JIPK. Volume 15 No 1. April 2023
Sinta 1 (Decree No: 158/E/KPT/2021)
e-ISSN:2528-0759; p-ISSN:2085-5842
Available online at https://e-journal.unair.ac.id/JIPK

JIPK
(JURNAL ILMIAH PERIKANAN DAN KELAUTAN)
Scientific Journal of Fisheries and Marine

Research Article

Effect of Dosages and Temperatures on Simalungun Red Ginger (*Zingiber officinale* var. Rubrum) Ethanol Extract Dipping on Masculinization of Zebra Fish (*Danio rerio*) as a Model Fish

Eka Fitria Febriani[1]*, Maheno Sri Widodo[2], and Abdul Rahem Faqih[2]

[1]Magister Aquaculture Department, Faculty of Fisheries and Marine Sciences, Brawijaya University, Malang, 65145. Indonesia
[2]Aquaculture Department, Faculty of Fisheries and Marine Sciences, Brawijaya University, Malang, 65145. Indonesia

OPEN ACCESS

ARTICLE INFO

Received: August 13, 2022
Accepted: September, 16 2022
Published: October 17, 2022
Available online: January 29, 2023

*) Corresponding author:
E-mail: efitria54@student.ub.ac.id

Keywords:
Testosterone Levels
Fish Reproduction
Sophoraflavonone G
Sitosterol Acetate

Abstract

Ornamental fish are known to be easily cultivated in aquarium or pond. Many people prefer male (MF) over female fish (FF) due to their aesthetic value and productivity which lead to better pricing. The aim of this study was to determine the effect of Simalungun Red Ginger (*Zingiber officinale* var. Rubrum) (SRG) on masculinization. This study used dipping method which was carried out using Simalungun Red Ginger (*Zingiber officinale* var. Rubrum) (SRG). The temperature treatments used in this study were 28°C and 32°C. For this research, an experimental study with a Completely Randomized Factorial Design (CRFD) method that had two independent variables such as different dosages and temperatures was used. From five dosages (0 mg/L; 5 mg/L; 10 mg/L; 15 mg/L; 20 mg/L) and three temperature treatments (ambient temperature (about 25°C; 28°C; 32°C), it was found that the highest male percentage of primary sexuality (88.33%), secondary sexuality (85%), and highest total testosterone levels (TL) (1.986 ng/L) were at the treatments with the highest dosages and temperature, while for the highest survival rate were at almost in all B treatments (5 mg/L) and b levels (28°C), in which the survival rate was 100%. For all of these results above, the results were significant.

Cite this as: Febriani, E. F., Widodo, M. S., & Faqih, A. R. (2023). Effect of Dosages and Temperatures on Simalungun Red Ginger (*Zingiber officinale* var. Rubrum) Ethanol Extract Dipping on Masculinization of Zebra Fish (*Danio rerio*) as a Model Fish. *Jurnal Ilmiah Perikanan dan Kelautan*, 15(1):15–30. http://doi.org/10.20473/jipk.v15i1.38271

1. Introduction

Currently, ornamental fish are popular in domestic and foreign markets. This is proven by the number of breeders who are starting to look at the potential of various types of fish cultivation activities in Indonesia. It should be noted that freshwater ornamental fish are also included in fishery commodities that can be cultivated continuously (Khomariah, 2021). However, ornamental fish enthusiasts sometimes prefers male fish (MF) over female fish (FF), because they are considered to have better aesthetic value and productivity, so the profits that they generate tend to be higher than cultivated FF (Mahfut and Sutyarso, 2020). Masculinization is needed to increase the number of MF populations. The masculinization technique that has been commonly used is the immersion of the embryo in the eyespot phase in a solution of hormone 17α-methyltestosterone (MT) (Herjayanto *et al*., 2019). From previous study, we know that the transition period of hermaphrodite gonads to testes or ovaries can occur on day 43 to day 71 (Maack and Segner, 2004). In genus Cyprinidae, sex reversal treatment can be given after hatching (Pandian and Sheela, 1995). According to Yusuf *et al*. (2014), at the age of two weeks (14 days) the skeleton of the larvae is only partially formed. Meaning at this age, the condition of the larvae is still considered vulnerable, as the organ to support its life is still under developed. Deswani (2019) also revealed that the heart allegedly started pumping fluid through the blood vessels on the 20th day and blood cells appears on the next day. Furthermore, blood vessels continue to develop throughout the embryo.

Recently, masculinization methods have been carried out on several species of fish using different materials. Masculinization is usually done by giving androgenic hormones to the fish gonad in its differentiation phase. However, nowadays the use of synthetic hormone MT as a masculinizing agent has been banned in aquaculture activities, because it is difficult to degrade naturally and may end up polluting the environment (Srikwan *et al*., 2020). Several other materials that can be used as an alternative to synthetic hormone replacement materials and are more environmentally friendly include extracts from plants. In previous studies, several extracts from plants have been shown to be able to increase testosterone levels (TL) in the blood of male organisms (Matondang *et al*., 2018). In previous research, ginger can increase sperm count as well as motility and has a stimulatory effect on serum testosterone level. Base on another research, ginger rhizome was traditionally used in Iran for enhancing male sexuality. A great number of studies these days have reported that some ginger extract can increase the weight of testis on individual and accessory reproductive glands (Bordbar *et al*., 2013). The mechanism which makes ginger proven to increase testosterone production is by increasing LH production, cholesterol levels in the testes, reducing oxidative stress, and lipid peroxidation in the testes. Ginger can also increase the activity of certain antioxidant enzymes, normalize the blood glucose, increase nitric oxide production, and blood flow in Leydig cells, testicular weight, and recycle testosterone receptors in gonad (Banihani, 2018).

Sex reversal is a technology used to artificially direct the development of sex to be the opposite by changing the male to female phenotype or vice versa. Generally, this technique is carried out before the fish undergoes clear gonadal differentiation between males and females at hatching (bipotential). Several methods that are often used in sex reversal are by injection, dipping, feed (oral), and bioencapsulation (natural feed and immersion) (Mangaro *et al*., 2018). The oral and dipping methods had different results in the number of males and females. The number of MF with the dipping method tends to be able to produce a higher percentage of males than the oral method. However, the survival rate for each treatment did not look much different, although it was found that the dipping method tended to cause lower mortality rate than the oral method (Hutagalung, 2020). Water temperature can also indirectly affect the metabolic processes in the fish body. The higher temperature can make the metabolism in the fish's body work faster (Nurcahyo, 2018). In the previous research, García-Cruz *et al*. (2020) stated that a strong temperature treatment can determine the sex of the fish. Sex ratios that tend to produce females are produced at low temperatures (<19°C), mixed sex ratios are produced at medium temperatures (20-28°C), and all male fish are produced at high temperatures (>29°C). Temperature-dependent sex determination can also be characterized through a genotypic form of sex determination. Where, it is found that the Y chromosome carries a copy of the anti-Mullerian hormone, which then acts as a sex determinant of male at a temperature that results in mixed sex.

The evidence that can be done to solve this problem is to utilize phytochemical compounds in Red ginger (*Zingiber officinale* var. Rubrum) (RG) as a masculinizing agent in ZF used in this study. RG is an Indonesian spice that can be easily found in markets and can have an important role in everyday life, especially in the health sector. The rhizome of RG is known to be much sought after because it has properties as herbal medicines. The use of this medicinal plant has also been widely tried to prevent or cure a disease. RG is also known to have several active compounds that have been shown to have antioxidant activity in it (Sujianto *et al*., 2021). Also, Donkor *et al*. (2018) stated that RG has androgenic activity. This is evidenced by the administration of RG extract which is proven to be able to increase the concentration of TT in serum. The TT hormone itself functions to control the process of spermatogenesis, maintain Sertoli cells and play a role in determining the quality of spermatozoa in male organisms.

ZF which has a native habitat in South Asian waters is one of the most widely used species in laboratories (Moorhead and Zeng, 2010). In various studies, ZF is used for developmental biology, biochemistry/molecular biology, cell biology, and neuroscience/neurology (Kinth *et al*., 2013). ZF are also increasingly common in being used for scientific research as a test animal model for vertebrate species because this fish has several advantages such as being easy to obtain and observed, high DNA similarity with humans, and sensitive to laboratory treatment (Nugroho *et al*., 2018). ZF's small size also allows these fish to be kept in large numbers in a limited space. The eggs laid by the fish are also desirable: high amount of eggs per cycle, transparent, does not stick to media, and rapid development which is especially sought after for researches, particularly the masculinization test which only takes 2-3 months (Hickman *et al*., 2017).

Masculinization through dipping with natural herbal ingredients are still rarely discussed, even more so with RG extract. The average literature related to the use of RG is still only being applied to mice (*Mus musculus* L.) and Wistar rats (*Rattus norvegicus*) in the medical field in oral form through feed. The articles that discuss this research include Aristiani *et al*. (2017) and Donkor *et al*. (2018). Therefore, this study was tested on ZF which is considered as a model fish comparable to mice (*Mus musculus* L.) and Wistar rats (*Rattus norvegicus*) to determine the effect of SRG on masculinization activities and able to determine the best dosage and temperature that can be used as a means of masculinization.

2. Materials and Methods

This research was conducted in October 2021-June 2022 at the Fish Cultivation Laboratory, Fish Reproduction Division, Building D 1st floor, Faculty of Fisheries and Marine Sciences, Brawijaya University Malang, Fisheries and Marine Resources Exploration Laboratory, Building A 1st floor, Faculty of Fisheries and Marine Sciences, Brawijaya University Malang, Biochemistry Laboratory, Central Laboratory Building Halal Center 1st floor, Islamic University of Malang, Pathology and Anatomy Laboratory, Faculty of Medicine, Brawijaya University Malang and Integrated Laboratory Installation of Dr. Saiful Anwar Hospital, Malang.

2.1 Material

The tools which used in this study included some glass jar (3 liters), blender (Miyako-101-GS, Indonesia), rotary evaporator (IKA 10, Germany), dropper, micropipette (Denshine-10-100ul-MikroTransfer, US), test tube (PyRex, US), oven (IKA-125 Basic Dry, Germany), aerator set, blower (Hiblow Takatsuki-HP-200, Japan), and shaker (IKA 8017400, Germany). Materials used in this study were Red Ginger (*Zingiber officinale* var. Rubrum) from Simalungun (Medan), n-hexane, 96% ethanol, 90% methanol, and 20 days old Zebrafish (*Danio rerio*).

2.1.1 Experimental design

This research is an experimental study with two independent variables: dosage and temperature. This study used five treatments of dosages, three levels of temperatures, and three replications (Table 1). This research used Completely Randomized Factorial Design (CRFD) to process the data analysis.

Table 1. Research layout of the experiments carried out

Treatments	Temperatures	Dosages	Replications		
			1	2	3
A	Control (a)	0 mg/l	1Aa	2Aa	3Aa
	28°C (b)		1Ab	2Ab	3Ab
	32°C (c)		1Ac	2Ac	3Ac
B	Control (a)	5 mg/l	1Ba	2Ba	3Ba
	28°C (b)		1Bb	2Bb	3Bb
	32°C (c)		1Bc	2Bc	3Bc
C	Control (a)	10 mg/l	1Ca	2Ca	3Ca
	28°C (b)		1Cb	2Cb	3Cb
	32°C (c)		1Cc	2Cc	3Cc
D	Control (a)	15 mg/l	1Da	2Da	3Da
	28°C (b)		1Db	2Db	3Db
	32°C (c)		1Dc	2Dc	3Dc
E	Control (a)	20 mg/l	1Ea	2Ea	3Ea
	28°C (b)		1Eb	2Eb	3Eb
	32°C (c)		1Ec	2Ec	3Ec

Description: A (0 mg/L); B (5 mg/L); C (10 mg/L); D (15 mg/L); E (20 mg/L).

2Bb	3Cc	3Da	2Ba	3Da	1Ac	2Aa	2Ac	1Bb
1Ca	3Ec	1Ab	3Ca	2Ec	1Db	3Ea	2Dc	1Cb
2Da	1Aa	2Ea	3Bc	3Dc	3Eb	2Ca	3Db	1Bc
3Ab	1Ba	2Eb	2Cc	2Ab	3Ac	2Bc	1Cc	1Ea
1Ec	1Dc	2Cb	3Aa	1Eb	3Bb	1Da	2Db	3Cb

Figure 1. Design layout

2.1.2 Container preparation

Preparation of the container was the first step that must be done before carrying out this research. The steps

were as follows: prepared an aquarium/pond that would be used as a treatment container, washed the treatment container with clean water and siphoning, dried for a day and night. The aquarium layout was in accordance with the research layout design. Installed the aerator set (aerator stone, aerator hose, and aerator faucet) in the aquarium as a broodstock container. Labeled each aquarium according to the research layout design. The 20 days old larvae was added to each aquarium according to treatment. The extract from each treatment was then put into the aquarium according to the research design and soaked for about 23 days. After 23 days, transferred all treatments from the aquarium to a mesh box that had been installed in a concrete pond according to the research layout (Figure 1).

2.1.3 Broodstock selection and spawning process

The ZF's broodstock used in this study came from Depok, West Java. The steps in the selection of the ZF's broodstock used were as follows: reduced the pond water by opening the pond outlet, bring the broodstock to the corner of the pond by herding it with a tool called seser, took healthy parents which had mature gonads as brood stock, used male to female ratio (2:1) and put that fish into two different aquariums and the MF and FF might be separated, aerated each aquarium, and acclimatized brood stock for about 24-48 hours (one to two days). For male ZF, the body color was known to look brighter and more attractive than the FF. The body shape was flatter with a slightly rounded stomach. For female ZF, the body tended to be wider and thicker, the belly also bulges. In FF that have matured gonads, the stomach will look very rounded and the eggs inside the stomach can be seen faintly from the outside of the body (transparent) (Pratama, 2021). Avdesh *et al.* (2012) also added that the female ZF have ovipositor (Figure 2); an organ that helps in the process of laying eggs.

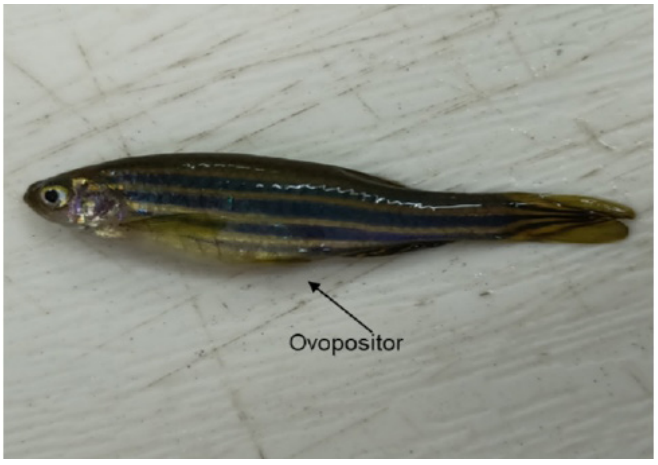

Figure 2. Ovopositor in female ZF

The process of spawning ZF is carried out *en masse*. The steps taken in spawning ZF were as follows: prepared an aquarium with a size of 40x60x30 cm which had been filled with water. Place the under gravel as a separator for male and female breeders and the eggs to be produced, male and female brood of ZF were selected in a ratio of 2:1 and put in one aquarium, after spawning occurred, the eggs was scattered under gravel, took the brood stock and separated it from the egg aquarium, all fertilized eggs were then hatched in separate aquariums. The eggs usually takes about 17-24 hours to hatch. Hatched larvae were then reared until it reached 20 days of age. The fecundity of ZF can be said to be quite a lot, it is because this fish can lay eggs easily. The number of fecundities from ZF is around 150-300 eggs during the breeding season and on the outside of breeding season, it reaches 100-200 eggs.

2.1.4 Extraction

RG used in this study came from Simalungun, Medan, North Sumatra. The extraction method according to Ghasemzadeh *et al.* (2016) and Benjamin *et al.* (2020) was as follow; RG was rinsed with clean water and dried under shade at room temperature, the dried plant samples were ground into a fine powder. Two kilograms of wet RG only produced 250 grams of dry RG sample, therefore, the sample needed to be dissolved in 1 L n-hexane, 1 L 96% ethanol, and 1 L methanol according to Benjamin *et al.* (2020) with a ratio (0,25:1) as follow; the weight of the powder samples (250 grams each) was extracted through maceration using a 0,25:1 ratio sample mixture, i.e., 250 grams of the sample was dissolved in 1 L of n-hexane for 48 hours with shaking and continuous stirring for proper extraction. The macerated bi-herbal sample was then sieved with a shaker and concentrated using a rotary evaporator. Then, the sample was macerated again and dissolved in 1 L of 96% ethanol for 48 hours, then sieved with a shaker and concentrated using a rotary evaporator. Finally, the sample was macerated again and dissolved in 1 L of 90% methanol for 48 hours, then sieved with a shaker and concentrated using a rotary evaporator. Furthermore, the three extracts obtained were cooled using a temperature conducive before further use.

2.1.5 Phytochemical analysis

Extracts of RG (0.5 grams/sample) were prepared in a test tube for testing several phytochemicals such as alkaloids, flavonoids, triterpenoids/steroids, saponins, and tannins (Rante *et al.*, 2020).

2.1.6 Thin Layer Chromatography (TLC)

Each sample that had become a thick extract (with n-hexane, 96% ethanol, and 90% methanol solvent) which was suspected to contain Sitosterol was tested by TLC with acetic

acid, methanol, and ethyl acetate (6:3:1) as eluent in a tubular chamber with a volume of 38.5 cm^3. Next, the Rf value was calculated and compared with the Rf value obtained from the results of pure Sitosterol in TLC. The eluents of acetic acid, methanol and ethyl acetate were used in this phase because they were commonly used eluents and were able to dissolve steroid compounds on TLC plates (Forestryana and Arnida, 2020; Agustin *et al*., 2021).

2.1.7 Electrochemiluminescence Immunoassay (ECLIA)

This method was carried out with a 1:1 dilution as the test sample. Briefly, the sample of the RG extract that was ready to be tested in a vacuum tube was pipetted 1 mg and mixed into 3 mL of aquadest. After that, the sample was tested using hormone antibodies directed against the related protein contained in the sandwich test format to detect derivative testosterone (DTT) in the sample. For the ECLIA method, the specificity and sensitivity were 99.9% and 94.5%, respectively. So, the test results can be said to have high validity compared to other hormone testing methods (Kolesova *et al*., 2021).

2.1.8 Liquid Chromatography Mass Spectroscopy - Quad Time of Flight (LCMS-QTOF)

LCMS analysis was performed on the ethanol extract of RG extract. LCMS has the advantage of a wider analysis range. Although there are also disadvantages, which require further analysis to ascertain the structure of the compound. The mass spectroscopy (MS) detector uses the QTOF (Quad Time of Flight) type. Sample ionization using electrospray ionization (ESI) method with positive ion mode [M+H]+. This causes ionization process where the sample gets an additional H atom, which is positively charged, so that the m/z read is equivalent to the molecular weight plus 1 Da (Fathoni, 2021).

2.1.9 Test parameters

2.1.9.1 Histology test

In order to determine the testicular development of ZF, testicular histology test was carried out. Staining of testicular histological preparations using Haematoxylin and Eosin staining was performed by histotechnic method (Pratiwi and Manan, 2015).

2.1.9.2 MF percentage

According to Matondang *et al.* (2018), calculating the percentage of male sex (MS) can be done using the following formula:

$$\text{MS (M\%)} = \frac{\text{Number of MS}}{\text{Number of all fishes}} \times 100\%$$

To calculate the percentage of male, it is done by looking at the secondary characteristics in the morphology of the fish and also looking at the primary characteristics by performing gonadal surgery.

2.1.9.3 Total testosterone level

Hormonal measurements were carried out using gonadal samples. The use of gonadal samples can also be used as another option for monitoring steroid levels and is considered very attractive because it can be collected quickly and has high validity. The first step was to perform gonadal surgery on MF and FF. Each gonad was placed on a porcelain dish and grinded it by pounding the gonads with a mortar until smooth. Dissolved the smoothen gonads with distilled water as an isotonic solution. Finally, put it in a vacuum tube and tested it with ECLIA (Revisa, 2016; Kolesova *et al*., 2021).

2.1.9.4 Survival rate

Dipping research can also be continued by checking the survival rate. Survival rate can be referred to as the percentage of survival of the dipping larvae. The higher percentage value of the survival rate can be considered as an optimal outcome. The factors that can affect survival rate is the quality of the waters and its parameters of fish habitat/ living environment (physical and chemical) (Nurcahyo, 2018). According to Kumar *et al.* (2018), survival rate can be calculated by the following formula:

$$\textit{Survival Rate} = \frac{\text{Number of live larvae}}{\text{Number of all stocked larvae}} \times$$

For survival rate calculations in this study, it was carried out after the larvae had finished being treated with dipping (before they were transferred to the rearing pond).

2.2 Analysis Data

Data collection techniques were carried out through direct observation followed by analyzing the graphs and tables of the ZF test results (*D. rerio*) from each treatment dose and temperature given. Statistical analysis and quantitative data collection in this study were carried out using a two-way ANOVA in accordance the design of Completely Randomized Factorial Design (CRFD) used with 95% confidence level ($\alpha = 0.05$) using five treatments, three levels, and three replications for each treatment. If the results of the variance showed a significant effect or very significantly different (F count > F table), then the analysis was followed by Duncan's Multiple Range Test (DMRT) used Ms. Excel 2013. Meanwhile, for the qualitative data collection in this study was carried out using a descriptive method.

3. Results and Discussion

3.1 Phytochemical Analysis

Phytochemical analysis was carried out on each

Table 2. Phytochemical test results from each RG extract

Type of RG Extract	Alkaloids	Flavonoids	Triterpenoids/Steroids	Saponins	Tannins
N-hexane	+++	+	++	-	-
96% Ethanol	-	++	+++	-	-
90% Methanol	-	+	+	+	++

extract of RG to analyze and prove the presence of steroid compounds qualitatively, which is thought to be able to influence the masculinization process.

It can be concluded that the n-hexane extract of RG contains alkaloids, flavonoids, and triterpenoids/steroids (Table 2). The ethanol extract of RG contained flavonoid compounds and triterpenoids/steroids. The methanol extract of RG contains flavonoids, triterpenoids/steroids, saponins, and tannins. From this description, it can be concluded that different extracts will also produce different contents. Phytochemical screening here is an early stage which is considered to be able to provide an overview of the content of certain compounds present in the natural materials studied (Dewatisari *et al*., 2020). Nguyen *et al*. (2019) also argued that the phytochemical content of plants or materials generally varies. This certainly allows the plant or material to have different special compounds, including the presence of alkaloids, flavonoids, triterpenoids/steroids, saponins, and tannins in the tested material. Due to the different content in each material, it will produce different properties and biological effects from each material to be used. D'Arrigo *et al*. (2021) revealed that some plants generally have bioactive such as polyphenols and especially flavonoids. Flavonoids are known to be able to recognize androgen and estrogen hormone receptors. Flavonoids are also said to have a lower binding affinity for any single receptor of the hormone. Flavonoids can interact with one or more estrogen and androgen hormones by showing additive binding effects. Several flavonoids have been considered as phytoestrogens capable of inhibiting the endocrine system, both because of their interaction with estrogen receptors and their ability to activate or deactivate the estrogen response *in vitro*. Several previous studies have suggested that flavonoids can also provide androgen-related signaling pathways through androgen receptor activation.

3.2 Thin Layer Chromatography (TLC)

TLC analysis was also performed on each extract of RG to analyze the similarity of the ethanolic extract of RG used with steroids suspected of Sitosterol and supports qualitative phytochemical analysis that had previously been carried out. In this case, the pure Sitosterol standard will be compared with each extract of RG obtained as a comparison material. The TLC results of each extract obtained were different. In the n-hexane extract, the TLC results obtained were 0.64. Meanwhile, the 96% ethanol extract and 90% methanol extract obtained TLC results of 0.93.

It can be concluded that the n-hexane extract of RG has a retention factor (Rf) value of 0.64 (Table 3), which is smaller than the pure standard used which has an Rf value of 0.96. Meanwhile, the ethanol and methanol extracts of RG had an Rf value of 0.93. Rf value of ethanol extract and the methanol of RG here had a slight difference with the pure standard. This means that the ethanol and methanol extracts have a close resemblance to the pure standard used. It can be concluded that both ethanol extract and methanol extract can be used in this study, because they have close similarities to the pure standard. However, the ethanol extract was the selected extract, because in the phytochemical analysis results, ethanol extracts tended to only contain two ingredients, namely flavonoids and also triterpenoids/steroids, unlike methanol extract. The TLC testing under UV light of 254 nm was not fluorescent than the TLC testing under UV light of 366 nm (Figure 3). According to Shashikala *et al*. (2018), the compound that contained steroids can be fluorescent in the 366 nm. Although, it looked clearer than in the 254 nm, the stain appeared was still unclear.

Table 3. TLC test results from each RG extract

Type of RG Extract	Rf Value of Material that Suspected Contains Sitosterol	Rf Value of Pure Standard
N-hexane	0.64	
96% Ethanol	0.93	0.96
90% Methanol	0.93	

If the Rf value obtained is close to the standard Rf value of β-sitosterol, then the compound can be said to be similar or close to the pure standard (Pramesti *et al*., 2021). Based on the results of previous studies on curcumin, if the Rf value in the sample is close to the same, this can prove that the concentration value of the compound analyzed in the sample is more or less similar to the value of the compound content in the pure standard (Permatasari *et al*., 2021). Pebe *et al*. (2022) also revealed that TLC is the most effective method because it can save operational costs for analysis on a large number of samples, such as sample screening. The general principle of TLC is to separate a mixture due to the movement of the solvent across a flat surface. These components will migrate

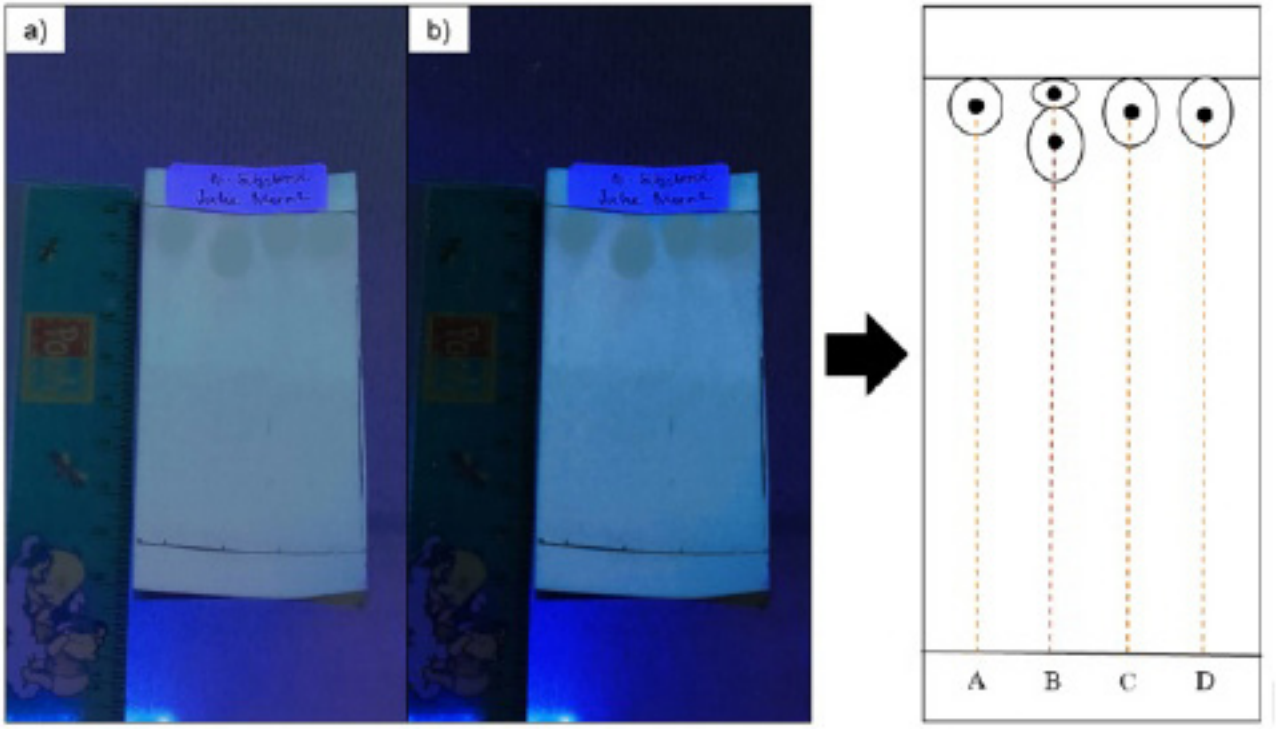

Figure 3. TLC plat of each RG extract; A (Standard); B (RG n-hexane extract); C (RG ethanol extract); D (RG methanol extract)

at different rates depending on their solubility, adsorption, molecular size, charge, and elution. In order to support this test, it is recommended to carry out several forensic analysis tests such as confirmatory tests.

3.3 Liquid Chromatography Mass Spectroscopy - Quad Time of Flight (LCMS-QTOF)

The ethanol extract of RG was also analyzed using the LCMS-QTOF method to determine the content of these ingredients. This is of course done to support and ensure the results of the previously carried out TLC whether it is true that Sitosterol compounds are contained in the ethanol extract of RG. The results of the analysis were that in 10 grams of ethanol extract of RG, there were compounds of Stigmast-5-en-3beta-ol, acetate (Sitosterol Acetate), and Kushenol F (Sophoraflavonone G) (Table 4). That result was supported by the ESI results of LCMS-QTOF. The result showed that ethanol extract of RG was positive (+) containing Stigmast-5-en-3beta-ol, acetate (Sitosterol Acetate), and Kushenol F (Sophoraflavonone G).

Table 4. LCMS-QTOF test results of RG ethanol extract

Compound	IUPAC's Name	Result
Kushenol F	Sophoraflavo-none G	Positive (+)
Stigmast-5-en-3be-ta-ol, acetate	Sitosterol Ace-tate	Positive (+)

Kushenol F (Sophoraflavonone G) is the active compound co-existing with three compounds, oxymatrine, trifolirhizine, and β-sitosterol (Hwang *et al*., 2005). Flavonoid compounds are found in almost all parts of the Rhizophora plant and are the main active ingredients in the Rhizophora class. Roots are known to contain various flavonoid compounds. One of these flavonoid compounds is Kushenol F (Sophoraflavonone G) (Sun *et al*., 2020). In particular, Kushenol F and Sitosterol have strong binding affinity for their receptors. Because Sitosterol is also known to be a type of cholesterol (Wang *et al*., 2021). Supported by Nurazizah *et al*. (2020), sitosterol is part of the lipid which is an important component of the membranes in several cells of the body, which are several plasma lipoproteins that can also function as precursors for the synthesis of steroid hormones. In the research of Hu *et al*. (2022), challenges faced regarding androgen regulation by flavonoids in particular may include exploration of target mechanisms and individual heterogeneity. It is also mentioned that nutritional interventions using flavonoids can also improve the symptoms of androgen disorders in an individual.

3.4 In Silico

The ethanolic extract of RG was also analyzed *in silico* with PyRx application to determine the content of these ingredients. This is certainly done to support the results of the LCMS-QTOF which had previously been carried out on the

ethanol extract of RG.

Table 5. In silico results of Sitosterol Acetate and Kushenol F with Progesterone

Compounds	Binding Affinity (kcal/mol)
Testosterone	-7.4
Sitosterol Acetate	-7.2
Methyltestosterone	-7.1
Kushenol F (Sophoraflavonone G)	-7.1

TT and MT compounds were used as control comparisons from the binding values obtained (Table 5). It can be concluded that the binding value of Sitosterol Acetate obtained is bigger than the binding value of TT and smaller than MT. Where, for Kushenol F (Sophoraflavonone G) obtained is bigger than the binding value of TT. However, this compound has the same binding value as MT. Saputri *et al.* (2016) said that binding affinity is a measure of the ability of a substance/drug to bind to a receptor. The smaller the binding affinity value is, the higher the affinity between the receptor and the ligand will be. On the other hand, the higher the binding affinity value is, the lower the affinity between receptors will be. If they are the same, it is suspected that the ability of these compounds to bind to receptors is comparable. Previously, Kellenberger *et al.* (2008) also revealed that the binding affinity value can greatly affect the stability of the interaction between the ligand and the receptor. The binding affinity value will generally be chosen from the most negative. A negative value indicates the smallest energy used by the receptor to interact with the ligand, so it is assumed that the interaction can take place spontaneously. The smaller the binding affinity value is also thought to be able to produce a more stable interaction between the ligand and the receptor.

3.5 Electrochemiluminescence Immunoassay (ECLIA)

The Sitosterol content in the ethanol extract of RG was also analyzed using the ECLIA method to determine the content of these ingredients in 1 mg/L RG extract. This is certainly done to support the results of the TLC and LCMS-QTOF, which were previously carried out. Although LCMS-QTOF is able to predict the content of compounds in the material. In fact, the method has not been able to analyze the amount of compound content in it quantitatively. The results of the ECLIA analysis of (DTT) such as Sitosterol in 1 mg/L ethanol extract of RG was 0.069 ng/ml. This compound is suspected to be Sitosterol, because Sitosterol is a DTT, which may be read in the ECLIA test. From these results it can be estimated that the total administration of RG extract which containing Sitosterol in each treatment (Table 6). In treatment A (0 mg/L) was suspected to contain 0 ng/L Sitosterol; treatment B (5 mg/L) was suspected to contain 0.345 ng/L of Sitosterol; treatment C (10 mg/L) was suspected to contain 0.690 ng/L of Sitosterol; treatment D (15 mg/L) was suspected to contain 1,035 ng/L of Sitosterol; treatment E (20 mg/L) was suspected to contain 1,380 ng/L of Sitosterol.

Table 6. ECLIA test results of RG ethanol extract in each treatment

Treatments	Dosages (mg/l)	Alleged Content of Hormones Suspected of Sitosterol (ng/ml)
A	0	0
B	5	0.345
C	10	0.690
D	15	1.035
E	20	1.380

Hormones that play a role in the sexual development of male individuals are steroid hormones. Steroid hormones are known to function in the process of spermatogenesis, the development of external reproductive organs, and secondary sex characteristics and as a component in the formation of cholesterol. Cholesterol itself is also known to be a precursor in androgen biosynthesis. Here, Sitosterol is known to be a steroid hormone that has a structure similar to cholesterol (Fitriyah and Isyaturriyadhah, 2021). The circulating water system can also accumulate steroid levels and produce a cumulative masculinizing effect. This is in accordance with previous research, where increasing the dose of steroid hormone was proven to be able to produce male individuals in intensive culture ponds (with strong aeration) compared to culture ponds with water flow aeration systems (Thanasupsin *et al*., 2021).

3.6 Histology of Fish Gonad

Histological tests were also carried out on the gonads to analyze if there was a differentiation in the shape of the gonads in ZF treated with dipping. It can be seen that there was a change in the gonads of ZF which were treated with dipping (Figure 4). There were striking differences in male and female gonads and intersex (Figure 4c). Individuals showing no external phenotype, but possessing testes without visible ovarian tissue or individuals showing external signs of being male, but having a fully functional ovotestis (individuals producing fertilized eggs) are said to be intersex male individuals. In this case, the body color is not enough to determine the morphology of the male gonads internally, therefore to predict the presence of ovotestis, surgery and histology are needed (Scarsella *et al*., 2018). The frequent occurrence of intersex symptoms and other gonadal disorders

in fish has been shown to be partly due to exposure to aquatic waste. The presence of ovotestis is a good indicator of the presence of endocrine-blocking chemicals and may reflect a sign that clear from the intersex condition. Therefore, when evaluating the potential effects of endocrine-blocking chemicals, histological investigations of the male gonads should be considered to observe ovotestis appearance. This is because these altered gonads often appear normal upon external examination, but are not. Histopathological signs of intersex conditions can range from mild to severe according to many factors such as the number, maturity, and distribution of oocytes in normal testicular tissue (Abdel-Khalek, 2017). In the testes, the sperm ducts are seen crossing the medial hilum region, and running along the dorsal and ventral surfaces of the gonads in the sperm sinus. Known for differentiated (ovotestes) gonads that are active in spermatogenesis, the gonads consist entirely of spermatogenic tissue and seminiferous tubules filled mostly with spermatozoa with few spermatocytes or spermatids without recognizable female germ cells or oocytes of any stage of development or ovarian stromal tissue (Choi *et al*., 2014).

The results of the ZF's ovotestis histology performed (Figure 5), ovotestis with an active spermatogenesis system can generally be categorized as a male individual, because there are no female germ cells in the ovotestis organ. According to Edgecombe (2020), some fish generally undergo a process of differentiated gonochorism during juveniles. This is no exception to one of the teleost fish such as the Cyprinidae family. According to García-Cruz *et al*. (2020), fish with differentiated gonochorism does not tend to change back to the female phase after passing the bipotential stage, if the fish are not fish capable in hermaphrodite reproduction. However, in some cases, it was found that there was fish with an ovotestis system which can carry carrier genes such as anti-Mullerian genes, that will make their offspring have organs similar to their predecessor.

3.7 MF Percentage

The number of males in ZF was also calculated to analyze the total number of MF in the dipping treatment. In this case, the calculation of the percentage itself was carried out by two kinds of observations, namely by observing the primary sexuality of the ZF by performing gonadal surgery and observing the secondary sexuality by looking at the morphology of the fish's body.

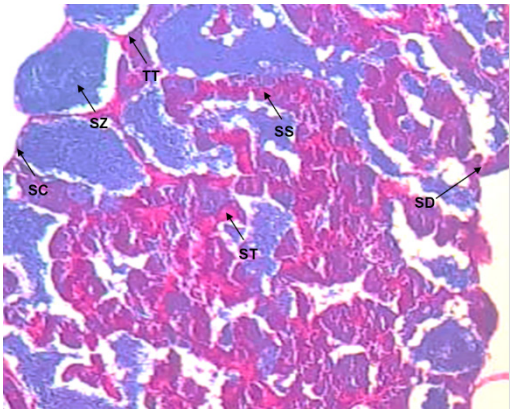

Figure 5. Intersex ZF's gonad histology: TT (testicular tissue); SZ (spermatozoa); SC (sinus channels); SS (spermatocyte); ST (spermatid); SD (sperm duct)

The Dc and Ec treatment had a significantly different effect with the Db and Eb treatments (Figure 6). The Db and Eb treatments had a significantly different effect with the Cc, Da, and Ea treatments. The Cc, Da, and Ea treatments had a significantly different effect with the Cb. The Cb treatments had a very significantly different effect with the Aa, Ab, Ac, Ba, Bb, Bc, and Ca. This proves that the results of the percentage of males through the observation of primary sexuality in ZF have increased in each dosage and temperature treatment. In general, the interstitial tissue of the gonads produces sex steroid hormones in response to hormonal messages from

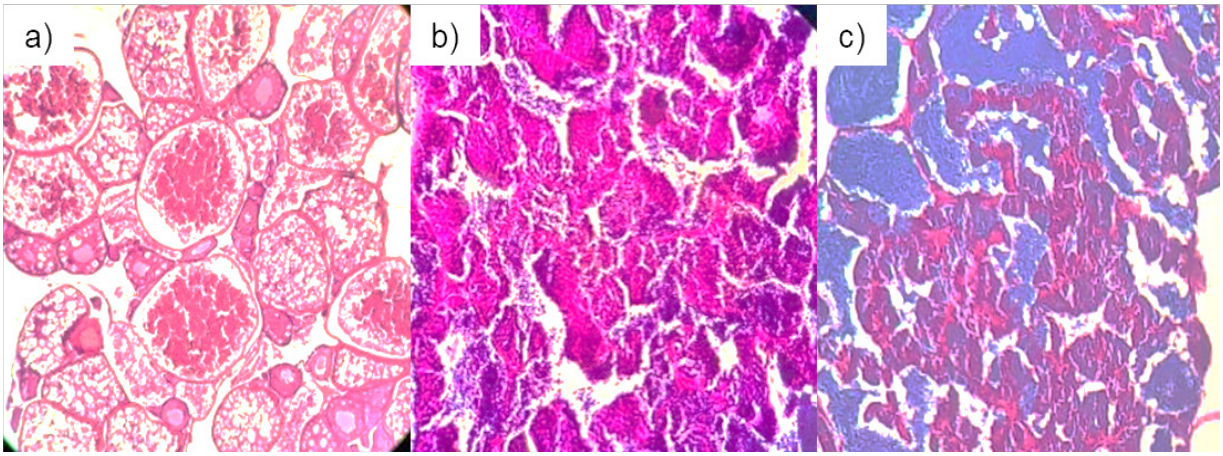

Figure 4. Histology of ZF's gonad: a) female; b) male; c) intersex

the pituitary gland at the base of the brain. This pituitary-gonadal axis controls the expression of sexuality which includes the development, maturation and release of gametes (sperm and ovum) in response to the environment. In this way, the sexual cycle of fish is closely related to environmental conditions. So that in this situation, reproductive problems that arise can be overcome through environmental manipulation such as salinity, light, temperature or by giving sex hormones directly (Subandiyono and Hastuti, 2021). Sex determination was dependent on a strong temperature treatment in which the sex fate of the gonads was determined in response to the temperature experienced during the first week of hatching, which was considered a critical period for determining sex. Sex ratios that tend to produce females are produced at low temperatures (temperatures producing females, <19°C), mixed sex ratios are produced at medium temperatures (temperatures producing mixed sexes, 20-28°C), and all offspring are male produced at high temperatures (male-stimulating temperatures, >29°C). Temperature-dependent sex determination can also be characterized by a genotypic form of sex determination. It was found that the Y chromosome carries a copy of the anti-Mullerian hormone, which then acts as a MS determinant at temperatures resulting in mixed sex (García-Cruz *et al*., 2020). Simó-Mirabet *et al*. (2018)

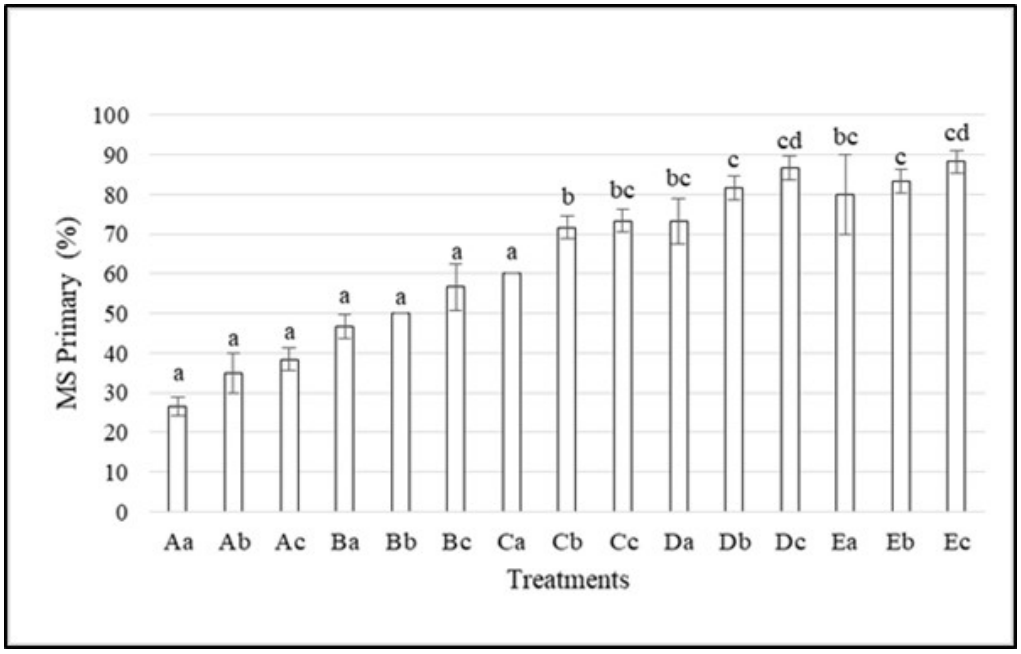

Figure 6. MF percentage (primary) of each treatment

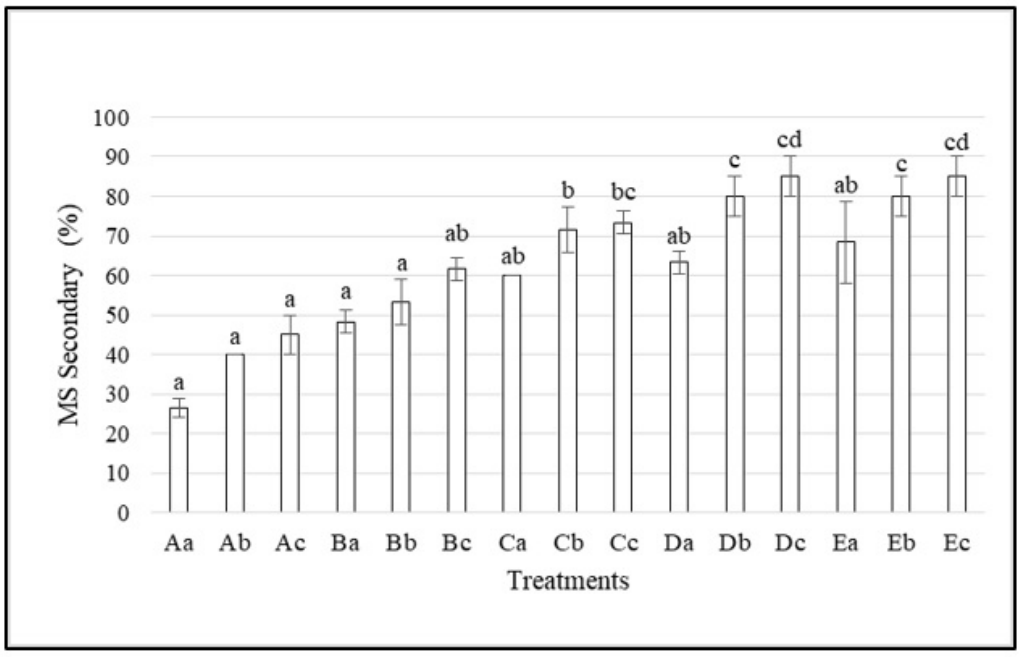

Figure 7. MF percentage (secondary) of each treatment

have also suggested that compounds contained in plants are also capable of triggering different effects depending on circulating levels of steroids on gonadal development and sex ratio. Plasma levels of gonadal sex steroid hormones normally circulate during the reproductive season. Sex steroids are considered to be major regulators of natural sex change and play an important role in gonadogenesis in teleost fish. The measured steroid levels are also known to be within the normal range for actively reproducing fish, but tend to be low during the non-reproductive period. Steroid levels are also known to increase gradually through the process of gametogenesis, along with gonadal growth and can decrease suddenly thereafter.

The Dc and Ec treatment had a significantly different effect with the Db and Eb treatments (Figure 7). The Db and Eb treatment had a significantly different effect with the Cc treatments. The Cc treatment had a significantly different effect with the Cb treatments. The Cb treatment had a significantly different effect with the Bc, Ca, Da, and Ea treatments. The Bc, Ca, Da, and Ea treatment had a significantly different effect with the Aa, Ab, Ac, Ba, and Bb treatments. This proved that the results of the percentage of males through the observation of secondary sexuality in ZF had increased in each dosage and temperature treatment. In accordance with the statement of Jiang *et al.* (2022), administration of androgen hormones (male hormones) with the right dosage can cause inhibition

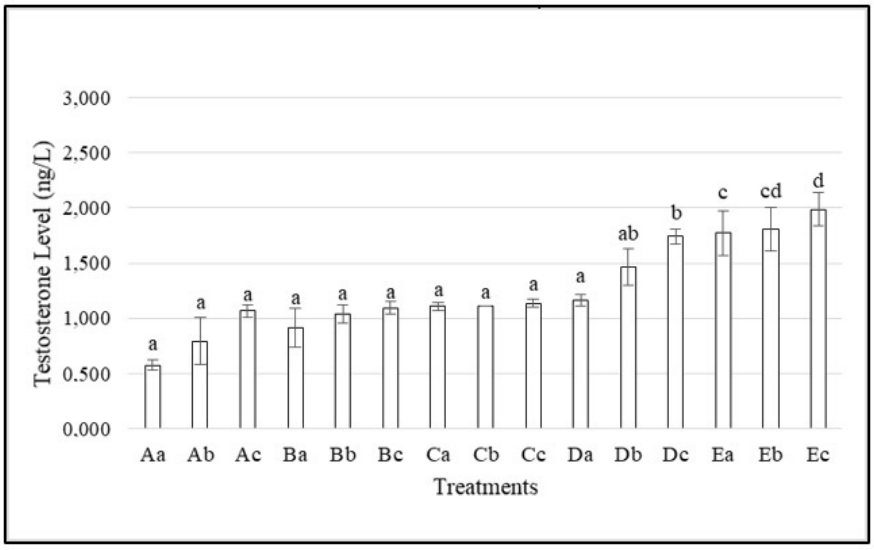

Figure 8. Total TL of each treatment

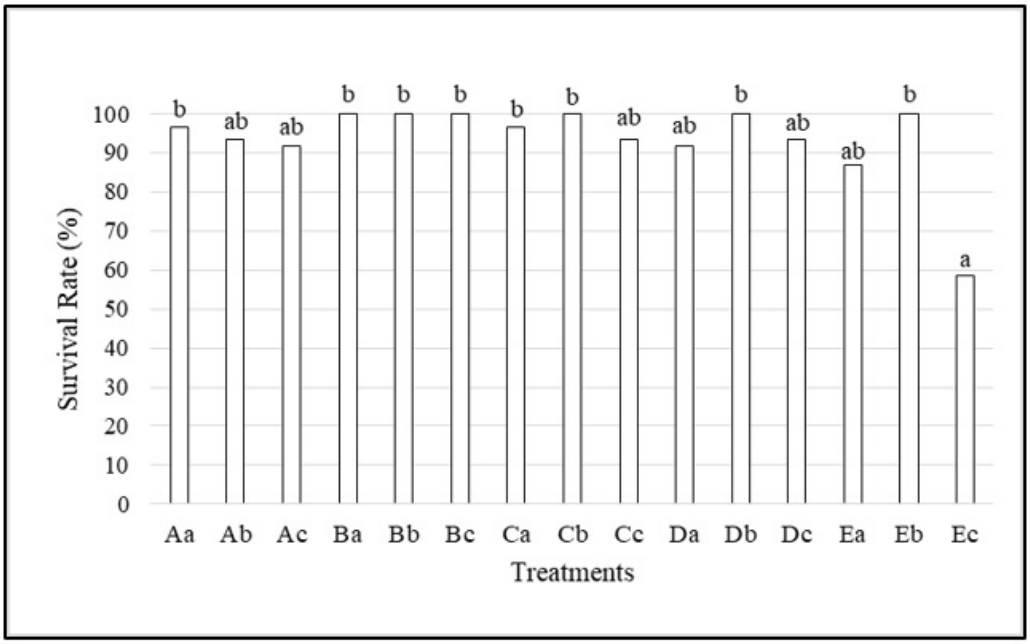

Figure 9. Survival rate of each treatment

of ovarian formation and development of testes so that the gonads will differentiate into testes. The success of masculinization (male process) was influenced by the accuracy of the technique of manipulation of environmental factors that affect steroid production. The right time to do fish sex reversal was during the sex differentiation of an individual. Ayuningtyas *et al.* (2015) revealed, sex differentiation (sex change) is the process of the gonads before they differentiate into testes or ovaries according to genetics influenced by the environment. Subandiyono and Hastuti (2021) added that the influence of hormones secreted by the testes has a role in secondary sexual signs. This is because MF usually develop sexual characteristics in terms of morphology, color, and aggressiveness by being influenced by androgen hormones produced by the testes.

3.8 Total Testosterone Levels

The analysis of TL in ZF was also carried out to analyze the total amount of male hormones contained in the fish's body after being given dose and temperature treatment. In this case, the analysis of TL is carried out by performing gonadal surgery on ZF to then be tested for TL using the ECLIA method.

The Ec treatment had a significantly different effect with the Eb treatments (Figure 8). The Eb treatment had a significantly different effect with the Ea treatments. The Ea treatment had a significantly different effect with the Dc treatments. The Dc treatment had a significantly different effect with the Db treatments. The Db treatment had a significantly different effect with the Aa, Ab, Ac, Ba, Bb, Bc, Ca, Cb, Cc, and Da treatments. Factors that could affect TL were generally age, body weight, stress, and blood sugar levels. A decrease in TL below the normal limit is known to cause various disorders, both physical and psychological/mental, which will affect the quality of life of the organism (Devita and Amran, 2019). Motosko *et al.* (2018) added that the addition of TT and masculine hormonal therapy has several purposes. One of the goals is to produce masculine traits internally (hormones) and develop masculine secondary sexual characteristics. TT can generally stimulate and cause suppression of estrogen and can further promote the formation of male gonads in females. Gharaei *et al.* (2020) also revealed that the TT hormone is the main male androgen in teleost and a precursor to 11-Ketotestosterone, which is mostly produced by the testes. TT is known to be a sex hormone that plays a role in germ cells and is present at the time of differentiation of male larval embryo cells and then used in the body during maturation or physiological development. In the previous study, the normal TL found in ZF is 0.05 ng/ml.

3.9 Survival Rate

The percentage of survival rate was also calculated for ZF to analyze the total number of fish that lived from the initial dipping treatment to the rearing process. The Aa, Ba, Bb, Bc, Ca, Cb, Db, and Eb treatments had significantly different effect with the Ab, Ac, Cc, Da, Dc, and Ea treatment (Figure 9). The Ab, Ac, Cc, Da, Dc, and Ea treatment had a significantly different effect with the Ec treatments. This proves that the survival rate of ZF increase significantly at each dosage and temperature treatment. Exposure to high environmental temperatures can cause an individual to experience stress and develop heat illness. The most acute phase of heat illness is the occurrence of heat stroke. Heat stroke has the potential to occur in individuals who live for a long time in relatively high temperatures. Individuals who experience heat stroke are at risk of experiencing organ failure or even death (Hifumi *et al.*, 2018). This is supported by the individual stages which are still larvae (20 days) where according to Koyama *et al.* (2020), the larval phase is a critical phase because at this stage the larvae are still adjusting to the external environment and making improvements to new organs. This is also supported by the statement of Hayati *et al.* (2020) which stated that the higher the dosage of exposure to the substance given to fish can make the toxicity levels of the substance higher. It is usually characterized by the death of the organism but is not supported by abnormalities or malformations.

4. Conclusion

The conclusion obtained from this study is that for the RG extract used is from the Simalungun, Medan, North Sumatra. The purest extract content was found in the SRG ethanol extract. Not only that, in the TLC test, it was also known that the ethanol extract which was suspected to contain Sitosterol was similar to the pure standard of Sitosterol. LCMS-QTOF stated that the compound contained in the ethanol extract of SRG, which contains the compound Sophoraflavonone G (SFG) and Sitosterol Acetate (SA). In addition, an *in silico* study was also conducted on the compound SFG and SA will be tested with TT and MT. From these data, it can be seen that the binding value of SA (-7.2 kcal/mol) is bigger than TT and smaller than MT. Meanwhile, for SFG (-7.1 kcal/mol) obtained bigger than the value of TT binding and has the same as MT. Several conclusions that obtained from this study, the treatment of different dosages and temperatures can affect the percentage of males based on observations of primary and secondary sexuality in ZF, total TL in ZF and also survival rates in ZF.

Acknowledgment

Writers would like to appreciate to the Mr. Maheno Sri

Widodo and Mr. Abdul Rahem Faqih for their supports to all research activities. To the Mr. Muchlis Zainudin Arif, I would like to appreciate for the facilities during a research study in Fish Cultivation Laboratory, Fisheries and Marine Science, Brawijaya University for 10 months. To the Ms. Bonick Kartini Lonameo, I would like to appreciate for the facilities during a research study in Microbiology Laboratory, Central Laboratory of Life Sciences, Brawijaya University for 3 months. To the Ms. Rakhmawati Fahmiy, I would like to appreciate for the facilities during a research study in Biochemical Laboratory, Halal Center, University of Islam Malang for 3 months.

Authors' Contributions

The contributions of each author are as follows, Eka; collected the data, compiled the manuscripts and designed figures. Mr. Maheno and Mr. Faqih; compiled the main conceptual ideas and critical revision of articles. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

Funding Information

This research and publication were funded by the authors personally.

References

Abdel-Khalek, A. A. (2017). Chronic exposure to water of Lake Qaroun induced metal-related testicular damage and endocrine disruption in male fish. *Biological Trace Element Research*, 185:197-204.

Agustin, R., Oktaviantari, D. E., & Feladita, N. (2021). Identifikasi hidrokuinon dalam sabun pemutih pembersih wajah di tiga klinik kecantikan dengan metode kromatografi lapis tipis dan spektrofotometri uv-vis. *Jurnal Analis Farmasi*, 6(2):95-101.

Aristiani, P., Sutyarso, & Busman, H. (2017). Effect of red ginger ethanol extract (*Zingiber officinale* Roxb. Var. Rubrum) on epididymal spermatozoa in cyproterone acetate induced mice (*Mus musculus* L.). *Jurnal Biologi Eksperimen dan Keanekaragaman Hayati*, 4(1):17-22.

Avdesh, A., Chen, M., Martin-Iverson, M. T., Mondal, A., Ong, D., Rainey-Smith, S., Taddei, K., Lardelli, M., Groth, D. M., Verdile, G., & Martins, R. N. (2012). Regular care and maintenance of a zebrafish (*Danio rerio*) laboratory: an introduction. *Journal of Visualized Experiments*, 69(4196):1-8.

Ayuningtyas Q. S, Junior, M. Z., & Soelistiawaty, T. D. (2015). Alih kelamin jantan ikan nila menggunakan 17α-metiltestosteron melalui pakan dan peningkatan suhu. *Jurnal Akuakultur Indonesia*, 14(2):159-163.

Banihani, S. A. (2018). Ginger and Testosterone. *Biomolecule*, 8(119):1-8.

Benjamin, G. O., Oshomoh, E. O., Steve, A. O., & Osayi, O. K. (2020). Reproductive potency of methanolic bi-herbal (*Zingiber officinale* and *Chrysophyllum albidum*) extract on masculine wistar rats. *The Journal of Phytopharmacology*, 9(5):333-341.

Bordbar, H., Esmaeilpour, T., Dehghani, F., & Panjehshahin, M. R. (2013). Stereological study of the effect of ginger's alcoholic extract on the testis in busulfan-induced infertility in rats. *Iranian Journal of Reproductive Medicine*, 11(6):467-472.

Choi, Y., Park, M., Lee, K., Oh, C., & Park, H. (2014). Reproductive characteristics of the humbug damselfish, *Dascyllus aruanus*, in Chuuk Lagoon, Micronesia. *Ocean Science Journal*, 49(4):411-418.

D'Arrigo, G., Gianquinto, E., Rossetti, G., Cruciani, G., Lorenzetti, S., & Spyrakis, F. (2021). Binding of androgen- and estrogen-like flavonoids to their cognate (non) nuclear receptors: a comparison by computational prediction. *Molecules*, 26(1613):1-24.

Deswani, Desmarnita, U., & Mulyanti, Y. (2019). Asuhan Keperawatan Prenatal dengan Pendekatan Neurosains. Malang: Wineka Media.

Devita, H., & Amran, V. Y. A. (2019). Efek rokok terhadap kadar follicle stimulating hormone (fsh) pada pria. *Indonesia Jurnal Kebidanan*, 3(1):11-17.

Dewatisari, W. F. (2020). Perbandingan pelarut kloroform dan etanol terhadap rendemen ekstrak

daun lidah mertua (*Sansevieria trifasciata* Prain.) menggunakan metode maserasi. *Prosiding Seminar Nasional Biologi di Era Pandemi COVID-19*, 6(1):127-132.

Donkor, Y. O., Abaidoo, C. S., Tetteh, J., Darko, N. D., Atuahene, O. O., Appiah, A. K., Diby, T. &. Maalman, R. S. E. (2018). The effect of *Zingiber officinale* (ginger) root ethanolic extract on the semen characteristics of adult male wistar rats. *International Journal of Anatomy and Research*, 6(3):5481-5487.

Edgecombe, J. (2020). Research report: The role of gene duplication and neofunctionalization in the sexual plasticity of teleost fish. Dunedin: University of Otago.

Fathoni, A. (2021). Characterization of phytochemicals and chemical compounds of patat leaves (*Phrynium capitatum*) as wrapping materials for pesor doclang. *EduChemia*, 6(1):13-25.

Fitriyah, A., & Isyaturriyadhah. (2021). Efek asam lemak omega 3, omega 6 dan kolesterol sintetis terhadap reproduksi puyuh jantan (*Coturnix coturnix japonica*) dalam meningkatkan pendapatan peternak. *Agriptek: Jurnal Agribisnis dan Peternakan*, 1(1):1-6.

Forestryana, D., & Arnida. (2020). Phytochemical screenings and thin layer chromatography analysis of ethanol extract jeruju leaf (*Hydrolea spinosa* L.). *Jurnal Ilmiah Farmako Bahari*, 11(2):113-124.

Ghasemzadeh, A., Jaafar, H. Z. E., & Rahmat, A. (2016). Variation of the phytochemical constituents and antioxidant activities of *Zingiber officinale* var. rubrum Theilade associated with different drying methods and polyphenol oxidase activity. *Molecules*, 21(780):1-12.

García-Cruz, E. L., Yamamoto, Y., Hattori, R. S., de Vasconcelos, L. M., Yokota, M., & Strüssmann, C. A. (2020). Crowding stress during the period of sex determination causes masculinization in pejerrey *Odontesthes bonariensis*, a fish with temperature dependent sex determination. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 245:1-7.

Gharaei, A., Jorjani, H. E., Harijani, J. M., & Miandare, H. K. (2020). Effects of *Tribulus terrestris* extract on masculinization, growth indices, sex determination and steroid hormones level in zebra fish (*Danio rerio*). *International Aquatic Research*, 12(1):22-29.

Hayati, F., Nugraha, A. T., Ansori, R., & Faizah, W. S. (2020). Toksisitas ekstrak daun pegagan (*Centella asiatica* L. Urb.) pada embrio ikan zebra (*Danio rerio*). *Prosiding Seminar Nasional Hasil Penelitian dan Pengabdian Masyarakat dengan tema "Kesehatan Modern dan Tradisional"*, 124-131.

Herjayanto, M., Carman, O., & Soelistyowati, D. T. (2019). Maskulinisasi ikan pelangi Iriatherina werneri Meinken, 1974 menggunakan hormon 17α-metiltestosteron melalui perendaman embrio. *Jurnal Akuatika Indonesia*, 4(2):31-37.

Hickman, D. L., Johnson, J., Vemulapalli, T. H., Crisler, J. R., & Shepherd, R. (2017). Commonly used animal models. In M. A. Suckow & K. L. Steward (Ed.), Principles of animal research for graduate and undergraduate students. (pp. 117-175). Cambridge: Academic Press.

Hifumi, T., Kondo, Y., Shimizu, K., & Miyake Y. (2018). Heat stroke. *Journal of Intensive Care*, 6(30):1-8.

Hu, X., Li, X., Deng, P., Zhang, Y., Liu, R., Cai, D., Xu, Q., Jiang, X., Sun, J., & Bai, W. (2022). The consequence and mechanism of dietary flavonoids on androgen profiles and disorders amelioration. *Critical Reviews in Food Science and Nutrition*.

Hutagalung, R. A. (2020). Pengaruh perbedaan metode sex reversalmenggunakan tepung testis sapi terhadap maskulinisasi ikan nila merah (*Oreochromis niloticus*). *Manfish Journal: Marine, Environment, and Fisheries*, 1(1):9-14.

Hwang, J. S., Lee, S. A., Hong, S. S., Lee, K. S., Lee, M. K., Hwang, B. Y., & Ro, J. S. (2005). Monoamine oxidase inhibitory components from the roots of *Sophora flavescens*. *Archives of Pharmacal Research*, 28(2):190-194.

Jiang, Y., Luo, H., Hou, M., Chen, J., Tao, B., Zhu, Z., Song, Y., & Hu, W. (2022). Aromatase inhibitor induces sex reversal in the protogynous hermaphroditic rice field eel (*Monopterus albus*).

Aquaculture, 551(30):737-960.

Kellenberger, E., Foata, N., & Rognan, D. (2008). Ranking targets in structure based virtual screening of three dimensional protein libraries: methods and problems. *Journal of Chemical Information and Modelling*, 48(5):1014-1025.

Khomariah, N. E. (2021). Implementasi pemasaran ikan hias "Sub Betta Brothers" melalui social media dan digital marketing. *Bernas: Jurnal Pengabdian Kepada Masyarakat*, 2(1):196-201.

Kinth, P., Mahesh, G., & Panwar, Y. (2013). Mapping of zebrafish research: a global outlook. *Zebrafish*, 10(4):510-517.

Kolesova, O., Tomassetti, F., Cerini, P., Finucci, D., Turchetti, G., Capogreco, F., Bernardini, S., Calugi, G., & Pieri, M. (2021). Evaluation of eclia antigen detection tests as screening methods for COVID-19 in comparison with molecular analysis. *Irish Journal of Medical Science*, 1-5.

Koyama, T., Texada, M. J., Halberg, K. A., & Rewitz, K. (2020). Metabolism and growth adaptation to environmental conditions in *Drosophila*. *Cellular and Molecular Life Sciences*, 1-29.

Kumar, P., Biswas, G., Ghoshal, T. K., Kailasam, M., & Vijayan, K. K. (2018). Embryonic and larval developments of brackish water catfish, *Mystus gulio* (Hamilton and Buchanan, 1822) induced with human chorionic gonadotropin and consequent larval rearing. *Aquaculture Research*, 49(7):2466-2476.

Maack, G., & Segner, H. (2004). Life-stage-dependent sensitivity of zebrafish (*Danio rerio*) to estrogen exposure. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology*, 139(1-3):47-55.

Mahfut, & Sutyarso. (2020). Aplikasi tanaman kolam dalam budidaya ikan cupang di kelompok tani Sumber Makmur II, Dusun Tasik Madu, Desa Talang Jawa, Kecamatan Merbau Mataram, Lampung Selatan. *LPPM UNILA*, 10(10):1-6.

Mangaro, R., Sinjal, H. J., & Monijung, R. D. (2018). Maskulinisasi dengan menggunakan metode perendaman dan oral terhadap perubahan kelamin ikan nila (*Oreochromis niloticus*). *Jurnal Ilmiah Platax*, 6(1):117-122.

Matondang, A. H., Basuki, F., & Nugroho, R. A. (2018). Pengaruh lama perendaman induk betina dalam ekstrak purwoceng (*Pimpinela alpina*) terhadap maskulinisasi ikan guppy (*Poecilia reticulata*). *Journal of Aquaculture Management and Technology*, 7(1):10-17.

Moorhead, J. A., & Zeng, C. (2010). Development of captive breeding techniques for marine ornamental fish: a review. *Reviews in Fisheries Science*, 18(4):315-343.

Motosko, C. C., Zakhem, G. A., Pomeranz, M. K., & Hazen, A. (2018). Acne: a side effect of masculinizing hormonal therapy in transgender patients. *British Journal of Dermatology*, 180:26-30.

Nguyen, T., Talbi, H., Hilali, A., Anthonissen, R., Maes, A., & Verschaeve, L. (2019). *In vitro* toxicity, genotoxicity and antigenotoxicity of *Nigella sativa* extracts from different geographic locations. *South African Journal of Botany*, 126:132-141.

Nugroho, R. A. (2018). Mengenal mencit sebagai hewan laboratorium. Samarinda: Mulawarman University Press.

Nurazizah, N., Nabila, A. I., Adriani, L., Widjastuti, T., & Latipudin, D. (2020). Kadar kolesterol, kreatinin, urea darah dan kolesterol telur ayam sentul dengan penambahan ekstrak buah mengkudu yang disuplementasi Cu dan Zn. *Jurnal Nutrisi Ternak Tropis dan Ilmu Pakan*, 2(1):9-18.

Nurcahyo, W. (2018). Parasit pada ikan. Yogyakarta: UGM Press.

Pandian, T. J., & Sheela, S. G. (1995). Hormonal induction of sex reversal in fish. *Aquaculture*, 138(1-4):1-22.

Pebe, M. A. P. (2022). Uji konfirmasi morfin dengan metode KLT. *HUMANTECH: Jurnal Ilmiah Multi Disiplin Indonesia*, 1(7):867-876.

Permatasari, D. A. I., Icsvanditra, G., & Mahardhika, M. P. (2021). Analisis kadar kurkumin jamu kunyit asam menggunakan metode klt-densitometri. *Prosiding Seminar Informasi Kesehatan Nasional* (*SIKesNas*), 264-269.

Pramesti, N. K. A., Putri, N. P. V. G., Dewi, I. A. M.

L., Moreira, M. V., & Antarini, L. R. (2021). Identifikasi dan penetapan kadar kinin ekstrak kulit batang kina (*Cinchona succirubra*) secara KLT-densitometri. *Jurnal Ilmiah Medicamento*, 7(2):118-128.

Pratama, A. R. (2021). Pemberian pakan alami berbeda terhadap pertumbuhan dan kecerahan warna ikan zebra (*Branchyndanio rerio*). *Jurnal Pendidikan Fisika dan Sains (JPFS)*, 4(1):1-7.

Pratiwi, H. C., & Manan, A. (2015). Teknik dasar histologi pada ikan gurami (*Osphronemous gouramy*). *Jurnal Ilmiah Perikanan dan Kelautan*, 7(2):153-158.

Rante, T. R. K., Simbala, H. E. I., & Mansauda, K. L. R. (2020). Skrining fitokimia dan potensi antioksidan dari ekstrak daun tumbuhan ekor tikus (*Stachytarpheta jamaicensis* L) dengan metode 1.1 Diphenyl-2-Picrylhydracyl (Dpph). *Jurnal MIPA*, 9(2):91-96.

Revisa, D. (2016). Volume Luteinizing Hormone (LH) Ikan Nila (*Oreocrhomis niloticus*) jantan ditinjau dari nilai indeks kematangan gonad. Malang: Universitas Brawijaya.

Saputri, K. E., Fakhmi, N., Kusumaningtyas, E., Priyatama, D., & Santoso, B. (2016). Docking molekular potensi anti diabetes melitus tipe 2 turunan zerumbon sebagai inhibitor aldosa reduktase dengan autodock-vina. *Chimica et Natura Acta*, 4(1):16-20.

Shashikala, B., Suma V., M., & Suchitra, P. (2018). Quality control constraint of Guizotia abyssinica Cass, source of medicinally useful edible oil seeds. *The Journal of Phytopharmacology*, 7(5):431-436.

Scarsella, G. E., Gresham, J. D., & Earley, R. L. (2018). Relationships between external sexually dimorphic characteristics and internal gonadal morphology in a sex-changing fish. *Journal of Zoology*, 305(2):133-140.

Simó-Mirabet, P., Felip, A., Estensoro, I., Martos-Sitcha, J. A., de las Heras, V., Calduch-Giner, J., Puyalto, M., Karalazos, V., Sitjà-Bobadilla, A., & Pérez-Sánchez, J. (2018). Impact of low fish meal and fish oil diets on the performance, sex steroid profile and male-female sex reversal of gilthead sea bream (*Sparus aurata*) over a three-year production cycle. *Aquaculture*, 490:64-74.

Srikwan, P., Niamhom, B., Yagi, T., & Thayanukul, P. (2020). Characterization of methyltestosterone degrading bacteria isolated from tilapia masculinizing ponds: metabolic intermediate, glucose amendments effects, and other hormones transformation. *Water, Air, & Soil Pollution*, 231(498):1-15.

Subandiyono, & Hastuti, S. (2021). Aplikasi manajemen pemberian pakan induk pada pembenihan Ikan Lele (*Clarias gariepinus*). Semarang: Tiga Media.

Sujianto, S., Iriani, E. S., Setiadi, A., Syukur, C., & Rizal, M. (2021). Pursuing sustainable ginger production and supply performance in Central Kalimantan Province. IOP Conference Series: *Earth and Environmental Science*, 892(102063):1-8.

Sun, C. P., Yan, J., Yi, J., Zhang, X., Yu, Z., Huo, X., Liang, J., Ning, J., Feng, L., Wang, C., Zhang, B., Tian, X., Zhang, L., & Ma, X. (2020). The study of inhibitory effect of natural flavonoids toward ***β***-glucuronidase and interaction of flavonoids with ***β***-glucuronidase. *International Journal of Biological Macromolecules*, 143:349-358.

Thanasupsin, S. P., Cheang, L., & Math, C. (2021). Ecological risk of 17α-methyltestosterone contaminated water discharged from a full water recirculating earthen masculinization pond. *Human and Ecological Risk Assessment: An International Journal*, 27(6):1-20.

Wang, Z. Z., Jia, Y., Srivastava, K. D., Huang, W., Tiwari, R., Nowak-Wegrzyn, A., Geliebter, J., Miao, M., & Li, X. (2021). Systems pharmacology and *in silico* docking analysis uncover association of CA2, PPARG, RXRA, and VDR with the mechanisms underlying the Shi Zhen Tea formula effect on eczema. *Evidence-Based Complementary and Alternative Medicine*, 2021(8406127):1-17.

Yusuf, D. H., Sugiharto, & Wijayanti, G. E. (2014). Perkembangan post-larva ikan nilem *Osteochilus hasselti* C. V. dengan pola pemberian pakan berbeda. *Scripta Biologica*, 1(3):185-192.