00)	0.86 (0.01)	x	0.88 (0.03)	0.28 (0.13)
GR(L)	Not estimable	0.99 (0.00)	0.89 (0.01)	x	-0.03 (0.20)
K	0.34 (0.03)	-0.06 (0.04)	0.37 (0.03)	0.02 (0.07)	x

Chapter 4

Tilapia selected for growth in brackish water has more efficient osmoregulation and increased growth rate in saline conditions

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Abstract

Salinity tolerance varies among tilapia species and salinity-tolerant strains can be produced through selective breeding. Breeding programs in Indonesia have produced salinity tolerant tilapia with improved growth rates in brackish water. Here we investigate the physiological adaptations of these selected tilapia using offspring from the fifth generation of Sukamandi strain. To understand which physiological traits are associated with genetic differences we selected three different parent groups by comparing their individual breeding values for freshwater and brackish water growth. Based on their breeding values, we selected brackish water specialists (B), freshwater specialist (F), and generalists (G). Progenies from the three groups with an average weight of ~100 gr were stocked in both brackish (25ppt) and freshwater tanks for a growth trial of 28 days. On day 28 we measured body weight, blood ion concentrations, haematocrit, and Na^+/K^+-ATPase concentration in intestine and gill. A total of 99 samples from the three most extreme families within each group were sent for lab analysis of blood ion and tissue enzyme concentrations. Growth was significantly reduced in brackish water tanks, compared to freshwater. Across all groups, brackish water reared fish had higher Na^+ and Cl^- concentrations in blood plasma while haematocrit levels were not affected by salinity. For Na^+/K^+-ATPase concentration the interaction between genetic group and salinity environment was significant in both gill and intestine. In brackish water, "B" fish had lower levels of Na^+/K^+-ATPase in both intestine and gill. We conclude that selection for growth in brackish water results in a more efficient osmoregulation with lower Na^+/K^+-ATPase concentrations, higher blood ion concentrations, and higher DGC compared to selection for growth in freshwater.

Key words: Tilapia, Salinity, EBVs, Na^+/K^+-ATPase, blood ion concentrations

4.1. Introduction

Salinity tolerance varies among tilapia species. Blue tilapia (*Oreochromis aureus*) and *O. mossambicus* have higher salinity tolerance than Nile Tilapia (*Oreochromis Niloticus*) (Cnaani & Hulata, 2011; Popma & Masser, 1999). Salinity tolerance also varies among fish in the same species. This variation is beneficial to select fish that have higher salinity tolerance from a population. A study by Thoa et al. (2016) showed that selection under medium salinity produced genotypes that can perform well in both freshwater and brackish water ponds. The heritability for body weight at harvest was reported moderate to high in both freshwater and brackish water ponds. A breeding program for a salinity tolerant tilapia strain has been conducted in Indonesia to meet the farmers needs in brackish water areas using a black tilapia strain called Sukamandi strain. This strain has been selected for four generations from the hybridization between Blue tilapia and a local tilapia strain that originated from GIFT (Setyawan et al., 2015). In brackish water ponds, black tilapia is more preferred particularly due to lower mortality rate in the first period after stocking compared to red tilapia which is also salinity tolerant but has a more visible red colour making it more susceptible to predators.

Selection for higher salinity tolerance in tilapia normally uses growth in brackish water as the main trait (de Verdal et al., 2014; Luan et al., 2008; Ninh et al., 2014). Higher salinity tolerance has a significant effect on tilapia growth in brackish water. Nile tilapia shows about 60% slower growth when reared in sea water compared to freshwater environment (Cnaani & Hulata, 2011). In addition to lower growth, reproduction is also reduced in high salinity environment (Cnaani & Hulata, 2011; Popma & Masser, 1999). The energy and protein requirement for maintenance increases by a factor of 1.7 and 1.8, respectively, in salinity of 12 ppt compared to freshwater (Hien et al., 2022). Furthermore, salinity also influenced egg fertilization and incubation, yolk sac absorption in fertilised eggs, early embryogenesis, swim bladder inflation and early stage of larva development (Boeuf & Payan, 2001).

Reduced tilapia growth in saline environment is attributed to more active osmoregulation in brackish water to maintain homeostasis. According to Boeuf and Payan (2001), there are four mechanisms of interaction between osmoregulation and growth: hormonal stimulation leading to reallocated energy, the differences in standard metabolic rate, changes in food intake, and changing digestibility. Osmoregulation involves several organs. Gills, kidney and intestine are the main organs for osmoregulation and ion transport to maintain homeostasis (Evans, 2008). The branchial epithelium is the main ion transport site with a surface that is around four times greater than that of the body (Prunet & Bornancin, 1989). In general, freshwater and seawater fish have different mechanisms to respond to osmotic challenges. Freshwater fish are hyperosmotic to their environment solution and will experience osmotic gain of water and diffusional loss of ions. To maintain homeostasis, they will excrete relatively large volumes of dilute urine and actively take up ions (Na^+, Cl^-) from the environment across the gill epithelium. Whereas sea water fish will actively ingest seawater to counteract osmotic loss of water and excrete small volumes of isotonic urine (Evans, 2008). Active secretion of ions (Na^+, Cl^-) takes place in the gills. In Nile tilapia as a less salinity-tolerant tilapia, hypersaline condition at 21 ppt significantly increased the frequency of anomalies in gills. In addition, there were some histopathological changes such as chloride cell hypertrophy, epithelial lifting, structure alteration, and primary lamellae cells aggregation (Azevedo et al., 2015). In a mix of one-third seawater with two-thirds freshwater, the plasma sodium and chloride in tilapia increased significantly as a response to the osmotic pressure (Prunet & Bornancin, 1989). During a salinity challenge, tilapia expressed more Na^+K^+-ATPase to meet physiological demands in pumping ions across the epithelial gill membrane (Lee et al., 2003).

To study salinity tolerance we investigated physiological responses in freshwater and brackish water for 3 groups of fish selected as Brackish Water specialists (B), Fresh Water specialists (F), and generalists (G). We used fish from the fifth generation of Sukamandi strain that were selected on the difference of their breeding values for performance in brackish water and performance in freshwater. We hypothesize that the more salinity tolerant fish will use less energy for maintaining homeostasis when kept in brackish water and show faster growth than less tolerant fish. We therefore expect that offspring of

brackish water specialists will show different physiological responses than offspring of freshwater specialists when exposed to a brackish water and freshwater environment.

4.2. Materials and methods

We used parent fish from the Sukamandi strain (Yu et al., 2022) that had been selected for growth in brackish water for 5 generations and tested in a genotype by environment experiment to estimate the genetic correlation for growth in fresh water and brackish water (described in Chapter 3). Adaptation was judged on the comparison of their genetic merit for growth in brackish water and freshwater. The experiment was conducted in indoor fish tanks at the Research Institute for Fish Breeding, Sukamandi, Indonesia. The experimental protocol followed the ethical guidelines on animal welfare stipulated in the Ministerial Decree of Ministry of Marine Affairs and Fisheries, Indonesia Number 6/PERMEN-KP/2020 about fish welfare in aquaculture (MMAF, 2020).

4.2.1. Selection of parents and fry production

Parents to produce progeny for the experiment were selected from 1,391 candidates of the 5th generation of the Sukamandi strain breeding program (Setyawan et al., 2022). Estimated breeding values for harvest weight in both freshwater (EBVfw) and brackish water (EBVbw) were available on all selection candidates. EBV were estimated based on own performance in freshwater and harvest weights of 1,308 sibs grown in brackish water. All details on the data collection and EBV estimation are described in Setyawan et al. (2022).

We selected three groups of 10 parents each, using the EBVfw and EBVbw as follows:

Generalists (G) = maximum (EBVfw + EBVbw)

Brackish water specialists (B) = minimum (EBVfw – EBVbw)

Freshwater specialists (F) = maximum (EBVfw – EBVbw)

The Generalists group was selected to be the best candidates for harvest weight across the two environments. Thus, we selected parents whose sum of EBVfw and EBVbw was highest. To investigate differences between fish that are genetically better adapted to brackish

water and fish that are better adapted to freshwater we selected specialists for brackish water and for freshwater. Here, B fish are those with the lowest (most negative) value for EBVfw minus EBVbw, and conversely, F fish those with the highest (most positive) value. Values of mid parent means are given in table 1. In brackish water, brackish water specialists are expected to have 15.6g higher harvest weight than freshwater specialists, at an average harvest weight of 290g (Setyawan et al., 2022). Similarly in freshwater, freshwater specialists would be expected to have 21.8g higher harvest weight than brackish water specialists.

Table 1. Total number of records, rearing days and mean of parents EBVs for growth in brackish water and freshwater for in each group.

Environment	Group	Records			mid-parent mean EBVs	
		Stock	Harvest	Lab analysis	All 5 families	Top 3 families
Brackish water	G	29	24	15	39.9	40.3
	B	30	26	17	4.6	9.5
	F	29	21	15	-11.0	-3.5
Freshwater	G	29	29	17	25.6	24.5
	B	31	31	17	-11.5	-16.0
	F	30	30	18	10.3	18.2

Each group of selected parents consisted of 5 males and 5 females. Within each group of selected parents, 5 families were produced by different parent pairs. Fertilised eggs were collected from the female mouth on day seven after pairing and incubated in an indoor hatching jar for 3-5 days until eggs hatched and larvae were freely swimming on the surface. Then, larvae were stocked into outdoor hapas (2x2 m^2), situated in an earthen freshwater pond. During rearing period (106 days), we fed them twice daily using a commercial powder feed with a 30% protein content at the rate of 10-15% of body weight until tagging size of around 10g per fish was reached. Fish received a PIT (Passive Integrated Transponder) tag and standard length, and stocking weight were recorded. All 15 families were then kept communally for 10 weeks in a freshwater pond until body weight reached on average 100g.

4.2.2. Growing condition

We collected 24 tagged fish per family from the freshwater pond and stocked them in 20 indoor tanks of 2x1m^2 filled with freshwater (80 cm depth). Fish from the different families were divided over the 20 tanks at a density of 18 fish per tank (Supplementary table S1). After one day of adaptation, we recorded their identification, standard length and body weight. On the same day we started the acclimation process to high salinity by increasing salinity to 10 ppt in 10 out of the 20 tanks. The next day, salinity increased to 15 ppt in the morning and to 20 ppt in the afternoon. On the third day, salinity was increased to 25 ppt where it remained during the rest of the experiment. On the 4th day, 180 fish were removed for a separate experiment. The remaining fish were kept until day 28 at a density of 9 fish per tank. The 10 tanks with freshwater and the 10 tanks with brackish water were on separate recirculation systems. Dissolved oxygen concentration, pH, water temperature and salinity were measured daily during the experiment period. Fish were fed a commercial feed with a dietary protein level of 28% at the rate of 3-5% of bodyweight.

4.2.3. Data collection

4.2.3.1. Weight and length

Fish were measured on day 1 for standard length (SL1) and body weight (BW1). On day 28, fish were again measured for standard length (SL28) and body weight (BW28).

4.2.3.2. Blood ion concentration and haematocrit

After weighing on day 28, a blood sample was collected from the caudal fin into 2 ml heparin blood collection tubes with a 23gauge needle which had been dipped in 50units/ml heparin in PBS solution. For the ion concentration measurements (Na^+, K^+ and Cl^-), 1 to 1.5 ml of blood was centrifuged at 10.000 rpm for 3 minutes. After centrifugation, plasma was transferred to a new tube and stored at -80 °C. Blood ion concentration analysis was performed by Bogor Agricultural Institute (Bogor, Indonesia). The concentrations of sodium (Na^+), potassium (K^+) and chloride (Cl^-) were obtained using flame photometry using an automatic analyser as described in Schales and Schales (1941).

Haematocrit values were measured by the microhematocrit method (Siwicki et al., 1994). We filled microhematocrit capillary tubes containing heparin to around three quarter of the tube by the blood capillary pressure. Then we blocked one side with wax and centrifuged using microhematocrit centrifuge at a speed of 5400 rpm for 1 minute. We obtained the haematocrit value by measuring the percentage of platelet cells in the blood volume using a microhematocrit reader.

4.2.3.3. Na^+/K^+-ATPase

On day 28, samples of gill and intestine were taken to measure Na^+/K^+-ATPase concentration. Gill tissue was collected from the third lamella on the left side of each fish, cutting around 1-2 cm of tissue from the middle of the gill arch and cleaned with phosphate-buffered saline (PBS). Tissue was stored in 1.5 ml tube with 1 ml SEI buffer containing 150 $mmoll^{-1}$ sucrose, 10 $mmoll^{-1}$ EDTA, 50 $mmol^{l-1}$ imidazole at pH 7.3, and frozen at -80^0C (McCormick et al., 2009). Intestine tissue 2 cm in length was collected at around 2 cm above the rectum. After cleaning with PBS, tissue was cut in 4 pieces and stored in a 1.5 ml tube with 1 ml SEI buffer. Gill and intestine samples were analysed by the Indonesian Primate Research Center of Bogor Agricultural Institute for Na^+/K^+-ATPase concentration using ELISA kit "fish Na-K-ATPase" from My Bio Source (San Diego, MBS 044830). This kit was based on Na^+K^+-ATP antibody and Na^+K^+-ATP antigen interactions (immunosorbency) and using the horseradish peroxidase (HRP) colorimetric detection system to detect Na^+K^+-ATP antigen targets in the sample. Then, Na^+/K^+-ATPase concentration were measured using a calibration curve to calculate concentration in mmol per liter (mmol/L).

4.2.4. Data analysis

We used standard length and body weight data at the end of experiment to evaluate the growth in the selected groups. We used individual BW1 and BW28 values to calculate DGC as in Bureau et al. (2000):

$$DGC = \frac{\mathrm{BW}_{28}{}^{\frac{1}{3}} - \mathrm{BW}_{1}{}^{\frac{1}{3}}}{days}\ x\ 100$$

We removed abnormal values for blood ion concentrations (Na, K, Cl), haematocrit percentage, and Na^+/K^+-ATPase concentration in gill and intestine based on the references (Sun et al., 1994, Kammerer et al, 2020, Soegianto et al., 2017., Ranzani et al., 2004, and Azevedo et al., 2015). We performed normality test to check the distribution of the remaining data using Shapiro-Wilk normality test. Values outside the range of ±3.5 standard deviations from the mean were considered outliers and removed for all traits.

Differences between selected groups and testing environments were determined by two-way analysis of variance (ANOVA) for all traits using $P<0.05$ as the criterion for rejecting the null hypothesis of no effect by the tested factor. When significant interaction of groups and environments was detected, we applied Tukey's pairwise comparisons using the "emmeans" r-package to examine differences between groups and environments. All analysis were performed using R version 4.2.

4.3. Results

The number of fish stocked was 178, between 29 and 31 per combination of selected group (G,B and F), and treatment (brackish water or freshwater) (Table 1). At day 28 we measured weight, length, and haematocrit on the surviving 161 fish, 17 were lost to mortality. Survival in brackish water was 80.7%, 71 out of 88 fish were collected. In freshwater environment survival was 100% and we collected 90 fish on day 28. Samples from the three most extreme families within each group (n = 99) were send for lab analysis of blood ion and tissue enzyme concentrations.

Water parameters were measured daily during the experiment. Salinity in brackish water tanks reached 25 ppt on day 3 and remained close to 25 ppt thereafter (Figure 1). Morning and afternoon temperature in freshwater and brackish water tanks showed small fluctuations with an average of morning temperature of 28.1±0.6 in brackish water and 28.3±0.4 in freshwater. Afternoon temperatures were only 0.2 to 0.3 degrees higher. The average of daily dissolved oxygen (DO) in brackish water was 4.9±0.2 mgl^{-1} and in freshwater was 4.7±0.2 mgl^{-1}. Daily pH in brackish water was 7.7±0.2 and in freshwater was 7.7±0.2.

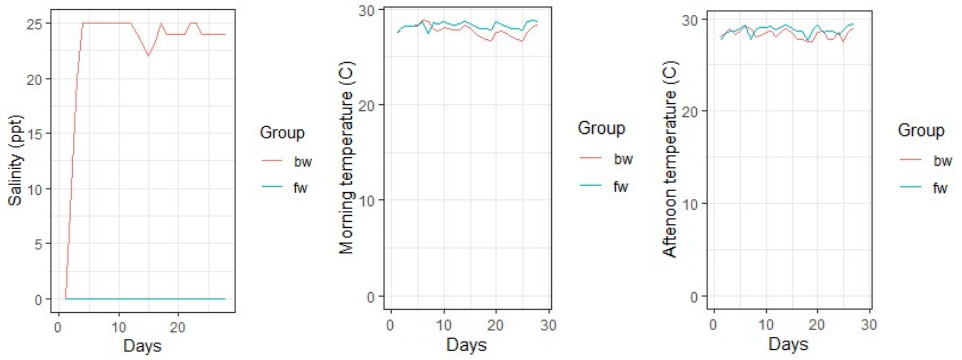

Fig 1. Salinity fluctuation (left) and water temperature in the morning (center) and afternoon (right) during experimental period.

4.3.1. Growth performance

4.3.1.1. Standard length

One value for SL28 was removed from the B group in freshwater because it was 9 cm shorter than the SL1 for this fish. Initial standard length (SL1) was in the same range in all groups and salinity treatments ($p>0.05$, Table 2). On average, Freshwater grown fish were longer at 15.7 cm compared to 15.2 cm for brackish water fish.

Table 2. Mean (±SD) of standard length of tilapia by genetic group and salinity treatment.

Standard length	Group	Brackish water			Freshwater		
		N	Mean ± SD	CV	N	Mean ± SD	CV
SL1 (cm)	G	29	13.9±1.0	7.1	29	13.9±0.9	6.3
	B	30	13.8±1.0	7.1	31	13.7±0.7	5.3
	F	29	14.1±1.2	8.6	30	13.6±0.9	6.9
	Mean	88	13.9±1.1	7.6	90	13.7±0.8	6.2
SL28 (cm)	G	24	15.4±1.1	7.2	29	15.8±1.1	6.8
	B	26	15.2±1.3	8.8	30	15.7±1.6	10.1
	F	21	15.1±1.6	10.9	30	15.7±1.6	9.9
	Mean*	71	15.3±1.4	8.9	89	15.8±1.4	9.0

* Significant different between brackish water and freshwater ($P<0.05$)

4.3.1.2. Body weight

No abnormal values our outliers (±3.5 SD) were found for stocking weight (BW1) and harvest weight (BW28). Average stocking weights per group ranged from 96.1 to 105.7 g and harvest weights from 133.3 to 158.8 g but were not significantly different between groups ($p>0.05$) (Table 3).

Table 3. Mean ± SD of body weight (g) of tilapia and coefficient of variation (%) by genetic group and salinity treatment.

Body weight	Group	Brackish water			Freshwater		
		N	Mean ± SD	CV	N	Mean ± SD	CV
BW1 (g)	G	29	101.5±18.3	18.0	29	105.7±16.3	15.5
	B	30	99.0±21.8	22.0	31	98.2±17.0	17.3
	F	29	104.9±23.5	22.4	30	96.1±20.0	20.8
	Mean	88	101.8±21.2	20.8	90	99.9±18.1	18.1
BW28 (g)	G	24	149.0±22.2	14.9	29	158.8±33.9	21.3
	B	26	140.5±25.0	17.8	31	141.9±41.3	29.1
	F	21	133.3±38.3	28.7	30	143.4±43.2	30.1
	Mean	71	141.2±29.0	20.6	90	147.9±40.0	27.1

4.3.1.3. Daily growth coefficient (DGC)

We had 161 observations for DGC analysis, two of which were outliers (±3.5 SD) leaving 159 records. Average DGC was lower in brackish water. The DGC per group ranged from 1.4 to 2.2 in brackish water, and from 2.0 to 2.4 in freshwater (Table 4). Tukey's pairwise comparisons showed that F in brackish water grew significantly slower ($P<0.05$) than F and G in freshwater. DGC of the other 2 genetic groups did not differ between brackish water and freshwater.

Table 4. Mean ± SD of DGC of tilapia and coefficient of variation (%) by genetic group and salinity treatment.

Group	Brackish water			Freshwater		
	N	Mean ± SD	CV	N	Mean ± SD	CV
G	24	2.2±0.4	16.4	29	2.4±1.2	48.1
B	26	2.0±0.8	40.9	31	2.0±1.2	60.3
F	20	1.4±0.8	55.1	29	2.3±1.0	43.9
Mean	70	1.9*±0.7	39.3	89	2.3±1.1	50.6

* significant effect of environment on growth rate (P<0.05)

4.3.2. Blood ion parameters and haematocrit

Table 5. The mean of Na^+, Cl^- (mmol/L), haematocrit (%) and their coefficient of variation (%) across all tilapia groups in brackish and freshwater

Blood parameters	Group	Brackish water			Freshwater		
		N	Mean ± SD	CV	N	Mean ± SD	CV
Na^+	G	14	175.5±3.4	1.9	16	167.2±4.1	2.5
	B	15	176.9±4.0	2.3	17	165.2±4.3	2.6
	F	11	173.5±5.8	3.3	18	165.8±4.9	3.0
	Mean*	40	175.5±4.5	2.6	51	166.1±4.5	2.7
Cl^-	G	15	182.0±11.5	6.3	17	169.1±15.0	8.9
	B	17	189.8±10.2	5.4	17	166.8±10.4	6.3
	F	15	179.1±12.8	7.2	18	167.1±11.2	6.7
	Mean*	47	183.9±12.2	6.6	52	167.7±12.1	7.2
Haematocrit	G	20	33.6±3.0	9.0	28	33.4±3.1	9.2
	B	21	32.5±3.4	10.3	28	32.8±4.1	12.4
	F	17	32.3±3.1	9.7	24	33.2±3.4	10.2
	Mean	58	32.8±3.2	9.7	80	33.1±3.5	10.6

* significant different between brackish water and freshwater (P<0.05)

Abnormal values of Na^+ were removed from the B group in brackish water (n = 2) ; from the G group in brackish water (n = 1) and in freshwater (n = 1); and from the F group in brackish

water (n = 4). All Cl^- data were inside the normal values. Most of the values of K^+ were removed as they were outside the expected range of normal values. K^+ was therefore removed from the analysis. Abnormal values of haematocrit were removed from the B group in brackish water (n = 5) and in freshwater (n = 3); from the G group in brackish water (n = 4); and from the F group in brackish water (n = 4), and in freshwater (n = 5). After removing abnormal data no outliers were found using the threshold of ±3.5*SD.

Growing tilapia in brackish water resulted in higher Na^+ and Cl^- concentration in blood plasma than in freshwater ($p<0.05$). This effect was consistent across the three groups, no interaction between environment and group was found. Haematocrit levels did not differ between brackish water (32.3 to 33.6%) and freshwater (32.8 to 33.4). Also, group and the interaction between group and environment did not affect haematocrit level.

4.3.3. Enzyme concentration

Table 6. Intestine and gill Na^+/K^+-ATPase concentration (mmol/L) across tilapia groups in brackish and freshwater

Na^+/K^+-ATPase (mmol/L)	Group	Brackish water			Freshwater		
		N	Mean ± SD	CV	N	Mean ± SD	CV
Intestine	G	15	435.1±65.5	15.1	14	500.7±61.3	12.2
	B	15	452.5±79.4	17.5	13	455.1±64.6	14.2
	F	7	531.9±84.2	15.8	14	445.0±82.9	18.6
Gill	G*	15	435.7 ^{a} ±51.2	11.8	15	545.5 ^{a} ±36.3	6.7
	B	14	458.5 ^{ab} ±64.9	14.2	15	452.7 ^{b} ±56.0	12.4
	F*	7	535.6 ^{b} ±64.2	12.0	14	456.0 ^{b} ±76.6	16.8

*significant differences between environments for this group, ^{a,b} significant differences between groups within the same environment.

A total 82 results were received from the 99 samples send for enzyme analysis. Abnormal values of Na^+/K^+-ATPase concentration in intestine were removed from the B group in freshwater (n=2); from the G group in freshwater (n=1), and from the F group in freshwater

(n=1). In gill, one abnormal value was removed from the B group in brackish water, and one from the F group in freshwater. After removing abnormal data no outliers were found using the threshold of ±3.5*SD.

The interactions between environment and tilapia group significantly influenced the Na^+/K^+-ATPase concentration in both gill and intestine. B fish had lower Na^+/K^+-ATPase concentration in intestine and gill than F fish in brackish water environment. In freshwater, F fish had lower Na^+/K^+-ATPase concentration than B in intestine and gill. Generalists had the lowest Na^+/K^+-ATPase concentration in intestine and gill in brackish water, and had the highest Na^+/K^+-ATPase concentration in freshwater.

4.4. Discussion

Many Tilapia species are known to be more tolerant to salinity than most farmed freshwater fish. The best growth is expected at isosmotic condition (Semra et al., 2013), however for the Sukamandi strain showed the better growth in 16 ppt compared to freshwater (Setyawan et al., 2022). Previously we have shown that this Sukamandi strain has salinity tolerance genes introgressed from blue tilapia (Yu et al., 2022). However, higher than isosmotic salinity will initiate physiological processes that will require the allocation of energy to osmoregulation (Boeuf & Payan, 2001; Tseng & Hwang, 2008), and reduce growth and feed efficiency as has been shown in *Oreochromis niloticus* (Chourasia et al., 2018). Higher salinity significantly reduced hepatosomatic index and muscle water content as well as protein and triglycerides in muscle (Semra et al., 2013). In our experiment standard length was 3% smaller after 28 days in brackish water compared to freshwater ($P<0.05$) across all groups (Table 2). Body weight was also smaller in brackish water for all groups, but these differences were not significant (Table 3). It appears that 28 days rearing was not sufficient to see significant differences in harvest weight. But growth rate was significantly reduced in brackish water (Table 4). Although the interaction was not significant, we see the biggest difference between environments for the F group. F appear to suffer more growth reduction than G and B.

Reduction in fish growth is related to stressful rearing conditions in hypo or hyperosmotic environments and leads to physiological changes and changes in blood ion concentrations. When water salinity increases, the levels of Na^+ and Cl^- in serum of tilapia also increase (Morgan et al., 1997; Vonck et al., 1998). Fish maintain an internal balance of ions with respect to the environmental salinity by osmoregulation. Na^+ and Cl^- are actively transported through the epithelial membranes during this process (Evans, 2010). Increasing salinity elevated sodium and chloride and after day 4 reach steady-state level (Morgan et al., 1997). Our data show that Na^+ and Cl^- in serum has higher values in brackish water than in freshwater after 28 days ($P<0.05$; Table 5). Fish face osmotic loss and diffusional gain of ions across the gill in hyperosmotic condition and they compensate with actively ingesting seawater, excreting low volume of blood-isotonic urine and active secretion of NaCl in gills (Evans, 2008). Higher ion concentration in hyperosmotic condition or smaller differences in osmotic pressure between the internal and surrounding solution will reduce the osmoregulation process and requires less energy for regulation. The effect of group was not significant but in brackish water we see a decreasing trend of the blood ion concentrations from B to F with G fish in between. In freshwater, the B group shows the lowest concentrations, but differences are very small. It appears that the F expend the most energy on osmoregulation given their lowest sodium and chloride concentrations as well as the lowest values for growth in brackish water.

Changes in ion concentrations due to salinity stress requires changes in the Na^+/K^+-ATPase concentration. This enzyme has an important role as a key enzyme for maintaining osmotic pressure during osmoregulation process (Sáez et al., 2009). It has a main function as ion pump, but it is also functions as a signal transducer to modulate cell polarity and can affect cell motility and gene expression among other functions (Li & Langhans, 2015). The change in concentration of this enzyme in response to osmotic change depends on the salinity tolerance that varies between species. *Oreochromis mossambicus* has a high salinity tolerance, showing a response that is similar to marine species, with increasing plasma osmolality in hyperosmotic environments (Zhu et al 2018). Fish that tolerate higher osmolality will require less osmoregulation and therefore less Na^+/K^+-ATPase. In this study,

the different groups did show different levels of Na^+/K^+-ATPase but the effect of group was not significant (Table 6). However, the pattern of differences between groups was very similar in both gill and intestine tissue. The interaction between environment and group significantly influenced the Na^+/K^+-ATPase concentration of the offspring in both tissues. In brackish water, the B group shows lower enzyme concentration in both intestine and gill compared to the F group. It seems that in this strain the B group has higher salinity tolerance, as indicated by their faster growth in brackish water, and they appear to do this at higher blood ion concentration and with producing less enzyme to maintain their osmoregulation process. This suggests that the salinity tolerance of B is not obtained from higher Na^+/K^+-ATPase but rather from the overall physiology of the fish maintaining its function under higher tissue ion concentrations.

Growth is a very complex trait, affected by salinity but also by many other factors that can obscure the effects of salinity. More specific physiological measures (Na^+, K^+, Cl^-, and Na^+/K^+-ATPase) show their effects more strongly because they are affected mostly by salinity and less by other factors. The F group showed higher enzyme concentration than the B group and significantly higher than the G group indicating that the F group has lower salinity tolerance. Fish with less tolerance to salinity will produce more enzyme and the enzyme activity requires energy to maintain their osmotic balance (Kültz, 2015). The osmoregulation process can consume a great deal of energy to pump ions through the basolateral side of the transporting epithelia in gill (Boeuf & Payan, 2001). Howarth et al. (2012) showed that the expression of Na^+/K^+-ATPase consumed up to 2/3 of the energy expenditure of a cell. Osmotic pressure above the threshold that can be tolerated will cause physiological stress that can reduce the fitness of the fish (Evans & Kültz, 2020). This condition of hyper-osmolality induces the cellular stress response (CSR) to stabilize, repair and protect macromolecular damage using reallocated metabolic energy (Kültz, 2005). This reallocation of energy is expected to reduce growth which we observed as lower DGC in the F group compared to B in the brackish water environment (Table 4). The F group is also showing a very high enzyme concentration which suggests that energy is reallocated away from growth.

The design of the high-low experiment using the high genetic contrast based on harvest weight EBVs was effective as a strategy to study physiological differences between genetically different groups. The DGC, Na^+ and Cl^- show clear pattern of physiological effects from selecting different groups based on the contrast of their EBVs for harvest weight in different environments. This contrast was designed to make groups that react most different to the environments. Using offspring from genetically selected parents allowed us to test the effect of selection on these physiological processes. With this approach, we can reduce cost and resources compared to genetic analysis that uses for instance a genotype by environment interaction study. Such a study would involve phenotyping at least 2,000 fish or at least 20-25 fish per family and 100 families to have a reasonable estimate (Sae-Lim et al., 2016). However, if we want to study the genetics of these physiological traits, we will need to have all measurements and laboratory analysis on all fish to estimate a genetic correlation. With a high-low design based on breeding values we can observe how physiological traits are correlated with genetic differences for a limited number of expensive laboratory measurements. This also allows elimination of traits that are not related without measuring thousands of fish. This design allows for understanding how a population responds to a specific environment. Each group has positive interaction with growth expressed in DGC. The G group which has highest genetic values also generated highest DGC in both environments. The B group which has higher genetic values in brackish water also has higher DGC than in freshwater, and on the contrary for the F group which has higher DGC than the B group in freshwater. Further, with this design we can monitor some physiological effects of breeding program that generate a positive or negative effect to other traits. In our case, selection for the F group based on harvest weight EBVs will increase their osmoregulation efforts in brackish water which will require higher enzyme levels to pump ions through the gill epithelia.

In this study, we were not able to use the most extreme families of each group. Not all families spawn simultaneously therefore the contrast between the groups were reduced, lowering the power of our experiment. There are some strategies to increase the probability of successful mating in tilapia. The most common strategy is to select male and female

which have similar reproduction status. Maintaining parents in separated ponds between male and female and feeding with high crude protein at 30% for two weeks also increase the probability of successful mating. Using multiple females in a small mating hapa also successfully increased the number of families at the same period (Omasaki et al., 2016; Trọng et al., 2013). Multiple females' combined with one male reduces mortality of females due to aggressive interaction during mating period.

4.5. Conclusion

The high-low experiment is an effective strategy to evaluate the physiological differences between groups contrasted on their harvest weight EBVs. We show that Tilapia selected for higher growth in brackish water show a more efficient osmoregulation process than tilapia selected for higher growth in freshwater with higher DGC, as well as higher blood Na^+, Cl^- and lower Na^+/K^+-ATPase concentration in both gill and intestine. Therefore, continued selection in brackish water is expected to reduce the performance of these fish in freshwater. A high-low experiment with contrasting genetics can be relatively small and still show the interaction of genetics with environment, especially if traits are chosen carefully to be closely linked to the physiological processes that respond to the environmental perturbation.

4.6. Acknowledgements

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Supplementary Table

Supplementary table S1. Coequal distribution of fish from all groups in freshwater and brackish water tanks.

Group	Subgroup	Tank number in fresh and brackish water environments					
		male			female		
Generalists	G1	1	2	3	4	10	6
	G2	4	5	6	7	8	9
	G3	7	8	9	10	1	2
	G4	10	1	2	3	9	5
	G5	3	4	5	6	7	8
Brackish water specialists	B1	1	4	7	6	10	5
	B2	2	5	8	7	1	6
	B3	3	6	9	8	2	7
	B4	10	2	4	9	3	8
	B5	1	3	5	10	4	9
Freshwater specialists	F1	8	6	3	10	5	9
	F2	5	2	7	6	4	10
	F3	1	9	4	8	3	7
	F4	5	3	1	7	2	8
	F5	4	2	10	9	1	6

Chapter 5

The evolution of and progress achieved by a tilapia breeding program to re-vitalize unproductive brackish water aquaculture ponds in Indonesia

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Abstract

In this paper we evaluate the evolution of and genetic progress achieved by a tilapia breeding program during 6 generations of selection in brackish water in Indonesia between 2011 and 2022. The breeding program initially imposed 4 generations of a simple mass selection whereby selection candidates from the Sukamandi strain were stocked in brackish water ponds and selected parents for the next generation were transported back to a freshwater research facility in Sukamandi, West Java for production of next generation fry. However, high mortality occurring during growout and transportation from brackish water to freshwater resulting in a low number of selection candidates and increased risk of inbreeding. Therefore, a family-based nucleus breeding program in freshwater with sib testing in brackish water was predicted to be the better strategy. For generation 5 we produced 91 families and reared them in fresh water and brackish water. For the sixth generation we produced 102 families and reared them brackish water in both monoculture and co-culture with shrimp. We used best linear unbiased prediction (BLUP) to estimate fish breeding values and optimal contributions selection to manage genetic diversity and inbreeding rate for the next generation using a total of 5755 observations from generations 5 and 6 combined. Our results show that the Sukamandi strain grew larger in brackish water than in freshwater and that harvest weight in brackish water was not significantly different between monoculture and co-culture with shrimp. Heritabilities of harvest weight ranged from 0.32 to 0.53 in all environments. The regression coefficients between mid-parent estimates of breeding values for brackish water and freshwater with the phenotypic performance of their progeny were 1.04 and 1.05, respectively, indicating a low bias of the estimated EBVs of the parents. The accuracy of selection for brackish water was 77.66% and for freshwater was 65.65%. The estimated genetic gain for the fifth to sixth generation of the Sukamandi strain was 24.77 g for brackish water and the correlated response for freshwater was 19.45 g. We conclude that a nucleus breeding program is feasible, and that because the genetic correlation between freshwater and brackish water growth is only moderate (0.63 – 0.66), genetic evaluations for the Sukamandi strain should be conducted in brackish water ponds to maximise the selection response.

Key words: Tilapia, salinity, breeding program, BLUP, genetic trend

5.1. Introduction

Since the 1990's, recurrent crop failures on Indonesian brackish-water shrimp farms have caused serious financial problems for smallholder farmers resulting in abandoned ponds in many brackish water areas. Consequently, intensive shrimp farming is no longer viable for approximately two thirds of these farmers (MMAF, 2018). In response, many have shifted to growing tilapia or combining tilapia and shrimp production in rotational and co-culture strategies as a viable alternative production system in former shrimp ponds (Setyawan et al., 2022b) with much lower risk than shrimp monoculture (Rimmer et al., 2012). Currently, they produce commercially available Nile tilapia that grow normally in up to 7 ppt water (Azevedo et al., 2015), but farming this tilapia strain in higher salinity ponds is challenging due to low growth rates, poor survival and reproduction, and other physiological problems (Azevedo et al., 2015; Boeuf & Payan, 2001; Cnaani & Hulata, 2011; Popma & Masser, 1999). A more salinity-tolerant tilapia strain could make these alternative rotational and co-culture farming systems much more productive, reliable, and profitable.

To address this need, the Indonesian Ministry of Marine Affairs and Fisheries has been developing a new strain of tilapia specifically bred for performance in brackish water ponds: the Sukamandi strain, which is an inadvertent hybrid between Nile tilapia (*Oreochromis niloticus*) and Blue tilapia (*Oreochromis aureus*) (Yu et al., 2022). For the first four generations, the breeding program used simple mass selection on harvest weight measured directly on candidates grown in commercial brackish water ponds with the selected fish returned to a freshwater hatchery to produce the next generation (Setyawan et al., 2022a). The selection were conducted in farmers' ponds in different locations (Table 1). However, poor husbandry practices caused high mortality during the grow out period and transportation of candidates from commercial brackish water test sites to the secure freshwater mating site, resulting in a low number of candidates, selection intensity, and genetic improvement relative to a control line (Table 1).

Table 1 Estimated genetic gain in the first to fourth generation of Sukamandi strain

Generation	Family number	*Genetic gain (g)		Growth out (days	Location
		Male(mean)	Female(mean)		
G1	9	5.9(158.3)	10.6(121.4)	120	Karawang
G2	16	7.4(119.0)	3.3(90.7)	120	Indramayu
G3	13	11.3(45.5)	6.7(40.0)	120	Brebes
G4	16	15.1(231.8) – mixed		120	Cirebon
G5		39.6	38.8		

*The difference in harvest weight of progeny from selected parents and an unselected control

The initial approach to mitigating these mortality problems was to switch to a traditional bio-secure nucleus-based strategy with the production of full-sib families in the secure, freshwater location, sib-testing in brackish water ponds and pedigree-based mixed-model BLUP estimation of breeding values for candidates in the nucleus starting with the fifth generation (Fig. 1). Furthermore, adding pedigree records to the program made it possible to implement optimal contributions selection to minimize short-term inbreeding and associated inbreeding depression and long-term loss of allelic diversity over generations (Knibb et al., 2014). Similar family/nucleus-based strategies have been implemented in the GIFT tilapia breeding program in Philippines, producing a cumulative response of 67% improvement of growth rate over five generations in pond culture (Bentsen et al., 2017), in Nile tilapia in freshwater cages in Brazil producing 4% genetic gain per generation after five generations of selection (de Oliveira et al., 2016), and in freshwater cages in Brazil producing around 3.3% gain in daily weight gain after nine generations of selection (Yoshida et al., 2022).

However, producing a sufficient number of families for this new strategy proved challenging. For the first to fourth generations, we conducted single-pair mating of one male and one female in small mating hapas and tagged their progeny using external plastic tags to measure individual growth rate. During this period, almost half of the potential female parents died due to male aggression, and we were unable to produce enough families to effectively implement between-family selection. We attempted to address this problem

using a previously developed jaw cutting method of removing males' upper lip (Bentsen et al., 2012) and pairing males and females of approximately the same size in the mating hapas, but this strategy was not very successful. Parental fish mortality continued, and it took approximately three months to produce fewer than 30 families. Starting with the production of G5 from G4 parents, we further modified the mating strategy from one male and one female per mating hapa to multiple females in each hapa based on Trọng et al. (2013a), who used multiple females in a tank resulting in 85% successful mating within 20 days and Omasaki et al. (2016) who used a 1:3 ratio of males and females and produced 76 full-sib families. We also discontinued cutting the jaws of males (Strategy 1 in Table 2).

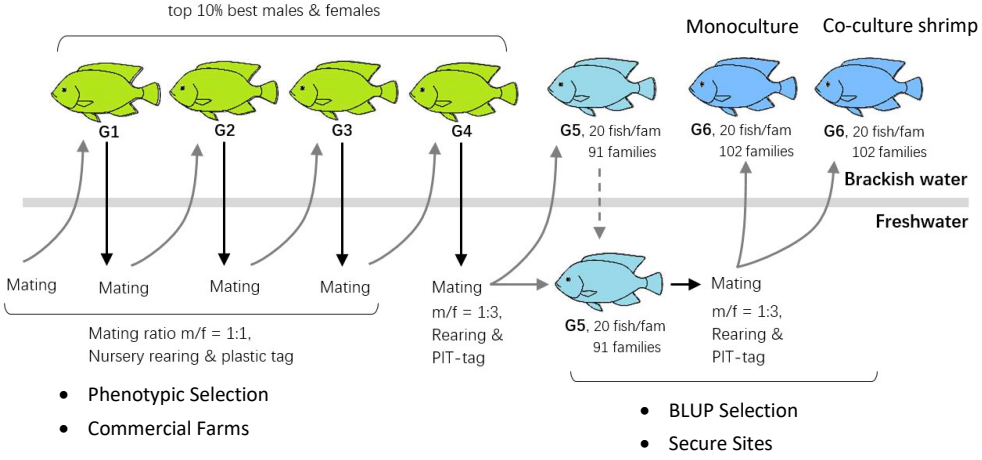

Figure 1. The development of a salinity tolerance tilapia breeding program for shrimp farming environment from the first to sixth generation.

The new strategy consisted of first stocking a single male into each mating hapa and adding 3 females to each hapa one day later. We conducted this mating process in 7-day cycles, removing full-sib groups of fertilized eggs from the mouths of spawned females and unspawned females at the end of each cycle, and replacing the male if no females spawned. We replaced males every two mating periods even if they did spawn to limit the number of half-sib families and the associated reduction in genetic diversity of the candidate population. Using this strategy, we produced a total of 117 families in three months, 91 of

which we used as G5 selection candidates after discarding duplicated families and families with insufficient numbers of larvae.

Table 2 The average of family production per harvest time in two optimised mating strategies

Harvest time	Number of families			
	Strategy 1 for G5			Strategy 2 for G6
	1st group	2nd group	3rd group	group 1
1st week	20	11	17	59
2nd week	6	5	11	51
3rd week	13	10	6	44
4th week	6	5	7	78
Total	45	31	41	232

Starting with G6 we further modified our approach (Strategy 2 in Table 2) by adding two sections of PVC pipe (4-inch diameter, 40 cm long) and a 1m x 3m diagonal curtain of polyester cloth as artificial shelters for the females to avoid male aggression. The curtain extended to the bottom of the hapa but there were some space in each corner that allowed females to move freely within the hapa (Fig. 2). Using this strategy, we produced 232 families in a month, 102 of which were used as G6 selection candidates after discarding duplicated families and families with insufficient numbers of larvae. For the fingerling nursery rearing, tagging and acclimation prior to sib testing in ponds, we used separate family methods described in Setyawan et al. (2022a).

In addition, we structured the G5 sib-testing trial as a genotype-by-environment (GxE) interaction experiment between fresh and brackish water ponds located in a secure research facility rather than commercial farms (Technical Implementation Unit for Brackish water Culture Karawang (-6.106192, 107.428710) and Research Institute for Fish Breeding (-6.371860, 107.623815). Both locations are in the West Java area close to the North Java

Sea. This experiment found a moderate genetic correlation for harvest weight of about 0.66 (Setyawan et al., 2022a), indicating that there is a substantial GxE interaction for this trait. Furthermore, the Sukamandi strain grew better in brackish water than in freshwater ponds, resulting in higher harvest weight, and daily growth coefficient (DGC) over the same growout period, as well as a higher genetic coefficient of variation (GCV) for harvest weight in brackish water. This higher GCV, sometimes called "evolvability" (Houle, 1992) represents the potential of a specific trait to respond to selection in a population (Cheung, 2017) and indicates that selection in brackish water ponds will produce more genetic gain than selection in freshwater ponds.

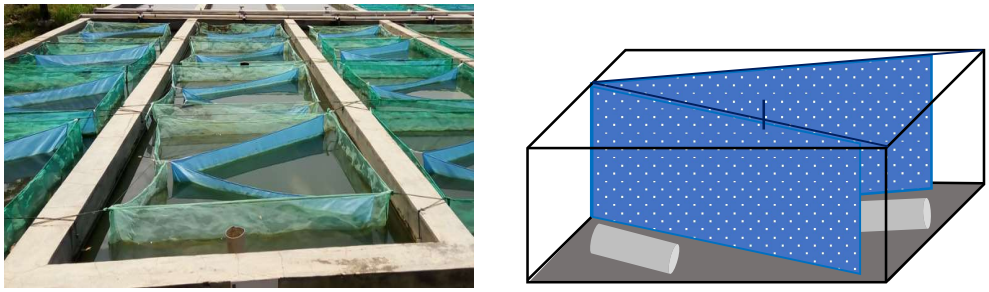

Figure 2. The picture and scheme of mating hapa using artificial shelter.

As previously mentioned, most of the brackish water tilapia farmers in Java Island, and Sidoarjo (Hukom et al., 2020) use a polyculture system with shrimp (Setyawan et al., 2022b). In G6, we conducted another GxE experiment using tilapia monoculture and tilapia/shrimp co-culture farming systems in brackish water ponds at the Technical Implementation Unit for Brackish water Culture Karawang to test the genotype by environment interaction between mono and co-culture systems, and found a genetic correlation close to unity (see Results below), indicating almost no GxE for mono- vs. co-culture.

Lessons learnt from these experiments using G5 and G6 are that our modified mating strategy makes it possible to produce sufficient numbers of families for a family or pedigree-based breeding program, that GxE with respect to salinity is substantial enough to require that performance data are collected in brackish water ponds, and that GxE with respect to mono- vs. co-culture can be safely ignored. Furthermore, conducting sib-testing in secure

test sites at research institutes rather than commercial farms has distinct advantages over in terms of security, management, and mortality.

In this study, we focus on estimating the genetic trend by combining all of the available data into a single pedigree-based BLUP analysis to evaluate the efficacy of the re-structured breeding program, the realized accuracy and bias of estimated breeding values, and the realized rate of inbreeding in the breeding program. We further discuss potential future modifications to the breeding program structure based on these results.

5.2. Materials and Methods

5.2.1. Data sets available and descriptive statistics

Figure 3 shows the pedigree structure of the available data, treating the generation three mass-selected parents as founders with unknown pedigree.

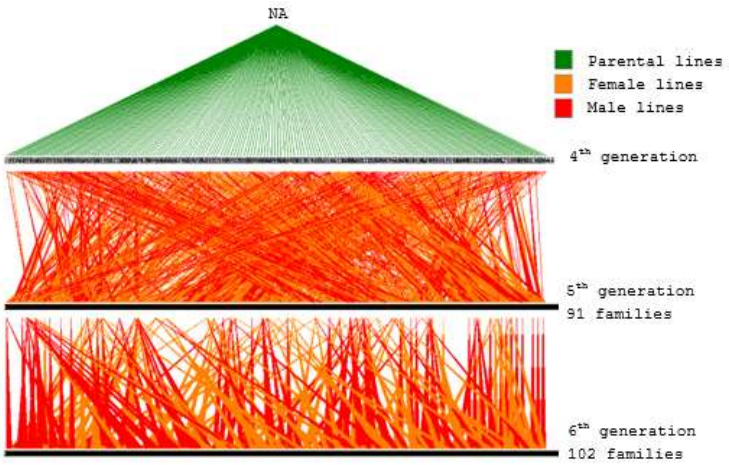

Figure 3 The pedigree of families in base parents and the successor generations.

Because the sib-testing for both the fifth and sixth generations of Sukamandi strain were designed as GxE experiments, the data collected in these trials have different structures, and for convenience we have given separate names for each of the four datasets produced by these experiments (Table 3). Specifically, the G5 experiment consists of data from only

monoculture in both brackish and freshwater (Datasets 1;2) whereas the data from the G6 experiment consists of both mono- and co-culture in brackish water only (Datasets 3;4).

Table 3 Structure of available data

Generation	Salinity	Monoculture	Coculture
G5	Fresh	Dataset 1	-
	Brackish	Dataset 2	-
G6	Fresh	-	-
	Brackish	Dataset 3	Dataset 4

The summary information of data structure on the pond environments, number of observations, initial weight at stocking time, the average of harvest weight, age at harvest and growth out periods are given in Table 4. The number of records available for the analysis ranged between 1308 and 1549 animals in all environments. The growout period lasted for 120-147 days in the G5 experiment and resulted an average harvest weight at around 238.1g in freshwater ponds, significantly lower than in brackish water at about 300.8g ($p<0.05$). whereas the growout period of 97-99 days in the G6 experiment resulted in lower harvest weight and similar size between monoculture and co-culture with shrimp at about 129.2g and 133.1g, respectively.

Table 4 . Total number of families (N), number of observations (n), mean of stocking weight (SW and SD) and its coefficient of variation (CV in %), mean of harvest weight (HW and SD) and its coefficient of variation (CV in %), harvest age (Age in days)

Dataset	Environment	N	n	SW(g)		HW(g)		Age	Days
				Mean	CV	Mean	CV		
1	Freshwater	91	1391	16.1±7.8	48.45	238.1±56.7*	23.81	258-298	125-147
2	Brackish water	91	1308	15.7±7.8	49.68	300.8±67.5*	22.44	243-274	120-126
3	Brackish water monoculture	102	1549	10.7±3.0	28.04	129.2±31.5	24.38	182-196	97-99
4	Brackish water co-culture	102	1507	10.9±3.1	28.44	133.1±31.0	23.29	182-196	97-99

* $p < 0.05$ Student-t-test comparing harvest weight in each environment.

5.2.2. Data pooling strategy

In order to combine these different datasets into a single trans-generational analysis to estimate the genetic gain in harvest weight in both fresh and brackish water, we performed several sub-analyses to estimate the genetic correlations between harvest weight for various pairwise combinations of the four datasets. All of BLUP analyses were performed using ASReml-R version 4 (Butler et al., 2017).

First, to estimate the heritabilities of harvest weight in each of the salinity environments and the phenotypic and genetic correlations between the two environments, we used the G5 datasets (1 and 2), to fit a bivariate model that treated harvest weight in fresh and brackish water as separate traits (Model 1 below, which was also fit as part of (Setyawan et al., 2022a), but is repeated here for completeness). This model included the fixed effects of replicate ponds, and sex and age nested within ponds. We attempted to account for common environmental effects (c^2) during the separate family rearing stage up to tagging size, but that model failed to converge, most likely due to the almost entirely full-sib structure of the data, so we fit the following model:

Model 1. $$y_{ijkl} = \mu + Pond_i + Sex(Pond)_{i,j} + Age(Pond)_{i,k} + a_l + e_{ijkl}$$

where y_{ijkl} is vector of harvest weight in fresh and brackish water; μ is overall mean; $Pond_i$ is a categorical fixed effect that accounts for both pond and batch effects (I = 1-3 for brackish water, and 4-6 for freshwater); $Sex(Pond)_{i,j}$ is a fixed effect for sex (j = male or female) nested within pond; $Age(Pond)_{i,k}$ is harvest age nested within pond; a_l is the random additive genetic effect of the l^{th} individual; and e_{ijkl} is the random residual effect. The main term of interest here is the genetic correlation (i.e. GxE interaction) because this effect determines whether or not we can legitimately treat the two traits as a single trait in subsequent models.

Similarly, we estimated the genetic correlation (i.e. GxE interaction) between mono and co-culture farming systems in G6 (Datasets 3;4) using a bivariate model of harvest weight in mono- and co-culture systems. This model (Model 2), included cage as a fixed effect because

in this experiment we used 54 separate 3x5 m^2 hapas to prevent shrimp from escaping from the system. In this case, the model did converge when we includes a common environmental effect (c^2), and we fit the following model:

Model 2. $$y_{ijklm} = \mu + Pond_i + Sex(Pond)_{i,j} + Age(Pond)_{i,k} + cage_l + c^2 + a_m + e_{ijklm}$$

where the Pond and Sex effects are as above: $cage_l$ is the fixed effect of cage (l = 54 cages); c^2 is random effect of family-specific hapas during nursery rearing; and e_{ijkl} is the random residual effect. As above, for this study, the main term of interest is the genetic correlation (i.e. GxE interaction) because this effect determines whether or not we can treat the two traits as a single trait in subsequent models.

Further, we used Dataset 1 and the combined Datasets 3 and 4 to fit another bivariate model (Model 3 below) to investigate genetic parameters and GxE interaction of harvest weight in brackish water ponds between the two generations. In this model, we included the cage effects and stocking weight as part of the fixed effects, but applied them only to the G6 data using the at() syntax in ASReml-R. Again, we tried to include a common environmental effect (c^2) in this model, but it failed to converge, so we fit the following model:

Model 3. $$y_{ijklm} = \mu + Pond_i + Sex(Pond)_{i,j} + Age(Pond)_{i,k} + Stockweight + at(trait, 2): Cage_l + a_m + e_{ijklm}$$

where the Pond and Sex effects are as above, but coded such that pond effects are unique across generations (i.e. effectively pond-year contemporary groups); $Stockweight$ is fixed effect of fish body weight as stocking time; and $at(trait, 2): Cage_l$ is the fixed effect of cage applied only to the combined harvest weight data from the sixth generation using the at() syntax of ASReml.

5.2.3. Genetic gain between generations

Finally, to estimate the genetic gain for harvest weight in both fresh and brackish water across generations and the genetic correlation between the salinity environments using all of the available data, we pooled the brackish water data across generations (G5 and G6; Datasets 2, 3 & 4) and fit a bivariate model (Model 4 below) of these pooled data and the G5 freshwater data (Dataset 1). In this case, the model did converge when we included a random effect to account for common environmental effects (c^2), resulting in the following model:

Model 4. $$y_{ijklm} = \mu + Pond_i + Sex(Pond)_{i,j} + Age(Pond)_{i,k} + Stockweight + at(trait, 2): Cage_l + c^2 + a_m + e_{ijklm}$$

where all of the fixed effect are as above and together account for contemporary groups based on the unique coding of pond effects between the two generations, and cage effects are applied only to the G6 brackish water data using the at() syntax of ASReml-R (Datasets 3 and 4 above).

For all of the models above, we calculated heritabilities $h^2 = \frac{\sigma_A^2}{\sigma_P^2 + \sigma_E^2}$. We also estimated individual-level inbreeding coefficients for all fish by using the inbreeding() function of the GeneticsPed R-package (Gorjanc et al., 2007).

5.2.4. Accuracy, bias, and genetic gain

We estimated the bias and realized accuracy of our breeding values by plotting the mid-parents EBVs of the parents for each generation as a predictor of the mean harvest weight of their progeny in fresh and brackish water ponds where the regression coefficient and correlation estimate bias and accuracy respectively. Finally, we evaluated genetic gain for selection on harvest weight in brackish water between the fifth and sixth generations as well as the correlated response in freshwater ponds based on the change in the mean EBVs.

5.3. Results

5.3.1. Data pooling

Table 5 summarizes the variance component analyses from all four of the models we fit to different combinations of datasets. Here we focus on the genetic correlation estimates and their implications for pooling the various datasets in order to construct a combined, cross-generational dataset suitable for quantifying genetic progress in both fresh and brackish water. Model 1 was intended as a single-generation (G5, datasets 1 and 2) analysis of the GxE between freshwater and brackish water ponds, and indicates that the genetic correlation is significantly different from unity based on an approximate 95% confidence interval (estimate ±1.96 SE) and low enough to indicate that if the breeding program were to select on freshwater performance alone, genetic improvement in brackish water would be about 66% of the improvement achieved in freshwater, and lower than desired.

Model 2 was intended as a single-generation (G6, datasets 3 and 4) analysis of the GxE between mono- and co-culture in brackish water, and indicates that the genetic correlation of near unity is high enough to justify pooling these two datasets into as single, brackish water dataset.

Model 3 was intended to estimate the more complex genetic correlation between growth in brackish water both across generations and culture systems, with the aim of pooling datasets 2, 3, and 4, to represent brackish water and thereby increase the power of a trans-generation analysis. Based on the results from Model 2, we expected this genetic correlation to be very high because it consists of a combination of the near unity of the mono vs. co-culture analysis of G6 with what we thought we be a high trans-generational genetic correlation in brackish water. While the Model 3 genetic correlation is lower than anticipated, the standard error is much higher than any of the others we estimated, and the approximate 95% confidence interval (estimate ±1.96 SE) for this estimate includes one. We therefore, concluded that pooling datasets 2,3, and 4 was justifiable, and fit Model 4 treating these pooled data as brackish water performance and dataset 1 as freshwater performance.

Table 5 The additive genetic variance (σ^2_A), total phenotypic variance (σ^2_P), heritability (h^2), genetic coefficient of variation (GCV), genetic correlation (rg), and phenotypic correlation (rp) estimated from bivariate analyses of harvest weight in different salinities and culture systems.

Model	Trait	Salinity	Culture System	Dataset	σ^2_A	σ^2_P	h^2	GCVa	rg	rp
1	1	Fresh	Monoculture	1	790.57	2104.70	0.38	11.8	0.66±0.10	0.24±0.05
	2	Brackish	Monoculture	2	1048.47	2958.76	0.35	10.8		
2	1	Brackish	Monoculture	3	256.29	794.71	0.32	12.4	0.99±0.03	0.37±0.06
	2	Brackish	Coculture	4	294.77	710.13	0.42	12.9		
3	1	Brackish	Monoculture	2+3	1394.04	2989.36	0.47	12.4	0.63±0.19	0.28±0.09
	2	Brackish	Co-culture	4	318.8	755.08	0.42	13.6		
4	1	Fresh	Monoculture	1	805.31	2045.36	0.39	11.9	0.61±0.09	0.28±0.05
	2	Brackish	Mono + Coculture	2+3+4	815.03	1541.77	0.53	15.7		

5.3.2. Variance components and inbreeding

We also tested the significance of each fixed effect in the final model between fresh and brackish water records (Model 4). The WALD statistic test in ASReml indicated that all fixed effects have a significant contribution to the model ($p < 0.05$, Table 6), indicating that they all should be included in the final trans-generational model for the pooled data.

Table 6. Tests of significance for all fixed effects fit in Model 4

Source	df	Sum of Sq	Wald statistic	Pr(Chisq)
Environment	2	13481.61	13481.61	< 10-5
Stock weight	2	1583.56	1583.56	< 10-5
Pond	6	3921.53	3921.53	< 10-5
Sex:Pond	8	2054.98	2054.98	< 10-5
Age:Pond	8	31.10	31.10	1.35·10-4
at(trait,2):Cage	53	245.56	245.56	< 10-5
Residual	NA	1.000	NA	NA

Two of the models we fit (Model 1; Model 4) provide direct comparisons of variance components between fresh- and brackish water production systems. In both of the analyses, harvest weight showed more additive genetic variance in brackish water than in freshwater, but the residual environmental variation in freshwater was higher than in brackish water in Model 1 and lower than in brackish water in Model 2. This resulted in very similar heritabilities and GVCs for harvest weight in brackish water between models, but a substantially higher heritability estimate for fresh water in Model 4 relative to Model 2.

The heritabilities of harvest weight were moderate in all environments, ranging from 0.323 to 0.529. The evolvabilities of harvest weight (GCV) were also moderate in all environments, ranging between 10.8 and 15.7, indicating that selection on this trait in either brackish or fresh water should produce significant genetic gain in the selection environment. The

between salinity environments genetic correlation was similar in Models 1 and 2 (0.66, 0.61 respectively), indicating that selection in either environment would produce a substantial correlated response in the other environment, but there would likely be considerable re-ranking of candidates across environments. Robertson (1959) suggested that genetic correlation between two environment is biologically important if the GxE interaction is lower than 0.8.

Our analysis of inbreeding estimated all individual-level inbreeding coefficients as zero, but this must be interpreted with caution because these estimates assume that all G3 "founders" are unrelated due to the truncated pedigree available. However, these estimates do indicate that our implementation of optimal contributions selection in the selection of parents for G5 and G6 has been able to completely avoid consanguineous matings up to this point.

5.3.3. Genetic gain, bias and accuracy

Based on EBV estimates from the final model (Model 4) using the pooled data from all brackish water trials, we plotted the average EBVs for harvest weight in both fresh and brackish water across the generations we evaluated. Because there are no pedigree records before G4, this model treats these fish as founders (see Fig. 4) with a mean EBV of zero by definition. The G5 generation showed very little genetic improvement from the previous generation in both environments, but the G6 generation showed a very substantial gain.

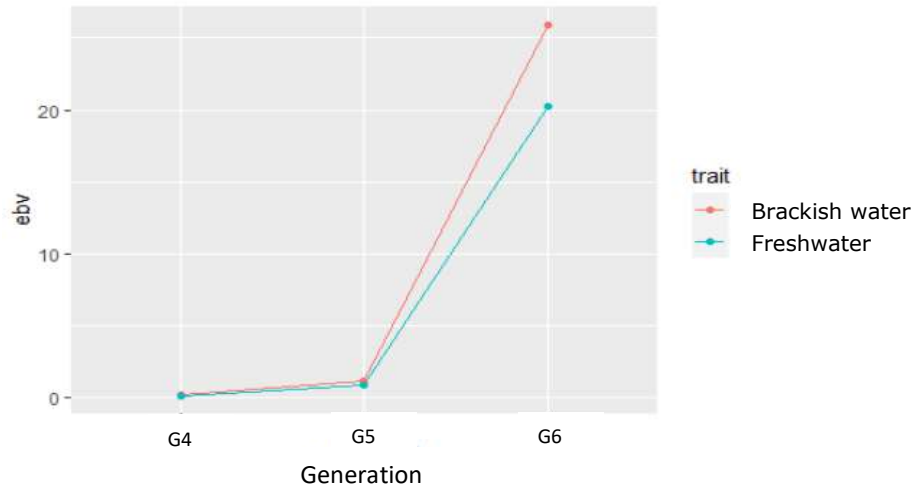

Figure 4 The estimated genetic gain in brackish water and freshwater ponds based on EBVs from Model 4

To test for bias in the genetic evaluation and estimate the realized accuracy of selection, we estimated the dispersion bias as the slope of a regression analysis using mid-parents EBVs as a predictor and phenotypic progeny harvest weight as the dependent variable. If this slope deviates significantly from one, breeding value estimates are biased. The slope for the brackish water environment was 1.05 and in freshwater environment was 1.04 (Table 7; Fig5).

Table 7 The coefficients of regression between harvest weight and mid-parent EBVs in fresh and brackish water ponds

Environment	Source	Estimate	Std. Error	T value	Pr(>|t|)	accuracy
Brackish water	Intercept	103.93	0.3502	296.75	2e-16	77.66%
	Mid-parent EBVs	1.05	0.0129	81.52	2e-16	
Freshwater	Intercept	236.35	0.383	617.72	2e-16	65.65%
	Mid-parent EBVs	1.04	0.021	49.67	2e-16	

We also estimated the accuracy of selection based on Spearman's correlation (Fig. 5). The accuracy of selection showed moderate values (Table 7). The accuracy of EBVs estimation in brackish water was higher at 77.66% than in freshwater at about 65.65%.

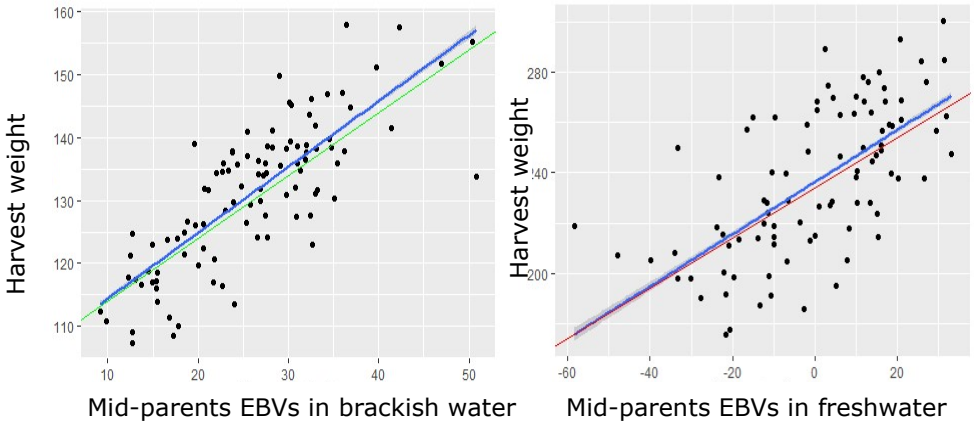

Figure 5 A plot between mid-parents EBVs and harvest weight (HW) of its progeny in brackish water (left) and freshwater (right) with the accuracy of selection are 77.66% and 65.65%, respectively (Spearman's correlation). The green and red lines are reference lines (the slope = 1).

5.4. Discussion

5.4.1. The evolution of the breeding program to date

Initially, the Indonesian Ministry of Marine Affairs and Fisheries adopted a simple mass-selection approach to selectively breeding a salinity-tolerant tilapia strain that evaluated fry produced in a freshwater hatchery on commercial farms, selected candidates based entirely on their own-performance records on those farms, and returned the selected candidates to fresh water for reproduction based on 1:1 pairings of males and females. However, low mating success and high mortalities during mating, growout at unsecure sites, and during transportation of candidates from commercial brackish water test sites to the secure freshwater mating site were serious problems, and we implemented a series of changes.

5.4.2. Mating protocol

We observed that trying to mate one male and one female resulted in males engaging in aggressive sexual behaviour resulting in the deaths of many females. Under more natural conditions, male tilapia establish mating territories based on hierarchies that involve aggressive interactions (Gonçalves-de-Freitas et al., 2019; Rossi et al., 2019). Limited space and spawning substrate can also increase dominance hierarchies of male cichlid fishes (Maruska, 2014). Previous studies have shown that this natural behaviour in an artificial situation causes bodily injuries, social stress, and changes in energy expenditure (Gonçalves-de-Freitas et al., 2019) and that male aggression during sexual behaviour has a positive correlation with male-specific protein (MSP) level in the serum (Machnes et al., 2008). Our initial attempt to solve this problem was to cut males' jaws to reduce the damage to females during the mating period in G3-G4, a commonly used procedure for tilapia breeding programs (Bentsen et al., 2012). This reduced the number of female deaths slightly, but not completely in one male to one female mating hapas.

Starting with G5, we modified our mating strategy by stocking our mating hapas with one male and three females and adding short sections of PVC pipes to provide females with refuges from male aggression. Having multiple females in the same hapa significantly reduced mortality during the mating period. Keller-Costa et al. (2015) showed that chemical communication via male urinary pheromones occurs during spawning and these hormones induce ovulation in females. Females that are not in the same reproductive status as the male are not triggered by these pheromones and avoid males. Moreover, female Nile tilapia prefer less aggressive males and tend to avoid highly aggressive males (Rossi et al., 2019). Having multiple females in the mating hapa allows the male to focus on females in reproductive condition and reduces mortality due to aggressive sexual behaviour. Shelters allow females that are not in reproductive condition to hide and avoid male aggression.

For G6 we further added synthetic curtains to reduce contact between the male and females. In combination, these measures significantly reduced the time required to produce families from three to one month (Table 2). Trọng et al. (2013a) also found that group matings of one male with 10 females resulted in successful spawning in 20 days.

Because tilapia females brood their young in their mouths, if the mating hapas are checked frequently, full-sib families for the breeding program can be isolated by removing fertilized eggs from the females' mouths and incubating them in separate containers. Based on our experience, we recommend that other tilapia breeding programs adopt our modified methods, which are more successful and eliminate the need to cut the jaws of males.

5.4.3. Genetic evaluation and selection strategy

The initial breeding strategy from G1 to G4 used a simple mass selection using small number of selection candidates based on their own performance records (Fig 1). Implementing this breeding program structure was relatively easy and inexpensive. After four generations, this breeding strategy resulted estimated cumulative responses for harvest weight of 39.6g for male and 38.8g for females based on a comparison of selected and control lines (Table 1). We improved our tilapia breeding strategy in G5 and G6 by individually tagging fish and implementing pedigree-based BLUP linear mixed models analysis to better estimate breeding values and to implement optimal contributions selection (Meuwissen, 1997) to select the best parents for future generations (Fig. 1). Implementing a mixed model to estimate breeding values in the selection process potentially increases selection response about 20-30% over simple mass selection (Gall & Bakar, 2002). Most of BLUP-based breeding programs to improve harvest weight in aquaculture achieve about 13% improvement per generation (Gjedrem & Rye, 2018) compared to about 10% in G1 to G4 of our breeding program.

5.4.3.1. Family production, selection intensity, and inbreeding

Using our improved mating strategy, we were able to create 91 G5 and 102 G6 candidate families, and impose sufficient selection intensity to produce substantial genetic gain while completely avoiding matings between relatives in the short-term. However, a sufficient number of families is crucial for maintaining genetic diversity and controlling the rate of inbreeding over the long term. As a "rule of thumb," 50 families is generally considered minimally sufficient to constrain inbreeding rate to under 1% per generation (Bentsen & Olesen, 2002). Having a larger number of selection candidate families also increases genetic

gain due to increasing selection intensity at the same rate of inbreeding (Sonesson & Ødegård, 2016). Increasing the family number is important, but it is also increases the cost of raising families separately up to tagging size. Additional research is required to optimize family number for the breeding program.

5.4.3.2. Test sites and GxE

Because of the husbandry issues, poor security, and mortality associated with on-farm testing and transportation of candidates, we implemented a freshwater nucleus breeding strategy in for G5 using BLUP on sib data from brackish water ponds (Fig. 1). We intended to insulate selection candidates from stressful conditions related to high variation in the salinity of brackish water ponds and transportation to the mating site in freshwater. Trọng et al. (2013b) applied a similar approach for Nile tilapia selection for harvest weight using sib- testing in river-cages and a secure nucleus in ponds. If the nucleus environment is similar enough to the commercial production environment, the between environment genetic correlation will be close to one (i.e. no GxE) and we could simply select the best fish in nucleus. On the other hand, if the nucleus environment differs markedly from the production environments, the between-environment genetic correlation will be lower than one, and sib-testing is required.

Our G5 results showed that Sukamandi strain grows faster in brackish water than in freshwater ponds (Setyawan et al., 2022a). Apparently, the brackish water environment is not a stressful condition for this strain, which also can be seen from its high survival rate of 77.07% in brackish water ponds (Setyawan et al., 2022a). Furthermore, the genetic correlation between fresh and brackish water growth is substantially lower than one (0.66). This suggests that sib-testing in brackish water is necessary because genetic gain in fresh water would only be 66% effective in brackish water. In addition, the additive genetic variance in brackish water was higher than in freshwater, which should increase the rate of genetic gain.

After performing the G5 experiment at a more secure site with better husbandry practices, we were able to return the candidates to the freshwater breeding site with minimal

mortality, and select among them using BLUP EBVs that incorporated their own records. We also tested G6 in two different farming systems which are commonly practiced by shrimp-tilapia farmers: monoculture and coculture with shrimp. Coculture between tilapia and shrimp is widely farmed in brackish water ponds (Cruz et al., 2008; Ferreira et al., 2015; Rosales et al., 2019), and it is the most practiced farming system in Central and East Java (Setyawan et al., 2022b). In this study, the genetic correlation between growth in monoculture and coculture system was 0.99 (Table 5). This extremely high genetic correlation indicates that there is almost no genotype by environment interaction for these two environments indicating that selection process in monoculture system will also produce parents that have a similar progeny performance in coculture systems.

5.4.3.3. Optimal contributions selection

Parent contribution to the selection candidate population can be managed effectively a breeding strategy using optimum contribution selection (OCS) method (Meuwissen, 1997). This method is an effective way of finding a balance between the optimum genetic gains and the rate of inbreeding (Wang et al., 2017). OCS is effective to control inbreeding rate over the generations (Yoshida et al., 2020). The change from mass selection to pedigree-based BLUP also allowed us to implement optimal contributions selection and completely avoid short-term inbreeding so far.

5.4.3.4. Evaluation of the improved breeding program

Genetic gain and genetic parameters

Figure 4 is based on the trans-generational analysis of pooled data (i.e. Model 4) and shows that the rate of genetic gain between G5 and G6 when we used pedigree-based BLUP to estimate the breeding values for brackish water harvest weight of G5 parents using Model 2 was much higher than the genetic gain between G4 and G5 when we used mass selection of G4 parents using only own-records for brackish water harvest weight. Furthermore, in agreement with our variance component estimates and the modest genetic correlation

between fresh and brackish water harvest weight, the response to selection in brackish water harvest weight is larger than the response to selection in fresh water harvest weight.

Model 4 also resulted a high estimated heritability of harvest weight at 0.53 in brackish water environment and shows a highest evolvability (GCV) at 15.7 (Table 5). GCV indicates the ability of particular trait to react to selection pressure (Cheung, 2017; Houle, 1992).

Bias and accuracy of breeding values

The breeding values estimated using Model 4 are nearly unbiased with regression coefficients between mid-parent EBVs for brackish water and freshwater on their progeny performance very close to 1 (Table 7, Fig. 5). The accuracy of selection based on Model 4 was 77.66% and 65.65% in brackish water and freshwater, respectively, and the difference between them is due to a combination of factors. First, the heritability of harvest weight in brackish water (0.529) is considerably higher than in fresh water (0.394), and accuracy is directly related to heritability. Second, after pooling the datasets, many more fish had records for harvest weight in brackish water than in freshwater.

5.4.3.5. Next steps for the future of the breeding program

Future responsibility for, infrastructure, and design of the breeding are currently in flux due to a major re-organization of the Indonesian research and innovation system, particularly the formation in 2019 of the National Research and Innovation Agency (Indonesian: Badan Riset dan Inovasi Nasional, BRIN), a cabinet-level government agency that fuses many pre-existing scientific agencies into it's a single body. Prior to this re-structuring, the development of the salinity-tolerant Sukamandi tilapia strain was led by the Research Institute for Fish Breeding (BRPI, Balai Riset Pemuliaan Ikan in Indonesian) under the Ministry of Marine Affairs and Fisheries, and all six generations of selection candidates have been produced in their freshwater hatchery. The first four were tested at commercial farm sites, which proved unsatisfactory, and some of the G5 and all of G6 fish were tested at the Technical Implementation Unit of Brackish Water Culture Karawang, and Aquaculture Production Business Service Center, Karawang through collaborative agreements to use their secure brackish water ponds suitable for both experimental work and ongoing genetic

evaluations. Given the differences in the missions and responsibilities between research organizations (BRIN) and production and dissemination facilities (BRPI, Karawang), new arrangements and agreements must be developed, and these will influence the structure of the breeding program.

While fingerlings of the Sukamandi strain grow well in brackish water up to 25 ppt, we observed that fertilization and early embryonic development is inhibited at salinities above 10-15 ppt, so a low salinity or freshwater hatchery capable of separately rearing families to tagging size such as the facilities at BRPI in Sukamandi are required. The optimum salinity for fertilization and hatching rate of Nile tilapia is at around 9 ppt at temperature 27^0C (Hui et al., 2014). However, this makes it necessary to move fingerlings to brackish water test sites for evaluation, and depending on whether or not practical and biosecurity considerations allow for returning them to the hatchery for spawning, a biosecure nucleus site to manage the candidate population and sib-testing to estimate their breeding values. Either way, given the substantial GxE between freshwater and brackish water either candidates themselves or their sibs must be evaluated in brackish water. However, testing candidates in brackish water ponds must also take into account physical security to prevent theft, bio-security and transportation-related mortality when returning candidates to the freshwater nucleus. It is, therefore, crucial to either provide a secure brackish water breeding facility with access to fresh water for spawning to allow the tested fish to be used as candidates and estimate breeding values using their own performance records or to maintain a secure nucleus population in freshwater and estimate their breeding values using sib data to avoid having small numbers of selection candidates as in the first to fourth generations of the program.

The high heritability of harvest weight in brackish water indicates that the Sukamandi strain harbours sufficient additive variance to support a response to selection in future generations using either approach.

Considering that a majority of brackish water farmers in Indonesia grow tilapia in a co-culture system which on average has a higher salinity at around 20-25 ppt than in our ponds

(± 16 ppt), the future selection should be conducted at the same salinity or higher than the current farming system. However because the genotype by environment interaction between mono- and co-culture is virtually non-existent, selection in monoculture will produce a similar performance as in co-culture system to keep things as simple as possible.

5.5. Conclusion

The Indonesian breeding program for salinity tolerant tilapia to re-purpose failed shrimp ponds has been evolving and improving through the development of a more efficient mating strategy to increase the selection intensity and the adoption of pedigree-based BLUP to increase the accuracy of estimated breeding values, substantially increasing the response to selection. The substantial degree of GxE between fresh and brackish water requires either candidate testing or sib-testing in brackish conditions. A traditional, freshwater nucleus-based breeding program is feasible. However, if candidates tested at secure brackish water testing site can be either sagely returned to the nucleus or a brackish water hatchery can produce fingerlings in fresher water, a traditional nucleus may not be necessary. There is no GxE interaction between monoculture and co-culture systems in this strain and the selection process can be conducted in monoculture systems for its simplicity. The improved breeding strategy using P-BLUP resulted a genetic gain for around 24.8g for the sixth generation of Sukamandi strain in brackish water ponds and a correlated response in freshwater for around 19.5g.

5.6. Acknowledgements

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Chapter 6

General Discussion

6.1. Introduction

Brackish water areas offer an enormous potential for aquaculture in Indonesia, which are utilized mainly for shrimp production as this is the most valuable aquaculture species. However, recurrent farming failures caused by shrimp diseases have driven shrimp farmers to adopt tilapia production and develop a different farming system. To be successful, improvement of tilapia for unpredictable conditions, especially salinity levels, in the shrimp farm environment is essential. The results presented in this thesis provide insight into the application and improvement of the selection method to improve salinity tolerance of tilapia for brackish water aquaculture. In chapter 2 we found that there is a lot of interest to adopt tilapia in shrimp farms as an alternative species, and polyculture is the most adopted system. Poly-rotation farming system provides the highest gross profit. In chapter 3, we show that we have successfully selected a tilapia strain that grows well in brackish water. We also evaluated the genotype by environment (GxE) interaction between the breeding nucleus, which is in freshwater, and the commercial production system in brackish water. The difference between these two environments is not just the salinity but also other farm conditions are different, such as the abundance of phytoplankton and zooplankton, the variation and composition of natural foods, light intensity, etc. In chapter 4, we investigated the physiological responses of progeny of high contrast groups based on their breeding values as Fresh Water specialists (F), Brackish Water specialists (B) and generalists (G) when exposed to a brackish water and freshwater environments. Tilapia selected for higher growth in brackish water have more effective osmoregulation than tilapia selected for higher growth in freshwater. In chapter 5, we estimated the genetic trend across generations based on BLUP selection. Using Pedigree BLUP in tilapia breeding resulted in a low bias and high accuracy of selection. Finally, in this chapter, findings in Chapter 2 to 5 are used to discuss how to develop an effective and feasible tilapia breeding program in Indonesia for salinity tolerance.

6.2. Farming tilapia in shrimp farm environments and its challenges

Tilapia is the best candidate species for brackish water farming due to several advantages. Several species in the genus Oreochromis, commonly referred to as tilapia, have high salinity tolerance, low mortality, rapid growth, and are easy to farm (Cnaani & Hulata, 2011; El-Sayed, 2006; Popma & Masser, 1999; Stickney, 1986; Suresh & Lin, 1992; Watanabe et al., 1985). As a result, tilapia has been adopted in brackish water ponds as an alternative species for shrimp. Our result in Chapter 2, in particular in Banten, showed that most shrimp farmers have shifted to tilapia due to its simplicity to farm and with lower risk than farming shrimp. Based on the response, more than half of the shrimp farmers in this regency were badly hit by recurrent farming failures. The simplicity of tilapia farming was also shown in an experiment using a total of 22 trials adopting tilapia in shrimp ponds in Aceh, Indonesia, which resulted in good growth and survival at salinities below 20 ppt (Rimmer et al., 2012). In addition, a rotational farming system between shrimp and tilapia also has been applied as an effective biological control when the use of antibiotics and other chemicals in controlling shrimp pathogens becomes ineffective (Paclibare et al., 1998). It appears that tilapia can cope well with the high salinity environment because they can grow and reproduce in brackish water ponds. During the first to fourth generation of selection we found spawned females holding eggs or larvae in their mouth while in brackish water ponds at salinity between 20 and 25 ppt. The number of larvae observed were however much lower than in freshwater ponds. A study by Mashaii et al. (2016) using 444 spawning events showed that Nile tilapia had successful reproduction at 11.5 ppt with spawning being most frequent at a female body weight between 60g and 500g. Another species of tilapia, *Tilapia guineensis* also has successful reproduction throughout the year without interruption in Ivory Coast brackish water at salinity of up to 15 ppt (Legendre & Ecoutin, 1989). A clutch-removal management technique by collecting of eggs or newly hatched fry form the mouths of female tilapia at regular intervals, followed by artificial incubation is technically feasible to produce tilapia seed in salinities up to 18 ppt (Suresh & Lin, 1992).

Adopting tilapia in a shrimp farm environment provides economic returns to the farmers. This financial profit is crucial for small scale farmers that according to (MMAF, 2022) constitute more than 65% of brackish water farmers in Indonesia. This group of farmers is economically more vulnerable to farming failures; therefore, they adopted tilapia which is

considered a less valuable farmed species than shrimp, but it has lower risk. High growth rate and low mortality rate in tilapia can generate significant profit in shrimp ponds. In Aceh, around 64% of ponds generated a profit from mixed tilapia shrimp farming that was comparable to traditional shrimp culture (Rimmer et al., 2012). The profit of shrimp farming can also be increased by using a rotation system (Paclibare et al., 1998). Approximately a quarter of shrimp farmers in West Java and Central Java have adopted a mono-rotation system in which they grow tilapia in the rainy season and shrimp in the dry season (Setyawan et al., 2022). In this thesis I found gross profit in the poly-rotation farming system to be nearly double the gross profit of mono-rotation system (Chapter 2). Therefore, I conclude that the poly-rotation farming system should be systematically promoted by fisheries extension services as a solution for small scale farmers that experience frequent shrimp farming failures.

Beside the opportunities above, farming tilapia in brackish water ponds faces several challenges related to high salinity that results in reduction in growth, survival, reproduction and other physiological functions (Boeuf & Payan, 2001; Cnaani & Hulata, 2011; Cruz et al., 2008; Popma & Masser, 1999). Brackish water ponds are a hyperosmotic condition for tilapia. Literature generally indicates that low growth performance at high salinity concentrations is related to osmoregulation that is needed to maintain the internal balance of salt content (Cnaani & Hulata, 2011; Cruz et al., 2008; küçük et al., 2013). Osmoregulation is an energy demanding process: it is estimated to consume more than 25% of the total energy obtained from feed (Cnaani & Hulata, 2011). Unfortunately, rather than eating more feed to acquire more energy, tilapia tend to reduce their feed consumption in high salinity. It seems that stress under such condition leads to reduction in appetite and more illness. A study by Kammerer et al. (2010) showed that cortisol concentration, an essential component of the stress response, increased rapidly by three hours after increasing salinity and remained elevated for three days. Allocated energy for osmoregulation in combination with reduction in feed consumption during hyperosmotic stress contribute to low growth rate. In chapter 4, I observed that the average DGC in brackish water was significantly lower

than in freshwater. To improve growth rate, a high salinity tolerant strain should be optimized to use the energy from feed for growth rather than for osmoregulation.

To summarize, farming tilapia in shrimp ponds has several advantages that contribute to farm profit for small scale farmers. However, tilapia suffer from hyperosmotic stress which results in lower growth rate. The farm profit could be increased if a high salinity-tolerant tilapia that requires less energy for osmoregulation was used.

6.3. Tilapia breeding program for high salinity ponds

Low risk and potential income from farming tilapia in shrimp ponds have attracted more farmers to adopt tilapia in brackish water (Rimmer et al., 2012). Fortunately, a huge area with potential for aquaculture in brackish water is available. According to MMAF (2022), the percentage of land usage for aquaculture is only 10.2% out of the potential area of 2.8 million hectares. Most of the unused land in the brackish water area can probably be used for tilapia farming. A study in Maros Regency, South Sulawesi Province in Indonesia, showed that in the rainy season, around 8% of brackish water ponds can be classified as highly suitable for farming tilapia, and the remaining 92% as moderately suitable. Whereas in dry season, around 72% of brackish water ponds is still moderately suitable and only 28% of the area is classified as not suitable for farming tilapia (A. Mustafa, 2020).

The lack of availability of salinity-tolerant tilapia strains is a serious impediment for tilapia farming in brackish water ponds. So far, most shrimp farmers are growing freshwater tilapia. A survey involving 224 shrimp farmers in four regencies in Java Island showed that 75% of the respondents are farming a freshwater tilapia strain. Farmers reported that growing freshwater tilapia in a hyperosmotic environment is a main factor contributing to the very low survival rate. The two salinity-tolerant tilapia strains which have been systematically distributed by the Ministry of Marine Affairs and Fisheries, i.e. Salina and Srikandi, have been reported to have a better productivity compared to freshwater tilapia. These two brackish water tilapia strains were developed using hybridization between Nile tilapia and a more saline tolerant tilapia strain. The Salina strain uses red tilapia (*Oreochromis sp*.) females which has higher salinity tolerance than Nile tilapia (MMAF, 2014). Whereas the

Srikandi strain is a hybrid between Nile tilapia and the Sukamandi strain that I studied. This strain contains salinity tolerance genes originating from blue tilapia (Yu et al., 2022). However, the harvest yield of these two salinity-tolerant tilapia strains is reported as inconsistent between many different brackish water ponds. High salinity and large fluctuation in salinity are typical conditions for traditional shrimp ponds and can be a challenge for these strains. The currently adopted tilapia strains need to be developed further through an improved breeding program to increase growth performance in brackish water environment.

In addition to the Salina and Sukamandi strains, several tilapia breeding programs have successfully produced more tolerant strains for brackish water ponds. In Vietnam, a synthetic population of tilapia from the GIFT strain, Taiwan strain and NOVIT4 (GIFT-derived) strain has been selected for growth in brackish water (15-20 ppt) since 2007, resulting in a genetic gain per generation of 10 to 13.3% over four generations of selection from 2008 to 2011 (Ninh et al., 2014). Another study on Nile tilapia that had been selected over five generations in medium salinity (10-20 ppt), reported an increase of harvest weight of 7% per generation (Thoa et al., 2016). In the Philippines, a Molobicus strain was selected for high salinity tolerance and growth rate. This strain was produced in 1999 using a series of backcrosses between the hybrid (*O. mossambicus* x *O. niloticus*) and *O. mossambicus* (Bartie et al., 2020; de Verdal et al., 2014).

In Indonesia a breeding program for salinity tolerant tilapia was initially conducted with red tilapia. The first generation of selection resulted in a genetic gain of 33.1g in males and 11.7g in females (Robisalmi & Dewi, 2014). However, this program was terminated because red tilapia is not preferred by farmers. Red fish are easily visible in brackish water and the behaviour of tilapia seeds to swim in a group near the water surface attracts predators resulting in significantly lower survival rates than black colour tilapia. The Sukamandi strain which has black colour is therefore more accepted by the farmers.

A salinity-tolerant tilapia strain is crucial to exploit the huge potential of aquaculture production in brackish water ponds in Indonesia. Selection is an effective way to produce this required strain. In the next section, I will discuss the design of such a tilapia breeding program to achieve this goal.

6.4. A design for a sustainable tilapia breeding program in Indonesia

Feasibility is a key factor for a successful breeding program. This is because a breeding program is very complex, and needs a lot of investment and commitment (Mueller et al., 2015). It is very challenging to carry out all activities from mating to selection of the breeding candidates in each generation, in particular in developing countries. Technical aspects and facilities often become main obstacles. In addition, qualified breeders and geneticists are in short supply, and genetics as well as genomics education are not well developed in developing countries (Cutiongco-de la Paz et al., 2022). It is also difficult to maintain a long-term commitment for the program. In many cases, genetic improvement programs stop to operate or move no further forward after a few years due to political changes, lack of funding, and the preference to generate outputs within a short time period (Hartmann & Linn, 2007). Community based breeding programs (CBBP) have been shown to be a viable strategy to implement breeding programs for small ruminants in smallholder production systems (Haile et al., 2020; Mueller et al., 2015). However, CBBP does not seem to be a viable solution for tilapia for several reasons. First, holding tilapia broodstock and selection candidates in one place (farm or pond) is less complicated than for livestock. Second, many tilapia females can be spawned at the same time in a relatively small pond and can each produce hundreds of progeny. Third, fish cannot be individually recognized and therefore ownership is difficult to establish, making community-based breeding problematic.

In Indonesia, one of the main issues has been the change in political direction resulting in termination of the program. To the best of my knowledge, all breeding programs for economically important freshwater fish such as tilapia, catfish and carp in Indonesia were organised by Directorate General of Aquaculture, under the Ministry of Marine Affairs and Fisheries. They used to share the progress of their program in an annual meeting that was organised by the Directorate General of Aquaculture. However, this annual meeting was

stopped in 2014 because of policy changes. Since then, it has been challenging to monitor tilapia breeding programs in Indonesia. Thus, for the sustainability of genetic improvement in the next generations, I believe that the program needs to involve a group of farmers which have a strong commitment to the breeding program. Direct benefit of the program to their farm will encourage them to continue the program. Focus group discussions during the survey in Chapter 2 identified potential groups of farmers that have a solid cooperation, have dedicated ponds for seed preparation for their group and are ready to run a breeding program with supervision from qualified breeder and fisheries extension services.

Nevertheless, an applicable, inexpensive and effective design for the selection of a salinity-tolerant strain needs to be developed to ensure the sustainability of the program. Next, I will discuss a feasible breeding design for the Sukamandi strain following the basic steps of breeding program design according to Figure 1 in Oldenbroek and van der Waaij (2014). The very first step is defining the production system. The production environment for the selection candidates should be similar or close to the commercial production system to maximise the potential genetic of the obtained strain. Chapter 2 clearly showed that most farmers adopt tilapia in polyculture with shrimp, in particular in East Java and Central Java. The average salinity in their polyculture system is higher (20-25ppt) than in the ponds where selection candidates from the fifth generation of the Sukamandi strain were grown (16 ppt, Chapter 3). This suggests that future selection should use a higher salinity that is more similar with the commercial production ponds. Fortunately, our estimation showed that the genotype by environment (GxE) interaction between monoculture and polyculture is almost absent (Chapter 5), meaning that the holding environment for selection candidates can be a monoculture system and it is not worthwhile to evaluate sib performance in a polyculture system. A similar study on Nile tilapia showed a genetic correlation of body weight of 0.92 between holding pond in brackish water (15-20 ppt) and testing pond in freshwater (Thoa et al., 2016). Our estimates showed a lower genetic correlation of harvest weight between brackish water and freshwater of 0.65 (Chapter 3). This indicated that selection can be conducted in freshwater but the sib information from brackish water is required. Separate

breeding programs are necessary if the genetic correlation is less than 0.61 (Mulder et al., 2006).

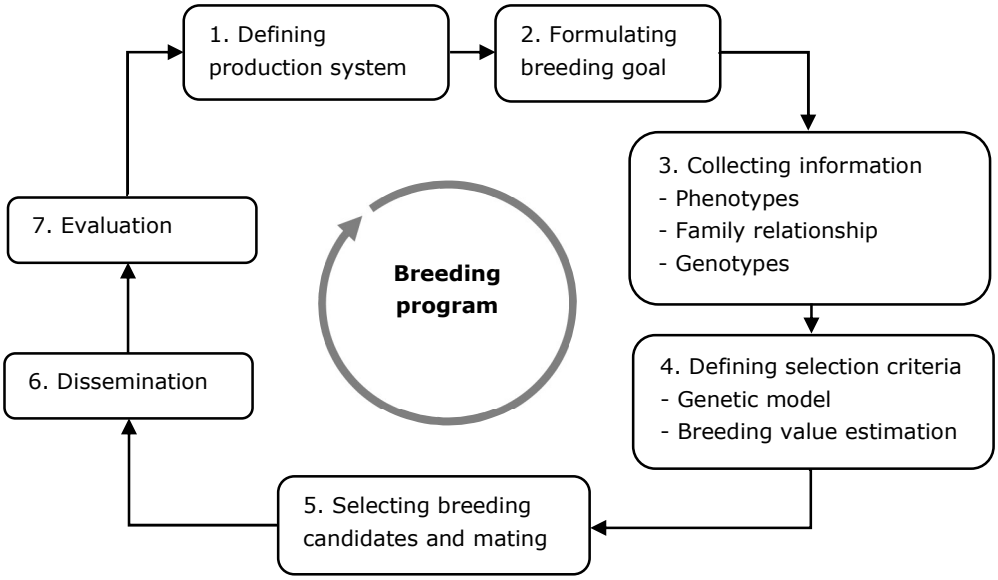

Figure 1. Basic scheme to develop and organise a successful breeding program (Oldenbroek & van der Waaij, 2014).

Definition of the desired traits for improvement is the next step. Focussing on harvest weight as the most important trait makes it simpler to involve the farmers in the next generation of selection, and this will bring several advantages. Harvest weight has a strong positive correlation with other traits, which means that selection on this trait will also improve those correlated traits. Body weight has a strong genetic correlation with fillet weight at 0.99 (Rutten et al., 2005), 0.96 (Nguyen et al., 2010; Thodesen et al., 2012), body depth at 0.95 and body width at 0.82 (Nguyen et al., 2010). Body weight also has a large genetic correlation with fillet yield at 0.74 (Rutten et al., 2005). Increasing growth expressed as higher harvest weight is likely to increase feed efficiency (Besson et al., 2016; de Verdal et al., 2018). Body weight is a trait which is easy to measure and can be recorded by the farmers directly in the pond. The heritability of harvest weight in Sukamandi strain is relatively high at 0.35-0.38 (Chapter 3) which means that selection will produce an obvious genetic gain in the next generation. Growth expressed as harvest weight has a big economic

impact and is relatively easy to record on selection candidates (Gjedrem & Robinson, 2014). The very first breeding program on tilapia organized by ICLARM in Philippines in 1986 was also primarily focused on growth (Gjedrem, 2012). In theory, a significant genetic improvement of tilapia growth can be achieved in five years or five consecutive generations (Dey & Gupta, 2000). Therefore, the potential economic impact and a noticeable higher growth rate after e.g., three more generations of selection in the Sukamandi strain will draw more attention from all stakeholders and government to support this program.

In general, adding pedigree information will increase the accuracy of the estimated breeding values. Harvest weight is relatively easy for farmers to record on their farm and family relationship information can be obtained when using PIT tags. Further, on farm measurement can be improved using image analysis. Pictures can be easily collected by the farmers. Then, a data technician needs to transform the information from each picture using image analysis software before using them in the genetic analysis. A recent study by Gulzari et al. (2022) using a dedicated machine for image collection resulted in high phenotypic and genetic correlations between measured and predicted phenotypes at 0.98 and 0.996 for harvest weight, and 0.93 and 0.99 for fillet weight. A simple image recording method can be applied in the Sukamandi program, and this technique can significantly reduce handling time and mortality due to handling stress. Another potential source of information is DNA sequence or genotype information that can be used to develop a genomic selection program. In the sixth and fifth generation of Sukamandi strain, we collected fin clips for future genomic evaluation which is not included in this thesis. Using genomic information can increase the accuracy of breeding value estimation by up to 34% compared to only using pedigree information (Barría et al., 2021). However, fin clips cannot be easily collected by the farmers without potential contaminant. Genomic analysis is also expensive and a cooperation with third parties is required to collect and analyse the sample for breeding value estimation. Finally, the Nagoya protocol sets strict limitations on the use of biological material outside the country of origin. For these reasons, I see less opportunities for genomic selection in our breeding program for salinity tolerant tilapia in Indonesia.

Overall, an improved tilapia breeding program using P-BLUP selection is a feasible and cost-effective strategy for the Sukamandi strain. Focus on growth related traits using a simple image collection method is also feasible and can be implemented on farm. Further, involving shrimp-tilapia farmers is a potential solution for a sustainable breeding program in the future. How to disseminate the results of the breeding program to the farmers will be discussed in the next section.

6.5. Dissemination and fry production for brackish water farms

The availability of high-quality tilapia fry specifically for a saline environment is a major bottle neck for farming tilapia in brackish water ponds. The current salinity-tolerant tilapia strains are not well distributed, and it is very difficult to find these strains in the market even in Java and Sumatra islands. These two islands are the largest seed producers for all fish species in Indonesia producing 46% (Java) and 44% (Sumatra) of the total seeds in Indonesia in 2019 (MMAF, 2022). In addition, total tilapia seed production in freshwater ponds is second after catfish at 40.7 billion. However, this number is far from sufficient to meet the farmers' needs for their ponds. The availability of tilapia fingerling in the market is limited by the number of fish producers and their seed production capacity. The number of tilapia producers in Indonesia is much lower than producers of catfish and carps. The biological aspect of tilapia reproduction seems to be the main reason that discourages fish farmers to produce tilapia seed. Tilapia has a relatively large egg size with low absolute fecundity between 678 to 2,086 eggs per female per spawn, depending on body weight (Shoko et al., 2015). The fecundity ranged from 178 to 1898 eggs at the average body weight between 78 and 501g (Duponchelle et al., 2000). Whereas African catfish has the average fecundity at around 25,664 eggs per female (Lawrence Ejeh, 2017). This reproduction aspect significantly influences the lower production capacity of tilapia seed compared to other species. In addition, the seed price of tilapia and other commercial species at the same size is relatively similar.

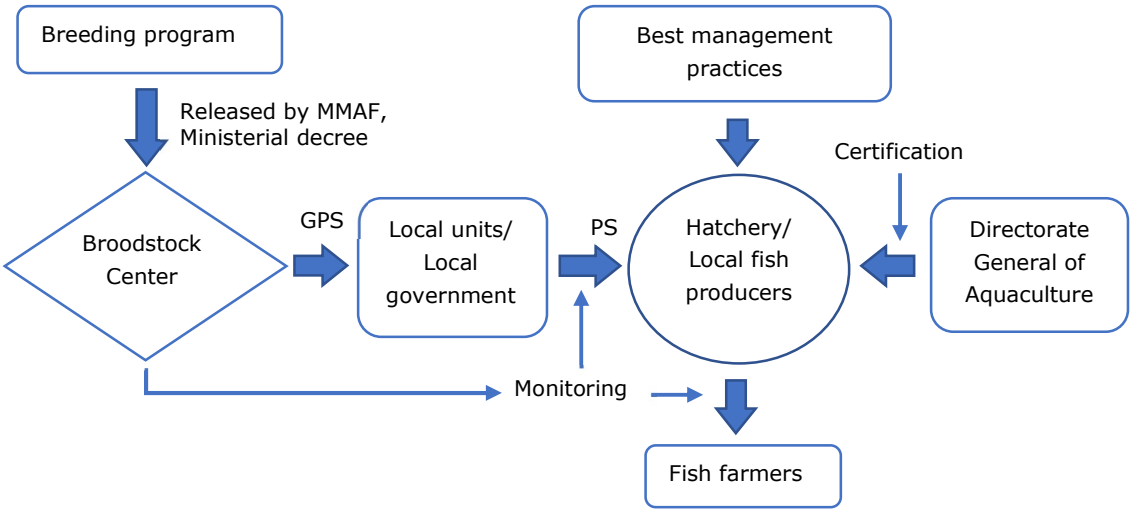

Figure 2. The scheme for the dissemination of qualified strains to fish farmers in Indonesia. GPS: grandparent stock, PS: parent stock, BMFP: best management practices for fish production.

A successful distribution of the genetically improved tilapia strains will benefit the seed availability in the market. However, the distribution of salinity-tolerant tilapia seeds faces some serious obstacles. We have produced Srikandi strain as a hybrid between Nile tilapia female and Sukamandi strain that has an obvious greater growth rate after sixth generations of selection. The distribution of this strain follows the dissemination scheme implemented by the MMAF (Fig. 2). This scheme is meant to control the distribution and the quality of the strain. However, the multiplication of the selected broodstock to produce grandparent stock (GPS) was not always successful due to time and budget constraints, which caused an insufficient number of parent stock (PS) that are progeny of the GPS. As a result, the released strains are not available in every regency to meet the farmers' needs. The scheme in Figure 2 should be simplified to allow wide distribution of a new strain without neglecting the seed quality. A simple scheme consisting of a certified breeder or breeding institution, groups of farmers as multipliers, and fish producers will reduce cost and time due to administration and procedures. Broodstock quality and distribution should be monitored directly by the breeding institution or certified breeders. Further, close cooperation with

the group of farmers with a direct interest in the breeding program is required to avoid selling unrelated fish in place of the high-quality strain which often occurs with a strong market demand. In this scheme, the government through the Directorate General of Aquaculture can play its role in monitoring the distribution of each strain by organising annual stakeholder meetings where all actors in the value chain participate.

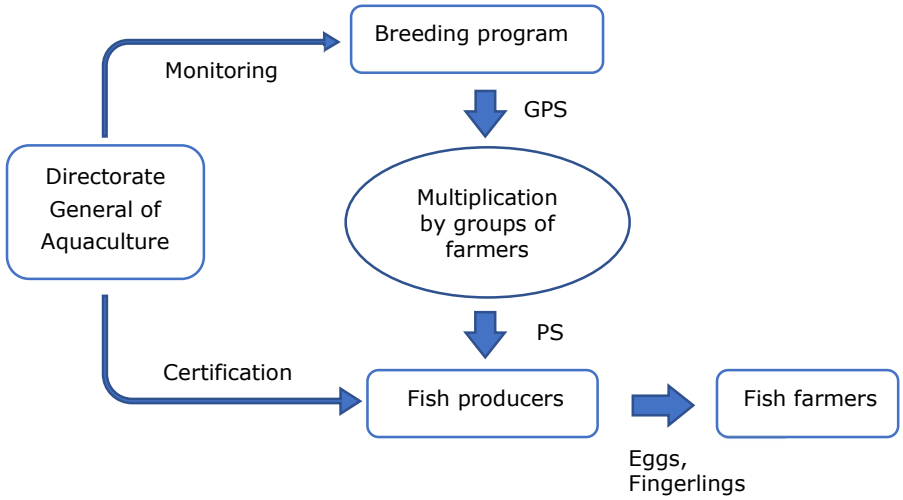

Figure 3. The simplified scheme for the dissemination of qualified strains to fish farmers. GPS: grandparent stock, PS: parent stock.

Seed production of the Sukamandi strain could be increased further to meet the market demand through several strategies. First, by optimizing the production capacity using adjustments in tilapia reproduction such as implementing a shelter that has successfully increased the number of spawned females (Chapter 2), using a high-quality fish feed, using the most productive broodstock between 7 months and 24 months old, and using a certified broodstock to ensure the quality of the broodstock. Second, by using an optimum mating combination. Currently, the fish producers in Indonesia use the common standard with 100 males and 300 females. I have shown that the number of females can be increased using a ratio of 1 male to 5 females. It remains to be tested if this ratio also works for larger numbers of fish. Third, by properly monitoring the distribution routes to ensure that the fish farmers receive a high-quality seed.

6.6. Concluding remarks

The brackish water area offers a huge potential for aquaculture of shrimp in combination with tilapia. In this thesis, I highlight the three important factors for achieving this goal, determining the most suitable farming system, establishing a feasible breeding program to produce salinity-tolerant tilapia, and making high-quality seed available in the market through an effective distribution strategy. Farmers' experiences of shrimp farming failures in the past led them to develop a poly-rotation system. This system can secure the farmers' livelihood through a lower failure risk and more productive ponds compared to farming only shrimp year-round. The use of a lower shrimp density in this system will reduce the risk of disease outbreaks, reduce effluent, and reduce the risk of land and environmental degradation which will benefit sustainable farming. In addition, these studies provide knowledge on the progress and improvement of the previous breeding strategy by using BLUP selection which is still an achievable and cost-effective strategy to be implemented in Indonesia. I suggest a feasible breeding program which involves groups of farmers who receive direct benefits from the program, which can encourage them to support the sustainability of the program. However, in addition to the salinity tolerance, the availability of tilapia fingerling is, and will remain a major bottle neck for tilapia farming in brackish water ponds unless a more simplified strategy for the dissemination of high-quality seeds to shrimp-tilapia farmers is adopted.

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Summary

Aquaculture in crucial for global food and nutrition security in the future. Therefore, aquaculture needs to increase its production in a sustainable way to meet the fast-growing population. Brackish water area has a great potential for the future of aquaculture production in Indonesia, which is the third largest fish producer in the world. However the development of shrimp farming, the most common farmed aquaculture species in brackish water, has been impeded by diseases outbreaks and has caused repeated farming failures and financial problems. Farming tilapia has been adopted as a viable solution to secure farmers' livelihood. At the same time, farming tilapia in brackish water also contributes to solve the problem of a shortage of freshwater ponds. The government of Indonesia has developed two salinity-tolerant tilapia strains to meet the farmers' need. Nevertheless, the current tilapia strains for brackish water are reported as having inconsistent performance in many different ponds. In addition, there is lack of availability of tilapia seeds in the market due to unsuccessful dissemination and distribution of these strains. Therefore, an improvement of the current breeding program and adjusting of the current dissemination strategy are required. The aims of this thesis are to provide an insight into the commercial production system in brackish water ponds, a feasible strategy to develop a selective breeding program that is cost-effective and sustainable for many generations, and to provide an effective dissemination strategy to distribute the selection results to shrimp-tilapia farmers.

In **Chapter 2** we organized focus group discussions involving 224 farmers from four provinces in Java. A questionnaire consisted of a set of structured questions was used to collect general information on shrimp-tilapia farmers and the characteristic of theirs ponds, the developed farming systems, and their income status. From the collected information we calculate farm-level gross profits for each farm in four different farming systems. We show that poly-rotation and polyculture are the most used farming systems, and that the poly-rotation is the most profitable system despite this system requiring a higher investment and more farm inputs. We also show that the farmers' decision to adopt a

farming system was influenced by their past experiences of farming failures. We suggest that the poly-rotation system should be systematically promoted using government policy, involving fisheries extension services to actively assist the implementation of this system in brackish water ponds.

In **Chapter 3**, we study the genetic parameters and genotype by environment (GxE) interaction between brackish water and freshwater ponds. We also improve the breeding strategy of the Sukamandi strain using pedigree BLUB selection, to include sib information from brackish water ponds to estimate the breeding values of selection candidates kept in freshwater. We show that brackish wates ponds provide a better support for fish grow, resulting in higher growth performance. The higher growth in brackish water ponds is likely not due to salinity per se, but more likely the effect of other differences between the pond environments. The heritability of traits are moderate in both environment ranged from 0.35 to 0.50 and the genetic correlations between brackish water and freshwater were between 0.65 and 0.75. We show that substantial GxE interaction exists for growth between brackish water and freshwater which suggest that sib information from brackish water should be incorporated to select candidates kept in freshwater.

In **Chapter 4**, we investigated physiological responses in freshwater and brackish water to study salinity tolerance of the Sukamandi strain. We use three groups of progeny from contrasting parent groups. The three groups were 1) generalist which was selected to be the best candidates across the two environments, 2) brackish water specialist which have much higher EBV for brackish water compared to EBV for freshwater, and 3) freshwater specialist which have much higher EBV for freshwater compared to EBV for brackish water. After 28 days, brackish water significantly reduced fish growth, compared to freshwater. We also show that across all groups, brackish water reared fish had higher Na^+ and Cl^- concentrations in blood plasma compared to freshwater reared fish. Furthermore, in brackish water environment, the brackish water specialists had lower level of Na^+/K^+-ATPase in both intestine and gill, higher blood ion concentration and higher growth. These findings suggest that tilapia selected for higher growth in brackish water show a more efficient osmoregulation process than tilapia selected for higher growth in freshwater.

In **Chapter 5**, we study the evolution of salinity-tolerant strain breeding program and the progress achieved after six generations of selection in brackish water ponds. We show the improvement of breeding strategy in the fifth and sixth generation using pedigree-based BLUP selection resulting in genetic gain for harvest weight of 24.8g, much higher than genetic gain per generation in the first to fourth generation. Furthermore, our estimation show the accuracy of selection in brackish water and freshwater were 78% and 66%, respectively.

In **Chapter 6**, the general discussion, I discuss the implementation of tilapia production in brackish water ponds, suggesting an improvement of the breeding strategy to produce a better growth performance of salinity-tolerant tilapia. I discuss the characteristics of the commercial production system of farming tilapia in brackish water ponds. Furthermore, I discuss the improvement of breeding strategy using pedigree BLUP selection for the fifth to sixth generations using sib information from the commercial production system that resulted in higher genetic gain than the previous generations of selection that used phenotypes only. I also discuss a feasible breeding strategy taking into account the sustainability of the program, involving groups or farmers who are interested in the breeding program and will received direct benefits from the program. Lastly, I present concluding remarks and suggest a simplified dissemination strategy to distribute the breeding outputs to shrimp-tilapia farmers.