



The Masculinization of Guppy *Poecilia reticulata* (Peters. 1859) Using *Tribulus terrestris* (Linnaeus, 1753) Extract Through Immersion of Pregnant Female Broodstock and Larvae

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ABSTRACT

The technology of sex reversal in guppy (*Poecilia reticulata*) cultivation is a way to produce monosex males through masculinization techniques. *Tribulus terrestris* extract (TTE) provides a low-cost, eco-friendly alternative to synthetic hormones such as 17 α -methyltestosterone. Unlike synthetic agents that may leave endocrine-disrupting residues harmful to aquatic organisms and humans, TTE is natural, biodegradable, and reduces ecological risks, supporting safer and more sustainable aquaculture practices. TTE, which contains bioactive compounds such as saponins and protodioscin known to stimulate androgen production was used as a natural agent for masculinization. This study aimed to examine the effect of giving *Tribulus terrestris* extract on masculinization of guppies by immersing gravid female and in the fish larvae. A Completely Randomized Design (CRD) was used for treatments involving pregnant broodstock to ensure uniform conditions among individuals, whereas a Randomized Completely Block Design (RCBD) was applied to larval experiments to account for variations in age and developmental stage. The treatments for immersion gravid guppy were K- without TTE and 17 α -methyltestosterone, K+ treatment with 17 α -methyltestosterone 500 μ g/L, P1 with 5 mg/L TTE dose, P2 with 10 mg/L TTE dose, and P3 with TTE 15 mg/L dose. The treatment for immersion in the larval stage was K- without TTE and 17 α -methyltestosterone, K+ treatment with a dose of 17 α -methyltestosterone 8 mg/L, P1 with an TTE dose of 2.5 mg/L, P2 with an TTE dose of 5 mg/L, and P3 with an TTE dose of 10 mg/L. Masculinization was most effective at a dose of 15 mg/L (P3) for gravid guppy, producing 87.78 \pm 8.75% male individuals with a high survival rate. These results indicate that *Tribulus terrestris* extract can effectively promote male differentiation while maintaining normal growth and survival performance.

Keywords: Guppies, gonads, sex reversal, *Tribulus terrestris*

ABSTRAK

Teknologi pengarahannya kelamin dalam budidaya guppy (*Poecilia reticulata*) merupakan metode untuk memproduksi jantan tunggal melalui teknik maskulinisasi. Pemanfaatan ekstrak *Tribulus terrestris* (ETT) pada teknik maskulinisasi dapat dijadikan alternatif yang lebih murah dan ramah lingkungan dibandingkan dengan hormon sintesis seperti 17 α -methyltestosterone. Berbeda dengan agen sintetis yang dapat meninggalkan residu pengganggu endokrin yang berbahaya bagi organisme air dan manusia, ETT bersifat alami, mudah terurai, dan mengurangi risiko ekologis, sehingga mendukung praktik akuakultur yang lebih aman dan berkelanjutan. ETT mengandung senyawa bioaktif seperti saponin dan protodioscin yang diketahui dapat merangsang produksi androgen, sehingga digunakan sebagai agen alami untuk maskulinisasi. Penelitian ini bertujuan untuk mengkaji pengaruh pemberian ETT terhadap maskulinisasi guppy melalui perendaman induk betina bunting dan pada tahap larva. Rancangan yang digunakan adalah rancangan acak lengkap (RAL) untuk perlakuan pada induk bunting, sedangkan rancangan acak kelompok (RAK) diterapkan pada tahap larva. Perlakuan

perendalam ETT berbeda dosis pada induk bunting, yaitu K- : tanpa ETT dan 17 α -metiltestosteron, perlakuan K+ : 17 α -metiltestosteron 500 μ g/L, P1: ETT 5 mg/L, P2: ETT 10 mg/L, dan P3: ETT 15 mg/L. Sedangkan perlakuan perendaman pada tahap larva, K-: tanpa ETT dan 17 α -metiltestosteron, K+: 17 α -metiltestosteron 8 mg/L, P1: ETT 2,5 mg/L, P2: ETT 5 mg/L, dan P3: ETT 10 mg/L. Hasil penelitian menunjukkan bahwa maskulinisasi paling efektif pada dosis 15 mg/L (P3), menghasilkan 87,78 \pm 8,75% individu jantan dengan tingkat kelangsungan hidup yang tinggi. Hasil ini menunjukkan bahwa ekstrak *Tribulus terrestris* dapat secara efektif mendorong diferensiasi jantan sambil mempertahankan pertumbuhan dan kelangsungan hidup yang normal.

Kata kunci: Ikan guppy, gonad, sex reversal, *Tribulus terrestris*

1. Introduction

Guppy (*Poecilia reticulata*) is a popular freshwater ornamental fish among fish enthusiasts due to its beautiful color patterns and fins, and it is also one of Indonesia's export commodities (Bisht et al., 2020). According to the Directorate General of Aquaculture (2021), Indonesia's ornamental fish exports reached USD 36.4 million, with guppies contributing significantly to the market due to their high global demand.

This highlights the economic importance and urgency of developing sustainable masculinization technologies. Male guppies are more desirable because of their brighter coloration and slender body shape, making their cultivation more profitable than females (Lailatul et al., 2016; Herdegen-Radwan, 2022). Increasing export demand has driven the need for consistent production of high-quality male guppies (Directorate General of Aquaculture, 2021). However, the predominance of female offspring presents marketing challenges in guppy culture, often resulting in higher production costs and reduced profitability. (Sarida et al., 2011; Alam et al., 2024).

The predominance of female offspring in guppy culture presents marketing challenges, prompting the use of synthetic hormones for male production. However, due to their ecological and health concerns, natural alternatives such as plant-based extracts are being explored as safer options. Sex reversal techniques are commonly used to produce monosex populations by manipulating the gonadal differentiation phase, when the gonads are still bipotential and can develop into testes or ovaries. Producing all-male populations offers advantages such as faster growth rates and more attractive coloration in guppies (Saputra et al., 2018). This process can be conducted through immersion, injection, or oral administration of androgenic steroid hormones (Nurlina & Zulfikar, 2016). Among these methods, 17 α -methyltestosterone (MT) is the most widely used synthetic hormone.

Despite its effectiveness, the use of synthetic hormones such as 17 α -methyltestosterone and aromatase inhibitors in

aquaculture should be reduced because of their relatively high cost (KKP, 2014), potential to accumulate as endocrine-disrupting residues in aquatic environments, and carcinogenic risks to humans (Suseno et al., 2020). Therefore, developing natural, low-cost, and environmentally friendly alternatives is crucial for sustainable aquaculture. One promising candidate is *Tribulus terrestris* extract (TTE), a plant-based compound that has not been widely applied in fish masculinization studies.

Tribulus terrestris could be used as a natural supplement that enhances testosterone production. This herbal plant is reported to increase testosterone levels by influencing androgen metabolism (Ghosal & Chakraborty, 2020). It contains bioactive compounds such as protodioscin and protogracilin, which can enhance luteinizing hormone secretion and increase testosterone synthesis (Gharaei et al., 2020; Hassona et al., 2020). Because protodioscin stimulates testosterone production, it is hypothesized to promote male differentiation in guppies. Guppies (*Poecilia reticulata*) are ideal model species for such studies because of their short generation time, clear sexual dimorphism, and established use in aquaculture research.

Previous studies have shown that *T. terrestris* extract can increase male production in other fish species: immersion treatment resulted in 87% males in *Cichlasoma nigrofasciatum* (Cek et al., 2007) and 97% in *Poecilia latipinna* (Kavitha & Subramanian, 2011). Despite these promising results, application in guppies has not been systematically tested, particularly comparing broodstock and larval immersion methods. Although previous studies demonstrated high masculinization rates in other fish species, research on guppies remains limited. Therefore, this study aimed to evaluate the effectiveness of *Tribulus terrestris* extract at different doses on guppy sex reversal and growth performance through immersion at two critical stages: pregnant broodstock and larvae.

2. Materials and methods

The fish used for the broodstock sex reversal experiment were mature female guppies measuring 4–5 cm in length ($n = 30$) and male guppies measuring 3–4 cm ($n = 15$). All fish were purchased from a local farmer in Bogor, Indonesia. Then, fish acclimatized for seven days under controlled conditions, with water temperature maintained at 27–29 °C, pH 7.2–7.8, and dissolved oxygen levels between 5–6 mg/L. On the other hand, for sex reversal in the larval stage, larvae aged 0, 3, and 6 days from the breeding process of the parent fish are used, with 30 individuals per treatment.

The research design for sex reversal in female broodstock uses a completely randomized design (CRD), while for the guppy fish in the larval stage, a Randomized Completely Block Design (RCBD) is used. The research design was as follows: The research design for sex reversal of guppy fish in the parent stage consisted of five treatments with three replications each. These treatments included a negative control without *Tribulus terrestris* extract (TTE) (K-), a positive control using 17 α -methyltestosterone at 500 $\mu\text{g/L}$ (K+), and three TTE treatments, namely P1 with 5 mg/L, P2 with 10 mg/L, and P3 with 15 mg/L. Meanwhile, the research design for studying sex reversal in guppy larvae at different developmental stages (day 0, day 3, and day 6 after birth) involved an immersion technique with varying doses to identify the critical window of sex differentiation and to optimize the timing of immersion. Each treatment was replicated three times and consisted of a negative control without TTE (K-), a positive control using 17 α -methyltestosterone at 8 mg/L (K+), and three TTE treatments: P1 with 2.5 mg/L, P2 with 5 mg/L, and P3 with 10 mg/L.

2.1 Preparation of *Tribulus terrestris* Extract

A total of 100 g of *Tribulus terrestris* powder was weighed, placed into a glass container, and combined with 1 liter of 90% ethanol as the extraction solvent. The suspension was heated and continuously stirred in a water bath at 80°C for 2 hours, then allowed to cool and filtered through filter paper to collect the filtrate. The obtained filtrate was subsequently concentrated using a rotary vacuum evaporator set at 85 rpm and 45°C (Do et al., 2013; Sasikumar et al., 2014). The concentrated extract was transferred into a dark bottle and preserved at -20°C until further use.

2.2 Breeding of Parent Fish

The breeding process was conducted naturally using a male-to-female ratio of 1:2, with 15 male and 30 female broodstock. Breeding continued until the females showed clear signs of gravidity, indicated by the appearance of black abdominal spots. The broodstock were maintained in separate aerated tanks at a 1:2 ratio, and only the gravid female broodstock were immersed for 24 hours in static, aerated water as part of the treatment procedure.

2.3 Immersion of Test Fish

Females broodstock were selected 12 days after breeding, identified by the presence of black abdominal spots and a swollen belly. The selected broodstock were immersed in *Tribulus terrestris* extract solution for 24 hours in static, aerated water, then transferred to maintenance tanks and reared until they gave birth. Larvae obtained from these broodstock were selected at 0-, 3-, and 6-days post-birth and subjected to the same immersion treatment for 24 hours under static, aerated conditions. After immersion, the larvae were transferred to maintenance tanks and reared until they developed into guppy fry.

2.4 Guppy Fry Maintenance

The guppy fry was reared for 60 days to determine the percentage of male individuals. Newly hatched fry was fed live *Artemia* for the first 10 days, then gradually transitioned to a commercial diet containing 35% protein with fine particle size suitable for fry development. Feeding was conducted twice daily, in the morning 08:00 AM and afternoon 17:00 PM, to ensure adequate nutrition for growth and survival. During the rearing period, tanks were maintained under a 12 h light:12 h dark photo period with daily water exchange to maintain optimal water quality.

2.5 Histological Observation and Sex of Fish

Histological observation was conducted to confirm the phenotypic sex of guppies and assess gonadal differentiation. The fish were first weighed and measured, and the trunk region containing the gonads was dissected. Samples were fixed in 10% neutral buffered formalin (NBF) for 24 hours, then replaced with 70% alcohol for preservation. The preserved samples were sent to the Lampung Veterinary Center (Balai Veteriner Lampung) for histological preparation. Tissues were dehydrated through a graded ethanol series, embedded in paraffin, and sectioned at 5 μm

thickness. The slides were stained with hematoxylin and eosin (H&E) and examined under a compound microscope at 100× and 400× magnification to observe gonadal structure and confirm the sex of the fish.

2.6 Research Parameters

The parameters observed in the research are the percentage of male guppies, absolute length growth, absolute weight growth, and gonad histology. Sex identification of guppy fish was carried out by observing both primary sexual characteristics (gonads) and secondary

sexual characteristics (morphology). Histological examination of the gonads was conducted once, typically at the end of the study or during the 8th week, using five treatments with seven specimens per treatment. For morphological assessment, seven larvae were sampled from each aquarium out of the 20 larvae available. Secondary observations focused on external morphology to distinguish males from females. The morphological differences between male and female guppies are shown in **Figure 1**.

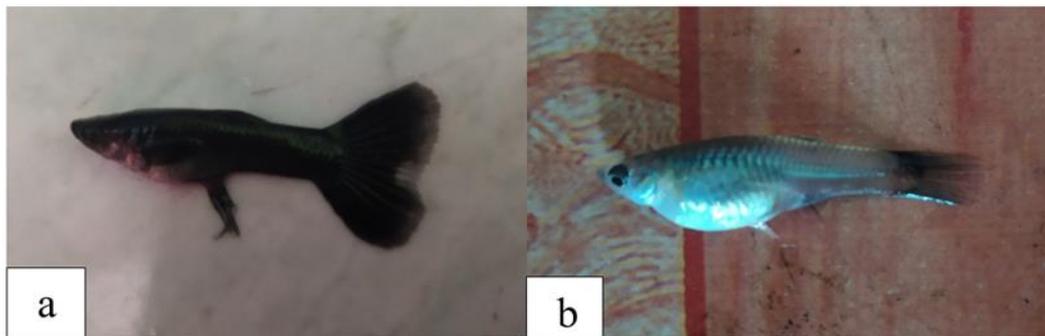


Figure 1. Identification of male and female guppy (*Poecilia reticulata*) based on morphological characteristics: a) Male guppies have a slender body shape, brighter coloration, and a longer, broader, and more expanded caudal fin, b) Female guppies have a larger body shape, coloration limited to the caudal fin, and a smaller caudal fin.

The parameters were calculated using the formulas presented below:

Percentage of male guppies (%) = $[\text{total of male juvenile} / \text{total of juvenile}] \times 100$

Percentage of female guppies (%) = $[\text{total of female juvenile} / \text{total of juvenile}] \times 100$

Absolute length growth (cm) = $[\text{final length} - \text{initial length}]$

Absolute weight growth (g) = $[\text{final weight} - \text{initial weight}]$

2.7 Data Analysis

The data analysis includes the percentage of male fish, percentage of female fish, absolute length growth, and absolute weight growth. Data arranged in a Completely Randomized Design (CRD) are analyzed using a one-way ANOVA, while data in a Randomized Completely Block Design (RCBD) are analyzed using a two-way ANOVA that includes the block factor. Before conducting ANOVA, data normality is assessed using the Shapiro–Wilk test and homogeneity of variance using Levene's test. If the block effect in RCBD is not significant, block-related results are omitted and it is simply stated that the block has no effect.

When ANOVA shows significant treatment differences, a Duncan test at the 95% confidence level is used to identify pairwise differences among treatments. The histological parameters of the gonads will be analyzed descriptively.

3. Results and Discussion

3.1. Percentage of Guppy Sex

The percentage of males in the sex reversal activity using females broodstock showed significant differences ($p < 0.05$). The highest percentage was obtained in treatment P3, which was $87.78 \pm 8.75\%$ (Table 1). According to Ghosal & Chakraborty (2020), *Tribulus terrestris* extract can increase testosterone levels and athletic performance. This is because *Tribulus terrestris* extract contains protodioscin, which can enhance the secretion of luteinizing hormone from the pituitary gland, the main stimulant hormone for testosterone production. *Tribulus terrestris* extract also contains several substances that are believed to be similar to steroids. Testosterone itself is a precursor for androgens and estrogens (Gharaei et al., 2020).

The percentage of females in sex reversal activities using gravid females showed significant differences ($P < 0.05$). The K-treatment, without the administration of *Tribulus terrestris* extract (TTE) and 17 α -methyltestosterone, exhibited a higher percentage compared to all other treatments. Guppy is an ovoviviparous fish, in which fertilization occurs internally, and embryos develop within the female's body until parturition. During this embryonic development, the gonads remain in an undifferentiated and labile state. At this stage, hormonal and

environmental influences mediated through the maternal body can affect the direction of gonadal differentiation (Lailatul et al., 2016). Therefore, immersion treatment of gravid females allows bioactive compounds from *Tribulus terrestris* extract to be absorbed and potentially transferred to the developing embryos, influencing gonadal differentiation. This rationale has been supported in previous studies where immersion of pregnant female guppies with natural extracts successfully altered sex ratios in the offspring (Syarif & Winardi, 2021).

Table 1. Sex percentage of guppy (*Poecilia reticulata*)

Treatments	Percentage of male (%)		Percentage of female (%) Broodstock immersion
	Broodstock immersion	Larval immersion	
K-	29.52 \pm 8.19 ^c	57.14 \pm 23.33 ^a	63.81 \pm 5.37 ^a
K+	60.32 \pm 4.49 ^b	57.14 \pm 0.00 ^a	29.40 \pm 8.09 ^{bc}
P1	42.06 \pm 6.83 ^{bc}	47.62 \pm 13.47 ^a	52.38 \pm 3.37 ^a
P2	47.62 \pm 17.82 ^{bc}	57.14 \pm 20.20 ^a	47.62 \pm 17.82 ^{bc}
P3	87.78 \pm 8.75 ^a	38.10 \pm 17.82 ^a	6.67 \pm 9.43 ^c

*Broodstock immersion: K-: a negative control without TTE, K+: a positive control: 17 α -methyltestosterone at 500 μ g/L, P1: TTE 5 mg/L, P2: TTE 10 mg/L, and P3: TTE 15 mg/L. Meanwhile, Larval immersion: K-: a negative control without TTE, K+: a positive control: 17 α -methyltestosterone at 8 mg/L, P1: TTE 2.5 mg/L, P2: TTE 5 mg/L, and P3: TTE 10 mg/L. Numbers followed by different superscript letters for each treatment indicate significantly different results ($p < 0.05$).

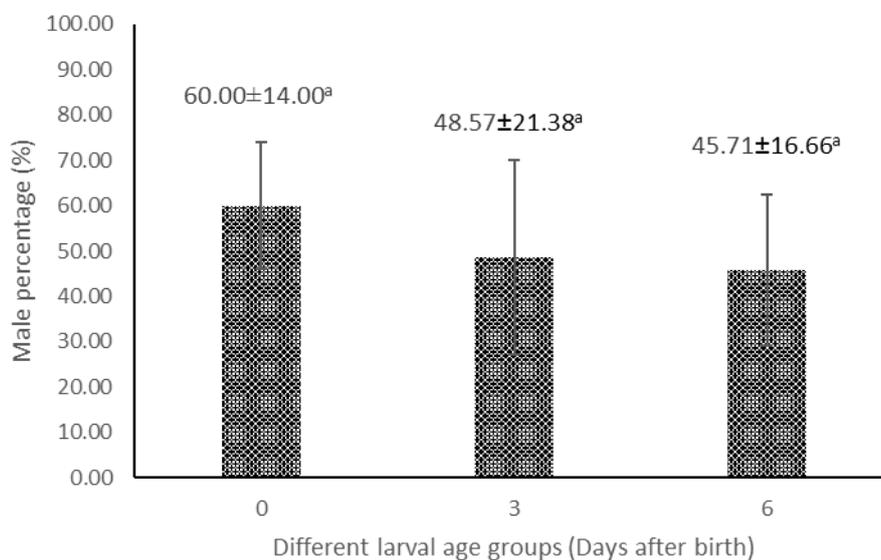


Figure 2. Male percentage of guppy (*Poecilia reticulata*) across age groups under larval immersion treatments. Numbers followed by similar superscript letters for each treatment indicate no significantly different results ($p > 0.05$).

TTE contains bioactive compounds such as steroidal saponins (protodioscin and protogracillin), flavonoids, and alkaloids, which are known to enhance androgenic activity during early development. These compounds

stimulate the hypothalamus–pituitary–gonadal (HPG) axis by increasing the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Consequently, the ovaries in gravid females may undergo altered

steroidogenesis, leading to increased conversion of cholesterol into testosterone and other androgens. The target organ is mainly the ovarian tissue, where steroidogenic enzymes such as aromatase (CYP19A1) may be downregulated, while androgen-related pathways (e.g., 17 β -HSD, StAR) are upregulated. This hormonal modulation increases androgen availability in the maternal bloodstream.

Sex reversal in larval guppies did not differ significantly from treatments applied to gravid females ($p > 0.05$) (Table 1). The control, K+ (17 α -methyltestosterone 8 g/L), and *Tribulus terrestris* extract (TTE) 5 g/L treatments produced approximately 57% males, with no significant differences among them. In contrast, TTE immersion at 2.5 g/L and 10 g/L resulted in 47.62% and 38.10% males, respectively (Table 1). The decline in male proportion at higher concentrations suggests a dose-dependent reversal effect, where excessive androgen exposure disrupts normal sex differentiation and promotes feminization—a phenomenon previously reported in similar studies (Hutagalung, 2020; Iskandar & Hasby, 2021; Phelps & Okoko, 2011; Soumokil et al., 2020).

The parameter of male guppy production in different age groups in this study showed no significant effect ($p > 0.05$), as seen in Figure 2. The highest production of male individual fish was obtained at 3 days old, resulting in 51.53% male individuals. The timing and method of TTE administration influence the masculinization response in guppies. Immersion of gravid females at 15 mg/L TTE was effective because the bioactive compounds (mainly protodioscin and other steroidal saponins) were transferred maternally to the developing embryos, allowing direct influence on gonadal differentiation before birth. In contrast, immersion of larvae (U0, U3, U6) did not yield significant differences. This is likely due to the fact that

once larvae are released, their gonadal differentiation may already be progressing rapidly, making it harder for phytosteroids to alter the sexual pathway. Moreover, larval absorption of active compounds during immersion is generally lower than maternal transfer during embryogenesis. This explains why gravid female immersion gave a stronger masculinization effect compared to larval immersion. At 6 days old, the production of male individuals resulted in 51.42% male individuals, while at 0 days old, the production of male individuals resulted in 37.14% male individuals. According to Himawati et al. (2018), younger larvae age contributes to higher production of male individuals compared to older larvae age.

3.2. Absolute Growth of Guppy Larvae

The parameter of absolute length growth performance of guppy fish fry with the administration of *Tribulus terrestris* extract had a significant effect ($p < 0.05$). The results showed that treatment K+ obtained the highest result, which was 2.76 cm, compared to other treatments (Table 2). The higher length observed in the K+ treatment (17 α -methyltestosterone) is likely due to the androgenic anabolic effects of methyltestosterone, which can enhance protein synthesis and muscle growth (Suseno et al., 2020; Sarker et al., 2022). These anabolic properties stimulate somatic development and energy utilization efficiency, resulting in greater body length compared to other treatments. Conversely, fish treated with *Tribulus terrestris* extract maintained comparable growth to the control group, consistent with recent studies showing that *T. terrestris* supplementation supports growth, metabolic activity, and gonadal development without suppressing somatic performance (Matter et al., 2024; Motlagh et al., 2025; Sarida et al., 2025).

Table 2. The absolute growth of guppy larvae under different doses of TTE immersion treatments at larval stage.

Treatments	Absolute growth	
	Length (cm)	Weight (g)
K-	2.63 \pm 0.16 ^{ab}	0.23 \pm 0.5 ^a
K+	2.76 \pm 0.06 ^b	0.18 \pm 0.02 ^a
P1	2.43 \pm 0.12 ^a	0.20 \pm 0.00 ^a
P2	2.49 \pm 0.03 ^a	0.17 \pm 0.02 ^a
P3	2.54 \pm 0.12 ^a	0.21 \pm 0.02 ^a

Note : Numbers followed by different superscript letters for each treatment indicate significantly different results ($p < 0.05$).

Table 3. The absolute growth of guppy larvae with TTE immersion treatment across different larval age groups.

Different larval age groups (Days after birth)	Absolute growth	
	Length (cm)	Weight (g)
0	2.65±0.18 ^a	0.20±0.01 ^a
3	2.49±0.14 ^a	0.21±0.05 ^a
6	2.57±0.09 ^a	0.19±0.03 ^a

Note : Numbers followed by similar superscript letters for each treatment indicate no significantly different results ($p > 0.05$).

Further, the absolute length growth performance of guppy fish fry refers to the change in length size over a specific period of time (Sanjaya et al., 2020). This performance is influenced by various factors, including internal and external factors. Internal factors include genetic traits, disease resistance, and food utilization ability. External factors include physical, chemical, and biological properties of the water, as well as food and temperature, which are the main external factors that can affect fish growth. The parameter of absolute length growth performance in different age groups in this study showed no significant difference ($p > 0.05$). The 0-day-old age group obtained a higher result of 3.10 cm compared to the other age groups. The absolute weight growth performance parameter in guppy fish larvae obtained insignificant results ($p > 0.05$). The treatment K- 0.23 g showed higher results compared to other treatments (Table 2). These findings reinforce the potential of *T. terrestris* as a natural, eco-friendly alternative to synthetic hormones in sustainable aquaculture. The correlation between growth performance and sex reversal is mainly influenced by hormonal regulation. Androgen stimulation (either through synthetic hormones or phytoandrogens such as *Tribulus terrestris* extract) tends to promote male differentiation. Male guppies generally exhibit a higher feed conversion efficiency and faster somatic growth compared to females, especially because energy allocation in males is directed more toward somatic tissues, while females must allocate additional energy toward gonadal and reproductive tissue development. Therefore, masculinization through sex reversal can indirectly enhance growth performance at the population level.

According to Warsono et al. (2017), it is stated that the normal daily growth rate is 2-3% for sizes ranging from 50-100 g and 0.7-1.5% for sizes ranging from 200-300 g. They also mention that the average weight gain of individuals decreases as their size and age increase. Growth can be influenced by the available space (habitat) and the fish's ability to utilize food. The absolute weight growth performance of the age groups in this study

showed no significant difference ($p > 0.05$). The results indicate that U3 age group obtained a higher weight gain of 0.21 g compared to other age groups (Table 3).

3.3. Histology of Gonad

The observation results of the gonads of 60-day-old fish clearly indicate that the suspected samples are male due to the presence of spermatozoa, spermatids, and spermatocytes (Figure 3A; 3D). In the suspected female samples, the presence of oocytes and oogonia is clearly visible (Figure 3B, 3E). Intersex individuals were also found in the histological observation of the gonads, as indicated by the presence of spermatocytes and oocytes (Figure 3C, 3F). At 60 days of age, the gonad tissue develops further, and spermatogonia begin to appear in the gonads of male individuals. According to Nowakowska et al. (2020), guppies at 60 days of age have shown advanced stage gonad development. In male guppies, the development of the gonopodium and body coloration indicates that the fish has reached sexual maturity. Based on histological observations, all types of germ cells at various stages of development have been observed in guppies: spermatogonia, spermatocytes, spermatids, and spermatozoa. In the female gonad tissue, oogonia and oocytes are present, but unlike males, female guppies at 60 days of age have not reached sexual maturity as the oocytes are still developing, and sexual maturity is reached at 90 days of age (Ruell et al., 2013; Kamaszewski et al., 2020). The presence of spermatocytes and mature spermatozoa in the P3 treatment (15 mg/L) confirms successful masculinization at the gonadal tissue level. These histological observations demonstrate that *Tribulus terrestris* extract effectively stimulated testicular differentiation, supporting the high proportion of males recorded in this treatment.

Several types of intersexual gonads were found in this study, characterized by the presence of both male (spermatocytes) and female (oocytes) germ cells in the same individual, which were more frequently observed in the K+ treatment (17 α -methyltestosterone) (Figure 3C; 3F). The

occurrence of intersex fish is likely caused by the presence of 17α -methyltestosterone, which is unable to direct the fish's sex towards male, resulting in an imperfect gonad differentiation process. Administration of low-concentration steroid hormones can lead to the formation of intersex individuals (Farias et al., 2023). This is due to the inability of exogenous steroids produced by tissues in the body, as well as internal genetic factors and physiological activities within the body (Ahmed et al., 2020). Such occurrences are often associated with hormonal overstimulation that disrupts the

normal process of gonadal differentiation, leading to partial development of both ovarian and testicular tissues. Previous research conducted by Om et al. (2003) found intersexual gonads in zebrafish larvae (*Danio rerio*) with 17α -methyltestosterone treatment (1 g/L), which resulted in an increase in vitellogenin levels at lower doses. Ramos-Júdez et al. (2020) also reported that higher doses of 17α -methyltestosterone in *Mugil cephalus* showed well-developed testicular tissue, including spermatozoa.

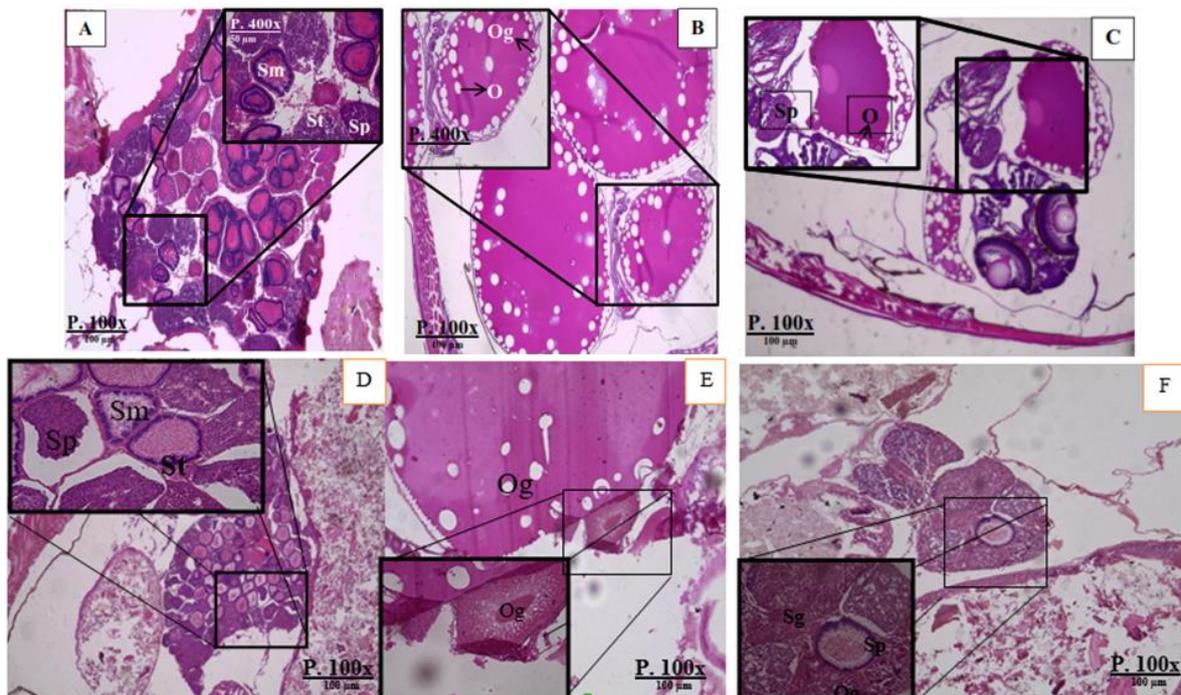


Figure 3. Gonad tissue of male, female and intersex guppies (*Poecilia reticulata*). A) Male gonad tissue soaked with *Tribulus terrestris* extract in pregnant broodstock (B) Female gonad tissue (C) Intersex gonad tissue (D) Male gonad tissue soaked with *Tribulus terrestris* extract in the larval stage (E) Female gonad tissue (F) Intersex Gonad tissue. Sm: spermatozoa; St: spermatids; Sp: spermatocytes; Og: oogonia; and O: oocyte. (100x & 400x magnification)

4. Conclusion

Immersion of guppy (*Poecilia reticulata*) female broodstock in *Tribulus terrestris* extract significantly enhanced masculinization, achieving 87.78% male production at a concentration of 15 mg/L. Growth performance improved relative to the control group, though differences among treatment dosages were not statistically significant. These results demonstrate that *T. terrestris* extract is an effective and environmentally sustainable alternative to synthetic androgens such as 17α -methyltestosterone, reducing potential ecological and health risks associated with

hormone use. This natural approach offers promising applications for sustainable guppy aquaculture and the ornamental fish industry. However, the absence of significant masculinization in larval immersion treatments suggests that future studies should optimize immersion protocols, elucidate the underlying molecular mechanisms, and evaluate long-term reproductive and progeny performance to fully realize the potential of this eco-friendly biostimulant.

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