



## Dose–Response Effects of Bovine Testis Extract Immersion on Masculinization, Survival, and Growth of *Betta splendens* Larvae

Ayu Winna Ramadhani<sup>1</sup>, Deva Achmad Irvanto<sup>1</sup>, Abdul Raheem Faqih<sup>1</sup>, R Adharyan Islamy<sup>1</sup>, Fitri Sil Valen<sup>2</sup>, Ahmad Syazni Kamarudin<sup>3</sup>, Norshida Ismail<sup>3</sup>, Veryl Hasan<sup>3,4,5\*</sup>

<sup>1</sup> Department of Fisheries and Marine Resources Management, Faculty of Fisheries and Marine Sciences, Aquaculture (Kediri Campus), Universitas Brawijaya, Kediri 64111, Indonesia

<sup>2</sup> Faculty of Agriculture, Fisheries and Biology, UBB Integrated Campus, Universitas Bangka Belitung, Bangka 33172, Indonesia

<sup>3</sup> School of Animal Science, Aquatic Science and Environment, Besut Campus, Universiti Sultan Zainal Abidin, Besut 22200, Malaysia

<sup>4</sup> Department of Aquaculture, Faculty of Fisheries and Marine, Universitas Airlangga, Surabaya 60113, Indonesia

<sup>5</sup> Research Group of Environmental and Fisheries Resources Management, Faculty of Fisheries and Marine, Universitas Airlangga, Surabaya 60113, Indonesia

Corresponding Author Email: [veryl.hasan@fpk.unair.ac.id](mailto:veryl.hasan@fpk.unair.ac.id)

Copyright: ©2026 The authors. This article is published by IETA and is licensed under the CC BY 4.0 license (<http://creativecommons.org/licenses/by/4.0/>).

<https://doi.org/10.18280/ijdne.210516>

### ABSTRACT

**Received:** 16 March 2026  
**Revised:** 18 May 2026  
**Accepted:** 25 May 2026  
**Available online:** 31 May 2026

#### Keywords:

androgen, *B. splendens*, dose–response, life below water, masculinization, survival rate

The increasing demand for male *B. splendens* in ornamental fish markets has driven the need for effective and safer methods of sex control in aquaculture. While synthetic hormones are widely used for masculinization, their potential environmental and health risks have encouraged the exploration of natural alternatives. This study evaluated the dose-dependent effects of bovine testis extract as a natural androgen source on sex ratio, survival rate, and growth performance of *B. splendens* larvae. A completely randomized design (CRD) was applied with four treatment dosages (0.04, 0.06, 0.08, and 0.10 mg/L), each conducted in triplicate. Larvae were exposed to the treatment through immersion for 24 hours and subsequently reared for 30 days under controlled conditions. The proportion of male fish increased dose-dependently from 36.51% at 0.04 mg/L to 85.00% at 0.10 mg/L, and Binomial Generalized Linear Model (GLM) analysis showed that treatment dosage significantly affected male proportion ( $p < 0.05$ ). In contrast, the survival rate decreased with higher dosages, with the lowest value (54.17%) observed at the highest treatment level, although the effect was not statistically significant ( $p > 0.05$ ). Specific growth rate (SGR) ranged from 3.37 to 4.18%/day and was also not significantly affected by treatment ( $p > 0.05$ ). The findings indicate a tendency toward a trade-off between masculinization efficiency and survival performance, suggesting that higher concentrations of bovine testis extract may influence larval survival during early development. From a practical perspective, intermediate dosages may provide a more balanced result by maintaining acceptable survival while achieving a favorable proportion of males. These results demonstrate the potential of bovine testis extract as a natural alternative to synthetic hormones, while highlighting the importance of dosage optimization in aquaculture systems.

## 1. INTRODUCTION

The ornamental fish industry has experienced rapid growth over the past decade, driven by increasing global demand for species with high aesthetic and economic value [1]. Among these, *B. splendens* is one of the most commercially important freshwater ornamental fish due to its vibrant coloration, aggressive display behavior, and adaptability to culture conditions. In commercial production systems, male individuals are generally preferred because they exhibit more attractive morphological characteristics, including brighter coloration, longer fins, and higher market value compared to females [2]. Consequently, controlling the sex ratio toward a

higher proportion of males has become a critical objective in ornamental fish aquaculture [3].

Sex reversal techniques have been widely applied to manipulate fish sex ratios, primarily through the administration of synthetic androgenic hormones such as 17 $\alpha$ -methyltestosterone. This compound has been extensively used due to its effectiveness in promoting masculinization and enhancing growth performance [4, 5]. Various delivery methods, including oral administration, injection, and immersion, have been reported to influence not only sex differentiation but also physiological responses such as growth, feed efficiency, and survival [6]. Despite its effectiveness, the use of synthetic hormones has raised increasing concerns

regarding environmental persistence, potential carcinogenic effects, and food safety issues, particularly in sustainable aquaculture systems [7, 8].

In response to these concerns, natural androgen sources have been explored as safer alternatives for sex control in fish. Bovine testis tissue, which is rich in endogenous testosterone, has been identified as a potential natural agent for inducing masculinization. Previous studies have demonstrated that extracts or meals derived from bovine testes can effectively increase the proportion of male fish in several species, including guppy and tilapia, with comparable results to synthetic hormones [9, 10]. The mechanism underlying this effect is associated with the absorption of exogenous testosterone during early developmental stages, which influences gonadal differentiation by suppressing ovarian development and promoting testicular formation [11].

However, while the masculinization potential of bovine testis-derived treatments has been documented, most existing studies focus primarily on achieving the highest percentage of males without adequately considering the associated impacts on survival and growth performance [12]. This limitation is critical, as increasing androgen exposure may simultaneously induce physiological stress, reduce survival rates, or alter water quality through increased organic loading and ammonia accumulation. From a production perspective, these competing effects suggest the existence of a trade-off between masculinization efficiency and overall culture performance.

Therefore, a more integrative evaluation is required to determine the optimal dosage that balances these competing outcomes. Understanding this trade-off is essential for developing practical and sustainable aquaculture strategies that maximize production efficiency while minimizing negative impacts on fish health and rearing conditions. Despite its importance, such optimization-based approaches remain limited, particularly for *B. splendens* culture systems.

Based on this background, the present study aimed to evaluate the dose-dependent effects of bovine testis extract on sex ratio, survival rate, and growth performance of *B. splendens* larvae. The immersion method was selected because it is practical and effective during the larval stage, enabling androgen absorption through the skin and gills while minimizing handling stress. Larvae at 7 days post-hatching were used because this period corresponds to the critical phase of gonadal differentiation, during which exogenous androgens can influence sexual development. Histological analysis was performed because external sexual characteristics are not yet clearly distinguishable at juvenile stages, making gonadal histology the most reliable method for sex identification. Furthermore, this study sought to identify an optimal treatment range that balances masculinization success and culture performance, thereby supporting more efficient and environmentally responsible aquaculture practices.

## 2. MATERIALS AND METHODS

### 2.1 Experimental design and fish rearing

This study employed a completely randomized design (CRD) consisting of four treatment levels of bovine testis extract dosage: 0.04, 0.06, 0.08, and 0.10 mg/L, hereafter referred to as treatments A, B, C, and D, respectively. Each treatment was conducted in triplicate. *B. splendens* larvae (average total length of  $0.545 \pm 0.02$  cm and an average body

weight of  $0.036 \pm 0.005$  g) at 7 days post-hatching were used as experimental animals. A total of 8 larvae were stocked in each aquarium unit. Larvae were reared under controlled conditions for 30 days following treatment application. Feeding and general maintenance procedures were conducted uniformly across all experimental units to minimize variability not associated with treatment effects.

### 2.2 Preparation of bovine testis extract

Fine bovine testis flour was subjected to solvent extraction using hexane (1:3 w/v) for 24 h to obtain a crude androgen extract. After filtration, the solvent extract was diluted into the treatment water according to the designated concentrations (0.04–0.10 mg/L). Thus, the experimental dosages referred to concentrations of bovine testis extract applied in the immersion media.

### 2.3 Preparation and application of bovine testis extract treatment

Bovine testis extract was used as a natural androgen source. The treatment was applied through an immersion method for 24 h, which has been reported as an effective exposure period for inducing sex reversal in fish. Larvae were immersed in treatment solutions according to their respective dosage levels in 5 L aquaria containing 3 L of water, with a stocking density of 8 larvae per aquarium. Continuous aeration was provided during immersion to maintain dissolved oxygen (DO) levels and ensure homogeneous dispersion of the extract. Water temperature was maintained at 27–28 °C under a 12 h light:12 h dark photoperiod, and larvae were not fed during the immersion period to avoid interference with androgen absorption. After immersion, larvae were gently rinsed with clean freshwater and transferred to rearing aquaria for 30 days under controlled conditions. Fish were maintained with continuous aeration and regular partial water exchange to maintain water quality, and were fed commercial feed three times daily according to larval requirements. All aquaria were randomly positioned to minimize environmental bias among treatments.

### 2.4 Histological analysis

Histological examination of gonadal tissue was conducted to verify sex differentiation. Tissue preparation followed standard histological procedures as described in published methods [13, 14]. Briefly, samples were fixed in Bouin's solution to preserve tissue structure, followed by dehydration through a graded ethanol series. The fixed tissues were embedded in paraffin, sectioned using a microtome, and mounted onto glass slides. Sections were then stained and observed under a light microscope to identify gonadal structures and confirm male or female differentiation based on established morphological characteristics.

### 2.5 Sex ratio determination

The percentage of male fish was calculated at the end of the experimental period using the following formula:

$$\text{Male percentage (\%)} = \left( \frac{\text{Number of male fish}}{\text{Total number of fish}} \right) \times 100 \quad (1)$$

Sex identification was based on gonadal observation through histological analysis.

## 2.6 Survival rate

Survival rate (SR) was calculated according to published methods [15] using the following equation:

$$SR (\%) = (N_t / N_0) \times 100 \quad (2)$$

where,  $N_t$  = number of fish at the end of the experiment and  $N_0$  = number of fish at the beginning of the experiment.

## 2.7 Specific growth rate

Specific growth rate (SGR) was calculated based on body weight using the following formula [15]:

$$SGR (\%/day) = [(\ln W_t - \ln W_0) / t] \times 100 \quad (3)$$

where,  $W_t$  = final average body weight (g);  $W_0$  = initial average body weight (g); and  $t$  = duration of the experiment (days).

## 2.8 Water quality monitoring

Water quality parameters, including temperature, DO, and pH, were monitored twice daily throughout the experiment using a portable multiparameter meter (AZ 86031; AZ Instrument Corp., Taichung City, Taiwan). To ensure measurement accuracy and address previous concerns regarding pH readings, the pH sensor was calibrated daily using a three-point calibration method with standard buffer solutions (pH 4.0, 7.0, and 10.0). Total ammonia nitrogen (TAN) was measured weekly using a salicylate-based commercial colorimetric test kit. Quality assurance and quality control (QA/QC) were maintained through duplicate measurements for each treatment and periodic instrument recalibration according to the manufacturer's instructions.

## 2.9 Statistical analysis

Sex ratio and survival data were analyzed using a Binomial Generalized Linear Model (GLM) with a logit link function because the response variables consisted of proportional outcomes (male/female and survival/death). Treatment dosage was included as a fixed factor, and each replicate aquarium was treated as the experimental unit. When significant effects were detected ( $p < 0.05$ ), pairwise comparisons among treatments were conducted using estimated marginal means. SGR data were analyzed using one-way ANOVA.

## 3. RESULTS

### 3.1 The sex ratio

The percentage of male *B. splendens* increased with increasing dosage of bovine testis extract. The highest proportion of males was observed in treatment D (0.10 mg/L), reaching 85%, followed by treatment C (0.08 mg/L) at 60%, treatment B (0.06 mg/L) at 41.27%, and treatment A (0.04 mg/L) at 36.51%. Binomial GLM analysis showed that treatment dosage significantly affected the male proportion of *Betta splendens* larvae ( $p = 0.039$ ). The highest male proportion was observed in treatment D (0.10 mg/L), while treatments A–C showed lower male proportions. Post hoc pairwise comparisons based on estimated marginal means showed that treatment D differed significantly from treatments A and B, while treatment C exhibited intermediate values and did not differ consistently from the other treatments. The observed trend suggests a positive relationship between increasing dosage of bovine testis extract and the proportion of male fish. Histological observations presented in Figure 1 confirmed gonadal differentiation between male and female fish following the experimental treatments. Raw count data for surviving fish and sex identification in each replicate are presented in Table 1.

### 3.2 Survival rate

The survival rate of *B. splendens* larvae tended to decrease with increasing dosage of bovine testis extract (Table 1). The highest survival values were observed in treatments A (0.04 mg/L) and B (0.06 mg/L), both reaching  $79.167 \pm 7.22\%$ . A moderate decline was recorded in treatment C (0.08 mg/L), with a survival rate of  $70.833 \pm 7.22\%$ , whereas the lowest survival was found in treatment D (0.10 mg/L), which decreased to  $54.17 \pm 7.22\%$ .

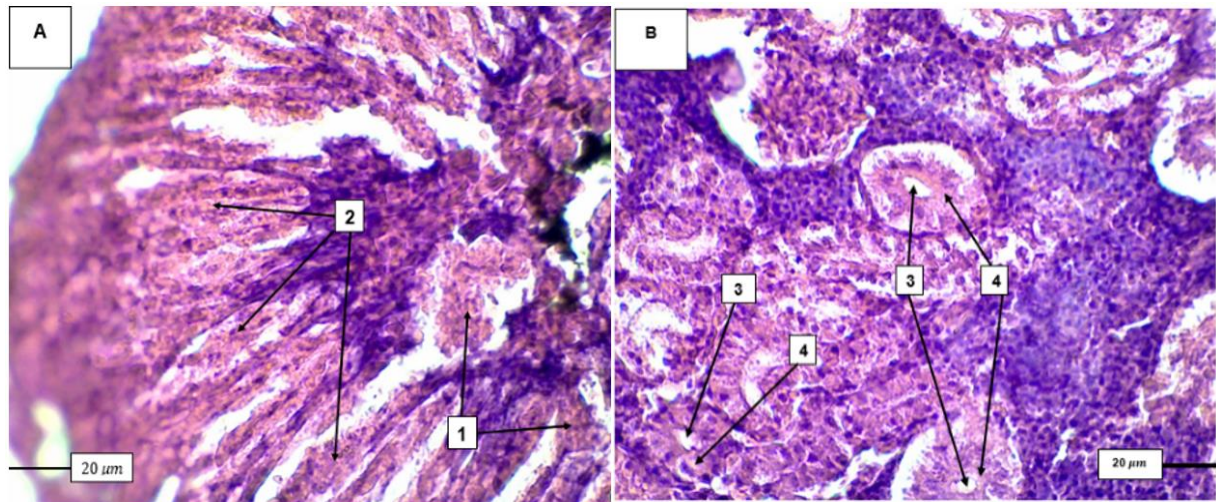
Although statistical analysis using a Binomial GLM indicated that treatment dosage did not significantly affect larval survival ( $p = 0.184$ ), the observed trend demonstrated a progressive reduction in survival at higher extract concentrations. This pattern suggests that increasing levels of bovine testis extract may exert physiological stress on larvae, potentially reducing their tolerance and overall viability during the rearing period.

The reduction in survival at higher dosages may be associated with excessive hormonal exposure during early developmental stages. In the present study, treatment D (0.10 mg/L) showed the most pronounced decline in survival, indicating that this concentration may approach the upper tolerance limit for *B. splendens* larvae under the experimental conditions.

**Table 1.** Effects of bovine testis extract dosage on sex ratio, survival rate (SR), and specific growth rate (SGR) of *B. splendens*

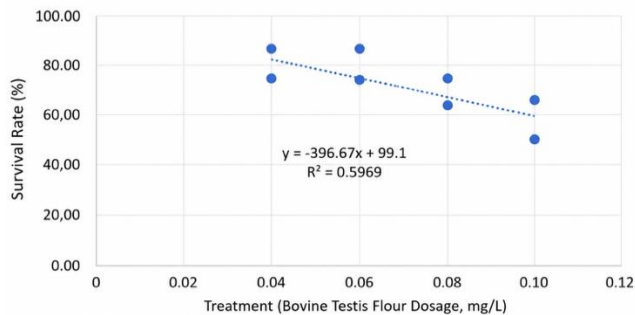
Treatment (mg/L)	Initial (Gender Not Yet Detected) (n)	Survivors (n)	Males (n)	Females (n)	Male (%)	Survival (%)	SGR (% day <sup>-1</sup> )
A (0.04)	24	19	7	12	36.51 ± 5.50 <sup>a</sup>	79.17 ± 7.22 <sup>a</sup>	3.37 ± 0.57 <sup>a</sup>
B (0.06)	24	19	8	11	41.27 ± 13.75 <sup>a</sup>	79.17 ± 7.22 <sup>a</sup>	3.74 ± 0.44 <sup>a</sup>
C (0.08)	24	17	10	7	60.00 ± 17.32 <sup>ab</sup>	70.83 ± 7.22 <sup>a</sup>	4.17 ± 1.04 <sup>a</sup>
D (0.10)	24	13	11	2	85.00 ± 13.23 <sup>b</sup>	54.17 ± 7.22 <sup>a</sup>	4.18 ± 1.04 <sup>a</sup>

Note: Values are presented as mean ± standard deviation (SD) of three replicates. Initial (n) represents the total number of fish at the beginning of the experiment (8 fish per aquarium × 3 replicates). Survivors, Males, and Females (n) represent cumulative raw counts across replicates for each treatment. Different superscript letters within the Male (%) column indicate significant differences among treatments according to Binomial Generalized Linear Model (GLM) analysis ( $p < 0.05$ ). Survival (%) and specific growth rate (SGR) did not differ significantly among treatments ( $p > 0.05$ ).



**Figure 1.** Histological structure of gonadal tissue in *B. splendens*. (A) Male gonad: (A1) spermatid, (A2) seminiferous tubules, (B) female gonad: (B3) oocytes, (B4) follicular cell  
Observed at 400× magnification

Despite the decreasing survival trend, all treatments maintained survival rates above 50%, indicating that the immersion treatments were still generally tolerated by the larvae. Furthermore, the absence of significant differences among treatments suggests that variability among replicates remained relatively high, thereby reducing statistical separation between treatment groups. The relationship between bovine testis extract dosage and larval survival is summarized in Table 1 and illustrated in Figure 2, which demonstrates a gradual decline in survival with increasing treatment dosage.



**Figure 2.** Dose-response relationship between bovine testis extract dosage and survival rate of betta fish

### 3.3 Specific growth rate

The SGR showed a slight increasing trend with higher treatment dosages (Table 1). The highest SGR value was observed in treatment D (0.10 mg/L) at 4.18%/day, followed by treatment C (0.08 mg/L) at 4.17%/day, treatment B (0.06 mg/L) at 3.74%/day, and treatment A (0.04 mg/L) at 3.37%/day. However, statistical analysis indicated that the differences among treatments were not significant ( $p > 0.05$ ), suggesting that bovine testis extract dosage did not have a statistically measurable effect on growth rate under the conditions of this study.

### 3.4 Histological observations

Male gonads were characterized by the presence of seminiferous tubules and spermatogenic cells, indicating testicular development. In contrast, female gonads exhibited

ovarian follicles containing oocytes surrounded by follicular cells. These structural differences were clearly observed under light microscopy (Figure 1), providing confirmation of sex differentiation results obtained from the experimental treatments.

### 3.5 Water quality parameters

Water quality parameters remained within measurable ranges throughout the experimental period (Table 2), although slight variations were observed among treatments. Temperature ranged from 27.216–27.358 °C, while DO varied between 7.754 and 7.904 mg/L. Water pH ranged from  $6.58 \pm 0.12$  to  $6.82 \pm 0.11$ . These values were rechecked using repeated measurements and calibration procedures, confirming relatively acidic conditions throughout the experimental period. Ammonia concentrations increased with increasing bovine testis extract dosage, ranging from 0.06 mg/L in treatment A to 0.16 mg/L in treatment D. Mean survival rates declined from 79.17% in treatments A and B to 54.17% in treatment D. Correlation analysis indicated that the narrow pH variation among treatments was not clearly associated with survival responses, whereas increasing ammonia concentrations tended to coincide with lower survival rates.

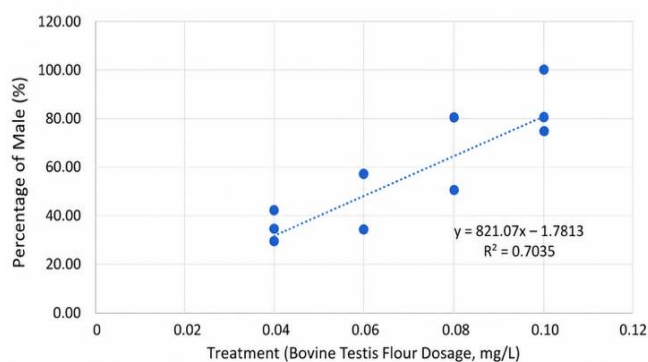
**Table 2.** Water quality parameters during the rearing of *B. splendens* under different bovine testis extract dosages

Treatment	Temperature (°C)	DO (mg/L)	pH	Ammonia (mg/L)
A (0.04 mg/L)	27.36 ± 0.12	7.90 ± 0.08	6.82 ± 0.11	0.06 ± 0.01
B (0.06 mg/L)	27.31 ± 0.10	7.89 ± 0.07	6.74 ± 0.09	0.07 ± 0.01
C (0.08 mg/L)	27.29 ± 0.11	7.75 ± 0.09	6.65 ± 0.10	0.14 ± 0.02
D (0.10 mg/L)	27.22 ± 0.13	7.85 ± 0.05	6.58 ± 0.12	0.16 ± 0.02

Note: Values are presented as mean ± standard deviation (SD). Temperature, dissolved oxygen (DO), and pH were measured twice daily throughout the 30-day experimental period ( $n = 60$  observations per treatment), while ammonia was measured weekly ( $n = 4$  observations per treatment). No statistical analysis was applied to water quality parameters; values are presented descriptively to support interpretation of biological responses.

#### 4. DISCUSSIONS

The present study demonstrates a clear dose-dependent effect of bovine testis extract on the masculinization of *B. splendens*, where the highest percentage of males (85%) was achieved at a dosage of 0.10 mg/L. This pattern indicates that increasing the availability of exogenous androgen sources enhances the probability of male differentiation during early developmental stages. As illustrated in Figure 3, regression analysis showed a positive relationship between dosage and male percentage ( $y = 821.07x - 1.7813$ ;  $R^2 = 0.7035$ ), indicating that treatment dosage accounted for approximately 70.35% of the variation in male proportion. Bovine testis extract is known to contain endogenous testosterone, which can be absorbed by fish larvae during immersion and subsequently influence the hormonal balance regulating gonadal differentiation [16]. An elevated androgen concentration relative to estrogen is likely to suppress ovarian development while promoting testicular formation, leading to a higher proportion of males [17].



**Figure 3.** Dose-response relationship between testis extract dosage and percentage of male betta fish

This mechanism is consistent with the general principle of sex differentiation in teleost fish, where the endocrine environment during critical periods of development determines gonadal fate. The effectiveness of natural androgen sources observed in this study aligns with previous findings reporting comparable masculinization success between bovine testis-derived treatments and synthetic hormones [18]. However, unlike synthetic compounds such as  $17\alpha$ -methyltestosterone, natural sources may exhibit more variable absorption and bioavailability, which can influence treatment efficiency depending on dosage and exposure conditions [19]. Despite the positive effect on masculinization, increasing the dosage of bovine testis extract was associated with a marked reduction in survival rate (Figure 3).

The lowest survival (54.17%) occurred at the highest dosage (0.10 mg/L), indicating that excessive exposure may impose physiological stress on larvae. As illustrated in Figure 2, regression analysis showed a negative relationship between treatment dosage and survival rate ( $y = -396.67x + 99.1$ ;  $R^2 = 0.5969$ ), indicating that increasing bovine testis extract concentration tended to reduce larval survival. One plausible explanation is the indirect effect of organic loading from bovine testis extract, which contributes to the accumulation of nitrogenous waste in the culture system. As protein-rich material decomposes, ammonia is released into the water, potentially reaching sublethal or toxic concentrations [20].

Ammonia is widely recognized as a critical water quality parameter in aquaculture systems, as elevated concentrations can impair gill function, reduce oxygen transport, and disrupt metabolic processes in fish [21]. In the present study, ammonia levels tended to increase with higher treatment dosages and coincided with lower survival rates. Although ammonia toxicity was not directly evaluated in this study, the observed increase in ammonia concentration may have contributed to physiological stress in larvae, particularly during early developmental stages that are more sensitive to environmental fluctuations.

Monitoring water quality is essential in aquaculture because environmental variables strongly influence fish physiological performance, stress responses, and overall production outcomes. Water chemistry parameters such as pH and ammonia can interact and affect biological processes, including metabolism, ion regulation, and survival [21]. In the present study, water quality generally remained within measurable ranges throughout the experimental period; however, pH and ammonia showed variation among treatments. The pH values ranged from  $6.58 \pm 0.12$  to  $6.82 \pm 0.11$ , indicating relatively optimal conditions compared with commonly recommended freshwater aquaculture conditions. Although the recorded pH values were lower than the commonly recommended range for *Betta* culture (approximately pH 6.5–7.5), *B. splendens* is known to possess relatively high tolerance to suboptimal environmental conditions. Water pH is recognized as a key factor affecting physiological and biochemical processes in fish, and deviations from suitable ranges may alter growth, stress responses, and health status [22]. Previous studies have shown that acidic conditions can influence fish performance through changes in physiological and hematological responses, particularly under culture-related stressors [23]. Acid–base balance in fish is primarily regulated through the gills, and deviations in water pH from optimal conditions may influence ion transport processes and contribute to physiological stress responses [24]. Nevertheless, *B. splendens* is recognized as relatively tolerant to fluctuating environmental conditions compared with many freshwater ornamental species, although prolonged exposure to highly acidic environments may still impose physiological stress [25]. Environmental conditions during early developmental stages may influence endocrine regulation and physiological responses associated with gonadal differentiation. Stress-induced cortisol elevation has been reported to interact with sex steroid pathways in teleost fish, potentially modifying androgen responsiveness and affecting sex differentiation outcomes. Therefore, the masculinization responses observed in the present study may reflect not only the direct androgenic effects of bovine testis extract but also the combined influence of hormonal exposure and rearing environment conditions.

Ammonia concentrations increased with increasing treatment dosage and coincided with reductions in survival rate. The highest ammonia concentration (0.16 mg/L) and lowest survival value (54.17%) were observed in treatment D. Elevated ammonia concentrations are known to impair respiration, disrupt metabolic function, and induce physiological stress in aquatic organisms, ultimately reducing survival under prolonged exposure conditions [26]. Although ammonia toxicity was not directly measured in the present study, the observed inverse trend between ammonia concentration and survival suggests that ammonia accumulation may have contributed to reduced larval

performance. The pH variation among treatments was relatively narrow and did not follow the same pattern as survival decline, indicating that ammonia accumulation may have exerted a stronger influence on biological responses than pH differences alone. Therefore, the interaction between water quality conditions and treatment dosage should be considered when interpreting the observed trade-off between masculinization efficiency and survival.

The contrasting responses between sex ratio and survival highlight an important trade-off in the application of natural androgen treatments. While higher dosages enhance masculinization efficiency, they simultaneously reduce survival performance, suggesting that maximizing one outcome may compromise the other. From a production perspective, this trade-off is critical because the optimal strategy is not necessarily the treatment that produces the highest percentage of males, but rather the one that achieves a balanced outcome between sex control and overall yield.

In terms of growth performance, the SGR showed a slight increasing trend with higher dosages, although the differences were not statistically significant. This indicates that bovine testis extract does not have a strong direct effect on growth under the conditions tested. The observed trend may be associated with the anabolic role of androgens, which can influence protein synthesis and growth processes, but the effect appears to be secondary compared to its role in sex differentiation [27]. The lack of significant differences suggests that growth is likely influenced by multiple interacting factors, including feed intake, metabolic efficiency, and environmental conditions.

Monitoring water quality is crucial for water exchange in aquaculture [28]. In this study, it measured during the measurements generally remained within ranges that support fish survival, although some variables, particularly ammonia and pH, showed similar values in every treatment. Water quality parameters remained within ranges generally considered suitable for *Betta* culture throughout the experimental period. Slight reductions in pH and increases in ammonia concentration were observed with increasing bovine testis extract dosage, which may reflect greater organic loading from the extract material. Among the measured parameters, ammonia showed a clearer inverse relationship with survival rate than pH variation, suggesting that nitrogenous waste accumulation may have contributed more strongly to physiological stress in larvae. Therefore, the interaction between water quality parameters and treatment dosage should be considered when interpreting the overall outcomes.

Taken together, the findings of this study suggest that bovine testis extract can be used as a natural alternative to synthetic hormones for sex control in *B. splendens*, but its application requires careful dosage management. The existence of a trade-off between masculinization efficiency and survival underscores the need for an optimization-based approach rather than a single-objective focus. From an aquaculture systems perspective, selecting an intermediate dosage may provide a more sustainable and economically viable strategy by maintaining acceptable survival rates while still achieving a favorable proportion of male fish.

## 5. CONCLUSIONS

Bovine testis extract demonstrated a dose-dependent effect on the masculinization of *Betta splendens*, with the highest

male percentage achieved at treatment D ( $85.00 \pm 13.23\%$ ). Binomial GLM analysis confirmed that treatment dosage significantly affected male proportion, while survival and growth performance were not significantly influenced by treatment. Although survival tended to decrease at higher dosages, intermediate treatments, particularly treatments B and C, provided a more balanced outcome by maintaining moderate-to-high male proportions (41.27–60.00%) together with acceptable survival rates (70.83–79.17%). Overall, the results support the potential use of bovine testis extract as a natural alternative to synthetic hormones in ornamental fish aquaculture.

## ACKNOWLEDGMENT

The authors would like to thank Universitas Brawijaya for providing support and research facilities that made this study possible.

## REFERENCES

- [1] Islamy, R.A., Mutmainnah, N., Putri, R.T., Valen, F.S., Kamarudin, A.S., Hasan, V. (2025). Cochineal powder as an Eco-Friendly carotenoid supplement to enhance coloration in *Betta splendens*. *Journal of Ecological Engineering*, 26(10): 116-125. <https://doi.org/https://doi.org/10.12911/22998993/205686>
- [2] Prasadi, O. (2019). Utilizing narrow land as a place for cultivating betta fish in Mertasinga, Cilacap. *Aksiologi Jurnal Pengabdian Kepada Masyarakat*, 3(2): 113. <https://doi.org/10.30651/aks.v3i2.1473>
- [3] Martínez, P., Viñas, A.M., Sánchez, L., Díaz, N., Ribas, L., Piferrer, F. (2014). Genetic architecture of sex determination in fish: Applications to sex ratio control in aquaculture. *Frontiers in Genetics*, 5340. <https://doi.org/10.3389/fgene.2014.00340>
- [4] Bautista, L.M., Tumbokon, B.L.M., Serrano, A.E. (2022). 17 $\alpha$ -Methyltestosterone enhances growth and reproductive performance of immature male Asian catfish (*Clarias macrocephalus*). *Israeli Journal of Aquaculture - Bamidgah*, 74. <https://doi.org/10.46989/001c.55654>
- [5] Ramírez, J.F.P., Alvarenga, É.R., Da Costa, F.F.B., Ferreira, M.P., et al. (2024). Reduction of methyltestosterone concentration in feed during masculinization of Nile tilapia (*Oreochromis niloticus*) in biofloc system. *Aquaculture*, 593: 741253. <https://doi.org/10.1016/j.aquaculture.2024.741253>
- [6] Huang, H., Liu, Y., Wang, Q., Dong, C., et al. (2024). Molecular and physiological effects of 17 $\alpha$ -methyltestosterone on sex differentiation of black rockfish, *Sebastes schlegelii*. *Genes*, 15(5): 605. <https://doi.org/10.3390/genes15050605>
- [7] Li, M., Zhang, N., Huang, Y., Pan, C., Dong, Z., Lin, Z., Li, C., Jiang, Y., Liang, Y. (2023). The effects of 17 $\alpha$ -methyltestosterone on gonadal histology and gene expression along hypothalamic-pituitary-gonadal axis, germ cells, sex determination, and hypothalamus-pituitary-thyroid axis in zebrafish (*Danio rerio*). *Environmental Toxicology*, 39(3): 1494-1504. <https://doi.org/10.1002/tox.24044>

- [8] Rivero-Wendt, C.L.G., Miranda-Vilela, A.L., Domingues, I., Oliveira, R., Monteiro, M.S., Moura-Mello, M.A.M., Matias, R., Soares, A.M.V.M., Grisolia, C.K. (2020). Steroid androgen 17 alpha methyltestosterone used in fish farming induces biochemical alterations in zebrafish adults. *Journal of Environmental Science and Health Part A*, 55(11): 1321-1332. <https://doi.org/10.1080/10934529.2020.1790954>
- [9] Hariati, A.M., Ananda, N.J., Afifah, F.O., Mahariawan, I.M.D. (2024). The effectiveness of fortification of various local natural ingredients in fish feed on the masculinization of tilapia (*Oreochromis niloticus*). *Jurnal Airaha*, 13(2): 191-199. <https://doi.org/10.15578/ja.v13i2.620>
- [10] Saputra, Y.F., Junaidi, M., Setyono, B.D.H. (2022). Masculinization of betta fish (*Betta sp*) using extracts of cow testicles trough immersion with different doses of immersion. *Indonesian Journal of Aquaculture Medium*, 2(2): 155-165. <https://doi.org/10.29303/mediaakuakultur.v2i2.1738>
- [11] Matsuyama, S., DeFalco, T. (2024). Steroid hormone signaling: Multifaceted support of testicular function. *Frontiers in Cell and Developmental Biology*, 11: 1339385. <https://doi.org/10.3389/fcell.2023.1339385>
- [12] Sayouh, M., Ali, M., Li, Y., Tao, Y.F., Lu, S.Q., Qiang, J. (2024). Differences in growth performance and meat quality between male and female juvenile Nile tilapia (*Oreochromis niloticus*) during Separate Rearing. *Animals*, 14(20): 2954. <https://doi.org/10.3390/ani14202954>
- [13] Kilawati, Y., Maimunah, Y., Widyarti, S., Amrillah, A.M., Islamy, R.A., Amanda, T., Atriskya, F., Subagio, F.R. (2025). Histopathological alterations of hepatopancreas and intestines in the vaname shrimp (*Litopenaeus vannamei*) infected by white feces disease (WFD). *Egyptian Journal of Aquatic Biology & Fisheries*, 29(2): 1235-1248. <https://doi.org/10.21608/ejabf.2025.419575>
- [14] Islamy, R.A., Hasan, V., Kamarudin, A.S., Mamat, N.B., Valen, F.S., Mutmainnah, N. (2025). Assessment of microplastic accumulation, genotoxicity, and gill histopathological alterations in wild herbivorous fishes from the Brantas River, Malang, Indonesia. *Inzynieria Ekologiczna*, 26(11): 1-16. <https://doi.org/10.12911/22998993/205979>
- [15] Islamy, R.A., Aisyah, D., Ramadhani, A.W., Ni'matullah, S., Setyowati, N.L., Mutmainnah, N., Valen, F.S., Kamarudin, A.S., Czech, M., Hasan, V. (2025). Functional feed development from alternanthera philoxeroides for herbivorous fish: Effects on growth performance, feed efficiency, and hematological response. *International Journal of Design & Nature and Ecodynamics*, 20(9): 2191-2199. <https://doi.org/10.18280/ijdne.200922>
- [16] Salam, N.I., Chadijah, A., Rahmi, R., Wahyu, F. (2021). Masculinization of betta fish (*Betta sp*) using cow testic flour through Artemia immersion. *Akuatikisle: Jurnal Akuakultur, Pesisir dan Pulau-Pulau Kecil*, 5(2): 45-48. <https://doi.org/10.29239/j.akuatikisle.5.2.45-48>
- [17] Amir, S., Shah, S.T.A., Mamoulakis, C., Docea, A.O., Kalantzi, O.I., Zachariou, A., Calina, D., Carvalho, F., Sofikitis, N., Makrigiannakis, A., Tsatsakis, A. (2021). Endocrine disruptors acting on estrogen and androgen pathways cause reproductive disorders through multiple mechanisms: A review. *International Journal of Environmental Research and Public Health*, 18(4): 1464. <https://doi.org/10.3390/ijerph18041464>
- [18] Eyni, H., Ghorbani, S., Nazari, H., Hajjalyani, M., Bazaz, S.R., Mohaqiq, M., Warkiani, M.E., Sutherland, D.S. (2021). Advanced bioengineering of male germ stem cells to preserve fertility. *Journal of Tissue Engineering*, 1220417314211060590. <https://doi.org/10.1177/20417314211060590>
- [19] Ojogoro, J.O., Scrimshaw, M.D., Sumpter, J.P. (2021). Steroid hormones in the aquatic environment. *The Science of The Total Environment*, 792148306. <https://doi.org/10.1016/j.scitotenv.2021.148306>
- [20] Edwards, T.M., Puglis, H.J., Kent, D.B., Durán, J.L., Bradshaw, L.M., Farag, A.M. (2023). Ammonia and aquatic ecosystems – A review of global sources, biogeochemical cycling, and effects on fish. *The Science of the Total Environment*, 907: 167911. <https://doi.org/10.1016/j.scitotenv.2023.167911>
- [21] Rudoy, D., Meskhi, B., Olshevskaya, A., Kozyrev, D., Shevchenko, V., Odabashyan, M., Teplyakova, S., Rybak, A. (2025). Analysis of Edaphic factors on the role of probiotics in the development of sustainable and productive aquaculture. *Fishes*, 10(9): 457. <https://doi.org/10.3390/fishes10090457>
- [22] Swain, S., Sawant, P.B., Chadha, N.K., Chhandaprajnadarsini, E.M., Katare, M. (2020). Significance of water pH and hardness on fish biological processes: A review. *International Journal of Chemical Studies*, 8(4): 830-837. <https://doi.org/10.22271/chemi.2020.v8.i4e.9710>
- [23] Da Paixão Lemos, C.H., Di Mambro Ribeiro, C.V., De Oliveira, C.P.B., Couto, R.D., Copatti, C.E. (2018). Effects of interaction between pH and stocking density on the growth, haematological and biochemical responses of Nile tilapia juveniles. *Aquaculture*, 495: 62-67. <https://doi.org/10.1016/j.aquaculture.2018.05.037>
- [24] Zimmer, A.M., Perry, S.F. (2022). Physiology and aquaculture: A review of ion and acid-base regulation by the gills of fishes. *Fish and Fisheries*, 23(4): 874-898. <https://doi.org/10.1111/faf.12659>
- [25] Hemmatzadeh, M., Mohammadiazarm, H., Ghasemi, S.A., Rezaie, A. (2024). Influence of humic acids on growth performance, bravery, stress resistance, antioxidant activity, and immune gene expression in male Siamese fighting fish (*Betta splendens*). *Aquaculture Reports*, 38: 102286. <https://doi.org/10.1016/j.aqrep.2024.102286>
- [26] Miranda, E.K.S., Garçon, D.P., Brazão, C.C., De Almeida, L.C.F., Bertini, G. (2025). Effects of ammonia on the survival and physiology of juveniles and adults of *Macrobrachium acanthurus* (Caridea: Palaemonidae). *Aquatic Toxicology*, 28: 0107261. <https://doi.org/10.1016/j.aquatox.2025.107261>
- [27] Dubois, V., Laurent, M., Boonen, S., Vanderschueren, D., Claessens, F. (2011). Androgens and skeletal muscle: cellular and molecular action mechanisms underlying the anabolic actions. *Cellular and Molecular Life Sciences*, 69(10): 1651-1667. <https://doi.org/10.1007/s00018-011-0883-3>
- [28] Hamzah, Albasri, H., Faridah, S., Mundayana, Y., et al. (2025). Analysis of the effectiveness of sand filters for raw water treatment in brackish water aquaculture environments. *Engineering Technology & Applied*

$N_t$	Number of fish at the end of the experiment, individuals
$N_0$	Number of fish at the beginning of the experiment, individuals
$W_t$	Final average body weight, g
$W_0$	Initial average body weight, g
$t$	Experimental duration, days
mg/L	Concentration (milligram per liter), $\text{mg L}^{-1}$
$^{\circ}\text{C}$	Temperature
pH	Acidity level (potential of hydrogen)
ANOVA	Analysis of Variance
LSD	Least Significant Difference

## NOMENCLATURE

CRD	Completely Randomized Design
SR	Survival Rate, %
SGR	Specific Growth Rate, $\% \text{ day}^{-1}$
DO	Dissolved Oxygen, $\text{mg L}^{-1}$