Evaluating the effects of 17 α-Methyltestosterone and nano chitosan on masculinization rate and growth performance in Nile tilapia (*Oreochromis niloticus*) using the immersion method

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ABSTRACT

The study aimed to evaluate the effectiveness of combining 17 α-Methyltestosterone (MT) with nano chitosan on the survival rate, masculinization rate, and growth performance of Nile tilapia (*Oreochromis niloticus*) using the immersion method. A completely randomized design was conducted, with five treatment groups receiving varying concentrations of MT and nano chitosan: chitosan + 1 mg MT/L (1 MC), chitosan + 1.5 mg MT/L (1.5 MC), chitosan + 2 mg MT/L (2 MC), chitosan + 2.5 mg MT/L (2.5 MC), and chitosan + 3 mg MT/L (3 MC). A control group was also included, with each treatment replicated three times. Seven-day-old fry were exposed to the MT solution for 2 h before being transferred to nurseries in hapas within earthen ponds at a stocking density of 1,500 fish/m^2 for a 60-day rearing period. After 2 h of hormone treatment and the 60-day rearing period, the 2.5 MC treatment exhibited the highest survival rate. Male ratios in the MT treatments ranged from 76.67% to 83.33%, significantly higher than in the control group (55.56%) ($P < 0.05$). Specifically, the male ratios in the 2.5 MC and 3 MC treatments were 82.2% and 83.3%, respectively, which were higher than those in the other MT treatments, however,these differences were not statistically significant ($P > 0.05$). While the mean weight and length of fish in the MT treatments were greater than those in the control group, these differences were also not statistically significant ($P > 0.05$). The study further revealed a positive correlation between the average weight of the fish and the hormone concentration. Based on these findings, a dose of 2.5 mg 17 α-MT/L in combination with nano chitosan is recommended for achieving optimal mono-sex male tilapia production.

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1. Introduction

Sex reversal in fish is a critical area of study with significant implications for aquaculture, fish farming, and conservation. This phenomenon, where an individual fish changes its sex from male to female or vice versa, occurs naturally in various species and can be influenced by environmental factors, social cues, and hormonal treatments (Piferrer, 2001b; Devlin & Nagahama, 2002). Globally, species such as groupers, wrasses, and clownfish are well-known for their sex-changing capabilities (Sadovy & Liu, 2008). In many countries, sex reversal is particularly relevant for species like the Asian sea bass (*Lates calcarifer*) and tilapia (*Oreochromis* spp.), which are vital to the local aquaculture industry (Mair et al., 1997). The ability to control sex differentiation through hormonal treatments has opened new avenues for optimizing fish production and maintaining ecological balance (Pandian & Sheela, 1995b).

Manipulating the sex of fish populations is essential for several reasons. In hatchery production, achieving a desired sex ratio can enhance reproductive efficiency and yield. For example, in species where one sex grows faster or reaches market size more quickly, producing monosex populations can significantly improve economic returns (Beardmore et al., 2001). In fish farming, controlling the sex ratio can prevent unwanted breeding, reduce competition for resources, and promote uniform growth (Mair et al., 1997). Additionally, in conservation, sex reversal can help restore endangered populations by ensuring a balanced sex ratio, which is crucial for successful breeding programs (Wedekind, 2002).

Tilapia farming plays a vital role in global aquaculture, providing a significant source of affordable protein and contributing to food security, especially in developing countries (FAO, 2020). In Vietnam, tilapia is a key species in freshwater aquaculture, supporting rural livelihoods and export markets due to its adaptability and high growth potential. The cultivation of mono-sex male tilapia is particularly important, as males grow faster and more efficiently than females, leading to higher productivity and profitability in aquaculture systems (El-Sayed, 2006). Controlling the sex ratio not only enhances growth performance but also minimizes issues associated with uncontrolled breeding and competition for resources (Beardmore et al., 2001).

Various methods have been developed to induce sex reversal in fish, including environmental manipulation, genetic techniques, and hormonal treatments. Hormonal treatments are the most widely used approach due to their effectiveness and relative ease of application (Pandian & Sheela, 1995b). Common hormones include androgens like 17 α-methyltestosterone (MT), which induce masculinization, and estrogens like estradiol, which promote feminization (Guerrero III, 1975). These hormones can be administered through feed, injections, or immersion baths, depending on the species and desired outcome (Piferrer, 2001b). Each method of sex reversal has its advantages and disadvantages: i) Environmental manipulation using techniques such as temperature changes are non-invasive and sustainable but can be less precise and effective; ii) Genetic techniques offer long-term solutions and high specificity but involve complex and costly procedures; iii) Hormonal treatments are cost-effective, easy to implement, and provide rapid results (Pandian & Sheela, 1995b). However, methods like hormone feeding can lead to inconsistent dosages, lengthy processing times, and high production costs (Piferrer, 2001b).

The hormone immersion method offers several advantages over the feeding method for fish sex reversal. It ensures uniform hormone uptake, especially during the early developmental stages, leading to more consistent and effective sex reversal outcomes (Piferrer, 2001a). This method also reduces the risk of environmental contamination from uneaten hormone-treated feed and minimizes labor costs associated with hormone-enriched diets (Pandian & Sheela, 1995a). Moreover, immersion treatment allows for precise control over hormone exposure time, improving the efficiency of the process (Beardmore et al., 2001).

The MT is a widely used androgen for inducing masculinization in fish. It is particularly effective in species where males are preferred for their growth rates, market value, or reproductive characteristics (Guerrero III, 1975). The dosage and method of administration vary depending on the species and desired sex ratio. Typically, MT is administered through feed during the early developmental stages when gonadal differentiation is most responsive (Piferrer, 2001b).

Nano chitosan, derived from chitosan, is a biopolymer known for its biocompatibility, biodegradability, and non-toxicity (Rinaudo, 2006). When combined with hormones, nano chitosan can enhance the effectiveness of sex reversal treatments (Rather et al., 2013). The nanoscale size increases the surface area and improves the delivery and absorption of hormones in fish (Kumar et al., 2004). Studies have shown that the combination of nano chitosan with hormones like MT can lead to more consistent and efficient sex reversal results (Zhao et al., 2013).

Given the potential advantages of combining MT with nano chitosan, there is a need to evaluate the effects of this combination on the masculinization rate and growth performance in Nile tilapia (*Oreochromis niloticus*) using the immersion method. This research aims to assess the efficacy and practicality of this approach, providing insights that could enhance fish production techniques and support sustainable aquaculture practices.

2. Materials and Methods

2.1. Broodstock development

The broodstock, weighing between 200 to 250 g, were sourced from the Aquatic and Crop Breeding Center, Saigon Agriculture Corporation, Vietnam. For spawning, the broodstock were paired in six mating hapas (12 m x 5 m x 1.5 m) at a ratio of 3 females to 1 male per square meter in earthen ponds (30 m x 50 m x 2 m). They were fed with a commercial floating pelleted feed containing approximately 30% crude protein, twice daily at 3% of their body weight.

2.2. Fry collection

After 15 days of pairing, fry were collected every seven days. Collection was done using a soft net early in the morning to minimize stress and mortality. The collected fry were acclimated in three composite tanks (1 m^3/tank) for one day, during which weak and dead fry were removed and the remaining fry were sorted by mesh size.

2.3. Preparation of hormone treated solutions

MT Solution: The 17 α-methyltestosterone hormone was obtained from Argent, Philippines. A stock solution was prepared by dissolving 400 mg of MT in 1 liter of 96% ethanol to achieve a nominal concentration of 400 mg/L.

MT + chitosan treatment solution (MC): Nano chitosan used in the present study was obtained from the Institute of Biotechnology and Environmental Research, Nong Lam University, Vietnam. To prepare the solution, 0.2 g of chitosan were dissolved in 10 mL of acetic acid and maintained at room temperature for 3 h. Subsequently, 40 mL of double-distilled water was added, and the mixture was stirred at 1500 rpm for 20 min using a magnetic stirrer. The pH of the solution was adjusted to 3.8 using NaOH (2 N), and distilled water was added to make a total volume of 100 mL. This solution was stirred at 1500 rpm for 20 min and then allowed to settle overnight at 4°C. For conjugation with MT, 20 mg of MT was dissolved in 2 mL of ethanol (96%) and added dropwise to the 100 mL chitosan solution while stirring continuously at 1500 rpm for 25 min. The resulting chitosan + MT (CS + MT) solution was stored at 4°C for later use.

2.4. Experimental design

The experiment was conducted in 5-liter glass tanks containing 3 liters of water, using a completely randomized design. The experiment included five MC concentrations of 1.0, 1.5, 2.0, 2.5 and 3 mg/L and a control group containing MT-free water. Each treatment was repeated three times. Following a one-day acclimatization in continuously aerated composite tanks, 750 tilapia fries were randomly stocked into the glass aquaria according to the MC concentrations and control conditions. Fish were exposed to the MT solution for two hours, after which the fry were transferred to nurseries in hapas (1 m x 1 m x 1 m) suspended in earthen ponds at a density of 1.500 fish/m^2 for 60 days. During this period, the fish were fed commercial floating pellets with varying crude protein levels: 40% (size 0.6 mm) from days 1 to 15, at a feeding rate of 10% of body weight, administered four times daily; 40% (size 0.6 - 1.5 mm) from days 16 to 30, at a feeding rate of 7% of body weight, administered three times daily; and 35% (size 2.0 mm) from days 31 to 60, at a feeding rate of 5% of body weight, administered twice daily.

2.5. Sex reversal

At the end of the experiment, 30 fish were randomly collected from each experimental replicate to determine their sex. Gonadal morphology was examined and recorded. Sex determination was carried out using the standard aceto-carmine gonad squashing technique as described by Guerrero III & Shelton (1974).

2.6. Growth performance

Every 30 days, 30 fish were randomly collected from each experimental replicate to measure mean growth (average weight and total length) and were then released back into the experimental hapas. Survival rates were recorded two hours after immersion in the MT solutions and at the end of the 60-day growth experiment.

2.7. Statistical analysis

Data were statistically analyzed using SPSS version 16.0. One-way ANOVA was performed, and Duncan's multiple range test (DMRT) was employed to determine significant differences between means at the 5% level of significance.

3. Results

3.1. Water quality parameters

The water quality parameters monitored throughout the experiment remained within the optimal range for Nile Tilapia, ensuring that external factors did not influence the outcomes of the treatments. The temperature ranged between

29.0 - 32.0°C, dissolved oxygen (DO) levels varied from 5.4 - 6.6 mg/L, pH levels were maintained between 6.4 - 7.4, and ammonia (NH_3) levels were kept below 0.25 mg/L (Table 1). These conditions are consistent with the standard environmental requirements for the species, confirming that the experiment's outcomes are likely attributable to the treatment effects rather than environmental stressors (Boyd, 1990).

Table 1. Water quality parameter during the experiment

Temperature (°C)	Dissolved oxygen (mg/L)	pH	NH_3 (mg/L)
29.0 - 32.0	5.4 - 6.6	6.4 - 7.4	< 0.25

3.2. Fish survival and sex ratio

The survival rates and sex ratios of the fish treated with various concentrations of MT and nano chitosan are summarized in Table 2. The survival rate at the day 60 did not significantly differ across treatments, indicating that the treatments were not toxic or detrimental to the overall health of the fish. However, the percentage of males varied significantly among the different treatment groups. The control group exhibited a male percentage of 55.56%, which aligns with the natural sex ratio expected in Nile Tilapia populations (Yamazaki, 1983). In contrast, the groups treated with combination of MT and nano chitosan showed a significant increase in male percentage, with the highest masculinization rate observed in the 3 MC group (83.33%). This trend indicates that the combination of MT and nano chitosan effectively enhances the masculinization process in Nile Tilapia, particularly at higher concentrations.

Table 2. Fish survival and mean percent of male of each combination of 17 α-Methyltestosterone and nano chitosan (MC) treatment groups and control group

Treatment	Number of analyzed fish (individual)	Survival rate after 2 h (%)	Survival rate at day 60 (%)	Male (%)	Female (%)
Control	90	100	87.78 ± 0.1	55.56 ± 5.1^{a}	44.44 ± 5.1
1 MC	90	100	89.04 ± 0.4	76.67 ± 10.0^{b}	23.33 ± 10.0
1.5 MC	90	100	85.96 ± 0.8	77.78 ± 8.4^{b}	22.22 ± 8.4
2 MC	90	100	85.71 ± 1.3	77.78 ± 1.9^{b}	22.22 ± 1.9
2.5 MC	90	100	93.00 ± 4.5	82.22 ± 3.3^{b}	17.78 ± 3.3
3 MC	90	100	86.60 ± 3.8	83.33 ± 3.8^{b}	16.67 ± 3.8

Values (mean of data for triplicate groups) with different superscripts in the same column are significantly different (one-way ANOVA and Tukey test, $P < 0.05$).

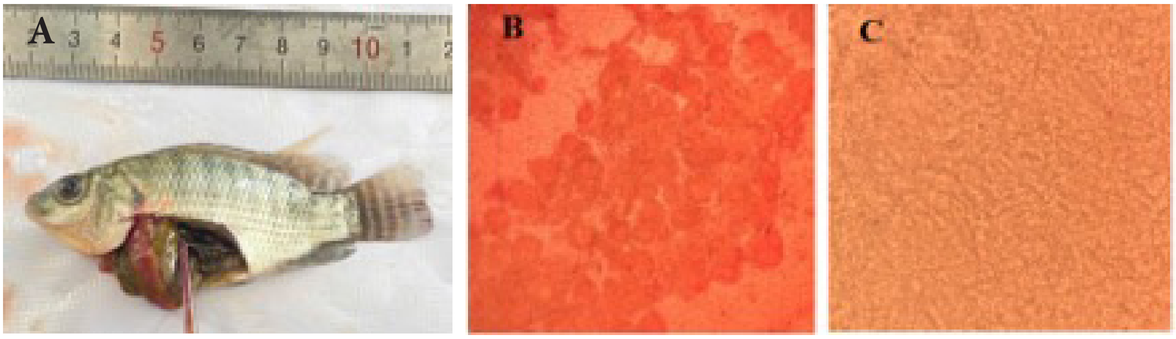

Figure 1. The observed gonad of male and female tilapia after 60 days of 17 α-Methyltestosterone treatment. (A) Gonad removal (B) Eggs, and (C) Testis under light microscope.

3.3. Growth performance

The growth performance of the experimental fish over the 60-day period is detailed in Table 3. All groups, including the control, displayed significant growth, with final weights ranging from 6.63 g in the 3 MC group to 5.17 g in the control group. The highest weight gain (WG) was observed in the 3 MC group (6.62 g), which also had the highest specific growth rate (SGR) at 10.53% per day. These results suggest that the combination of MT and nano chitosan not only promotes masculinization but also enhances growth performance, particularly at higher concentrations. This finding is consistent with previous studies indicating that MT can boost growth in fish by promoting feed efficiency and protein synthesis (Johnstone, 1985).

Table 3. The growth performance of experiment fish in 60 days after being exposed in different combinations of 17 α-Methyltestosterone and nano chitosan (MC) concentrations

Treatment	Initial weight (g)	Final weight (g)	Weight gain (g)	Specific growth rate (%/day)
Control	0.01 ± 0.0	5.17 ± 0.16	5.16 ± 0.16	10.73 ± 0.05
1 MC	0.01 ± 0.0	5.34 ± 0.43	5.33 ± 0.43	10.17 ± 0.14
1.5 MC	0.01 ± 0.0	5.40 ± 0.19	5.38 ± 0.19	10.20 ± 0.06
2 MC	0.01 ± 0.0	5.88 ± 0.48	5.87 ± 0.48	10.34 ± 0.14
2.5 MC	0.01 ± 0.0	5.21 ± 0.24	5.20 ± 0.24	10.14 ± 0.07
3 MC	0.01 ± 0.0	6.63 ± 0.37	6.62 ± 0.37	10.53 ± 0.11

Values (mean ± standard deviation of data for triplicate groups).

4. Discussion

Maintaining optimal water quality is crucial for the success of aquaculture operations. The parameters recorded in this study were within the optimal range for Nile tilapia, supporting the physiological processes necessary for growth and masculinization (El-Sayed, 2006). The stability of these parameters highlights the importance of proper management practices in experimental settings to minimize stress and potential confounding factors. Proper water quality management ensures that environmental conditions such as temperature, pH, dissolved oxygen, and ammonia levels are conducive to the health and growth of the fish (Boyd, 1990). This is essential as suboptimal water conditions can lead to stress, reduced immune function, and lower survival rates (Kubitza, 2004). Therefore, maintaining stable optimal water quality parameters throughout the experiment was a critical factor in the success of this study.

The combination of MT and nano chitosan significantly influenced the sex ratio of Nile tilapia. Higher MT concentrations (2.5 MC and 3 MC) resulted in a significant increase in the male ratio, demonstrating the effectiveness of this method in achieving the desired sex ratio for commercial purposes (Pandian & Sheela, 1995b). This finding is consistent with previous studies that have demonstrated the effectiveness of MT in inducing masculinization in fish species (Piferrer, 2001b). The high survival rates across all treatment groups indicate that the immersion method of hormone application combined with nano chitosan is a safe and effective approach for masculinization (Johnston et al., 2009). This hormone method minimizes stress and potential injuries, concerns often associated with other methods such as oral or injectable hormones (Hunter & Donaldson, 1983). Furthermore, nano chitosan has been shown to enhance the efficacy of hormone treatments by facilitating better absorption and sustained release of the hormone within the fish (Sánchez et al., 2012; Abd El-Naby et al., 2019; Wu et al., 2020). This synergistic effect likely contributed to the high masculinization rates observed in the study.

The enhanced growth performance observed in the treatment groups, particularly in the 3 MT group, aligns with previous findings that anabolic steroids can promote growth by enhancing protein synthesis and feed conversion efficiency (Sundararaj & Vasal, 1976; Guerrero III & Guerrero, 1988; Pandian & Sheela, 1995b). The use of MT in aquaculture has been associated with improved growth rates due to its anabolic effects, enhancing muscle development and overall body mass (MacIntosh & Little, 1995; Pandian & Sheela, 1995b; Zohar & Mylonas, 2001). Nano Chitosan may enhance growth performance by improving nutrient absorption and immune response (Sánchez et al., 2012; Manaf et al., 2020) . Chitosan has been reported to have various beneficial effects on fish health, including enhanced gut health, better nutrient uptake, and improved resistance to diseases (Gopalakannan & Arul, 2006). These properties make nano chitosan a valuable additive in feed and treatment protocols for aquaculture. The results of this study demonstrate the potential of combined MT and nano chitosan therapies in optimizing growth performance and production efficiency in Nile tilapia aquaculture. The significant improvements in growth performance observed suggest that this combination can be effectively used to enhance the productivity and profitability of tilapia farming operations (El-Sayed, 2006).

The findings from this study have important implications for aquaculture practices. The use of MT and nano chitosan can be integrated into existing farming protocols to improve both the growth rates and the desired sex ratios of Nile tilapia populations (Little et al., 2003). This can lead to more efficient production cycles and higher economic returns for fish farmers. Moreover, the safety and efficacy of the hormone immersion method provide a viable alternative to more invasive techniques, potentially reducing labor costs and improving animal welfare (Guerrero III & Guerrero, 1988). The integration of nano chitosan also offers additional benefits such as enhanced disease resistance and better overall health of the fish, contributing to sustainable aquaculture practices (Sánchez et al., 2012).

5. Conclusions

The combination of 17 α-Methyltestosterone and nano chitosan significantly improved the male sex ratio and survival rates of Tilapia. Their combination enhanced growth performance and optimizing production efficiency of Tilapia. Thus, applying this method into existing aquaculture protocols can increase productivity and profitability in Tilapia farming. A dose of 2.5 mg 17 α-MT/L in combination with nano chitosan is recommended for achieving optimal mono-sex male tilapia production.

Conflict of interest

The authors declare no conflict of interest.

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