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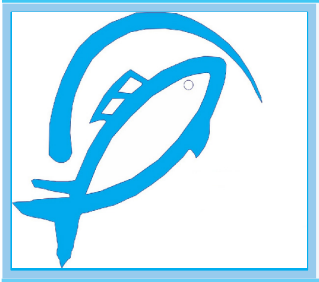

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Research Article

Feminization of Maroon Clownfish (*Amphiprion biaculeatus*, Bloch 1790) with 17β-Estradiol Hormonal Induction

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Abstract

As maroon clownfish (*Amphiprion biaculeatus*) is a protandrous hermaphroditic fish, feminization process with 17β-estradiol hormone can be applied to accelerate the female broodstock candidate supply for further spawning effort. This study aimed to evaluate the feminization of *A. biaculeatus* with 17β-estradiol hormonal induction. This study used a completely randomized design with several hormone dosage, namely P0 (without 17β-estradiol hormone induction), P1 (0.5 µg 17β-estradiol/g body), P2 (1.0 µg 17β-estradiol/g body), and P3 (1.5 µg 17β-estradiol/g body). These treatments were applied with three replications. Five fish composed of α-fish, β-fish, and three γ-fish were reared in each aquarium for 90 days with a flowing water system. The α- and β-fish were then removed, while the γ-fish was injected with hormone. *Otohime* pellet feed was fed three times a day until apparent satiation. The results showed that the 17β-estradiol hormone could induce 100% of the feminization process of male *A. biaculeatus*. The dosage of P3 obtained the lowest value percentage of red, green, blue (RGB), but showing the highest total of length and body weight (6.67±0.42 cm and 6.40±0.78 g, respectively), estradiol content (149.73±4.24 ρg/mL), GSI and HSI (0.38±0.07% and 3.59±0.49%), and glucose content (4.67±0.64 mg/dL), followed by more mature gonad profile than other treatments. This condition indicates that fish in P3 treatment has been reversed as functional female. The average survival rate for the treatment was as high as 60%. Therefore, the application of 17β-estradiol hormonal induction is effective for the feminization process in *A. biaculeatus* as a protandrous hermaphroditic fish.

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1. Introduction

Maroon clownfish (*A. biaculeatus*) is one of the marine ornamental fish included in the Pomacentridae family and is the third highest exporting-commodity in the world (Biondo, 2018). Therefore, the international demand of this fish is quite high. To fulfill this condition, natural catch is performed. Due to continuous exploitation by catching, this fish has become rare in its natural environment. Therefore, cultural production takes precedence over meeting the international market demand for environmental information (Prasetio and Kusrini, 2012). In addition, this fish has a unique reproduction system due to its social hierarchical system, protandrous hermaphroditism, and monogamous system (Madhu *et al*., 2013; Klann *et al*., 2021; Fitz *et al*., 2022). This condition marks the difficulties of mass artificial spawning, limiting the seed stock and broodstock candidate supply in culture production. Social hierarchy in the reproduction system of clownfish is influenced by various factors, namely body size, sex reversal, domination, aggressiveness, feed, environment, and genetics (Fitz *et al*., 2022).

Among those factors, sex reversal becomes a phenomenon that is controlled directly by social hierarchy (Sambroni *et al*., 2013). This phenomenon could help supply the female broodstock for sustainable reproduction intensiveness and productivity. Several studies have revealed that certain fish species can reverse their sexes, including clownfish (Nagahama *et al*., 2021). In fish, sex reversal can occur from male to female or mentioned as protandry hermaphrodite, and from female to male or called protogynous hermaphrodite (Kobayashi *et al*., 2013).

In clownfish, social hierarchy is composed of the giant female fish called α-fish, the giant male fish called β-fish, and a non-functional male fish or a non-breeder fish called γ-fish (Mitchel, 2003). In a natural population, if the functional female fish dies, the adult male fish will develop and reverse its sex as female broodstock. Furthermore, the giant male fish among non-breeder fish will become a new male broodstock (Casas *et al*., 2022). This mechanism is known as protandry hermaphrodite. Madhu *et al*. (2012) stated that male broodstock of *Amphiprion ocellaris* can reverse its sex to female when the non-breeder fish are introduced to the population to form a new pair. In cultivation condition, sex reversal can be induced with hormonal approach (Hoga *et al*., 2018). Feminization is defined as a technology to reverse sex development, one of them is estradiol hormone. According to Haq (2013), there are factors that influence the sex reversal success, namely treatment dosage, hormone types, treatment procedure and period, and duration. High dosage generally provides a shorter period, and vice versa. A factor discussed in this study is a hormone dosage applied in a short-term period.

Estrogen is one of the essential steroidal hormones that plays a role in sexual maturation and differentiation, including oogenesis, vitellogenesis, and testis development (Sambroni *et al*., 2013). Several studies have been conducted to confirm feminization in fish. Falahatkar *et al*. (2013) stated that 5 mg/kg of 17β-estradiol hormone injected into *Sturgeon stellata* with a 3-weeks duration was an optimum dose for feminization of five-months fish (40.9 ± 1.1 g). Moreover, dietary supplementation of 50 and 60 mg/kg of 17β-estradiol hormone obtained 100% female fish in Mexican snook (*Centropomus poeyi*) for 60 days (Vidal-López *et al*., 2019). Pereira *et al*. (2020) reported that the dietary supplementation of 100 mg/kg of 17β-estradiol was effective for 50-days post-larvae feminization of *Leporinus* at the 60th day. Therefore, 17β-estradiol hormone can be applied for the acceleration of sex reversal mechanism. During sex reversal in *A. bicinctus*, Casas *et al*. (2016) stated that a stable male gonad reduced its testis tissue and turned to ovarian tissue by 87%, 40 days after female fish were removed from the environment. Thuong *et al*. (2017) reported that male feminization to female occurred in *A. ocellaris* on the 60th day after incubation with 0.1 mgL-1 of 17β-estradiol for 15 days. This feminization success was marked by the gonad profile and hormone level alteration.

Kim *et al*. (2010) found different estradiol levels in *A. melanopus* male broodstock that experienced feminization. When a female broodstock existed, male broodstock had estradiol level at 182.1±30.2 ρg/mL, then increased to 893.1±55.3 ρg/mL after female removal and became higher after completely turning to female (1192.4±40.4 ρg/mL). This condition was also affirmed by the increased estrogen receptor and vitellogenin gene expressions in gonad and liver when fish developed as adult female. Furthermore, different estradiol injection dosage caused different estradiol levels. During the initial trial, the plasma estradiol level was 35.9±5.2 ρg/mL, then increased to 375.5±15.3 ρg/mL for 24 hours after 0.1 μg/g estradiol injection and continued to increase higher at 560.4±40.1 ρg/mL for 24 hours after 1 μg/g estradiol injection, before finally decreased. The higher the estradiol dosage, the higher the plasma estradiol level will be, resulting in a faster sex reversal process.

Recently, the success of feminization has been determined solely by the alteration of steroidal

hormones and the gonad histological profile. In fact, the success indicators of physiological mechanisms can be marked morphologically, histologically, hormonally, and genetically. Color is one of the morphological indicators related to the presence of ornamental fish. In addition, oxidative stress due to hormone injections is important in relation to survival. So far, color characters and oxidative compounds during feminization remain unidentified. Liu *et al.* (2017) explained that social hierarchy can also affect the ornamental fish's capability in color differentiation. Social hierarchy in sex reversal is reflected in an individual's color due to carotenoid concentrations. Cavraro *et al.* (2017) stated that color pattern is an intraspecific signal that leads to sexual dimorphism as carotenoids have roles in color pigmentation. Moreover, Glade *et al.* (2015) reported that oxidative stress inclination in gonad tissue could limit testosterone production. Alonso-Alvarez *et al.* (2008) mentioned that testosterone reduction could be maintained by increasing the carotenoid levels.

However, the feminization study in *A. biaculeatus* through estradiol hormone injection needs further investigation. Therefore, this study aims to evaluate the effectiveness of the feminization process on *A. biaculeatus* with 17β-estradiol hormone application. This is considered important to accelerate the availability of the broodstock under cultivation conditions so as to minimize the female broodstock limitation in social hierarchy. Hence, a sustainable development can be established to fulfill the increasing market demand.

2. Materials and Method

This study was performed from February to April 2021 at several laboratories namely Laboratory of Ornamental Fish and Laboratory of Environmental Health in Ambon Sea Aquaculture Fisheries Center, Laboratory of Chemistry and Laboratory of Zoology in Faculty of Mathematics and Natural Sciences, Pattimura University Ambon, and Laboratory of Center for Ornamental Fish Culture Research Depok.

2.1 Experimental Design

This study used a completely randomized design with four doses of 17 β-estradiol hormone, namely P0 (without 17β-estradiol hormone induction), P1 (0.5 µg of 17β-estradiol/g body), P2 (1.0 µg of 17β-estradiol/g body), and P3 (1.5 µg of 17β-estradiol/g body). Each treatment had three replications, so there were 12 experimental units.

2.2 Fish Rearing

The fish used were spawning products from a cultured condition and reared for 60 days after hatching. The spawned broodstock pair was collected from nature on Ambon Island, Maluku. The fish were reared with a flowing-water system inside a 40 x 40 x 30 cm aquarium equipped with aeration and 50 L water. Each aquarium was filled with five fish, containing α-fish (11-12 cm), β-fish (7-8 cm), γ1, γ2, and γ3-fish (5-6 cm). Overall, the fish samples used in this study were 60 fish. The α-, β-, and γ-fish were put and kept alive for two days in the same aquarium. The feminization treatment was performed by removing the α- and β-fish from each aquarium, and one of the γ-fish was injected with 17β-estradiol hormone, following the applied dosage. The injected γ-fish reared for 90 days was intended for sensitivity and aggressivity. Otohime commercial pellet was fed until apparent satiation three times a day (07.00, 12.00, and 16.00 GMT+9). A total of 15% feed was given per body weight.

2.3 Hormonal Injection

The 17β-estradiol hormone was retrieved from Sigma Chemical Co. (St. Louis, MO, USA). Before injection, the γ-fish was pulled from the aquarium with a wet towel to prevent its movement and measured its body weight to determine the hormone volume for injection. The hormone was diluted in 0.5 mL of 70% alcohol, before injecting it into the fish sample (Putra *et al.*, 2012). The fish was sedated with 200 mg/L of MS-222, injected intramuscularly on the right part of the dorsal fin, and then released back to the aquarium. The injection was performed following the applied dosage at 08.00 GMT+9. The fish was injected with the hormone three times at the 1st, 3rd, and 5th week of rearing.

2.4 Feminization and Growth Observations

To identify feminization visually, secondary sexual characteristics were observed, such as skin and fin color in each treatment. The skin and fin color were measured based on the red, green, blue (RGB) value with the RGB measure option in ImageJ 1.48f software. Color mapping around body area was grouped as skin zone 1, skin zone 2, anal fin, pectoral fin, caudal fin, ventral fin, and dorsal fin (Figure 1).

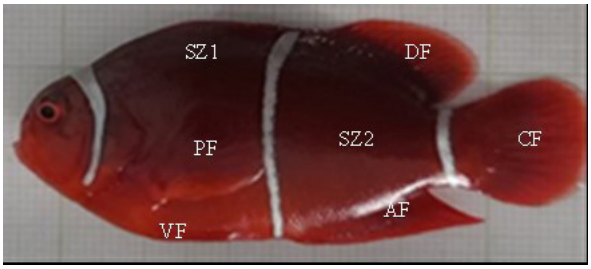


Figure 1. Color observation zone. SZ1: skin zone 1; SZ2: skin zone 2; AF: anal fin; PF: pectoral fin; CF: caudal fin; DF: dorsal fin; VF: ventral fin.

For growth, total length was measured with a vernier caliper (0.01 cm accuracy). Body weight was measured with digital scale (0.001 g accuracy). Total length and body weight were measured during the initial and final rearing period. The specific length growth rate was calculated with the formula:

SLGR (%) = [(ln Lt–ln L0)/t x 100] .....(Eq 1)

where:
SLGR = Specific length growth rate
Lt = total length on the final rearing period (cm)
L0 = total length on the initial rearing period (cm)
t = rearing period (day)

The specific weight growth rate was calculated with the following formula:

SWGR (%) = [(ln Wt–ln W0)/t x 100] .....(Eq 2)

where:
SWGR = Specific weight growth rate
Wt = body weight on the final rearing period (g)
W0 = body weight on the initial rearing period (g)

2.5 Estradiol Content Analysis

Hormone content was measured at the final rearing period. The fish were sedated with 200 mg/L of MS-222 (Salis *et al*., 2019). The 0.5 mL blood sample was taken from the caudal fin artery with a 1 mL syringe rinsed with Ethylenediaminetetraacetic acid (EDTA). The blood sample was stood for two hours and centrifuged at 3,000 rpm for 15 minutes. Blood plasma obtained from centrifugation was kept in a freezer at -20ºC for further analysis. The hormone concentration in the plasma was measured with enzyme-linked immunosorbent assay (ELISA) kit estradiol DRG diagnostics.

2.6 Measurement of Gonadosomatic Index (GSI), Hepatosomatic Index (HSI), and Gonad Profile Observation

After blood sampling, the fish was dissected to obtain its gonad and liver for further measurement. The gonad and liver weight were used for GSI and HSI calculations.

GSI (%) = (gonad weight/body weight) x 100 ..(Eq 3)

HSI (%) = (liver weight/body weight x 100) ..(Eq 4)

Gonad was documented on a millimeter-blocked paper with NIKON COOLPIX camera and observed its morphological condition. The gonad histology was prepared following Casadevall *et al.* (2009), Abol-Munafi *et al.* (2011), Ghosh *et al.* (2012), and Khoo *et al.* (2018).

2.7 Carotenoid Content Analysis

The carotenoid and glucose contents were analyzed at the final rearing period. The carotenoid content was analyzed using the spectrophotometric method modified from Ramamoorthy *et al.* (2010). After the fish was operated on to remove its gonad and liver, the fish skin and fin were taken separately, then air-dried for 48 hours. The dried samples were grinded with a blender and extracted with acetone. Total carotenoids were determined with AMV11 UV-Vis spectrophotometer at 260 nm wavelength.

2.8 Glucose Content Analysis

The glucose content was analyzed by spectrophotometer. The 0.05 mL of blood plasma, glucose standard, and distilled water were mixed in each reaction tube filled with 3.5 mL color reagent (acetic acid : Orto-toluidine = 94:6). These samples were heated in a covered water bath for 10 minutes at 100°C and cooled to a room temperature. The blood glucose content was also measured with the spectrophotometer at 635 nm after color reagent dilution (Wedemeyer and Yasutake, 1977).

2.9 Data Analysis

The RGB value, carotenoid content, total length, body weight, estradiol content, GSI, HSI, and glucose content were analyzed statistically with one way ANOVA at 95% confidence level using SPSS 22.0 software. If there was a significant difference among treatment data, Duncan's test would be performed further. The gonad profile was analyzed descriptively. To analyze the relationship of parameters and the feminization process with 17β-estradiol induction, a correlation analysis called product moment correlation was applied.

3. Results and Discussions

3.1 Results

3.1.1 Secondary sexual characteristics

Three non-breeder fish were reared in each aquarium, but several fish died due to aggressive behavior and were replaced with new samples, resulting in a longer period of observation. After 90 days of rearing, P2 treatment had the brightest color among other treatments. In this study, P0 and P1 treatments had the palest color (Figure 2).

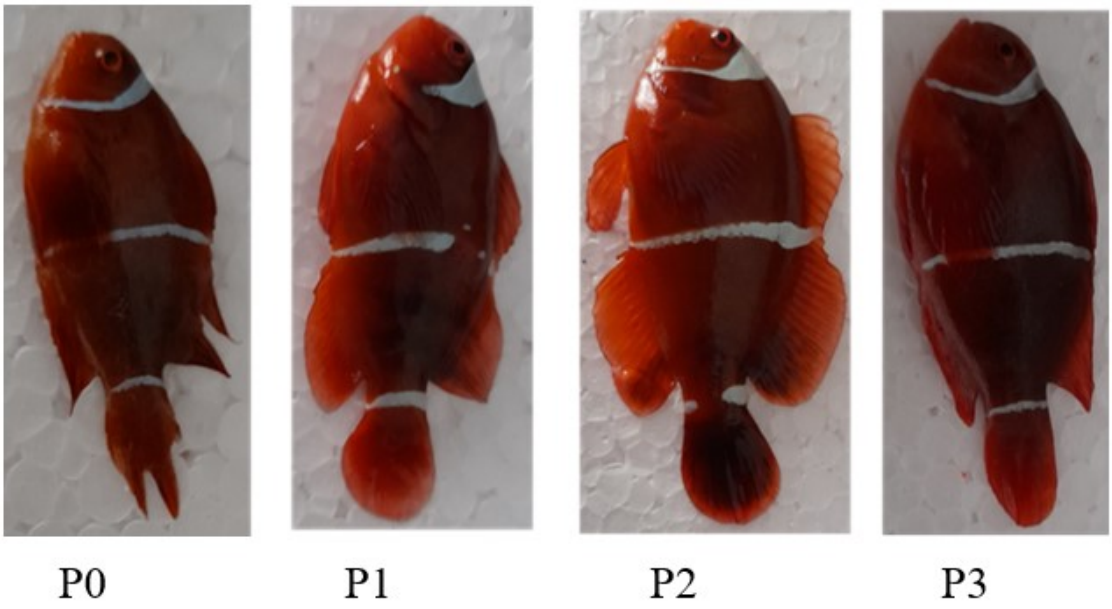


Figure 2. Visual display of *P. biacuelatus* after 17β-estradiol induction. P0: 0 mg/g body; P1: 0.5 mg/g body; P2: 1.0 mg/g body; P3: 1.5 mg/g body.

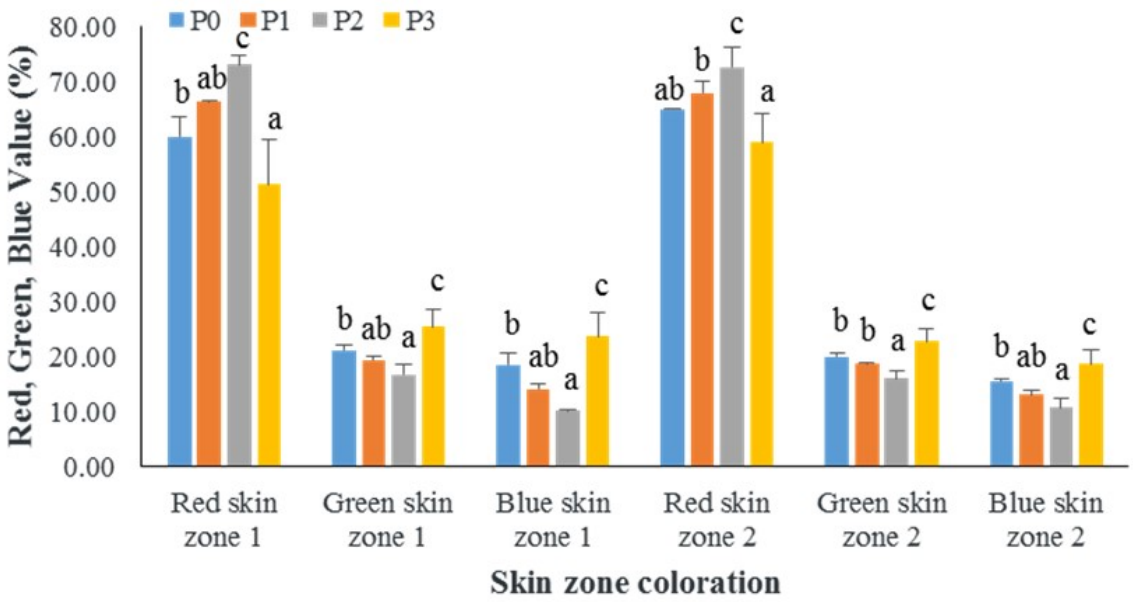


Figure 3. The RGB value of skin color in *P. biaculeatus* after 17β-estradiol induction. P0: 0 mg/g body; P1: 0.5 mg/g body; P2: 1.0 mg/g body; P3: 1.5 mg/g body. Different letters indicate a significantly different value among treatments.

Based on the statistical analysis, the 17β-estradiol hormone influences the RGB value in skin and fin ($p<0.05$). The red color percentage in skin zone 1 and 2, and all fin parts in P2 treatment obtained the highest value among other treatments (Figure 3 and Figure 4).

3.1.2 Carotenoids content

Statistical analysis indicates that the 17β-estradiol induction influences the carotenoid content ($p<0.05$). The P2 treatment obtained the highest carotenoids in skin and fins among other treatments (Figure 5). In zone 1 and 2 of fish skins, P2 treatment had average carotenoid contents of 5.60 ±1.74 mg/g and 8.99 ± 4.50 mg/g, respectively, while carotenoid contents in anal, pectoral, caudal, and ventral fins were

9.17 ± 2.82 mg/g, 9.29 ± 0.13 mg/g, 11.63 ± 1.72 mg/g, 8.78 ± 1.40 mg/g, and 9.92 ± 0.17 mg/g, respectively.

3.1.3 Growth rates

The total length and body weight increased during the rearing period. Based on the statistical analysis, the 17β-estradiol dosage influences the increased total length and body weight ($p<0.05$). The P3 treatment obtained the highest total length and body both at the initial and final rearing period compared to other treatments. In contrast, the specific growth rate in the P1 treatment obtained the highest value among other treatments (Figure 6). For total length, P3 treatment obtained a higher initial and final total lengths (5.99 ± 0.22 cm and 6.67 ± 0.42 cm, respectively), followed by P2 treatment (5.60 ± 0.12 cm and 6.17 ± 0.21 cm, respectively), and P1 treatment (5.32 ± 0.16 cm and 5.10 ± 0.36 cm, respectively). In the body weight, P3 treatment obtained a higher initial and final total lengths (4.97 ± 0.62 g and 6.40 ± 0.78 cm, respectively), followed by P2 treatment (4.33 ± 0.15 g and 5.62 ± 0.28 g, respectively), and P1 treatment (3.58 ± 0.53 g and 5.32 ± 0.52 g, respectively).

3.1.4 Estradiol content

Based on the statistical analysis, the 17β-estradiol induction influences the estradiol level in the fish ($p<0.05$). The estradiol level in P3 treatment obtained the highest value among other treatments (Figure 7). In comparison with the control, P3 treatment had a higher estradiol content (149.73 ± 4.24 ρg/mL), followed by P2 and P1 treatments at 134.78 ± 4.47 ρg/mL and 111.33 ± 9.04 ρg/mL, respectively.

3.1.5 GSI and HSI

Based on the ANOVA results, 17β-estradiol dosage influences the GSI and HSI. Duncan's test presented that P3 treatment has the highest GSI and HSI values among other treatments. (Figure 8). The P3 treatment had a higher HSI value (3.59 ± 0.49%), followed by P2 and P1 treatments at 2.82 ± 1.0% and 1.82 ± 0.52%, respectively. For GSI, P3 treatment also had a higher value (0.38 ± 0.07%), followed by P2 and P1 treatments at 0.30 ± 0.09% and 0.20 ± 0.03%, respectively.

3.1.6 Gonad profiles

Descriptively, the female gonad profile has been reversed from that of male fish in all treatments (Table 1). In the control treatment, the testes reversed to the ovary, as marked by the orange gonad color and long-deflated structure. In hormone treatments, the gonad was enlarged with an orange color and easily separated texture, mainly in P3 treatment, with a long and bulging structure. Gonad histology describes the maturing development and mature condition based on the number of oocytes. Similar to P3 treatment, P2 treatment also had a mature gonad with a maturity level of level IV,

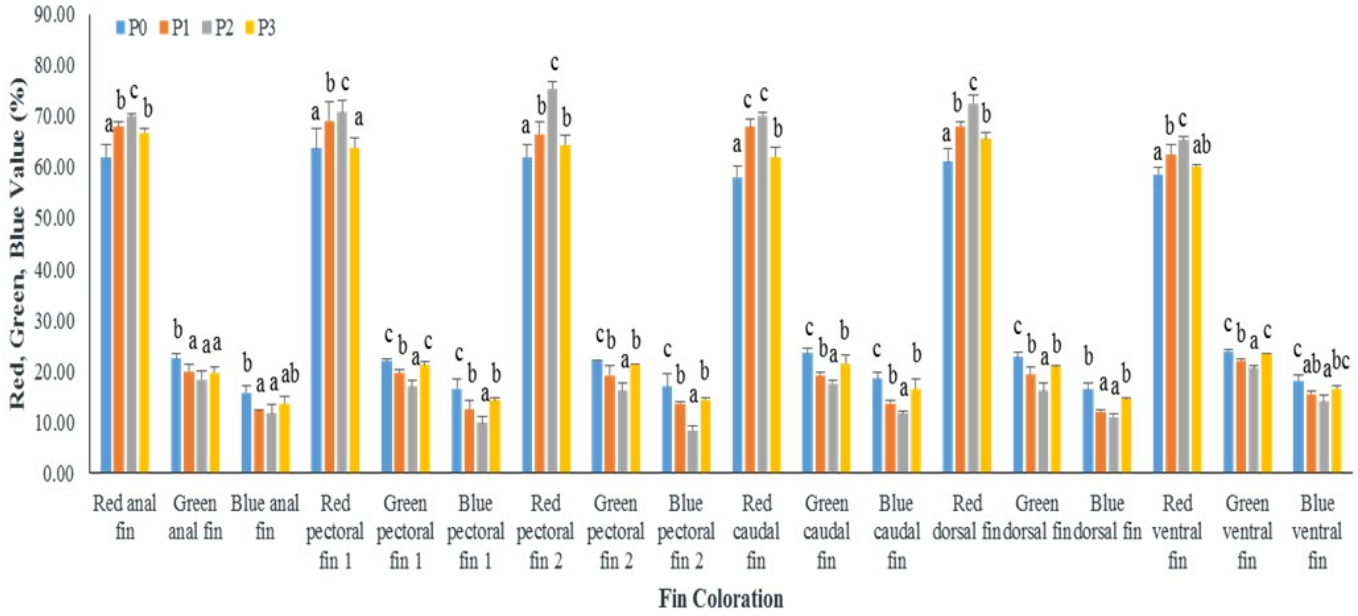
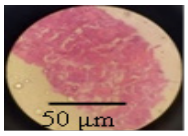
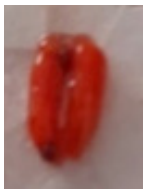
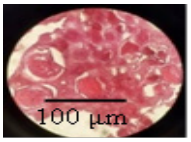
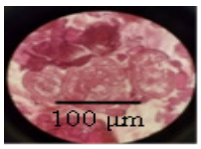
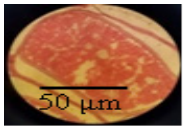


Figure 4. The RGB value of fin color in *P. biaculeatus* after 17β-estradiol induction. P0: 0 mg/g body; P1: 0.5 mg/g body; P2: 1.0 mg/g body; P3: 1.5 mg/g body. Different letters indicate a significantly different value among treatments.

as there was an oocyte at the perinucleolar phase and no cystic spermatocytes, spermatids, or sperm. The P1 treatment was similar to the control, whereas in a maturity level of level III, which contained perinucleolar oocyte phase, did not have cystic spermatocytes, spermatids, or sperm.

Table 1. The *P. biaculeatus* gonad profiles after 17β-estradiol hormonal injection

Treatment	Developmental Stage	Morphology describes	Characteristics	Gonadal maturation level	Histology profile	Gonadal status
P0	*Maturing*		Sub-adult gonad, containing primary spermatocyte and oocyte	III		Intersex
P1	*Maturing*		Gonad contains oocyte in perinucleolus phase. Spermatid declines and cysts presents without ovarian cavity	III		Female
P2	*Mature*		Gonad contains oocyte in perinucleolus phase. Gonad has no spermatocyte cyst, spermatid, and sperm	IV		Female
P3	*Mature*		Gonad contains oocyte in perinucleolus phase. Gonad has no spermatocyte cyst, spermatid, and sperm	IV		Female

Note: P0: 0 mg/g body; P1: 0.5 mg/g body; P2: 1.0 mg/g body; P3: 1.5 mg/g body

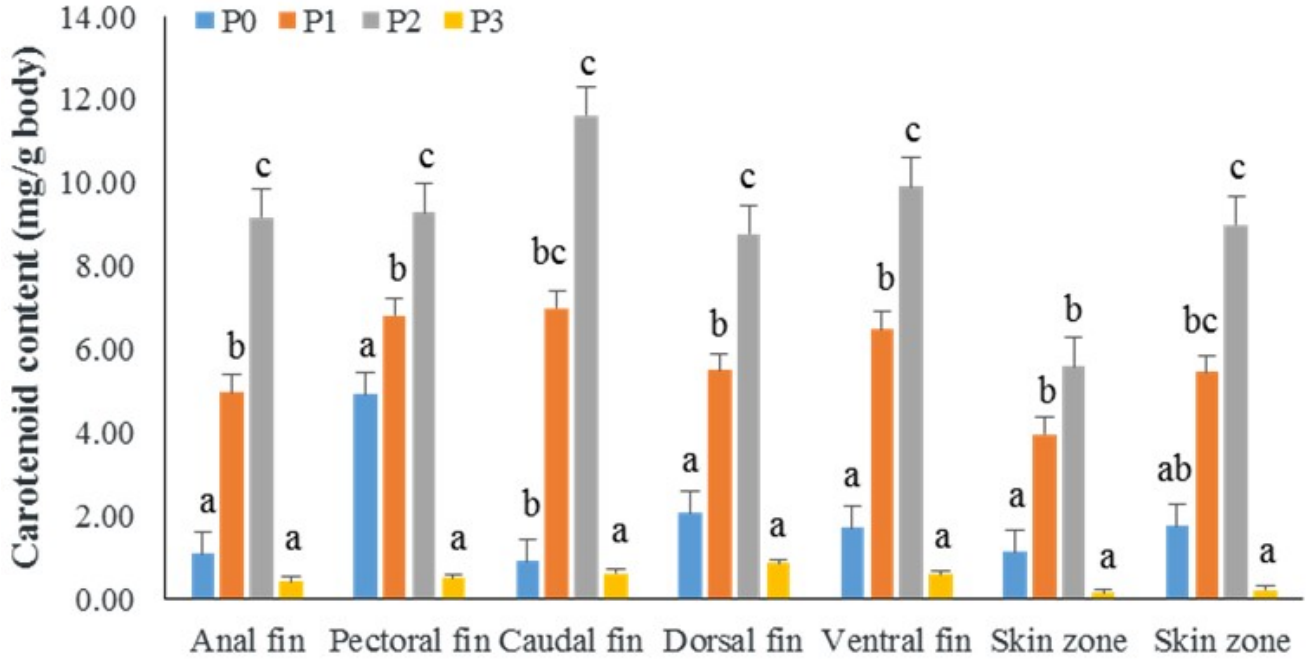


Figure 5. Carotenoid content of *P. biaculeatus* after 17β-estradiol induction. P0: 0 mg/g body; P1: 0.5 mg/g body; P2: 1.0 mg/g body; P3: 1.5 mg/g body. Different letters indicate a significantly different value among treatments.

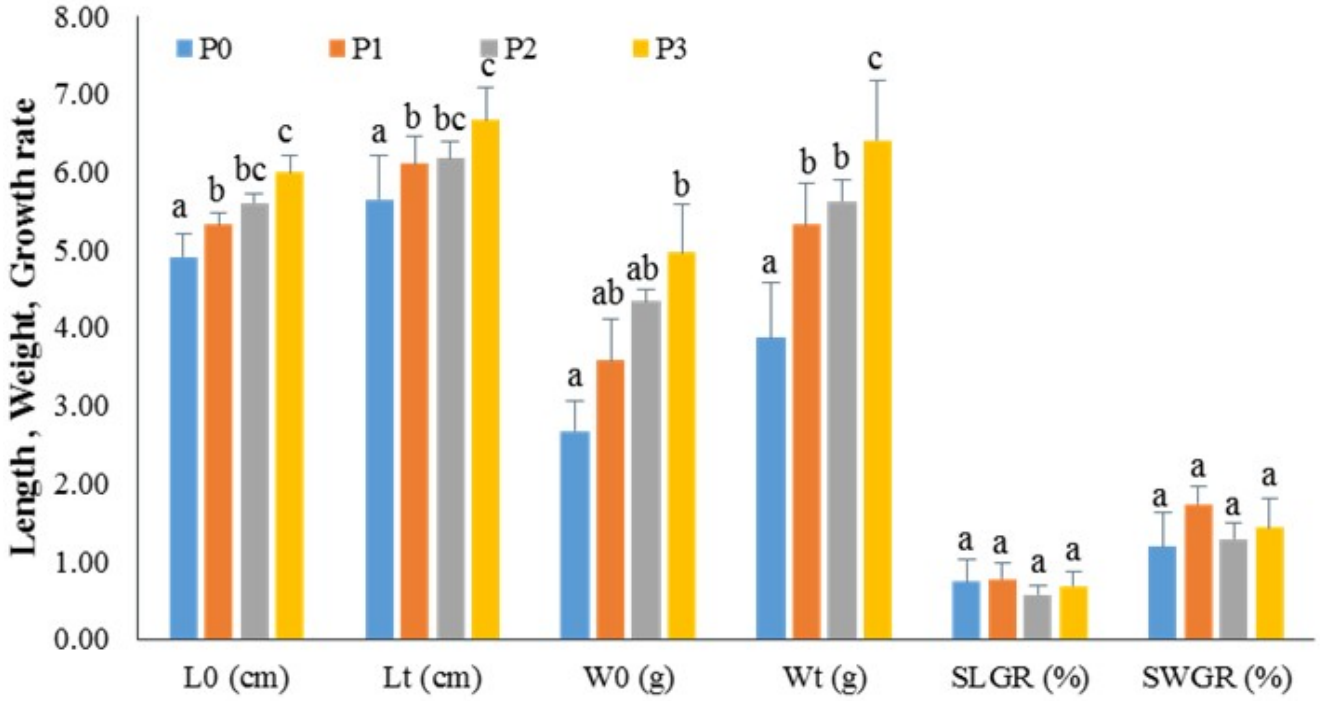


Figure 6. Total length, body weight, and specific growth rates of *P. biaculeatus* after 17β-estradiol induction. P0: 0 mg/g body; P1: 0.5 mg/g body; P2: 1.0 mg/g body; P3: 1.5 mg/g body; L_0 = total length on the initial rearing period (cm); W_0 = body weight on the initial rearing period (g); L_t = total length on the final rearing period (cm); W_t = body weight on the final rearing period (g); SLGR = Specific length growth rate; SWGR = Specific weight growth rate; Different letters indicate a significant different value among treatments.

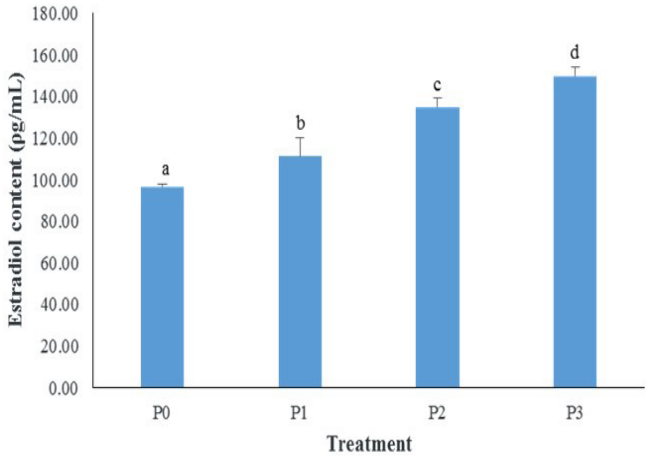


Figure 7. Estradiol content of *P. biaculeatus* after 17β-estradiol induction. P0: 0 mg/g body; P1: 0.5 mg/g body; P2: 1.0 mg/g body; P3: 1.5 mg/g body. Different letters indicate a significantly different value among treatments.

3.1.7 Glucose content

The fish samples had different glucose content among treatments ($p<0.05$). The P3 treatment has the highest glucose level compared to other treatments (Figure 9). The P3 treatment had a higher glucose content (4.67 ± 0.64 mg/dL), followed by P2 dan P1 treatments at 4.00 ± 0 mg/dL and 3.87 ± 0.12 mg/dL, respectively.

3.1.8 Treatment parameter correlation

Based on the correlation analysis, a significantly positive correlation was found among treatments. Estradiol content was significantly positively correlated with total length and body weight, with the r value of 0.909 and 0.850, respectively. A significant correlation was also found in estradiol content with glucose level (r = 0.769), HSI (r = 0.863), and GSI (r = 0.786). Therefore, the correlation analysis results indicate that estradiol level is positively correlated with total length and body weight, red color percentage, glucose level, HSI, and GSI.

3.2 Discussions

In hermaphrodite species, sex reversal is regulated by steroid hormones, namely androgen and estrogen (Li *et al*., 2019). Gemmel *et al*. (2019) explained that hormonally, steroidogenesis of androgen and estrogen occurs simultaneously with the proliferation, differentiation, and maturation of germ cells in the gonads. Through environmental signals in the form of social interactions, hormonal regulation takes place that regulates these mechanisms on the axis of the hypothalamus, pituitary, and gonads (HPG). Social interaction signals are received by the hypothalamus to release GnRH (gonadotropin-releasing hormone) and then transferred to the pituitary for the formation of FSH (follicle stimulating hormone) and LH (luteinizing hormone). FSH and LH are then taken to the sex glands and received by the FSH receptor (FSHR) and LH receptor (LHR). FSHR is responsible for germ cell proliferation and differentiation and steroidogenesis, while LHR is responsible for steroidogenesis and germ cell maturation. According to Liu *et al*. (2017), steroidogenesis includes the reversible synthesis of estrogen (E2) and 11-ketotestosterone from testosterone substrates.

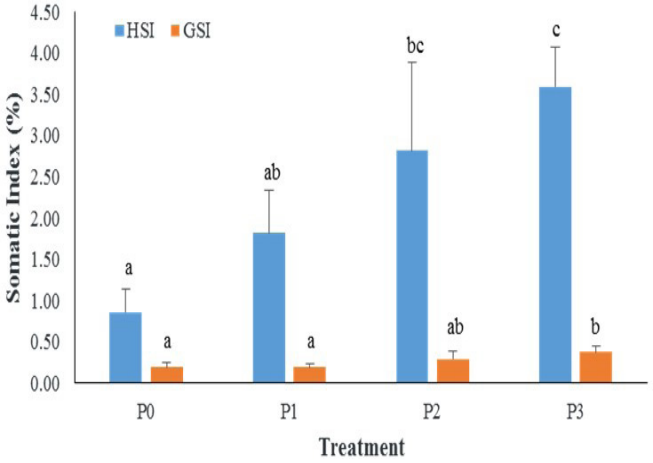


Figure 8. GSI and HSI of *P. biaculeatus* after 17β-estradiol induction. P0: 0 mg/g body; P1: 0.5 mg/g body; P2: 1.0 mg/g body; P3: 1.5 mg/g body. Different letters indicate a significantly different value among treatments.

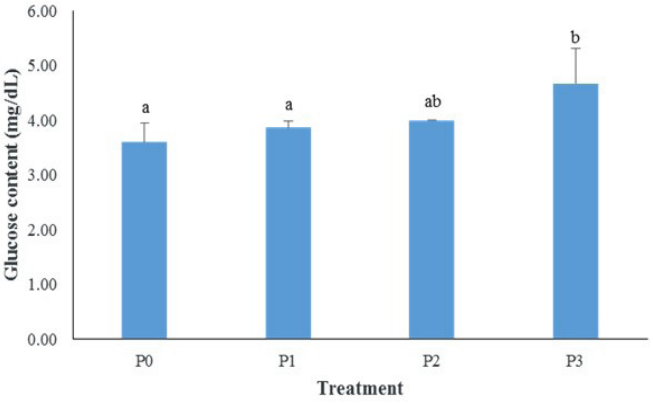


Figure 9. Glucose content of *P. biaculeatus* after 17β-estradiol induction. P0: 0 mg/g body; P1: 0.5 mg/g body; P2: 1.0 mg/g body; P3: 1.5 mg/g body. Different letters indicate a significantly different value among treatments.

Zhang *et al.* (2014) explained that endogenously, estrogen is produced by androgens through the conversion of cytochrome Aromatase P450, which is encoded by cyp19a1a/b in the fish. Tao *et al.* (2013) stated that in teleost species, aromatase and endogenous estrogens are expressed and synthesized in female gonads and act as natural inducers of ovarian differentiation. If the estrogen in the gonads is high, it will cause the fish to be female. The fish species show that sex reversal from male to female is associated with increased estrogen contents. Therefore, estrogen is an important factor for inducing ovarian development in the fish. Li *et al.* (2019) stated that fish sex reversal can be carried out by administering exogenous estrogen (E2). This is to increase estradiol levels to accelerate the sex change. In this study, the indicators of successful sex reversal can be observed morphologically and physiology.

In morphologically, P3 treatment had a low RGB value and showed a slightly darker color. The changing color indicates a feminization mechanism (Liu *et al*., 2017). Koski *et al.* (2015) stated that the individual ranking in a population is based on their position in the social hierarchy and is supported by physical and competitive fitness, such as size and color. This means that the fish with large body sizes and dark colors will have a high position in the hierarchical system. Thus, the different individual colors in this study indicate a ranking and a feminization process. These color differences are due to differences in hue that develop smaller with time and are likely due to an increased β-individual size (Ho *et al*., 2014). In addition, the difference in red color is also due to the exogenous supply of carotenoids. According to Vinkler and Albrecht (2010), carotenoids are used interchangeably in pigmentation for secondary sexual characteristics or in self-maintenance process. In this study, the 1.0 µg/g body obtained the highest carotenoid contents compared to other doses. Fish with high carotenoid contents had a brighter red color. Alonso-Alvarez *et al.* (2008) stated that the increased carotenoid contents was caused by a high testosterone content. According to Liu *et al.* (2017), high testosterone content produced male-sexed individual. However, the 1.5 µg/g body had a low carotenoid content and high estradiol content, thus reversing to female-sexed individual. Tian *et al.* (2012) also found that male guppies had a continuous deposition of carotenoid pigments to produce sexually attractive color appearances during the maturation period.

Besides that, total length and body weight differed significantly among treatments. Fitzgerald, (2020) stated that ranking changes from non-breeders to males and males to females showed opportunistic growth due to male or female removal from the group that suppressed the growth of their subordinates. It was also added that individuals who changed rank could grow faster than others. This condition occurred as a result of the high feed intake, which affected the status. According to Roux *et al.* (2019), the growth of clownfish includes allometric growth, as weight growth is more dominant than length growth. In addition, weight growth can be affected by gonadal development due to 17β-estradiol hormone induction, which enters the bloodstream and increases the vitellogenin secretion. Increased vitellogenin can cause gonadal growth, resulting in increased body weight.

In addition to body size, 17β-estradiol hormone induction also triggered the gonadal profile change in each treatment. In this study, estradiol played a role in ovarian development due to the different gonad profiles in the three treatments compared to the control. Descriptively, there were prominent changes in the gonadal structure of the fish due to estradiol hormone, both morphologically and histologically. Functional female tissue had been formed, and gonad structure had reached the mature ovary on the 90th day. This was similar to Kim *et al.* (2010) who showed that estradiol is a sex hormone regulator in immature *A. melanopus* for 90 days. Thuong *et al.* (2017) stated that 0.1 mg/L estradiol induction by immersion for 15 days could stimulate the feminization of *A. ocellaris* clownfish and reach gonadal maturity on the 60th day.

In physiologically, the highest estradiol content found in P3 treatment, while P2 and P1 content have the lowest estradiol content. High estradiol hormone in P3 treatment was caused by a high injection dose of 17β-estradiol. The higher injection dosage of 17β-estradiol hormone causes a higher plasma estradiol content. This condition was in line to Kim *et al.* (2010) who stated that exogenous estradiol addition could increase the estradiol contents in blood plasma of *A. melanopus*. This study revealed that the 1 µg/g dosage had a higher estradiol content than the 0.1 µg/g and 0 µg/g dosages at 6, 12, 24, and 48 hours after injection.

On the other hand, P3 treatment can increase rapid gonad maturation. It is in accordance with Santo *et al.* (2014), who stated that acceleration of the gonadal maturation process in *Osteochilus hasselti* was caused by the high injection dose of the 17β-estradiol. This means that the greater estradiol dose, the higher estradiol plasma content will be to accelerate the gonadal maturation. The increased estradiol content in plasma and acceleration of gonadal maturation by hormonal

injection can be caused by several factors, namely individual specificity, hormonal dosage, hormonal application time, and method (Haq *et al*., 2013). In this study, the individual samples had a size of 5-6 cm after differentiation with various hormonal doses and a three-time injection.

Gopurappilly *et al.* (2013) confirmed that sex change in clownfish is mediated by the brain. Mechanism of the sex change involves neuronal activity in the brain of male fish. It is transmitted along the hypothalamic-pituitary-gonadal axis (HPG). Casas *et al.* (2016) explained that at the brain level, aromatase plays an important role during sex change. Receptors in gonadal tissue receive hormone signals and complete the gonadal sex changes (Kobayashi *et al*., 2009). This process involves a complete reorganization of the gonad tissue. The functional male gonad is ovotestis, with the presence of both testicular and ovarian tissue (Casadevall *et al*., 2009). However, when the testis is mature, the ovary is in an immature phase with only oogonia and primary oocyte growth. When males begin to change sex, they enter a transitional phase characterized by progressive degeneration of the testes and proliferation of ovarian tissue (Li *et al*., 2019).

The increased estradiol content and accelerated gonadal maturation were due to hormone role in vitellogenin biosynthesis in the liver, secreted into the bloodstream and gonads for oocyte development (Reading *et al*., 2017). Kumar *et al.* (2015) also explained that the process of vitellogenesis and oocyte growth are indicators of gonadal development. Both are regulated by the hormone estradiol. According to Pham *et al.* (2012) and Pham and Nguyen. (2019), estradiol levels in plasma correlate with GSI, HIS, and ovary development in the fish. Morphologically and histologically, there were different gonad enlargements in the treatment group. This condition was similar to a study by Casadevall *et al.* (2009), who explained that the male gonad wall invagination was the first signal of sex reversal.

Feminization success in functional male fish is also indicated by the different gonadosomatic and hepatosomatic indexes. The fish induced with higher hormone doses had a high GSI and HSI values. Increased GSI and HSI indicate the vitellogenesis process and gonadal development based on the elevated number and granules of the yolk, resulting in oocyte diameter and volume enlargement, then increasing the GSI value. Lee and Yang (2002) explained that changes in estradiol content were positively correlated with oocyte development and an increased gonad maturity index value. In addition, HSI value increased with increasing doses of the hormone 17β-estradiol. This is in line with the research by Harlıoğlu *et al.* (2018) which stated that compared to controls, the GSI and HSI values in female crayfish injected with E2 had increased. According to Nunes *et al.* (2011), Pham and Nguyen (2019), and Kumar *et al*. (2022), the highest HSI values in *Sardina pilchardus*, *Siganus guttatus*, and *Planiliza parsia* were found in the vitellogenesis phase. Thus, the fish in this study were in the vitellogenesis process after hormonal injection. This condition was caused by the vitellogenesis mechanism in the liver.

To describe fish stress level due to 17β-estradiol induction, glucose content was measured. Based on the analysis results, P3 treatment had a high glucose content. This condition indicates that the fish experiences stress because of the injection dosage. In contrast, non-injected fish had a low glucose content. The correlation analysis presents a positive correlation between estradiol hormone and glucose content. Honeycutt (2018) and Geraghty and Kaufer (2015) stated that stress is closely related to the reproductive function of several species, including teleost fish. In this study, fish injected with estradiol had higher glucose contents than control. This result followed Falahatkar *et al.* (2013), who found that estradiol can significantly increase the plasma glucose concentration of *Acipenser stellatus* compared to the control fish. Therefore, estradiol influences the carbohydrate mechanism in *A. stellatus*. Estradiol causes hyperglycemia through the gluconeogenesis process in the liver or by increasing the intestinal enzyme activity responsible for glucose transmission or absorption. This is because glucose is an essential fuel for most metabolic activities, providing energy to meet metabolic demands.

4. Conclusion

The 17β-estradiol hormone influences the feminization of *A. biaculeatus*. The 1.5 mg of 17β-estradiol/g body could induce the feminization of *A. biaculeatus* γ-size, based on the low percentage of RGB values for skin and fin color, growth in total length and body weight, high estradiol hormone, elevated GSI and HSI, high glucose content, and sex maturity level of females with mature ovaries. Therefore, the 1.5 mg/g body of 17β-estradiol can be applied for *A. biaculeatus* sex-reversal.

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Authors' Contributions

The contribution of each author is as follows, HAS; provided idea, conducted research, processed data, and wrote original drafts. AOS; conceptualized, conducted methodology, analyzed data, and wrote partial drafts. MAS; conceptualized and corrected the draft. DTS; provided ideas, conceptualized, analyzed data, wrote partial drafts. LITAT and IE; conceptualized and corrected the draft. All authors discussed and contributed to the final manuscript.

Conflict of Interest

All the authors declared no conflict of interest upon the publication of this manuscript.

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References

Abol-Munafi, B. A., Norazmi-Lokman, N. H., Asma, N. A., Sarmiza, S., & Abduh, M. Y. (2011). Histological study on the gonad of the protandrous anemonefish (*Amphiprion ocellaris*). *Journal of Animal and Veterinary Advances*, 10(22):3031–3036.

Alonso-Alvarez, C., Pérez-Rodríguez, L., Mateo, R., Chastel, O., & Viñuela, J. (2008). The oxidation handicap hypothesis and the carotenoid allocation trade-off. *Journal of Evolution Biology*, 21(6):1789-1797.

Biondo, M. V. (2018). Importation of marine ornamental fishes to Switzerland. *Global Ecology and Conservation*, 15:1-10.

Casadevall, M., Delgado, E., Colleye, O., Monserrat, S. B., & Parmentier, E. (2009). Histological study of the sex-change in the skunk clownfish *Amphiprion akallopisos*. *The Open Fish Science Journal*, 2(1):55-58.

Casas, L., Saborido-Rey, F., Ryu, T., Michell1, C., Ravasi, T., & Irigoien, X. (2016). Sex change in clownfish: molecular insights from transcriptome analysis. *Scientific Reports*, 6(35461):1-19.

Casas, L., Parker, C. G., & Rhodes, J. S. (2022). Sex change from male to female: active feminization of the brain, behavior, and gonads in anemonefish. In V. Laudet and T. Ravasi (Eds), Evolution, development and ecology of anemonefishes. (pp. 117-128). Florida: CRC Press.

Cavraro, F., Gheno, G., Ganzerla, R., Zucchetta, M., Franzoi, P., & Malavasi, S. (2017). Habitat constraints on carotenoid-based coloration in a small euryhaline teleost. *Ecology and Evolution*, 8(9):4422-4430.

Falahatkar, B., Poursaeid, S., Meknatkhah, B., Khara, H., & Efatpana, I. (2013). Long-term effects of intraperitoneal injection of estradiol17b on the growth and physiology of juvenile stellate sturgeon *Acipenser stellatus*. *Fish Physiology and Biochemistry*, 40:365-373.

Fitz, K. S., Montes Jr, H. R., Thompson, D. M., & Pinsky, M. L. (2022). Isolation-by-distance and isolation-by-oceanography in Maroon Anemonefish (*Amphiprion biaculeatus*). *Evolutionary Applications*, 16(2):379-392.

Fitzgerald, L. M. (2020). What happened to nemo: Population dynamics of the orange clownfish, *Amphiprion percula* over an eight-year time gap on Kimbe Island, Papua New Guinea. Thesis. Thuwal: King Abdullah University of Science and Technology.

Gemmell, N. J., Todd, E. V., Goikoetxea, A., Ortega-Recalde, O., & Hore, T. A. (2019). Natural sex change in fish. *Current Topics in Development Biology*, 134:71-177.

Ghosh, S., Kumar, T. T. A., Gunasundari, S., & Balasubramanian. (2012). Reproductive biology of *Amphiprion nigripes* (Regan, 1908) at Lakshadweep archipelago: Implication for specific reef fish conservation in Asia. *Scientific Reports*, 1(1):2–5.

Glade, M. J., Smith, K., & Meguid, M. M. (2015). A glance at…nutritional antioxidants and testosterone secretion. *Nutrition*, 31(10):1295-

1298.

Geraghty, A. C., & Kaufer, D. (2015). Glucocorticoid Regulation of Reproduction. In W. E. Crusio, H. Dong, H. H. Radeke, N. Rezaei, O. Steinlein, J. Xiao (Eds.), Advances in experimental medicine and biology. (pp. 253-278). New York: Springer.

Gopurappilly, R., Ogawa, S., & Parhar, I. S. (2013). Functional significanceof GnRH and kisspeptin, and their cognate receptors in teleost reproduction. *Frontiers in Endrocrinology*, 4(24):1-13.

Haq, H. K., Santika, A., & Herawati, T. (2013). Pengaruh lama waktu perendaman induk dalam larutan madu terhadap pengalihan kelamin anak ikan Gapi (*Poecilia reticulata*). *Jurnal Perikanan dan Kelautan*, 4(3):117-125.

Harlıoğlu, M. M., Yonar, M. E., Harlıoğlu, A. G., Yonar, S. A., Farhadi, A. (2018). Effects of 17β-estradiol injection on the reproductive efficiency of freshwater crayfish, Astacus leptodactylus (Eschscholtz, 1823). *Journal of Applied Aquaculture*.

Ho, A. L. F. C., Zong, S., & Lin, J. (2014). Skin color retention after dietary carotenoid deprivation and dominance mediated skin coloration in clown anemonefish, *Amphiprion ocellaris*. *AACL Bioflux*, 7(2):103-115.

Hoga, C. A., Almeida, F. L., & Reyes, F. G. R. (2018). A review on the use of hormones in fish farming: analytical methods to determine their residues. *CYTA: Journal of Food*, 16(1):679-691.

Honeycutt, J. L. (2018). Environmental and endocrine regulators of stress effects in teleost fishes. Dissertation. North Carolina: North Carolina State University.

Khoo, M. L., Das, S. K., & Ghaffar, M. A. (2018). Growth pattern, diet and reproductive biology of the clownfish *Amphiprion ocellaris* in waters of Pulau Tioman, Malaysia. *The Egyptian Journal of Aquatic Research*, 44(3):233-239.

Kim, N. N., Jin, D. H., Lee, J., Kil, G. S., & Choi, C. Y. (2010). Upregulation of estrogen receptor subtypes and vitellogenin mRNA in cinnamon clownfish *Amphiprion melanopus* during the sex change process: Profiles on effects of 17β-estradiol. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 157(2):198-204.

Klann, M., Mercader, M., Salis, P., Reynaud, M., Roux, N., Laudet, V., & Besseau, L. (2021). Anemonefishes. In A. Boutet., B. Schierwater, B. (Ed.), Handbook of marine model organisms in experimental biology established and emerging. (pp. 443-459). New York: CRC Press.

Kobayashi, Y., Nakamura, M., Sunobe, T., Usami, T., Kobayashi, T., Manabe, H., Paul-Prasanth, B., Suzuki, N., & Nagahama, Y. (2009). Sex change in the gobiid fish is mediated through rapid switching of gonadotropin receptors from ovarian to testicular portion or vice versa. *Endocrinology*, 150(3):1503-1511.

Kobayashi, Y., Nagahama, Y., Nakamura, M. (2013). Diversity and plasticity of sex determination and differentiation in fishes. *Sexual Development*, 7:115-125.

Koski, J., Xie, H. & Olson, I. R. (2015). Understanding social hierarchies: The natural and psychological foundations of status perception. *Social Neuroscience*, 10(5)527-550.

Kumar, P., Arasu, A. R. T., Kailasam, M., Sukumarran, K, Subburj, R., Tyagraj, G., & Natarajan, M. (2015). Gonadal development and steroid hormone profile of wild caught grey mullet (*Mugil cephalus*). *Biological. Rhythm Research*, 4:601-610.

Kumar, P., Behera, P., Biswas, G., & Ghosha, T.K. (2022). Oocyte growth, gonadosomatic index, hepatosomatic index and levels of reproductive hormones in goldspot mullet *Planiliza parsia* (Hamilton, 1822) reared in captivity. *Indian Journal of Fisheries*, 69(1):84-96.

Lee, W. K., & Yang, S. W. (2002). Relationship between ovarian development and serum level of gonadal steroid hormones, and induction of oocyte maturation and ovulation in the cultured female Korean spotted see bass *Lateolabrax maculatus* (Jeom-nong-eo). *Aquaculture*, 207(1-2):169-183.

Li, M., Sun, L., & Wang, D. (2019). Roles of estrogens in fish sexual plasticity and sex differentiation. *General and Comparative Endocrinology*, 277:9-16.

Liu H., Todd, E. V., Lokman, M. P., Lamm, M. S., Godwin, J. R., & Gemmell, N. J. (2017). Sexual plasticity: A fishy tale. *Molecular Reproduction and Development*, 84(2):171-194.

Madhu, K., Madhu, R., & Retheesh. (2012). Broodstock development, breeding, embryonic development and larviculture of spine-cheek anemonefish, *Premnas biaculeatus* (Bloch, 1790). *Indian Journal of Fish*, 59(1):65-75.

Madhu, R., Madhu, K., & Retheesh, T. (2013). Breeding and seed production of clown fishes under captivity. *CMFRI*, 203-208.

Mitchell, J. S. (2003). Social correlates of reproductive success in false clown anemonefish; subordinate group members do not pay-to-stay. *Evolutionary Ecology Research*, 5:89-104.

Nagahama, Y., Chakraborty, T., Paul-Prasanth, B., Ohta, K., & Nakamura, M. (2021). Sex determination, gonadal sex differentiation, and plasticity in vertebrate species. *Physiological Reviews*, 101(3):1237-1308.

Nunes, C., Silva, A., Soares, E., & Ganias, K. (2011). The use of hepatic and somatic indices and histological information to characterize the reproductive dynamics of Atlantic sardine *Sardina pilchardus* from the Portuguese coast. *Marine and Coastal Fisheries: Dynamics, Management, and Ecosystem Science*, 3(1):127-144.

Pereira, T. S. B., Boscolo, C. N. P., & Batlouni, S. R. (2020). Use of 17β-estradiol for *Leporinus macrocephalus* feminization. *Boletim do Instituto de Pesca*, 46(2):1-7.

Pham, H. Q., & Nguyen, A. V. (2019). Seasonal changes in hepatosomatic index, gonadosomatic index and plasma estradiol-17β level in captively reared female rabbit fish (*Siganus guttatus*). *Aquaculture Research*, 50(8):2191-2199.

Pham, H. Q., Nguyen, A. T., Kjørsvik, E., Nguyen, M. D., & Arukwe, A. (2012). Seasonal reproductive cycle of Waigieu seaperch (*Psammoperca waigiensis*). *Aquaculture Research*, 43(6):815-830.

Prasetio, A. B., & Kusrini, E. (2012). Ikan hias laut: tantangan budidaya dan peluang bisnis. *Media Akuakultur*, 7(2):84-87.

Putra, I. G. D. K., Wardiyanto, & Tarsim. (2012). Hormon testosteron dan estradiol 17β dalam plasma darah induk betina ikan baung (*Mystus nemurus*). *Jurnal Rekayasa dan Teknologi Budidaya Perairan*, 1(1):71-78.

Ramamoorthy, K., Bhuvaneswari, S., Sankar, G., & Sakkaravarthi, K. (2010). Proximate composition and carotenoid content of natural carotenoid sources and its colour enhancement on marine ornamental fish *Amphiprion ocellaris* (Cuveir 1880). *World Journal of Fish and Marine Sciences,* 2(6):545-550.

Reading, B. J., Sullivan, C. V., & Schilling, J. (2017). Vitellogenesis in fishes. *Reference Module in Life Sciences*, 1-12.

Roux, N., Salis, P., Lambert, A., Logeux, V., Soulat, O., Romans, P., Frédérich, B., Lecchini, D., & Laudet, V. (2019). Staging and normal table of post-embryonic development of the clownfish (*Amphiprion ocellaris*). *Developmental Dynamic*, 284(7):545-568.

Salis, P., Lorin, T., Lewis, V., Rey, C., Marcionetti, A., Escande, M. L., Roux, N., Besseau, L., Salamin, N., Sémon, M., Parichy, D., Volff, J. N., & Laudet, V. (2019). Developmental and comparative transcriptomic identification of iridophore contribution to white barring in clownfish. *Pigment Cell and Melanoma Research*, 32(3):391-402.

Sambroni, E., Rolland, A. D., Lareyre, J. J., & Le Gac, F. (2013). FSH and LH have common and distinct effects on gene expression in rainbow trout testis. *Journal of Molecular Endocrinology*, 50(1):1-18.

Santo, A. P., Susilo, U., & Wijayanti, G. E. (2014). Perkembangan oosit induk *Osteochilus hasselti* c.v. yang diberi hormon estradiol-17β dan pakan dengan kadar protein berbeda. *Scripta Biologica*, 1(1):33-42.

Tao, W., Yuan, J., Zhou, L., Sun, L., Sun, Y., Yang, S., Li, M., Zeng, S., Huang, B., & Wang, D. (2013). Characterization of gonadal transcriptomes from Nile tilapia (*Oreochromis niloticus*) reveals differentially expressed genes. *PLoS One,* 8:e63604.

Thuong, N. P., Sung, Y. Y., Ambak, M. A., & Abol-Munafi, A. B. (2017). The hormone 17β-estradiol promotes feminization of juvenile protandrous hermaphrodite false clownfish (*Amphiprion ocellaris*). *Marine and Freshwater Behavior and Physiology*, 50(3):195-204.

Tian, H., Li, Y., Wang, W., Wu, P., & Ru, S. (2012). Exposure to monocrotophos pesticide during sexual development causes the feminization/ demasculinization of the reproductive traits and a reduction in the reproductive success of male guppies (*Poecilia reticulata*). *Toxicology and Applied Pharmacology*, 263(2):163-170.

Vidal-López, J. M., Contreras-Sánchez, W. M., Hernández-Franyutti, A., Contreras-García, M. de J., & Uribe-Aranzábal, M. C. (2019). Functional feminization of the Mexican snook (*Centropomus poeyi*) using 17β-estradiol in the diet. *Latin American Journal of Aquatic Research*, 47(2):240-250.

Vinkler, M., & Albrecht, T. (2010). Carotenoid maintenance handicap and the physiology of carotenoid-based signalisation of health. *Naturwissenschaften*, 97:19-28.

Wedemeyer, G. A., & Yasutake, W. T. (1977). Clinical methods for the assessment stress on fish health. Technical Paper of The U.S. Fish and Wildlife Service. Washington, D.C.: Department of The Interior Fish and Wildlife Service.

Zhang, Y., Zhang, S., Lu, H., Zhang, L., & Zhang, W. (2014). Genes encoding aromatases in teleosts: Evolution and expression regulation. *General and Comparative Endocrinology*, 205:151-158.