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IOP Conference Series: Earth and Environmental Science

PAPER • OPEN ACCESS

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To cite this article: G N Susanto *et al* 2021 *IOP Conf. Ser.: Earth Environ. Sci.* **674** 012057

IOP Conf. Series: Earth and Environmental Science **674** (2021) 012057 doi:10.1088/1755-1315/674/1/012057

Genital reversal of betta fish by immersion using steroid extract of sea urchins

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Abstract. Sea urchin, *Diadema setosum* is a potential fishery commodity with high economic value. It contains a steroid compound as an aphrodisiac in male monosexes (masculinization). In aquaculture usually used 17α-methyl testosterone, but it was difficult to decompose or it contaminated with carcinogens and pollutants. Therefore, it needs to be replaced with safer natural hormones from sea urchin. B*etta sp.* is an ornamental fish with high demand because of its colorful, diverse tail shapes, and price of male is higher than female, so that male cultivation is beneficial. This study aimed to utilize steroid compounds of sea urchin gonad extracts in masculinization of betta fish. In particular, it is to examine the steroid compounds by providing different doses and soaking times to the formation of male. The study was conducted using a completely randomized design method which was further classified into two stages. In first stage, the soaking dose was 0, 2, 4, 6 and 8 mg L^{-1} for 12 h, while in stage II, the immersion duration was 0, 12, 18, 24 and 30 h at a dose of 4 mg L^{-1} in larvae aged 2 weeks. Each treatment was repeated 3 times, and data were analyzed using Anova as well as with the LSD test at the level of 5%. The results showed that on the immersion in dose of 4 mg L^{-1}, the highest male individual of 84.10% was formed. Also, a dose of 4 mg L^{-1} was significantly different from the control and 6 mg L^{-1}, but it was not significantly different from that of 2 and 8 mg L^{-1}. The difference in immersion time significantly affected the success of male monosex formation and the duration of 12 h in dose of 4 mg L^{-1} sea urchin extracts showed the highest percentage of 84.00%.

1. Introduction

Sea urchins (Echinodermata: Echinoidea) is one of the fisheries commodities with shells and gonads has a high potential economic value [1, 2]. *Diadema setosum* is a type of sea urchin which has economic value and the body parts consumed are the gonads [3, 4, 5] as a source of nutritious food [3, 6, 7]. Some research from sea urchins also has been done including isolation antibacterial from gonad and visceral organs [8, 9], toxicity analysis [10], bioactive potential [11]. Sea urchin gonads contain omega-3 fatty acids which are effective in lowering cholesterol levels in the body [12]. Sea urchin gonads also contain 28 kinds of amino acids, vitamin B complex, vitamin A, minerals, omega-3 fatty acids, and omega-6 [7, 9, 12-15], while the shell has potential as an anticancer, antitumor, antibiotics [8] and antimicrobials [8-10, 16]. The portion of sea urchins that produced the highest yield was gonads, about 7.10% and the high yield of sea urchin gonads indicates that many bioactive components can be extracted by methanol solvent [5, 9]. One of the bioactive substances contained in sea urchins is a steroid compound, a type of testosterone hormone that functions as an aphrodisiac in male monosex formation (masculinization) in some members of fishes and crustaceans. Various studies also indicated that sea urchins contain high protein, low fat and are believed as an aphrodisiac. Based on bioactive analysis, the crude extracts of gonads and whole sea urchins contain bioactive compounds, such as alkaloids,

IOP Conf. Series: Earth and Environmental Science **674** (2021) 012057 doi:10.1088/1755-1315/674/1/012057

steroids, flavonoids, saponins and phenols [4, 5, 9]. Echinoderm and Mollusca gonads can produce steroids de novo and synthesis of these steroids is assisted by the enzyme cytochrome P-450 [17, 18].

Betta fish, *Betta* sp. is one of the ornamental fish found in around Indonesian waters. This fish has a great demand by fish lovers, because it has a proportional body shape, bright coloration of scales, diverse tail shapes, attractive colour, and aggressiveness in maintaining territory. Male betta fish have fins and colors that more attractive than female, therefore male monosexual cultivation is more profitable. In addition, the price of male is also more expensive than female. To increase the male production, it can be cultured by genital reversion. The monosexual cultivation method is one way to produce good quality and quantity of betta fish with relatively short time. The sex reversal process usually requires the use of synthetic hormones, which face difficulty in decomposing within the body. In addition, there have been reports of carcinogenicity, pollution and other dangerous side effects [19, 20, 21]. One most widely used is the synthetic androgen hormone, 17α-methyl testosterone (MT) [22], assumed to improve digestion, food absorption and conversion, alongside regulate sexual development and other physiological processes [23]. One alternative way to overcome the problem is used natural testosterone from steroid extracts of sea urchin gonads, other than use from sea cucumbers. Sea urchin extract is relatively easy to be absorbed by the body and it is not cause side effects. Based on biotechnological application, genital reversion is one technique to produce monosex individuals through administration of hormones to stimulate the desired fish sex [21, 23]. Furthermore, sex reversion to male is expected to accelerate growth, increase production as well as economic value, due to the diversion of reproductive energy to somatic growth activities [24, 25]. Administration in steroid hormone can change the sex of fish physiologically, but it only alters phenotype rather than its genotype. The most effective method is by immersing the larvae during the critical period of differentiation, in which the larva's is still in a bipotential state directing the formation of sex morphology, behavior and function [19]. Therefore, the study aimed to utilize steroid compounds of sea urchin gonad in male formation of betta fish larvae. While specifically, aiming to determine these steroid compounds by administering at an appropriate dose and immersion time in the formation of male monosexuals.

2. Materials and Methods

2.1. Time and Place

The research was conducted from April to August 2020, at the Aquatic Biology Research Laboratory, Faculty of Mathematics and Natural Sciences, University of Lampung. The research was carried out in two stages, the first stage was the extraction of steroid compounds of sea urchin gonads, determination of steroid compounds and bioactive substances contained in sea urchins. The second stage was to test the activity of steroid compounds by administering the steroid hormone at different doses and immersion time in Betta fish (*Betta* sp.) larvae.

2.2. Extraction of Sea Urchin Gonads

The extracted sea urchin gonads were categorized based on diameter and weight, in terms of species and age. This conditions were to determine gonad existence. The raw materials obtained from fisherman at Lampung Bay. Extract of sea urchin gonads (*D. setosum*) was obtained by maceration using methanol for 48 h with a ratio of 1:3 (weight/volume) of ingredients and solvent (weight/volume), then shaken using a shaker with a speed of 180 rpm for 72 h, then filtered and evaporated using a rotary vacuum evaporator at 37-40ºC. The extract obtained was then dissolved in distilled water to obtain a solution with a concentration of 4 mg. L^{-1} as a treatment material. The process of sea urchin gonads extraction was performed at the Integrated Laboratory and Technology Innovation Center, University of Lampung.

2.3. Animal Maintenance Test and Treatment

The processes include the morphological selection of betta fish larvae, based on length, body colour, organ completeness, as well as age. Betta fish larvae were used aged 12-14 days, then acclimatized in a fibre tanks for 3 days and were selected based on the morphological and movement characteristics. The selected larvae were treated by immersion in sea urchin gonad solution according to the doses and predetermined duration of immersion, then transferred to rearing tanks and maintained for 50 days. The specimens were fed twice a day with fish pellets and silk worms *ad libitum*.

2.4. Research design
The research was conducted with a Completely Randomized Design (CRD) comprising 5 treatments. The experiment was further classified into two stages. In stage I, the soaking at different doses of steroid extract of sea urchins at 0, 2, 4, 6 and 8 mg L^{-1} immersed for 12 h, while in stage II, the immersion duration was 0, 12, 18, 24 and 30 h at a dose of 4 mg L^{-1}. Also, each treatment was repeated 3 times with a density of 4 individuals per liter and maintained for 50 days.

2.5. Research parameters
The parameters in this study are:

1. Percentage of Males, where: J : percentage of males (%), A: number of male fish and T: number of fish samples

$$J\ (\%) = \frac{A}{T} \text{ x } 100\ \%$$

2. Survival Rate, where: SR : survival rate of fish (%), Nt : number of fish at the end of the study and No: number of fish at the beginning of the study

$$SR = \frac{Nt}{No} \text{x } 100\ \%$$

3. Water quality during maintenance

Water quality measurements include: pH measured using pH meter, water temperature using thermometer kit, and dissolved oxygen using DO meter.

2.6. Data analysis
The data including the percentage of sex ratio and survival rate, then processed for the analysis of variance (Anova), if there is a real difference the continued with LSD test (the smallest real difference with α = 5%) using SPSS 16 software.

3. Results and Discussions
3.1. Determination of steroid compounds and bioactive substances contained in sea urchins
The analysis of bioactive components was carried out using phytochemical methods. This method is used to determine the secondary metabolite contents of a material. Tests were only carried out on ethyl acetate and methanol extracts from sea urchin gonads, *D. setosum*. The results obtained indicated that the two types of extracts contained bioactive compounds from the steroid, triterpenoid and saponin groups (Table 1).

Table 1. The results of analysis of the bioactive component of sea urchin gonads (*D. setosum*) on the study and comparisons with several other similar studies

	Present study			**Akerina [13]**		**Sukiman [6]**	**Apriandi [7]**	
Solvents	ethyl acetate	methanol	n-hexane	ethyl acetate	methanol	methanol	methanol	n-hexane
Alkaloid	+	+	-	-	+	+	+	-
Flavonoid	-	-	+	+	-	-	+	+
Phenol	-	-	-	+	-	-	-	-
Steroid	+	+	+	+	+	-	+	+
Triterpenoid	+	+	+	+	+	-		
Saponin	+	+	+	+	+	-	+	+

Notes: (-) = not detected, (+) = detected

3.2. Effect different doses of extract gonad of sea urchin on male formation of betta fish (Betta sp.)
The results of different doses of extract gonad of sea urchins on sex formation and survival of betta fish during 50 days maintenance in the aquaria are shown in Table 2.

Table 2. The formation of sex and survival rate at different doses of extract gonad of sea urchins during 50 days rearing in the aquaria

Treatments (extract of gonad sea urchin doses)	Male formation (%)	Female formation (%)	Survival rate (%)
0 mg L^{-1}	40.16±6.00^a	59.83±6.00^a	69.44±9.62
2 mg L^{-1}	72.86±1.89^b	27.13±1.89^b	72.13±17.31
4 mg L^{-1}	84.10±16.75^b	15.90±16.75^b	49.99±8.33
6 mg L^{-1}	54.26±14.09^a	45.73±14.09^a	69.44±12.72
8 mg L^{-1}	65.26±16.82^b	34.73±16.82^b	66.67±16.66

Notes: different letters in the table indicate a difference between treatments (LSD test)

The results showed that on treatment dose of sea urchin gonad extract 4 mg L^{-1} was significantly different from that of 0 and 6 mg L^{-1}, but it wasn't significantly different compared to 2 and 8 mg L^{-1}. The significance value of each treatment $p > 0.05$. The highest percentage of males was 84.10% in the treatment dose of 4 mg L^{-1}, while the lowest at the control dose of 0 mg L^{-1} was 40.16% as seen in Table 2. The formation of female phenotypes in control (0 mg L^{-1}) was 59.83%, 2 mg L^{-1} (27.13%), 4 mg L^{-1} (15.9%), 6 mg L^{-1} (45.73%) and 8 mg L^{-1} was 34.73%. The significance value of each treatment $p > 0.05$. The results of ANOVA showed that giving different doses of extract gonad of sea urchins did not have a significant effect on the survival rate of betta fish. The highest survival was found in the treatment dose of 2 mg L^{-1} at 72.13%, while the lowest was in the dose of 4 mg L^{-1} at 49.99%.

3.3. Effect of immersion time in extract gonad of sea urchin on male formation of betta fish (Betta sp.)
The results of immersion time in extract of sea urchins on sex formation and survival of betta fish during 50 days maintenance in the aquaria are shown in Table 3.

Table 3. The formation of sex and survival rate at different immersion time in extract gonad of sea urchins dose of 4 mg L-1 during 50 days rearing in the aquaria

Treatments (immersion time)	Male formation (%)	Female formation (%)	Survival rate (%)
0 h	39.67±5.86^a	63.67±11.93^a	69.43±9.64
12 h	84.00±17.09^b	19.33±22.84^b	49.96±8.35
18 h	70.67±26.10^b	29.33±26.10^a	45.53±23.62
24 h	58.00±8.00^a	45.33±4.16^a	50.00±22.05
30 h	70.00±22.71^b	33.33±28.45^a	58.30±16.70

Notes: different letters in the table indicate a difference between treatments (LSD test)

The results of ANOVA indicated that the soaking time in the dose of 4 mg L^{-1} for 12 h showed a significant effect on the formation of male betta fish. The immersion time at dose of 4 mg L^{-1} was significantly different from 0 h (control) and 24 h with significance value of each treatment $p < 0.05$. The highest formation of male genital phenotypes was obtained from immersion in a solution of 4 mg L^{-1} for 12 h at 84.00%. The results also indicated that the difference in the length of immersion at a dose of 4 mg L^{-1} did not have a significant effect on the survival rate of betta fish. The significance value of each treatment $p > 0.05$.

In this study, the maintenance of water quality was observed in the form of degree of acidity (pH), temperature and dissolved oxygen (DO) content measured every 10 days during 50 days of maintenance. Maintenance of water quality can be seen in Table 4.

Table 4. Water quality measurements during 50 days maintenance

Parameters	Range		Tolerance Range
	Low	High	
pH	6,66	6,93	6,8-7[1)]
Temperature (°C)	26,53	27,53	25-30[2)]
DO (mg/L)	3,73	3,83	3,6-4[3)]

Sources: **1)**. Cholik [26], **2)**. Biokani [27], **3)**. Lubis [28]

The bioactive compound analysis showed similar to the study by Akerina [9] on the same sample of sea urchin gonads (*D. setosum*) which contain bioactive components of steroids, triterpenoids and saponins in extracts of n-hexane, ethyl acetate and methanol. Meanwhile, research by Sukiman *et al* [4] on whole extracts of sea urchins was only found alkaloid and no other bioactive compounds. The difference may occur due to the use of different solvents and extraction methods. The research by Apriandi [5] indicated base on bioactive analysis, crude and whole gonad extracts from sea urchins, *D. savignyi* contains bioactive compounds of alkaloids, steroids, flavonoids, saponins and phenols. Bioactive compounds derived from the steroid have potential as aprodisiac agents and sex reversal.

According to Zairin [23] giving low dose hormone can cause less optimal sex drive, while high dose can cause sterile and prolonged immersion cause a paradoxical effect. To distinguish the formation of male phenotypes, it can be seen from the physical characteristics of the tail, dorsal and anal fins which are longer and wider. In the female larvae the three types of fins are narrower and shorter. Male formation (masculinization) is possible because sea urchins contain high levels of protein. Padang *et al* [3] and Toha [13] stated that sea urchin gonads contain essential and non-essential amino acids. One of the important roles of amino acids in the formation of the androgen hormone, testosterone, serves to increase libido and spermatozoa formation, and can enter the bloodstream as a regulator of secondary sexual characteristics. This is consistent with the statement of Tupan and Silaban [2] that sea urchin gonads contain steroids, triterpenoids and saponins, which have been proven to be used in masculinization techniques. Based on the results, extract gonad of sea urchins at dose of 4 mg L^{-1} had a significant effect on the increase of male percentage by 84.10%. Another study by Lubis *et al* [28] with honey at a dose of 4 mg L^{-1} produced the highest male of betta fish at 77.33%. The function of sea urchin gonad extract is almost the same as other natural ingredients, such as sea cucumber that plays a role in increasing male phenotypes. Sea urchin is a fishery product with high protein content. The distinctive function of protein is to build and maintain cells and tissues of living things that cannot be replaced by other nutrients. According to Akerina *et al* [9] the part of sea urchins that produced the highest yield was gonads by 7.10% and the lowest was spines of 0.94%. The high yield of sea urchin gonads is thought to be influenced by the large amount of compounds that dissolve in methanol solvent. Methanol solvent can extract components derived from alkaloids, phenolic, carotenoids, tannins, sugars, amino acids and glycosides. In addition methanol solvent also has less polar properties than water, therefore it can destroy cell walls and cause components in destroyed and dissolved cells [29].

In *D. setosum* gonads there are 8 essential amino acids (lysine, methionine, phenylalanine, threonine, valin, arginine, tryptophane and histidine) and essential aminosemic acids (cystine) and non-essential amino acids such as aspartic acid, glutamic acid, glycine and serine, vitamin A and B complex, and minerals [7, 13]. Zinc (Zn) and selenium (Sn) are mineral compounds contained in sea urchin gonads and can affect the body's testosterone levels [6]. In addition, sea urchins have a secondary metabolite, naphthoquinone, which has anti-free radical effects [30]. Extract gonad of sea urchin contains active compounds from the steroid/triterpenoid group and saponins from the triterpenoid group [9]. According to Ruey-Sheng *et al* [31] steroids are androgenic hormones that play a role in determining the expression of male phenotypes. Therefore, the stimulation of this hormone can cause the character of fish larvae to become male (masculinization). The study used the immersion method to direct the male formation, because the hormones more effectively enter the body through the circulation and osmoregulation system in the body. Betta fish larvae used in the study were 12-13 days old by considering the critical period of sexual differentiation process, so that individual sex can be determined to either male or female. According to Handayani *et al* [32] there are factors that need to be considered in the process of directing sex, such as continuous hormone administration when the gonads have not been formed and the use of appropriate hormone doses. The results in the dose of 4 mg L^{-1} sea urchin extract solution for 12 h also showed the highest male formation by 84.00%. The longer the hormone immersion time is used, the lower of male formation is performed. This probably not all hormones are properly absorbed in the body. It is in accordance with the opinion of Zairin [23] that the weakness of the immersion method is not all absorbed hormones can reach the target organs.

Treatment by immersion in sea urchin extract solution for male sex reversal gave more optimal effect than without immersion. Giving hormones aims to disrupt the hormonal balance of the blood during the process of sex differentiation [23]. In the treatment without immersion in sea urchin extract solution, the formation of female genitalia was higher by 63.67%. There is no hormonal stimulation that affects the gonad differentiation process, therefore the formation of male and female sex occurs naturally. Meanwhile, treatment by immersion in sea urchin extract solution triggers hormonal stimulation which affects the differentiation into males. Sea urchin gonad extract contains active compounds that can penetrate the cell wall by inhibiting protein synthesis, causing changes in the composition of the cells. *Naphtaquinone* which is owned by sea urchins also has potential as anti-bacterial and anti-inflammatory similar to aspirin [33]. The length of soaking has no significant effect on the survival of betta fish. The highest survival rate was obtained in the control at 69.43%, due to the age of fish larvae which are susceptible to changes in environment and temperature. The factors influencing survival rates comprise of biotic factors, including population density and age, the organisms' adaptation ability to the environment as well as environmental abiotic factors [24, 25].

According to Effendi [34] temperature is an important factor in the metabolic process of water organisms, therefore sudden changes in temperature can disrupt their life and cause death due to increased toxicity of dissolved contaminants and decrease dissolved oxygen. The mortality of betta fish larvae occurred on the first day until the eighth day after treatment. The death larval is thought to be due to stress after transfer from the treatment tanks to the rearing tanks. According to Law *et al* [35] the pH value describes the acidity level of a water which is related to the concentration of carbon dioxide in the waters. The ideal pH for growth of betta fish ranges from 6.8-7.0 [26]. The pH of media was range from 6.66-6.93, it was in accordance with the life span required by betta fish. Respiration of organisms and faeces left over from feeding can cause the pH of the maintenance media to change every day. Lower pH value can cause fish easily stressed and attacked by disease, and reduce productivity and growth levels. According to Effendi [34] temperature can affect the rate of metabolism and respiration in aquatic organisms. Fish are classified as poikilothermal where the body temperature adjust to environmental temperature, therefore, all fish physiological processes are strongly influenced by temperature. Low temperatures will increase the toxicity of dissolved contaminants, reduce DO levels which may cause fish more susceptible to fungi and mortality [34]. The temperature was maintained at 26.53-27.53ºC and relatively stable. According to Biokani *et al* [27] betta fish prefer warm water with temperatures between 25-30ºC. Oxygen is needed as a source of energy to oxidize food substances that enter the body [36]. The fluctuations of dissolved oxygen in the rearing media can cause a decrease in appetite which will interfere on growth and stress of fish. Dissolved oxygen was maintained at 3.73-3.83 mg L^{-1}. The range is still considered normal, because the ornamental fish can tolerate DO in 3.6-4.0 mg L^{-1} [28].

4. Conclusions

The results of two types of extracts (ethyl acetate and methanol) from sea urchin gonads, *D. setosum* contained bioactive compounds from the steroid, triterpenoid and saponin groups. The highest male formation was found in the immersion dose of 4 mg L^{-1} sea urchin extract solution at 84.10%. The difference of immersion time significantly affected the success of male monosex formation. The duration of 12 h showed relatively high in male formation by 84.00%. There is no significant effect of different doses and immersion time on the survival rate of betta fish larvae (*Betta* sp.).

Acknowledgment
The research was fully sponsored by a grant from Research Institutions and Community Services (LP2M) University of Lampung, under Penelitian Dasar DIPA BLU Unila 2020 on behalf of G N Susanto. The authors are grateful to the Integrated Laboratory and Technology Innovation Center, University of Lampung for sea urchin extraction analysis.

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