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# Effects of Dietary Supplement with 17β-Estradiol on Growth Performance, Feminization Rate, and Gonadal Maturity of the Giant Freshwater Prawn, *Macrobrachium rosenbergii* All-Male Postlarvae

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**Abstract**

Monosex all-male culture of the giant freshwater prawn (*Macrobrachium rosenbergii*) maximizes aquaculture yield due to a male growth advantage, but direct hormonal treatment of grow-out populations poses significant food safety risks. This study evaluated the efficacy of dietary 17β-estradiol (E2) in inducing functional neo-females from a fully all-male postlarval population to support an indirect monosex seed production strategy. All-male postlarvae were fed diets supplemented with E2 at concentrations of 0, 50, 100, 150, and 200 mg/kg for 36 days, followed by a 150-day hormone-free post-treatment period to assess growth performance, feminization rates, and gonadal histology. E2 administration successfully induced feminization across all treatments, reaching a peak rate of 35.5% at 150 mg/kg, whereas the control group remained entirely male. During the 36-day treatment period, E2 supplementation transiently enhanced specific growth and survival rates but concurrently reduced feed conversion ratios. Notably, these physiological differences disappeared completely over the 150-day post-treatment phase. Histological assessments confirmed that E2-induced neo-females exhibited normal oogenesis, with gonadosomatic index (GSI) values and oocyte diameters similar to those of wild-caught females. This establishes a definitive, physiologically safe, and non-surgical protocol for producing the neo-female broodstock necessary to sustain high-yield commercial monosex populations.

**Keywords:** 17β-estradiol; feminization; gonadal development; *Macrobrachium rosenbergii*; monosex culture; sex reversal

**Key Contribution:** By uniquely using a verified all-male baseline to eliminate natural sex-ratio variance, this study definitively demonstrates that dietary 17β-estradiol produces functional, reproductively viable neo-females in *Macrobrachium rosenbergii* and that transient physiological side effects resolve completely post-treatment. These combined findings establish a safe, scalable, and non-invasive protocol for hatchery managers to develop the neo-female broodstock required for sustainable, hormone-free monosex aquaculture

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## 1. Introduction

The giant freshwater prawn, *Macrobrachium rosenbergii* (De Man, 1879), is a foundation of global inland aquaculture, particularly in tropical and subtropical regions of South and East Asia. This species is celebrated for its rapid growth rate, large attainable size, disease resistance, and high export value, making it a pivotal economic resource for many nations [1,2]. The global production trends indicate a steady increase, with a range from 131,000 t in 2000 to 294,000 t in 2020 [3]. In Vietnam, *M. rosenbergii* cultivation has seen substantial expansion, particularly in the Mekong Delta, where it represents a critical livelihood for local farmers and a significant contributor to the national aquaculture output [4].

Despite its economic success, the culture of *M. rosenbergii* faces distinct biological challenges, including those inherent to its sexual dimorphism and social structure. Male prawns exhibit a heterogeneous individual growth pattern driven by a complex social hierarchy consisting of three distinct morphotypes: blue claw, orange claw, and small males [4–7]. The dominant blue claw males aggressively suppress the growth of subordinate males, leading to significant size variation at harvest and reduced overall yield [5,7]. Conversely, female prawns generally exhibit more uniform growth but reach smaller maximum sizes than males because a substantial portion of their energy is diverted towards egg production rather than somatic growth [4,7]. Consequently, the culture of monosex all-male populations has been identified as a superior strategy, offering significantly higher productivity and profitability compared to mixed-sex or all-female cultures [4,8].

Establishing monosex populations can be approached through two primary hormonal methodologies: direct hormonal sex reversal or indirect breeding technologies. Direct treatment involves administering hormones to the entire production stock to alter their phenotypic sex [9,10]. However, this method is increasingly scrutinized due to food safety concerns regarding hormone residues in products that humans consume [5,11]. Alternatively, the indirect method is favored for sustainable aquaculture [9,10]; it involves creating sex-reversed broodstock – specifically, neo-females (genotypic males, ZZ, reversed to phenotypic females) – which are then mated with normal males (ZZ). This mating strategy (female ZZ × male ZZ) theoretically yields 100% all-male progeny (ZZ), eliminating the need for hormonal treatment of the grow-out stock and ensuring an environmentally safe and socially acceptable product [6,11,12].

Central to the indirect method is the successful feminization of genotypic males during their "labile period", a critical window of development where the gonad is undifferentiated and responsive to exogenous steroids [10,11,13]. Among various sex steroids, 17β-estradiol (E2), a natural estrogen, has demonstrated high potency in inducing feminization across a diverse array of aquatic species, including finfish and crustaceans [5,9,10,14]. In crustaceans, dietary supplementation with E2 has been shown to effectively promote female differentiation. For instance, studies on *Penaeus monodon* and *Litopenaeus vannamei* have reported successful skewing of sex ratios towards females following E2 administration [14–16]. In *M. rosenbergii*, partial success has been documented: E2-enriched diets administered to larvae and postlarvae (PL) resulted in significant feminization rates, although complete reversal remains elusive and dose-dependent [5,11]. Previous research indicates that feminization rates in *M. rosenbergii* PL can still be enhanced by administering higher doses of E2 [5,11]. However, while these foundational studies successfully evaluated macroscopic metrics such as growth performance, survival, and overall sex ratios, they did not investigate the underlying histological changes associated with gonadal development.

The efficacy of hormonal feminization is heavily dependent on the method of administration, dosage, and timing of treatment [9,17]. While immersion and injection are viable routes, dietary supplementation is often regarded as the most practical method for mass administration in aquaculture settings [7,10,17]. However, finding the optimal dosage is critical; insufficient doses may lead to intersex individuals or incomplete reversal [9], while excessive doses can induce toxicity, suppress growth, or increase mortality [9,13]. For example, high concentrations of E2 have been linked to retarded growth and lower survival rates in species like *L. vannamei*, suggesting a trade-off

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between feminization efficiency and physiological health [16]. Furthermore, the specific timing of the treatment must align with the species-specific window of sexual differentiation to be effective [10,12].

A significant limitation in previous studies on the feminization of *M. rosenbergii* is the use of mixed-sex populations in experimental trials. Exactly assessing hormone efficacy in such studies is often confounded by the failure to establish a definitive baseline sex ratio at stocking. Consequently, experimental outcomes are highly sensitive to initial population dynamics; indeed, previous studies have reported that natural sex-ratio variance caused their untreated control groups to range from 26% to 65% female [5,18]. To date, there is a paucity of research utilizing all-male PLs for feminization trials exclusively. Using an all-male population allows a precise evaluation of the feminization rate, as any female phenotype observed post-treatment can be attributed definitively to the hormonal intervention rather than to natural sex-ratio variation.

Therefore, this study aims to bridge these gaps by investigating the effects of dietary supplementation with 17β-estradiol on the growth performance, feminization rate, and gonadal development of all-male *M. rosenbergii* PL. By applying graded levels of E2 to a known male population, this research seeks to determine the optimal protocol for generating functional neo-females. The findings will provide essential data to optimize the production of all-male stocks, thereby supporting the sustainable expansion of the freshwater prawn industry in Vietnam and globally.

## 2. Materials and Methods

### *2.1. Ethics Statement*

All experiments involving *M. macrobrachium* comply with current Vietnamese animal welfare laws and were authorized by the Animal Ethics Committee of Nong Lam University, Ho Chi Minh City (Approval No.: 250618).

### *2.2. Postlarvae Preparation for Experiment*

This study was conducted at the Research Center for Aquatic Biotechnology (RECAB) of the Vietnam Academy of Fishery Sciences in Ho Chi Minh City, Vietnam, from March to September 2025. All-male PLs are derived from neo-females – genotypic males that have undergone functional sex reversal via microsurgical ablation of the androgenic gland during the juvenile stage. Upon reaching gonadal maturity, these neo-females are mated with normal males to produce PLs that are 100% genotypically male. The broodstock selected from neo-female and normal male populations were reared in composite tanks equipped with recirculating aquaculture systems and continuous aeration, with water temperature maintained at 28-30°C. The feeding regimen consists of two sessions daily: a morning ration of fresh feed (marine fish, mollusk meat, beef liver, etc.) at 5-10% of body weight, and an afternoon ration of formulated pellets (38-42% crude protein) at 3-5% of body weight. Gravid females are transferred to dedicated hatching tanks until the embryos develop into the grey stage. Post-hatching, the nauplii were reared in brackish water (12-14‰) under vigorous aeration and periodic water exchange and fed artemia and microencapsulated diets tailored to each developmental stage. After 25-30 days, the larvae metamorphose into PLs, which are subsequently harvested and gradually acclimated to freshwater for further use in the trial.

### *2.3. Diet Preparation*

17β-estradiol (E2, purity ≥98%) used in this trial was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The formulation and proximate composition of the diets are shown in Table 1.

**Table 1.** Formulation and proximate composition of the experimental diets.

| Ingredient Name | Ingredient (%) |
|---|---|
| Hydrolyzed fish meal | 30 |

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| | |
|---|---|
| Fish meal 65% protein | 15 |
| Soy protein concentrated | 25 |
| Milk powder | 3 |
| Egg powder | 6 |
| Wheat flour | 10 |
| Dicalcium phosphate | 0.7 |
| Vitamin and mineral premix | 0.6 |
| Stay C 35% | 0.1 |
| Gelatine | 3 |
| Fish oil | 4 |
| Astaxanthin | 0.1 |
| Filler | 2.5 |
| *Proximate composition* | % of dry matter |
| Crude protein | 45.1 ± 1.3 |
| Crude lipid | 13.9 ± 1.0 |

To prevent E2 hormone leaching during feeding, four experimental diets supplemented with E2 (50, 100, 150, and 200 mg/kg) were formulated by blending sieved dry ingredients with a gelatin-astaxanthin binder. The hormone was dissolved in fish oil to ensure uniform distribution within the lipophilic phase before mixing. The resulting composite was pelletized by cold microextrusion-spheronization into three size classes to accommodate the prawns' ontogenetic feeding requirements throughout the 36-day trial. The control diet was made in the same method without hormones. Pellets were cold-dried at 40°C for 24 hours and stored in sealed containers in a refrigerator at 4°C in the dark to protect the hormone from UV light during the trial period. For hormone analysis, all E2-supplemented and control feeds were sampled at days 0, 12, 24, and 36 ($n$=1 per group) and frozen at -80°C until hormonal analysis by liquid chromatography–tandem mass spectrometry (LC–MS/MS).

*2.4. Feminization Experimental Design*

The all-male PLs (mean body weight of 6.12±0.00 mg) used in the trial were taken from the same batch for all groups. The experimental design was completely randomized with four treatments (50, 100, 150, and 200 mg E2/kg) and a control (no hormone), each having four replicates. 500 PLs were randomly allocated to each of the twenty 100-liter conical composite tanks. Feeding was done by hand four times per day from day 1 through day 36. The initial feeding rate was 1 g per 500 PLs and was adjusted daily based on prawn consumption.

Tanks were provided with continuous aeration to ensure oxygen saturation and equipped with nylon shelters to mitigate cannibalism. Prawns were reared under a natural photoperiod, and tanks were siphoned every two days to remove organic debris and uneaten feed, thereby maintaining water quality. Physicochemical parameters, such as temperature, pH, and dissolved oxygen (DO), were recorded twice daily at 07:00 and 16:00 h. Additionally, total ammonia nitrogen (TAN) and nitrite concentrations were assessed biweekly. Throughout the 36-day trial, environmental conditions were maintained within optimal ranges for *M. rosenbergii* (temperature: 27–29°C; pH: 7.0–7.5; DO: >5.0 mg/L; TAN: 0.23 ± 0.29 mg/L; nitrite: 0.17 ± 0.32 mg/L).

To assess E2 accumulation, *M. rosenbergii* specimens were sampled on days 12, 24, and 36 ($n$= 70, 40, and 30 individuals per tank, respectively). Before analysis, the sampled individual was starved for 12 h to ensure complete gut evacuation; these specimens were subsequently excluded from survival calculations. All samples were frozen at –80°C until hormone analysis.

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At the end of the 36-day feminization trial, the tanks were drained through a collection sieve to harvest the remaining juveniles. All individuals were counted and weighed to determine survival rate (SR), final body weight (FBW, $W_t$), weight gain (WG), and specific growth rate (SGR). Survival and growth metrics were calculated as follows:

$$SR(\%) = \frac{N_t}{N_0 - N_s} \times 100$$

$$WG(g/ind.) = W_t - W_0$$

$$SGR(\%/day) = \frac{lnW_t - lnW_0}{t} \times 100$$

Where $N_0$ represents the initial stocking density, $N_t$ is the number of prawns at harvest, $N_s$ denotes the number of sampled individuals for hormone analysis, $W_0$ is the initial mean body weight, and $t$ is the experiment duration (36 days). The feed conversion ratio (FCR) was calculated by dividing the total dry feed intake by the total wet biomass gain.

*2.5. Post-Feminization Experiment*

Prawn growth in all groups was monitored for 150 days after feminization. After the feminization, all prawns from each 100-liter conical tank were randomly transferred to one of the 20 3-$m^3$ round tanks (4 replicates per group). Prawns were fed to satiation 3 times daily with juvenile feed (50% crude protein, 10% crude fat) without E2 supplementation. The initial feeding rate was set at 5% of the total tank biomass and adjusted daily based on actual feed intake. Feed amounts were monitored and recorded daily to calculate FCR by subtracting the remaining feed amount after the last feeding from the previous feed amount in the container. Thirty prawns were collected monthly from each tank for body weight.

The husbandry and tank management protocol were conducted as described above, with some modifications. Temperature, pH, and DO were recorded at 07:00 h every 3 days for the first 60 days and weekly thereafter. TAN and nitrite were monitored weekly for the first 60 days, and then fortnightly for the remaining 90 days. During the post-feminization stage, environmental conditions were maintained within optimal ranges for *M. rosenbergii* (temperature: 25–31°C; pH: 7.1–7.5; DO: >5.0 mg/L; TAN: 0.159 ± 0.028 mg/L; nitrite: 0.010 ± 0.004 mg/L).

After 60 days of the post-feminization period, the sex ratio for each treatment was determined based on macroscopic morphological characteristics. The sex of each prawn was observed to have the appendix masculina on the second pair of pleopods in males [19–21]. All live prawns were counted and weighed to determine SR, FBW, WG, and SGR as stated earlier. After sex differentiation, all juvenile males were removed, and all females were maintained in the original 3 $m^3$ round tanks used for the post-feminization experiment. The feminization rate of each group was then estimated from the number of prawns that survived at 60 days of the post-feminization stage by using the following formula:

$$\text{Feminization rate}(\%) = \frac{number\ of\ female\ prawn}{total\ number\ of\ sexed\ prawn} \times 100$$

At the end of the experiment, the female prawns were counted and weighed to determine SR, FBW, WG, and SGR as stated earlier.

*2.6. Gonadal Maturity and Histological Analysis*

To evaluate the gonadosomatic index (GSI) and oocyte diameter, four E2-treated female prawns nearing maturity (Stage III/IV) were randomly selected from each treatment group ($n$=1 per tank), euthanized via cold shock (ice bath), and the ovary was dissected out, weighed, and the gonadal tissues were cut into 1-2 mm sections. GSI was determined using the equation: GSI (%) = [$W_{ovary}$ / $W_{body}$] × 100. The remaining prawns were maintained until ovulation occurred. For comparative purposes, three wild-caught females were sourced from the Dong Nai River, Vietnam (10°52'55"N, 106°50'34"E) through a local supplier. These wild specimens, selected for body weights comparable

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to those of the experimental groups, were reared under standard broodstock conditions until full ovarian maturation, serving as a baseline for GSI comparisons with the E2-treated groups.

For histological analysis, gonadal tissues were fixed in Davidson's fixative for a minimum of 2 h, transferred to 50% ethanol, and embedded in paraffin wax. Sections (5–6 μm) were prepared using a microtome, mounted on glass slides, and stained with Hematoxylin and Eosin (H&E). Ovarian development was categorized into five stages – Oc1, Oc2, Oc3, Oc4, and mature oocytes (mOc) – following the classification by Meeratana and Sobhon [22]. Morphometric analysis was conducted under a light microscope (10–40× magnification). Thirty oocytes with complete nuclear profiles were randomly measured at each stage using an eyepiece micrometer, and data were reported as mean diameter ± standard deviation (SD).

### *2.7. Hormone Analysis*

Concentrations of E2 in prawn whole body and feed were analyzed using the LC–MS/MS combined with Dansyl chloride derivatization to enhance sensitivity and ionization efficiency [23,24]. The LC–MS/MS analysis was conducted at the Center of Analytical Service, Experimentation and Standards, Metrology, Quality of Ho Chi Minh City, Vietnam. Whole-body PL or juvenile samples (0.2 g) and feeds (2 g) were homogenized and extracted with 20 mL of methanol/water (8/2, v/v). After filtration, a 5 mL aliquot of the extract was diluted with 25 mL of water and purified using an EASY solid-phase extraction (SPE) cartridge preconditioned with methanol and water. The cartridge was washed with water, and estradiol was eluted with ethyl acetate, then evaporated to dryness under a nitrogen stream. The residue was reconstituted in 1 mL of 0.1 M $NaHCO_3$ buffer (pH 10.5), and Dansyl chloride solution (1 mg/10 mL) was added. The derivatization reaction was carried out at 60°C for 10 minutes. Subsequently, 2 mL of water and 9 mL of acetonitrile were added, followed by QuEChERS salt mixture (0.2 g NaCl and 0.8 g $MgSO_4$). The mixture was vortex-mixed and centrifuged at 4000 rpm. A 1 mL aliquot of the supernatant was further cleaned using dispersive SPE (dSPE) containing 150 mg $MgSO_4$ and 150 mg C18, and the final extract was transferred to LC vials for analysis. LC–MS/MS analysis was performed on a Thermo TSQ Endura triple quadrupole mass spectrometer equipped with an electrospray ionization ($ESI^+$) source. Chromatographic separation was achieved using a Poroshell 120 C18 column (50 × 4.6 mm, 2.7 μm) with a gradient elution of mobile phase A (water containing 0.1% formic acid) and mobile phase B (acetonitrile). Dansyl-derivatized estradiol was quantified in MRM mode using the precursor ion m/z 506.03 and product ions m/z 171.13 (quantifier) and m/z 156.13. The limits of detection (LOD) of E2 in prawn and feed were 5 ng/g and 1 μg/g, respectively.

### *2.8. Statistical Data Analysis*

The SR percentages were arcsine-square-root-transformed to ensure normality, although untransformed means are reported for clarity. Values of E2 analysis below the LOD were substituted with LOD/2 before statistical evaluation [25]. The effects of E2-dietary treatments and sampling intervals on growth metrics, FCR, SR, and tissue E2 concentrations were analyzed using a one-way repeated-measures ANOVA. A one-way ANOVA was used to evaluate differences in GSI. Where significant main effects were observed, the least significant difference (LSD) post-hoc test was applied to differentiate means ($p < 0.05$) [26]. Descriptive statistics are presented as mean ± SD or mean ± standard error (SE). These analyses were conducted using IBM SPSS Statistics (Version 22.0; IBM Corp., Armonk, NY).

To determine the influence of dietary E2 on feminization rates, adjusted count data were modeled using a generalized linear model (GLM) with a binomial distribution and a logit link function. Due to the complete absence of female phenotypes in the control group (0/1097) – a condition that led to complete separation in logistic regression – a small-count continuity correction of 0.5 was applied to stabilize parameter estimates. To control for the family-wise error rate associated

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with multiple testing, *p*-values were adjusted using the Holm-Bonferroni method [27]. These analyses were conducted in the program R version 4.3.3 [28].

## 3. Results

### *3.1. Dietary E2 Concentrations and Tissue Bioaccumulation in Postlarvae*

To verify the actual concentration of the administered hormone, experimental diets were analyzed via LC-MS/MS. The measured E2 concentrations in the supplemented feeds closely aligned with the nominal inclusion levels for the lower dosages, yielding actual mean concentrations of 58.0 ± 16.5 mg/kg and 104.4 ± 18.6 mg/kg for the 50 and 100 mg/kg diets, respectively. However, hormone incorporation appeared to plateau at higher inclusion rates; the nominal 150 mg/kg and 200 mg/kg diets exhibited actual E2 concentrations of 153.5 ± 21.7 mg/kg and 148.4 ± 40.0 mg/kg, respectively. A trace background level of E2 (1.39 ± 1.03 mg/kg) was detected in the control diet.

The corresponding whole-body accumulation of E2 in *M. rosenbergii* PLs exhibited a distinct temporal profile (Figure 1). From a baseline tissue concentration on Day 0, endogenous E2 levels on Day 12 showed slight, non-significant increases across all supplemented groups compared with the control ($p > 0.05$). Maximum bioaccumulation was observed on Day 24, where PLs receiving the nominal 200 mg/kg dietary dosage exhibited a pronounced and significant peak in tissue E2 concentration (19.4 ± 5.4 ng/g). This peak was significantly higher than that of the control and all other treatment groups ($p < 0.05$). By Day 36, despite continuous dietary exposure, whole-body E2 concentrations in all supplemented groups decreased markedly, returning to baseline levels (5–7 ng/g), with no significant differences among groups ($p > 0.05$).

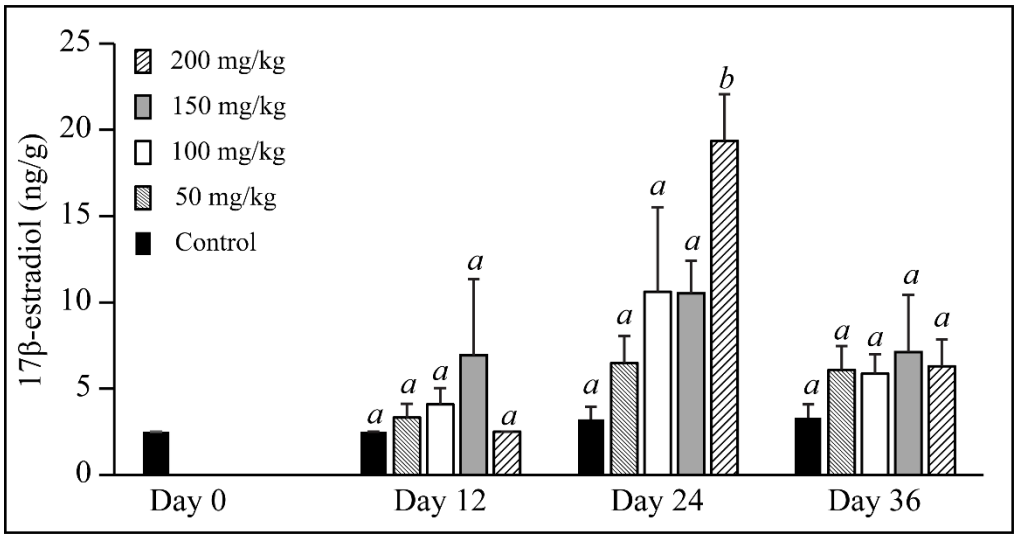


**Figure 1.** 17β-estradiol (E2) concentrations in *Macrobrachium rosenbergii* postlarvae following dietary supplementation with varying E2 levels throughout the experimental treatment period. Data are expressed as mean ± SE. Different letters indicate statistically significant differences between groups at a given time point (one-way repeated-measures ANOVA followed by LSD test; $p < 0.05$).

### *3.2. Growth Performance, Survival Rates, and Feed Efficiency*

Table 2 summarizes the growth performance of *M. rosenbergii* PL fed varying E2 concentrations. At the conclusion of the 36-day feeding trial, the control group recorded the lowest mean FBW (0.099 ± 0.014 g) and SGR (7.75 ± 0.39 %/day). Except for 100 mg/kg treatment, all E2-supplemented groups exhibited significantly higher mean FBW and SGR compared to the control ($p < 0.05$). However, no significant differences in growth metrics were detected among the various E2 dosages ($p > 0.05$). Long-term monitoring revealed that these initial disparities were temporary; by 150 days post-treatment, there were generally no statistically significant differences in FBW or other growth parameters between the treated and control groups or among E2 treatment (Table 2).

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**Table 2.** The growth rates of M. rosenbergii during 17β-estradiol treatment and the post-treatment period.

| Parameter | Treatment | Day 36 | Day 66 | Day 96 | Day 126 | Day 156 | Day 186 |
|---|---|---|---|---|---|---|---|
| FBW (g/ind.) | Control | 0.099 ± 0.014$^{a}$ | 0.90 ± 0.05$^{a}$ | 2.86 ± 0.50$^{a}$ | NA | NA | NA |
| | 50 mg/kg | 0.120 ± 0.004$^{b}$ | 0.83 ± 0.06$^{a}$ | 3.25 ± 0.83$^{a}$ | 19.2 ± 1.1$^{a}$ | 41.5 ± 1.5$^{a}$ | 59.8 ± 1.5$^{a}$ |
| | 100 mg/kg | 0.111 ± 0.009$^{ab}$ | 0.80 ± 0.13$^{a}$ | 2.55 ± 0.13$^{a}$ | 15.9 ± 1.0$^{b}$ | 35.5 ± 1.9$^{b}$ | 55.4 ± 1.7$^{b}$ |
| | 150 mg/kg | 0.129 ± 0.006$^{b}$ | 0.84 ± 0.11$^{a}$ | 2.64 ± 0.35$^{a}$ | 17.2 ± 1.8$^{a}$ | 37.9 ± 3.3$^{ab}$ | 59.3 ± 3.0$^{ab}$ |
| | 200 mg/kg | 0.123 ± 0.025$^{b}$ | 0.79 ± 0.10$^{a}$ | 2.56 ± 0.55$^{a}$ | 18.0 ± 1.9$^{a}$ | 39.8 ± 3.5$^{a}$ | 60.6 ± 3.7$^{a}$ |
| WG (g/ind.) | Control | 0.053 ± 0.013$^{a}$ | 0.80 ± 0.06$^{a}$ | 1.96 ± 0.52$^{a}$ | NA | NA | NA |
| | 50 mg/kg | 0.067 ± 0.009$^{a}$ | 0.71 ± 0.06$^{a}$ | 2.42 ± 0.88$^{a}$ | 16.0 ± 0.3$^{a}$ | 22.2 ± 0.3$^{a}$ | 18.4 ± 0.7$^{a}$ |
| | 100 mg/kg | 0.049 ± 0.009$^{a}$ | 0.69 ± 0.13$^{a}$ | 1.75 ± 0.14$^{a}$ | 13.4 ± 0.9$^{b}$ | 19.6 ± 0.9$^{b}$ | 19.8 ± 1.3$^{ab}$ |
| | 150 mg/kg | 0.067 ± 0.010$^{a}$ | 0.71 ± 0.12$^{a}$ | 1.80 ± 0.38$^{a}$ | 14.5 ± 1.5$^{ab}$ | 20.8 ± 1.5$^{ab}$ | 21.4 ± 1.2$^{b}$ |
| | 200 mg/kg | 0.066 ± 0.025$^{a}$ | 0.67 ± 0.09$^{a}$ | 1.76 ± 0.51$^{a}$ | 15.5 ± 1.6$^{a}$ | 21.7 ± 1.6$^{a}$ | 20.8 ± 2.9$^{ab}$ |
| SGR (%/day) | Control | 7.75 ± 0.39$^{a}$ | 7.59 ± 0.09$^{a}$ | 6.41 ± 0.18$^{a}$ | NA | NA | NA |
| | 50 mg/kg | 8.31 ± 0.09$^{b}$ | 7.47 ± 0.11$^{a}$ | 6.53 ± 0.27$^{a}$ | 6.41 ± 0.05$^{a}$ | 5.67 ± 0.02$^{a}$ | 4.95 ± 0.01$^{a}$ |
| | 100 mg/kg | 8.08 ± 0.24$^{ab}$ | 7.41 ± 0.23$^{a}$ | 6.30 ± 0.06$^{a}$ | 6.26 ± 0.05$^{b}$ | 5.57 ± 0.04$^{b}$ | 4.91 ± 0.02$^{b}$ |
| | 150 mg/kg | 8.52 ± 0.13$^{b}$ | 7.48 ± 0.22$^{a}$ | 6.33 ± 0.14$^{a}$ | 6.31 ± 0.08$^{ab}$ | 5.61 ± 0.05$^{ab}$ | 4.94 ± 0.03$^{ab}$ |
| | 200 mg/kg | 8.34 ± 0.53$^{b}$ | 7.39 ± 0.20$^{a}$ | 6.29 ± 0.21$^{a}$ | 6.35 ± 0.09$^{ab}$ | 5.64 ± 0.06$^{a}$ | 4.96 ± 0.03a |

[†] values represent the mean ± SD ($n$ = 4); NA: not available. Mean values in the same column with different superscripts differ significantly (results from one-way repeated measure ANOVA and LSD test, $p < 0.05$).

Regarding feed utilization during the feminization phase, E2-treated PL consumed less feed than the control group (Table 3). Statistical analysis indicated that the mean FCR in the control group was significantly higher than in the 150 mg/kg and 200 mg/kg groups ($p < 0.05$), though it did not differ significantly from the 50 mg/kg and 100 mg/kg groups ($p > 0.05$). These variations in feed efficiency diminished over time; no significant differences in FCR were observed between any groups at day 96 or throughout the 150-day post-treatment period ($p > 0.05$).

**Table 3.** The feed conversion ratio of *M. rosenbergii* during 17β-estradiol treatment and the post-treatment period.

| Treatment | Day 36 | Day 96 | Day 186 |
|---|---|---|---|
| Control | 3.80 ± 0.76[†a] | 1.62 ± 0.20$^{a}$ | NA |
| 50 mg/kg | 3.14 ± 0.44$^{ab}$ | 1.40 ± 0.29$^{a}$ | 2.52 ± 0.51$^{ab}$ |
| 100 mg/kg | 3.11 ± 0.32$^{ab}$ | 1.58 ± 0.06$^{a}$ | 2.50 ± 0.45$^{b}$ |
| 150 mg/kg | 2.76 ± 0.42$^{b}$ | 1.64 ± 0.19$^{a}$ | 2.07 ± 0.38$^{ab}$ |
| 200 mg/kg | 2.67 ± 0.21$^{b}$ | 1.69 ± 0.23$^{a}$ | 1.99 ± 0.19$^{a}$ |

[†] values represent the mean ± SD ($n$ = 4); NA: not available. Mean values in the same column with different superscripts differ significantly (results from one-way repeated measure ANOVA and LSD test, $p < 0.05$).

Survival rates (SR) at day 36 ranged from 79.5% to 89.9%, with the control group exhibiting the lowest survival and the 200 mg/kg group the highest (Table 4). The SR of the control group was significantly lower than that of the 100 mg/kg and 200 mg/kg cohorts ($p < 0.05$), whereas no significant difference was observed among the E2-treated groups. By day 96, the SR were largely comparable across groups, although the 50 mg/kg treatment showed significantly lower SR than both the control and the 200 mg/kg groups ($p < 0.05$). At the final assessment (150 days post-treatment), no significant differences in SR of E2-treated females were evident across the experimental treatments ($p > 0.05$).

**Table 4.** The survival rates of *M. rosenbergii* during 17β-estradiol treatment and the post-treatment period.

| Treatment | Day 36 | Day 96 | Day 186 |
|---|---|---|---|
| Control | 79.5 ± 7.7[†a] | 95.8 ± 1.5$^{a}$ | NA |

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| | | | |
|---|---|---|---|
| 50 mg/kg | 86.3 ± 2.4$^{ab}$ | 90.5 ± 1.5$^{b}$ | 65.8 ± 9.8$^{a}$ |
| 100 mg/kg | 88.5 ± 4.6$^{b}$ | 92.6 ± 3.5$^{ab}$ | 73.0 ± 8.0$^{a}$ |
| 150 mg/kg | 84.4 ± 4.5$^{ab}$ | 93.8 ± 2.6$^{ab}$ | 67.3 ± 4.7$^{a}$ |
| 200 mg/kg | 89.9 ± 4.1$^{b}$ | 94.5 ± 2.5$^{a}$ | 68.1 ± 4.9$^{a}$ |

† values represent the mean ± SD ($n$ = 4); NA: not available. Mean values in the same column with different superscripts differ significantly (results from one-way repeated measure ANOVA and LSD test, $p < 0.05$).

*3.3. Feminization Rate, Gonadal Maturity, and Histological Analysis*

The number of females and feminization rates across all treatments observed at the sex differentiation time are summarized in Table 5. The results showed that dietary E2 supplementation significantly increased the female-to-male ratio compared to control groups (binomial GLM; $p < 0.05$) (Table 5). The control group exhibited complete masculinization, with only 0.18% (0/1097 prawns; continuity-corrected to 2/1099) females observed. Conversely, all E2-treated groups showed substantially higher proportions of females, ranging from 27.9% to 35.5%. The highest feminization rate (35.5 ± 1.4%) was achieved in the nominal 150 mg/kg group. Pairwise comparisons (Holm-Bonferroni method) revealed that this rate was significantly greater than that of the 50 mg/kg (29.7 ± 1.4%) and 100 mg/kg (27.9 ± 1.3%) treatments ($p < 0.05$), but there was no significant difference between the 50 mg/kg and 100 mg/kg treatments ($p > 0.05$). Notably, the 200 mg/kg group showed a feminization rate of 33.1 ± 1.3%, which was not statistically different from that of the 50 and 150 mg/kg groups ($p > 0.05$) but significantly higher than that of the 100 mg/kg treatment ($p < 0.05$) (Table 5).

**Table 5.** Feminization rate (mean ± SE) and 95% confidence interval (CI) across different E2 treatment groups.

| Treatment | Females† | Total prawn† | Feminization rate (%)‡ | 95% CI‡ |
|---|---|---|---|---|
| Control | 0 | 1097 | 0.18 ± 0.13$^{a}$ | (-0.07, 0.43) |
| 50 mg/kg | 333 | 792 | 29.7 ± 1.4 $^{bc}$ | (27.01, 32.34) |
| 100 mg/kg | 328 | 851 | 27.9 ± 1.3 $^{b}$ | (25.34, 30.45) |
| 150 mg/kg | 404 | 736 | 35.5 ± 1.4 $^{d}$ | (32.72, 38.26) |
| 200 mg/kg | 405 | 819 | 33.1 ± 1.3$^{cd}$ | (30.51, 35.78) |

† number of females and total prawns of each treatment (a total of four tanks). ‡ feminization rate and 95% CI in each group after continuity correction. Mean values in the feminization rate column with different superscripts differ significantly (results from a binomial generalized linear model with a logit link function; Holm-Bonferroni method, $p < 0.05$).

Gonadal development in E2-treated females across all treatment groups appeared physiologically normal. Furthermore, statistical analysis revealed no significant differences in the mean GSI between any of the E2-treated groups and the wild-caught female ($p > 0.05$) (Table 6).

**Table 6.** The GSI values (%) of E2-treated female and wild-caught female prawns.

| 50 mg/kg | 100 mg/kg | 150 mg/kg | 200 mg/kg | Wild-caught female |
|---|---|---|---|---|
| 5.51 ± 0.48$^{a}$ | 6.34 ± 1.27$^{a}$ | 7.70 ± 1.99$^{a}$ | 6.23 ± 0.72$^{a}$ | 7.38 ± 1.13$^{a}$ |

Data in the tables are presented as mean ± SD ($n$=4 for E2-treated female and $n$=3 for wild-caught female). Mean values in the same row with different superscript letters differ significantly (results from one-way ANOVA and LSD test, $p < 0.05$).

Histological analysis of *M. rogenbergii* gonads at near maturity was performed to confirm phenotypic sex differentiation and assess the morphological integrity of the gonads following hormone treatment (Figure 2). Histological structure of the gonads confirmed that neo-females from

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all the E2 treatment groups underwent normal oogenesis consistent with the classification by Meeratana and Sobhon [22]. Ovarian tissue contained oocytes ranging from early previtellogenic stages (Oc1–Oc2), characterized by basophilic cytoplasm and chromatin condensation, to vitellogenic stages (Oc3–Oc4), distinguished by eosinophilic cytoplasm and lipid droplet accumulation. Fully mature ovaries were dominated by mature oocytes (mOc) exhibiting densely packed yolk granules and nuclear migration toward the animal pole, confirming the capacity of hormone-treated prawns to reach full reproductive maturity.

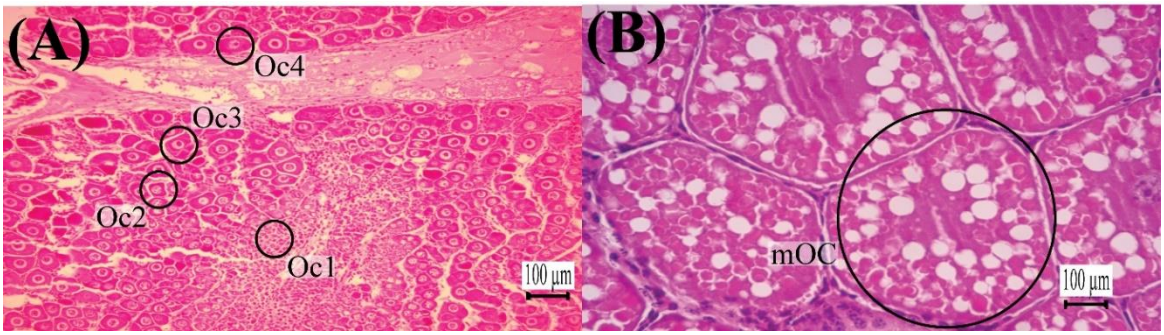


**Figure 2.** Histological structure of the ovary in E2-treated female *Macrobrachium rosenbergii*. (A) Ovary at stage III; (B) Ovary at stage IV; (Oc1: early previtellogenic oocytes; Oc2: late previtellogenic oocytes; Oc3: early vitellogenic oocytes; Oc4: mature vitellogenic oocytes; mOc: fully mature oocytes prior to ovulation.

Detailed oocyte diameter data are provided in Table 7. A comparative analysis of oocyte diameters across developmental stages demonstrated morphological characteristics and progression patterns consistent with those reported for natural females by Meeratana and Sobhon [22]. These findings confirm that ovarian maturation in neo-females parallels that of normal females.

**Table 6.** Oocyte diameter (µm) of E2-treated female and normal female *Macrobrachium rosenbergii*.

| Oc1 | Oc2 | Oc3 | Oc4 | mOc |
|---|---|---|---|---|
| 30.6 ± 7.1† | 64.7 ± 17.8 | 120 ± 12 | 168 ± 24 | 324 ± 93 |
| (15.2 - 41.0)‡ | (40.8 - 97.0) | (101 - 146) | (127 - 258) | (210 - 528) |
| 10 – 30# | 30 – 100# | 100 - 200# | 150 - 250# | 300 - 550# |

† *and* ‡ *mean* ± *SD and min – max*. # min – max of normal female prawns [22].

## 4. Discussion

### *4.1. Relating E2 Concentrations in Feed to the Hormone Bioaccumulation*

A notable finding of this study is the transient nature of E2 bioaccumulation in *M. rosenbergii* PL during continuous dietary exposure. LC-MS/MS feed analysis confirmed that the juveniles were consistently exposed to elevated exogenous steroid levels, though formulation efficiency plateaued at approximately 150 mg/kg; the nominal 200 mg/kg diet averaged 148.4 ± 40.0 mg/kg. Interestingly, despite the comparable actual hormone concentrations between the 150 mg/kg and 200 mg/kg diets, only the nominal 200 mg/kg group exhibited a significant accumulation peak at Day 24 (19.4 ± 5.4 ng/g). This may be attributed to the high variance (± 40.0 mg/kg) observed in the 200 mg/kg feed samples, suggesting that periodic exposure to highly concentrated, localized doses temporarily exceeded the PL's metabolic clearance capacity. The trace levels detected in the control feed (1.39 ± 1.03 mg/kg) were likely due to endogenous ingredient backgrounds or minor cross-reactivity and did not result in significant tissue accumulation relative to Day 0 baselines.

Most notably, the tissue accumulation observed at Day 24 was not sustained. By Day 36, E2 concentrations across all treatment groups had sharply declined, showing no statistically significant differences compared to the control group. This uniform reduction – occurring concurrently with continuous dietary E2 intake – strongly indicates that *M. rosenbergii* initiates a compensatory

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physiological response, likely upregulating hepatic or comparable metabolic pathways to degrade and excrete excess steroidal loads. Similarly, Rasheed, Tiwari, Reddy, Gupta and Rani [5] reported that there was negligible or no bioaccumulation of E2 hormone in the muscle of juvenile prawns fed diets supplemented with 25, 50, and 100 mg/kg. Also, E2 levels in the muscle of female PL did not change significantly in any of the treated groups or the control group. From an aquaculture and consumer safety perspective, this rapid clearance is highly advantageous. It demonstrates that dietary administration of E2 for feminization does not result in permanent hormonal bioaccumulation in juvenile tissues, significantly mitigating the risk of residual hormone following the treatment period.

*4.2. Efficacy of Dietary E2 on Growth Performance, Feed Efficiency, and Feminization Rate*

During the 36-day treatment period, E2 supplementation significantly improved FBW, SGR, and SR, while concurrently decreasing FCR compared with the control group. Importantly, these different growth, survival, and feed efficiency metrics were transient, disappearing entirely by the 150-day post-treatment period. The initial enhancement of FBW and SR in E2-treated groups aligns with findings that estrogenic steroids can exert anabolic effects and promote growth in many decapod species [5,14–16]. However, these results contrast with studies in other *Penaeus* shrimp, where E2 treatment has been shown to inhibit growth [14,29]. The impact of E2 on survival is also dose-dependent, with lower doses sometimes reducing viability while higher doses enhance it [29]. The temporary nature of these physiological alterations in the present study – evidenced by the equalization of growth and FCR during the 150-day post-treatment period – mirrors the compensatory growth phenomena observed in shrimp after the cessation of exogenous steroid administration [29]. Dietary E2 has also been hypothesized to function as an appetite stimulant [9]. Enhanced feed consumption and increased physical activity have been observed in E2-fortified groups, ultimately reducing FCR. These findings align with previous studies demonstrating the anabolic effects of E2 in *M. rosenbergii* [5] and *P. monodon* [15].

The observed feminization rates closely match the E2 concentrations quantified in the experimental diets. LC-MS/MS analysis revealed that dietary hormone incorporation plateaued at 150 mg/kg (actual concentration: 153.5 ± 21.7 mg/kg). The 200 mg/kg diet failed to retain additional hormone, resulting in a slightly lower actual concentration of 148.4 ± 40.0 mg/kg. Consequently, the lack of a significant increase in the feminization rate between the 150 mg/kg and 200 mg/kg groups reflects the actual hormone dosage ingested by the PLs rather than a physiological limitation. These data indicate that 150 mg/kg represents the practical saturation point for dietary E2 preparation, and formulations exceeding this threshold offer no further advantage in *M. rosenbergii* feminization protocols. The control group, which received no hormone, consisted entirely of males (0% females), confirming the all-male status of the initial population.

The present study confirms that dietary supplementation with E2 effectively induces gonadal feminization in juvenile *M. rosenbergii*. These findings partially align with those of Rasheed, Tiwari, Reddy, Gupta and Rani [5], who observed feminization rates of 26.7% and 32.0% at E2 dietary concentrations of 25 and 50 mg/kg, respectively. However, our outcomes contrast with their higher-dose results, in which 100 mg/kg yielded 45.8% females. Furthermore, previous studies on mixed-sex decapod populations have reported substantially higher conversion rates, such as 68.9% in *M. nipponense* fed 200 mg/kg E2 [30] and 71.9% in *P. vannamei* subjected to 2 mg/L E2 immersion [29]. This discrepancy is likely due to the use of an all-male postlarval cohort in the present experiment. Previous trials achieving high feminization rates predominantly utilized mixed-sex PLs. Because a baseline sex ratio at stocking is not feasible, it ranges widely from 26% to 65% female [5,18]. In contrast, the present study employed an exclusively all-male postlarval population, presenting a more rigorous biological barrier to complete feminization. Additionally, dietary E2 levels were deliberately kept low to minimize physiological and reproductive stress. As demonstrated by Hafiz, Hidayah, Yusdianatu, Ambak, Abol-Munafi and Ikhwanuddin [15] in *P. monodon*, steroid efficacy

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eventually reaches a biological plateau: increasing E2 levels from 200 to 400 mg/kg yielded an 8.8% increase in feminization, whereas increasing it from 800 to 1000 mg/kg yielded only a marginal 3.3% improvement. While complete (100%) feminization has been achieved in other species via high-dose immersion (1 mg/L in *L. vannamei*; Sugestya, Widodo and Soeprijanto [16]) or extreme dietary inclusion (1600 mg/kg in *P. merguiensis*; Ikhwanuddin, Bahar, Ma and Manan [14]), such aggressive treatments were avoided to preserve postlarval health. Furthermore, the 36-day experimental period may have been insufficient to fully maximize the sex-reversal effects of E2. Consistent with Macintosh, *et al.* [31], who established that the anabolic and sex-reversal impacts of steroidal treatments are highly proportional to exposure time, a prolonged treatment duration may be necessary to overcome the androgenic pathways in an all-male *M. rosenbergii* population. The efficiency of sex reversal may also depend on water temperature [32]. Further investigations are therefore warranted to improve these protocols, specifically by evaluating prolonged dietary exposure periods, increased hormone inclusion levels, and external environmental parameters.

Dietary supplementation with E2 proved to be an effective strategy for inducing functional gonadal development in neo-female *M. rosenbergii*. The histological progression of oogenesis in E2-treated individuals closely paralleled the developmental milestones typical of natural females, aligning with previous observations of hormone-induced gonadal maturation [9,30]. Specifically, the cellular structure of the ovaries – marked by the typical transition from previtellogenic to vitellogenic oocytes – demonstrates that exogenous E2 facilitates the formation of fully functional, rather than sterile or rudimentary, female gonads [9,33]. Furthermore, quantitative assessments of ovarian development, including GSI and oocyte diameter, revealed no significant deviations from wild-type females in this study or normal females from published data. During a normal reproductive cycle, the average GSI indices at ovarian stages III and IV in *M. rosenbergii* were 2.41% and 5.77%, respectively, while the oocyte diameters measured 250 μm and 360 μm, respectively [34]. Sagi, Soroka, Snir, Chomsky, Calderon and Milner [8] reported that oocytes typically expand from 20 to 650 μm in diameter, with GSI values concomitantly rising from approximately 0.2 to 8.0. In the present study, oocyte morphometrics across the five identified developmental stages mirrored the established baseline characteristics of natural females [22]. These results confirmed that the E2-dietary intervention induced normal ovarian differentiation without introducing histological abnormalities, as indicated by Dokkaew, Songdum, Prachom, Boonyung, Kitikiew, Khamphet, Waicharoen, Na-Nakorn, Paankhao, Uchuwittayakul and Kantha [33]. Ultimately, achieving a GSI and oocyte developmental profile comparable to that of wild or normal females confirms the reproductive viability of these neo-females. These findings substantiate the applicability of E2-mediated sex reversal for generating neo-female broodstock, representing a critical step forward in the sustainable production of all-male monosex populations and high-quality seedstock for the aquaculture industry.

## 5. Conclusions

According to our study results, dietary supplementation with E2 effectively redirects sexual differentiation in all-male *M. rosenbergii* PL, producing functional neo-females with normal ovarian maturation and only transient effects on growth and survival. By using an all-male population, this study eliminates the confounding effects of natural sex-ratio variance, thereby definitively quantifying hormone efficacy in crustacean models. Hatchery managers can apply this non-surgical dietary protocol to produce neo-female broodstock. This capability is critical for the indirect mass production of monosex all-male populations, maximizing harvest yields while avoiding food safety concerns associated with direct hormone application. While this method proved highly effective, a primary limitation remains the absence of progeny testing. Future research should investigate crossing E2-induced neofemales with normal males to empirically validate the generation of 100% all-male offspring.

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