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Sex Reversal of Juvenile Freshwater Crayfish (*Cherax quadricarinatus*) Influenced by Steroid Extract of Sea Cucumber and 17α-Methyltestosterone Hormone at Different Temperatures

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Abstract. Redclaw crayfish (*Cherax quadricarinatus*) is a freshwater crayfish with high economic value, which entices fish farmers to farm and increases its production. Males of this species have larger harvest size than females, so it is proposed to culture an all-male population for increasing yields. This research aims to find out the effect of sea cucumber steroid extract and 17α-methyltestosterone at different temperatures on sex reversal of juvenile *C. quadricarinatus*. This research was designed using factorial complete random design method. The treatments were observed in controlled temperatures at 27 °C and 31 °C, with a dose of sea cucumber steroid extracts (SCSE) of 50 mg · kg^{-1} and 50 mg · kg^{-1} of 17α-methyltestosterone. The results indicated that both SCSE and 17α-methyltestosterone were most effective at 27 °C on increasing the male percentage of juvenile crayfish, which were up to 75.16 % and 73.79 % respectively. These steroid hormones had a significant effect on decreasing the number of female individuals and also on increasing total length, daily weight gain, and growth of juvenile crayfish. However, they did not make a significant effect on survival rate, the percentage of intersex and feed conversion ratio of juvenile freshwater crayfish.

Keywords: 17α-methyltestosterone, freshwater crayfish, sea cucumber, steroid, temperature

INTRODUCTION

Redclaw freshwater crayfish (*Cherax quadricarinatus*) is a fishery commodity originating from Queensland, Australia and has been introduced to Indonesia since 1991. Crayfish culture is increasingly favored by farmers because it is adaptable to wide range of Indonesia environment and seasonal changes, so it has the potential to be cultivated anywhere and throughout the year. However, there is an obstacle in the growing stage (at seven to eight month of rearing), since the male crayfish grow faster and weighs more than the females ones [1].

In the development of aquaculture, one way to increase the production rate is through the cultivation of monosexual sex by directing sex reversal (genital reversal). Sex reversal is a way of reversing the direction of genital development of animals that should be directed male to female or vice versa. One of the sex reversal technique is masculinization, in which the genital development is directed from female to be male. This engineering technique is widely used in the process of masculinization of fish and some animal crustacean members, such as giant prawns, crabs, and freshwater crayfish. This technique is performed in a labile period before the gonads differentiate and are still sensitive to hormone treatment [2]. Sex reversal only alters phenotype rather than its genotype [3]. Sex reversal can be done by using natural or synthetic hormones. The choice of the hormone, whether androgen or estrogen, is according to the desired sex result, and should consider that it is not harmful to cultivated biota, consumers, and environment. The steroid hormone that widely used in genital reversals is 17α-methyltestosterone. The use of this synthetic hormone has a very high rate of success at 96 % to 100 %. However, it leaves residues that are potentially bad for human health, environment, and cultivated organisms [4]. Research needs to be done to obtain a natural steroid source that is safe for humans

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and environment. One approach is to exploit the regular hormones extracted from the viscera of sea cucumbers (*Holothuria scabra*). Liebermann-Burchard test and bioassay showed that the extract of sea cucumber is proven to contain a large amount of steroid. The largest yield obtained from 1 kg of wet viscera (21.28 g of crude extract) contains steroid in the form of testosterone with the amount of 6.124 $\mu g \cdot kg^{-1}$ [5]. Kustiariyah [6] stated that the extract of sea cucumber contains testosterone that can be used for sex reversal of fishery commodities, such as giant prawns [7–9], guppy fish [10, 11], where males are more valuable than females.

In addition to the steroid hormones, the process of sex gestation can also be triggered through environmental temperature manipulation. Temperature affects the rate of metabolism in the body [12]. Increased temperature can speed up the metabolism process so that the crayfish appetite also increases [13]. This certainly can help the absorption of steroid hormones contained in the feed to enter the body effectively. Optimal water temperature will affect various mechanisms in the body, especially during the period of sexual differentiation, which is very sensitive to hormonal stimulation and temperature. Conversely, if the temperature is too low, it will cause gonadal sensitivity in the formation of genitals to be slower [13].

In tilapia, the temperature at 28 °C caused the male individuals' formation up to 52.33 %, while at 36 °C produced male tilapia up to 81 % [12]. In channel catfish, the influence of temperature at 29 °C to 30 °C could give rise to forming males equivalent to 69.5 % [14]. Some of the results of these studies indicated that the temperature also affects the process of sex reversal. In this study, a combination of steroid hormone extracts of sea cucumber and 17α-methyltestosterone with different temperature variations on sex reversal of juvenile freshwater crayfish were conducted. This research is expected to answer the problem in freshwater crayfish cultivation, especially in the effort to increase production through monosexual cultivation with the natural hormone.

MATERIALS AND METHODS

Extraction of Sea Cucumber

The sea cucumber used for extraction were characterized by species and age, based on weight and length to determine the existence of testosterone. The raw material used was approximately 10 kg of mature sea cucumber, each was weighing around 300 g to 800 g with a length around 20 cm to 37 cm. Extracts of powdered sea cucumber were obtained by using solvents including n-hexane, diethyl ether, and methanol. After 24 h exposure in n-hexane, the extract was concentrated under low pressure at 30 °C by rotary evaporation. The diethyl ether extract was ready after 48 h, and then the solvent was removed by rotary evaporation at 35 °C. The methanol extract was ready after 72 h, and then the solvent was removed by rotary evaporation at 40 °C. Ether-methanol was formed by adding ether in order to separate the methanol-aqueous extract, and then the upper phase was separated by separating funnel. The upper phase was combined with n-butanol and aqueous extract was separated by separating funnel. Each extract was shaken by mechanical shaking at room temperature (25 °C). All processes were carried out on dark condition, and crude extracts were kept in the freezer [15].

Animal Maintenance Test and Treatment

Three hundred of juvenile freshwater crayfish, approximately 1 wk to 2 wk old or 2 cm to 2.5 cm of body length, were obtained from Gemma Farm cultivators in Klaten, Central Java. Juvenile crayfish morphologically had complete extremity organs (such as chelipeds and pleopods), healthy, and taken from well-selected brood-stocks. Before putting it in the container, juvenile crayfish were first weighed to determine their initial weight and then acclimated in the laboratory for 2 d to 3 d before being kept in a tub or aquarium.

The crayfish was fed with sinking pellet acquired from Gemma Farm. The feed composition was 40 % protein, 12 % water, 6 % fat and 3 % fiber. The hormone used was a natural steroid hormone from the extracts of sea cucumber innards. The sea cucumber extraction process was done in the laboratory of Organic Chemistry, University of Lampung, while the synthetic hormone used was type 17α-methyltestosterone from Biotech Argo-lab (Sidoarjo, Indonesia). The dose of both hormones was 50 $mg \cdot kg^{-1}$ of feed.

The hormones were added in separate pellet feed by adding 50 mg of each hormone. Each hormone was dissolved in 250 mL of 70 % alcohol and then sprayed evenly on the separate feed. After the hormone was sprayed, the feed was then stirred and dried for 1 d until all alcohol evaporated, then stored in a closed container. Pellet feed for the control group did not include steroid hormones and was only treated with various temperatures. Treatment feeding was done for 50 d. Each treatment consisted of three replications. The amount of feed given was 8 % to 10 % of the total weight of juvenile crayfish. The frequency of feeding was as much as two times a day, which is in the morning and in the evening.

Juvenile freshwater crayfish were reared for 50 d. During nursery rearing, juveniles were observed under a stereoscopic microscope to determine sex, using the presence or absence of the genital openings at the base of the third pair of walking legs (females), genital papillae at the base of the fifth pair of walking legs (males), or both structures in an individual (intersex) [13].

Each container was given enough aeration as to keep the water quality always optimal for the growth of crayfish. Substitution of water was done as much as 30 % to 50 % of total water volume every 3 d by basic water siphoning or dirt settling, and then the substitute water was added to the volume of water as before. Water quality parameters measured were temperature, pH, dissolved oxygen content (DO) and hardness. Observation on temperature, DO, and pH of the water was performed every 3 d, while the hardness was measured at the end of the study. These individuals were maintained in individual plastic tanks containing 20 L of dechlorinated tap water (pH: 7.2–7.9, DO: 3.9 mg · L^{-1} to 5.3 mg · L^{-1}, hardness: 83 mg · L^{-1} as $CaCO_3$ equivalents), under continuous aeration and natural photoperiod. The water temperature was maintained at 26 °C to 31 °C.

Morphology of Gonopod of Juvenile Freshwater Crayfish with Scanning Electron Microscopy (SEM)

SEM analysis was done by preparing the samples of freshwater crayfish specimen using a freeze dryer. The samples were sprinkled as thinly as possible on a carbon-coated specimen holder with the aim at attaching the sample on the specimen holder and then coated with sputter coater, which is the surface coating process of the sample to strengthen the electrical conductivity. SEM works through electron beam where the electron on the sample will be reflected and captured by a secondary electron detector and converted into an image. The coating was done with the gold material (gold coating) at a current of 20 mA for 60 s. The coating sample was then mounted on stage holder for SEM analysis. Stage holders containing samples were inserted into the chamber and retrieved by SEM type ENOV 10 (Integrated Laboratory and Technology Innovation Center, University of Lampung).

Research Design

This research was arranged with factorial completely randomized design method, consisting of temperature factor of two levels (27 °C and 31 °C) and hormone factor of two levels (steroid extract of sea cucumber and 17α-methyltestosterone) with each control, six treatments and each treatment with three replications. The treatments are as follows in Table 1.

TABLE 1. Experimental groups and treatments.

Experimental Groups	Treatments
KT. 27	Control maintained at 27 °C
KT. 31	Control maintained at 31 °C
ST .27	Administration of 50 mg · kg^{-1} of sea cucumber steroid extract in feed maintained at ± 27 °C
ST .31	Administration of 50 mg · kg^{-1} of sea cucumber steroid extract in feed maintained at ± 31 °C
MT .27	Administration of 50 mg · kg^{-1} of 17α-methyltestosterone in feed maintained at ± 27 °C
MT .31	Administration of 50 mg · kg^{-1} of 17α-methyltestosterone in feed maintained at ± 31 °C

The Percentage of Male Formation in Each Group

The percentage of successful formation of the male sex is calculated using the formula [4]:

$$J\ (\%) = \frac{number\ of\ male\ crayfish}{number\ of\ crayfish\ samples} \times 100\%$$

Survival Rate

The survival rate of juvenile crayfish is calculated using the formula [16]:

$$SR = Nt/No \times 100\%$$

SR: crayfish survival rate (%)
Nt: number of juvenile crayfish at the end of the study
No: number of juvenile crayfish at the beginning of the study

The Growth Rate of Daily Weight

The growth rate of daily weight is calculated using the formula [16]:

$$GR = \frac{Wt - Wo}{t}$$

GR: daily growth rate ($g \cdot d^{-1}$)
Wt: average weight of crayfish on day t (g)
Wo: average weight of crayfish on day 0 (g)
t: maintenance time (d)

Growth or Weight Gain

The growth or weight gain is calculated using the formula [16]:

$$\Delta W = Wt - Wo$$

W: weight gain (g)
Wt: weight at the end of the study (g)
Wo: weight at the beginning of the study (g)

Feed Conversion Ratio

The feed conversion ratio is calculated using the formula [16]:

$$FCR = \frac{F}{(Wt + D) - Wo}$$

FCR: feed conversion ratio
F: the amount of feed given during treatment (kg)
Wt: crayfish weight at the end of treatment (kg)
D: dead crayfish weight during treatment (kg)
Wo: weight at the beginning of treatment (kg)

Data Analysis

The data, including the formation of sex, life, daily growth rate, weight gain, and feed conversion ratio were then processed for the analysis of variance. If there is a real difference, the analysis will continue to the LSD test (the smallest real difference) using SPSS 19 program by IBM SPSS Inc., USA.

RESULTS AND DISCUSSION

Results

The complete results of the effect of natural steroid hormone extracts of sea cucumber and synthetic hormone of 17α-methyltestosterone at different temperatures to the male formation in juvenile freshwater crayfish are shown in Table 2.

TABLE 2. The proportion of individual males, survival, daily growth rate, weight gain, and feed conversion ratio of juvenile freshwater crayfish feed treated with sea cucumber steroid extracts and 17α-methyltestosterone at different temperatures for 50 d of treatment.

Treatment	Percentage of Male Individuals (%)	Survival (%)	The Growth Rate of Daily Weight ($g \cdot d^{-1}$)	Weight Gain (g)	Feed Conversion Ratio
KT.27	42.06 ± 8.36^{a}	23.33 ± 10.41^{a}	1.26 ± 0.25^{a}	1.56 ± 1.0^{a}	2.98 ± 0.51^{a}
KT.31	50.07 ± 5.51^{a}	80.00 ± 15.00^{a}	0.86 ± 0.09^{a}	6.87 ± 4.90^{a}	3.94 ± 1.83^{a}
ST.27	75.16 ± 6.01^{b}	75.00 ± 8.66^{a}	1.53 ± 0.02^{b}	11.10 ± 1.33^{b}	3.25 ± 0.73^{a}
ST.31	62.5 ± 12.5^{b}	50.00 ± 10.00^{a}	1.45 ± 0.10^{b}	11.02 ± 4.06^{b}	2.15 ± 0.19^{a}
MT.27	73.79 ± 6.18^{b}	56.67 ± 10.41^{a}	1.00 ± 0.20^{a}	6.72 ± 3.44^{a}	3.02 ± 0.20^{a}
MT.31	53.33 ± 5.77^{b}	53.33 ± 5.77^{a}	1.15 ± 0.9^{a}	8.95 ± 3.97^{a}	2.06 ± 0.31^{a}

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

The Proportion of Freshwater Crayfish Males

The result showed that the highest percentage of juvenile males was found in the treatment of steroid hormone from sea cucumber at 27 °C (ST.27) by 75.16 %, while the lowest percentage of males was found in control treatment at 27 °C (KT.27) by 42.06 % (Table 2). The result of analysis of variance with 95 % confidence level showed that giving steroid hormones and different temperatures produced a significant influence ($p < 0.05$). The results of the analysis proved that giving steroid hormones and different temperatures gave a positive response in increasing the percentage of male freshwater crayfish.

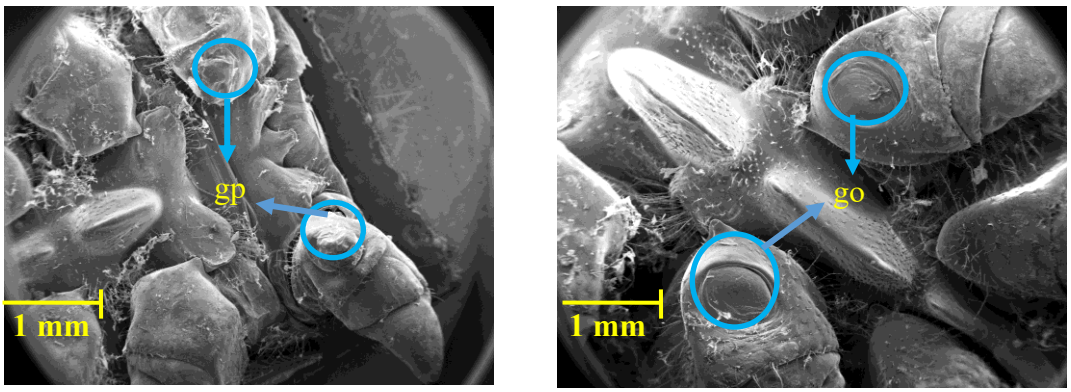

FIGURE 1. Photograph of scanning electron microscopy of the sex formation on male (left) and female (right) of juvenile freshwater crayfish (*Cherax quadricarinatus)*. The genital papillae (gp) on the males are located at the base of the fifth pair of walking legs (pereiopods) and the genital openings (go) on the females are located at the base of the third pair of walking legs (pereiopods)

Survival

Based on the analysis of the variance of each treatment, it was found that the highest survival rate was found in the control treatment at 31 °C (KT.31) by 80.00 %, while the lowest survival rate was in the temperature control treatment at 27 °C (KT.27) by 23.33 % (Table 2). The results of analysis of variance with 95 % confidence level showed that giving steroid hormones and different temperatures did not produce a significant effect ($p > 0.05$).

The Growth Rate of Daily Weight

Based on the observation for 50 d, the highest daily weight gain of freshwater crayfish was obtained at the treatment of steroid extract of sea cucumber at temperature 27 °C (ST.27) by 1.53 g · d^{-1}, while the lowest daily weight gain of crayfish was found in the control treatment at 31 °C (KT.31) by 0.86 g · d^{-1}. (Table 2). The results of analysis of variance with 95 % confidence level indicated that giving steroid hormones and different temperatures produced a significant influence ($p < 0.05$).

Weight Gain

Based on observation for 50 d, the highest crayfish weight was obtained in the treatment of steroid extract of sea cucumber at temperature 27 °C (ST.27) by 11.10 g, while the lowest crayfish weight was found the in control treatment at 27 °C (KT.27) by 1.56 g (Table 2). From the results of analysis of variance with 95 % confidence level, it was indicated that giving steroid hormones and different temperatures produced a significant influence ($p < 0.05$).

Feed Conversion Ratio

Based on observation for 50 d, the lowest feed conversion value of freshwater crayfish was obtained in the treatment of 17α-methyltestosterone hormone at 31 °C (MT.31) by 2.06 g, while the highest crayfish feed conversion ratio was found in the temperature control treatment at 31 °C (KT.31) by 3.94 g (Table 2). From the results of analysis of variance with 95 % confidence level, it was shown that giving steroid hormones and different temperatures did not produce a significant effect ($p > 0.05$).

Discussion

Several success factors in the sex reversal of the males are the presence of steroid hormones and the optimal maintenance environment. In this study, the hormone used was a natural hormone derived from sea cucumber steroid extracts and a synthetic one, which was 17α-methyltestosterone with a dose of 50 mg · kg^{-1} of feed for both hormones. This dose was sufficient to produce the male majority in juvenile freshwater crayfish culture.

Steroid extract of sea cucumber is a hormone that provides the effect of masculinity because it can increase the amount of testosterone in the crayfish body. This is in line with research by [9] on juvenile males of prawns, which also employed sea cucumber steroid extract. In the research, the highest yield of testosterone hemolymph was found at a dose of 25 mg · L^{-1}.

The treatment with 17α-methyltestosterone gave the male percentage of 73.79 %, which was lower than one of sea cucumber steroids. These results were different from the research with 17α-methyltestosterone treatment dose of 50 mg · kg^{-1}, which resulted in a smaller percentage of males of 59.96 % in the same animal [17]. However, other research showed that 17α-methyltestosterone treatment in 20 d old prawn larvae at 35 mg · kg^{-1} dose feed for 30 d resulted in 80.91 % males [7].

The dose of hormones that were too low caused the process of sex reversal lasted less perfect. Likewise, if the dose were too high, there would be a tendency that the fish would become sterile [18]. Therefore, the appropriate dose is required. In this study, a dose of the hormone 50 mg · kg^{-1} of feed simply provided a high percentage of males when compared with controls. The administration of steroid hormones in a small amount to individuals that have not develop gonads yet will specifically affect the hypothalamus regularly during the critical phases of gonadal development and sex determination of males. Suspected testosterone affects neurons through the preoptic hypothalamus section with synapses secreted in gonadotropin-releasing factor [7].

The natural hormone of sea cucumber steroid extracts was capable of giving a higher percentage of males when compared with the use of 17α-methyltestosterone. This could be an answer for the problem in the cultivation, in which in the synthetic hormone, the residual properties will have a negative impact on human well-being. Sea cucumber steroid extracts can be an alternative option to substitute the synthetic hormone 17α-methyltestosterone.

In addition to hormonal influences, the right environmental temperature can affect the process of forming individual males in freshwater crayfish. Crayfish is a poikilothermic animal in which its body temperature rises and falls according to ambient temperature [19]. Therefore, all physiological processes of crayfish are influenced by ambient temperature. Water temperature affects the behavioral response, metabolic and reproductive processes [20].

In this study, in addition to the use of hormones, different temperature treatments were also given, which were 27 °C and 31 °C. The ST.27 treatment was given steroid extract of sea cucumber at 27 °C, while the

MT.27 was given 17α-methyltestosterone at 27 °C, giving the male percentage 75.16 % and 73.79 % respectively. This result is similar to that of [10, 11] at a temperature of 27 °C, which resulted in the highest percentage of male guppy by 92.7 %. In the process of sex reversal, environmental factors are made in such way that it can affect the transcription of sex determinants [21]. By making the environment predominant of saturated androgen hormones, the hormonal balance will be disrupted, so the phenotypic sex determination will further lead to the formation of a male individual.

In Table 2, it was known that the presence of steroid hormones in feed and different temperatures cause the percentage of survival of crayfish to increase. From this results, it was proven that the existence of steroid hormone in the diet and the different temperatures had a positive effect on increasing the percentage of crayfish survival, although not significant. The treatment of sea cucumber steroid extract and different temperature could give the value of survival equal to 75 %, which was higher if compared to control treatment in which only equal to 23.33 % at 27 °C. Previous research showed that sea cucumbers have useful bioactive components for the medical and health fields; consisting of triterpene glycosides (saponins), chondroitin sulfates, glycosaminoglycan, sulfated polysaccharides, sterols (glycosides and sulfates), phenolics, peptides, cerebrosides, and lectins [22]. Sea cucumber extract has biological and pharmacological activities such as anti-cancer, anti-coagulant, anti-hypertensive, anti-inflammatory, antioxidant, and anti-tumor [23]. Sea cucumber extract also has antibacterial and antifungal activity. The methanol extract of the sea cucumber *Holothuria scabra* and acetyl acetate have inhibitory ability against bacteria *Pseudomonas aeruginosa* and *Bacillus cereus* [24]. *H. scabra* sea cucumber extract also has antifungal activity against *Candida albicans* and furthermore, the crude extract of sea cucumber can improve stamina of the body [25]. Our previous works indicated that the sea cucumber steroid hormone is able to improve crayfish body health and give a positive response to its livelihood.

The giving of sea cucumber extract had a significant effect on the increase of daily weight of crayfish. This further reinforced the opinion that the steroid hormone can stimulate metabolic activity, thus stimulating the growth of crayfish. In addition to having androgenic properties, testosterone turned out to have anabolic properties that can spur muscle growth [26]. Sea cucumber steroid extract contains androgen hormones just like methyltestosterone (MT), so it has anabolic properties that can stimulate growth [27]. Steroid hormone could also affect the growth of both male and female fish [28].

Table 2 showed that the treatment of sea cucumber steroid hormone at 27 °C (ST.27) showed a higher weight gain by 11.10 g compared to the control treatment at 27 °C (KT.27) by 1.56 g. This showed that the extract of sea cucumber that was given could increase crayfish weight gain because the hormone that was mixed in the feed was able to cooperate in growth acceleration.

The final weights obtained gave different effects. This is suspected because the steroid hormones increase androgen content, so that animal aggressiveness increase and affects the rate of metabolism in the body. The increased rate of metabolism affects appetite by increasing it, and in the end, the growth rate will also increase. This is also demonstrated by [28] research in which steroid hormones could affect the growth of both male and female fish. However, in the freshwater crayfish growth, there was a difference between male and female individuals that were seen as characteristic [29].

Other factors besides hormones were additional substances contained in the sea cucumber. The content of muscle collagen in the sea cucumber reaches 80 %, while other protein content reaches 86.6 % [30]. Sea cucumbers also contain many growth factors that can stimulate cell and tissue regeneration [31].

In Table 2, 17α-methyltestosterone hormone at 31°C (MT.31) was able to produce a lower feed conversion ratio (FCR) of 2.06 when compared with the treatment of steroid sea cucumber (ST) and control (KT). It shows the use of the hormone 17α-methyltestosterone is still better when compared with other treatments. Administration of hormones 17α-methyltestosterone and temperature variations is able to make the conversion value of feed on juvenile freshwater crayfish to be efficient. As a comparison, tilapia treated with 17α-methyltestosterone and temperature were able to give feed conversion ratio of 2.48 [32].

The hormone 17α-methyltestosterone in addition to regulating the sexual development of fish also improves digestion, food absorption, feed conversion as well as other physiological processes [33]. The administration of sea cucumber steroid extracts and 17α-methyltestosterone will stimulate metabolic activity so that crayfish will have a high appetite.

CONCLUSION

The results of this study conclude that feeding sea cucumber steroid extract and 17α-methyltestosterone with a dose of 50 mg · kg^{-1} each to freshwater crayfish (*Cherax quadricarinatus*) have the most noteworthy effect on sex reversal to male individuals at 27 °C by 75.16 % and 73.79 % respectively. Sea cucumber steroid extract is more effective in sex reversal to male individuals of freshwater crayfish (*Cherax quadricarinatus*) when compared to 17α-methyltestosterone at both 27 °C and 31 °C respectively.

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