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Optimization of 17α-Methyl Testosterone Dose in *Artemia* Nauplii and Rotifer using Enrichment Technique

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ABSTRACT

Background: The present study explored the possibility of using live feed, as vector for supplementing the androgenic hormone in aquaculture seed production.

Methods: The study consisted of five treatment doses of 17 α-methyl testosterone namely; control (without hormone), T1 (20 mg L^{-1}), T2 (40 mg L^{-1}), T3 (60 mg L^{-1}) and T4 (80 mg L^{-1}) and each were triplicated. The doses were tested in the live feed of *Artemia* nauplii and rotifer by following completely randomized design. The *Artemia* nauplii (*Artemia salina*), collected by hatching the *Artemia* cyst and 5-8 days old rotifers, collected from mass culture unit of rotifer, were released in their respective treatment doses at 100 and 800 individuals/ml, respectively.

Result: The study found significant difference in accumulation of hormone in the tissues of live feed at different time intervals. In *Artemia*, significantly higher amount of 17α-MT (15.33±0.09 mg L^{-1}) was observed in 80 mg L^{-1} group at 24 h. In rotifer, significantly higher value of 17 α-MT (17.42±0.06 mg L^{-1}) was recorded in 60 mg L^{-1} group at 6 h. The level of 17α-MT in *Artemia* nauplii and rotifer started showing a decreasing trend in 24 h and 6 h, respectively. Overall the findings of the present study support that supplementation of hormone through live feed is possible as live feed accumulated 17 α-MT in its tissue after enrichment which can be further explored for producing all-male population in a more sustainable way.

Key words: *Artemia*, Androgenic hormone, Bio-enrichment, 17 α-methyl testosterones, Live feed, Mono-sex seed production, Rotifer.

INTRODUCTION

The recent fish production trend shows a significant contribution by aquaculture to the world fish production especially by freshwater aquaculture. As per the latest FAO report, freshwater aquaculture alone contributes around 88% of the world's farmed fish (FAO, 2020). In order to improve the quality seed production and better fish production the sector has started using various chemical derivatives like antibiotics and hormones in the recent times (Emeka *et al*., 2014). In fish many of the phenotypic traits such as growth rate, colour pattern, *etc*. were sex based which lead to the development of mono-sex seeds, either all male or all female population. Further, the development of captive seed production systems - hatcheries - paved the way for egg or uniform yolk-sac larvae collection which opened the gate way for development of mono-sex seed production (male) using 17α-methyltestosterone through oral administration (Gale *et al*., 1996). In the past decades, many authors have reported that hormone is a naturally-occurring or synthetic androgens and estrogens which shown growth-promoting effect in many cultured fishes (Pelissero and Sumpter, 1992; James and Sampath, 2006). In the recent years, monosex seed production technology has become a common practice among the various animal production sectors (Bardhan *et al*., 2021). However, the hormone residue is creating various negative impacts to the natural ecosystem which making the hormone based sex reversal as a much challenging task in the recent times (Azizi-lalabadi and Pirsaheb, 2021). Therefore, delivering of potent hormones to the fish larvae through suitable vectors -without leaching to the environment- may reduce the negative impacts of the hormone-based mono-sex seed production.

Alternative to commercial diet, live feed - a natural diet rich of nutrients - has gained an importance in hatcheries and started playing a pivotal role in larviculture. Among the various live feed, rotifer and *Artemia* are widely used due to their availability, low cost, ease of culture and nutritional biochemical composition (Kolkovski *et al*., 1997; Sorgeloos and Lavens,1996). Additionally, these live feed act as a very good carrier of supplying essential nutrients (PUFA) and hormones (androgens or estrogens) to the target animal through enrichment process.

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Therefore, the best alternative option for supplementing the hormone for monosex seed production is live feed enrichment process. In commercial feed, a particular dose is selected as optimal dose for all male production, however, it is very difficult to achieve that particular dose in live feed. Therefore, it is essential to standardize the dose of hormone in live feed through enrichment process, before recommending them as potential carrier for hormone-based mono-sex seed production in future. Keeping this in mind, the study was aimed to optimize the 17α-methyl testosterone hormone dose in *Artemia* nauplii and rotifer using enrichment process.

MATERIALS AND METHODS

Experimental site and design

The present experiment was conducted at the Krishnagiri Barur-Center for Sustainable Aquaculture, Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Tamil Nadu and India and research was carried out in summer 2021. The study consisted of five treatment doses of 17α methyl testosterone namely; control (without hormone), T1 (20 mg L^{-1}), T2 (40 mg L^{-1}), T3 (60 mg L^{-1}) and T4 (80 mg L^{-1}) and each were triplicated. The doses were tested in *Artemia* nauplii and rotifer by following completely randomized design.

Decapsulation of *Artemia* cyst

The cyst of *Artemia* strain *(Artemia salina)* was procured from Tuty Enterprises, Tamil Nadu, India. The procured cysts (Dynasty TM, Salt Lake City, UT, USA) were hatched out by following standard method (Sorgeloos *et al.,* 1986) on daily basis. First, 3 g cyst were decapsulated using sodium hypochlorite solution (at 15 mL/g cyst) for 15 min, filtered through nitex screen (50 µm) and washed with fresh water. To ensure complete removal of chlorine, the cysts were treated with 0.1% sodium thiosulphate. The washed cysts were then transferred to a conical *Artemia* hatching jar containing 4 L of seawater (30 ppt), with vigorous aeration, and maintained at 28°C for 24 h under 1000 lux light for hatching. After 24 h, the hatched-out larvae were harvested using 50-micron nitex screen and used for enrichment study.

Rotifer culture

Pure culture of rotifer (*Brachionu s calyciflorus),* procured from live feed culture unit, Bharathidasan University, Tamil Nadu, India, was maintained at Krishnagiri-Barur Centre for Sustainable Aquaculture. First it was cultured in a 1000 ml flask and was periodically up-scaled to 500 litre Fibre-reinforced plastic (FRP) tanks for mass culture. The culture was maintained by feeding them with yeast @ 0.15 g/l and *Chlorella vulgaris* (1.5-2.5×10^6 cells/ml) during morning (08.00 AM) and evening (17.00 PM) times, respectively (Akter *et al.,* 2013). Once, the culture density reached 250-450 individuals/ml, the rotifer were collected and used for enrichment process.

Enrichment of *Artemia* nauplii and rotifer

The enrichment process was carried out in plastic buckets, each of 3 L capacity and provided with aeration and each treatment were triplicated. The enrichment process of androgenic hormone, 17 α-methyl testosterones (MT), (Sigma Chemical Company, St Louis, MO, USA) in the *Artemia* and rotifer was carried out by following the standard method (Sorgeloos *et al.*, 1986). The freshly hatched out *Artemia* nauplii and 6-8 days old rotifer were used for enrichment process. The hormone (17 α- MT) and ethanol mixture was prepared by following the method of (Stewart *et al.,* 2001) with slight modifications. Firstly, a stock solution of hormone-ethanol mixture (20000 ppm) was prepared. This stock solution of 20000 ppm was prepared by dissolving 1000 mg of 17 α-MT in 50 ml of 70% ethanol. 1 ml of this stock solution contains 20 mg of 17 α-MT which was further used for treatment doses preparation. A pre-determined volumes of stock solution such as 1 ml, 2 ml, 3 ml and 4 ml were added to individual replicate buckets to obtain the treatment doses of T1 (20 mg L^{-1}), T2 (40 mg L^{-1}), T3 (60 mg L^{-1}) and T4 (80 mg L^{-1}), respectively. In control group, 2 ml of 70% ethanol without hormone was added. After the preparation of enrichment medium, rotifer (800 individuals/ml) and *Artemia* nauplii (100 individuals/ml) were introduced into the respective treatment medium and kept for different durations of enrichment.

Sample collection, preparation and hormone analysis

After enrichment, samples were collected at different time intervals. The hormone-enriched *Artemia* nauplii and rotifer were harvested processed by following the standard method (Sandifer and Smith, 1985), Samples of 10000 *Artemia* nauplii and rotifer taken from each replicate on 6, 12, 18, 24, 30, 36 and 42 hrs (for rotifer up to 24 hrs only collected).Testosterone levels were measured using enzyme-linked immunosorbent assay kit (ELISA) method. Testosterone was extracted using CALBIOTECH company kits following the manufacturer's instruction manual and ELISA reader (Model No is ER-180) was used (Jester *et al.*, 2014; Liu *et al.*, 2013; Jiang *et al.*, 2011).

Statistical analysis

The collected data were statistically analysed using a statistical program SPSS version 28.0. Significant difference between the mean values were tested using repeated one-way analysis of variance by keeping dose and time as independent variables (each dose vs different time intervals; each time interval vs different doses). Tukey's test was used to rank the mean values and the statistical significance for the test was set as $p>0.05$.

RESULTS AND DISCUSSION

In both live feed, the different durations enrichment found significantly higher 17 α-MT content at different doses. Similarly, *Daphnia magna* enriched with canola oil for different durations displayed significant difference in the lipid content between enriched and non-enriched daphnia (Fereidouni *et al.*, 2013). In *Artemia* nauplii, 17α -MT was in the range of 0.35-0.50 mg L^{-1} before the commencement of experiment (0^{th} h). The study did not find any significant

variation in 17α-MT hormone of *Artemia* kept in control group at different time intervals and it was in the range of 0.35-0.49 mg L^{-1}. Similarly, no significant difference was found at 0th hour in the *Artemia* kept in different dose groups, however, as the time increased, *Artemia* started accumulating 17 α-MT in its tissues at different rates (Table 1). In general, a normal *Artemia* has lower quantity of steroids, for example 17 α-estrodiol, than the enriched *Artemia* (Martin-Robichaud *et al*., 1994) and it may be the reason for lower levels of 17 α-MT detection in the control group of the present study.

The increasing trend of 17α-MT augmentation in *Artemia* was noticed up to 24 h. Significantly higher amount of 17α-MT (15.33±0.09 mg L^{-1}) was observed in 80 mg L^{-1} group at 24 hours (Table 1). Similarly, Martin-Robichaud *et al*., (1994) reported that *Artemia* nauplii enriched in different concentrations of 17 α-estrodiol was displayed significantly higher accumulation of 17α-estrodiol after 24 hours of enrichment. The study examined the assimilation of 17 α-MT at different time points, up to 42 hours, to know highest point of accumulation and starting point of withdrawal period. Interestingly, the study found maximum accumulation at 24 hours and, thereafter, a reduction in 17 α-MT assimilation has been noticed which may be due to higher metabolic activities and excretion activities (Navarro *et al*., 1991; Dhert *et al*., 2001). Similarly, *Artemia* enriched with estradiol (E2) showed increasing trend and stable up to 24 hours (Contreras-Sánchez *et al*., 2004). In an another study, when *Artemia* enriched with ascorbic acid (AA) at different durations found maximum absorption at 18 hrs and the AA content was started declining after 18 hrs of enrichment (Noshirvani *et al*., 2006).

In rotifer, different doses and time intervals investigation found a significant difference in hormone accumulation. 17α-MT levels in rotifer ranged from 0.37 to 0.47 mg L^{-1} at 0th hr. The study did not find any significant differences in the 17 α-MT hormones of rotifer kept in the control group at various time intervals and it was in the range of 0.36-0.49 mg L^{-1}. Similarly, there was no significant difference in the 17 α-MT level among the rotifers kept in different treatment doses at 0th hour.

At 6 hours, the level of 17-MT was significantly higher in all treatment groups. Further, the dose vs time analysis revealed that rotifer accumulated statistically higher quantum of 17 MT at 6 hrs than the 12 hrs (Table 2). After 6 hrs, a decreasing trend was noticed in 17 MT accumulations and significantly higher value of 17 α-MT (17.42±0.06 mg L^{-1}) was recorded at 6 hrs in 60 mg L^{-1}. Previous studies on rotifer reported maximum accumulation of n-3 HUFA within 12 hrs of enrichment (Imada *et al*., 1979; Watanabe *et al*., 1983)

Table 1: *Artemia* enriched with different doses of 17 α-MT for different durations.

	0 h	6 h	12 h	18 h	24 h	30 h	36 h	42 h	P value (Dose *Time)
0 mg L^{-1}	0.45±0.06	0.41±0.09^{1}	0.44±0.02 ^{1}	0.49±0.17 ^{1}	0.41±0.015^{1}	0.43±0.09^{1}	0.35±0.05^{1}	0.38±0.04^{1}	0.420
20 mg L^{-1}	0.43±0.05 ^{a}	1.35±0.0.15^{ab1}	2.55±0.15^{b12}	5.47±0.22^{cd2}	7.48±0.14^{d2}	7.42±0.08^{d2}	4.25±0.11^{c2}	3.60±0.15^{bc2}	0.000
40 mg L^{-1}	0.45±0.01^{a}	1.97±0.04$^{ab\ 1}$	6.15±0.65^{bc2}	8.10±009^{cd23}	9.56±0.02^{d23}	8.41±0.03^{cd2}	7.54±0.02^{c3}	4.65±0.00^{bc2}	0.001
60 mg L^{-1}	0.50±0.01^{a}	8.13±0.01^{bc2}	10.35±0.08^{cde3}	11.06±0.08^{e34}	12.19±0.02^{e3}	11.30±0.01^{e3}	9.69±0.01^{cd4}	7.67±0.15^{b3}	0.000
80 mg L^{-1}	0.35±0.01^{a}	9.53±0.18 ^{c2}	12.60±0.01^{cd4}	12.85±0.04^{d4}	15.33±0.09^{e4}	12.68±0.05^{d3}	10.63±0.01^{cd4}	8.20±0.22^{b3}	0.000
P value (Time*Dose)	0.382	0.000	0.005	0.000	0.000	0.000	0.000	0.000	

In each row, mean values with different alphabet superscripts differ significantly at p<0.05, where each hormone dose was tested at different time intervals (dose*time).

In each column, mean values with different numerical superscripts differ significantly at p<0.05, where different hormone doses were tested against each time interval (time*dose).

Table 2: Rotifer enriched with different doses of 17 α-MT for different durations.

	0 h	6 h	12 h	18 h	24 h	P value (Dose*Time)
0 mg L^{-1}	0.45±0.08	0.49±0.06^{a1}	0.36±0.05^{1}	0.39±0.09^{1}	0.38±0.05^{1}	0.096
20 mg L^{-1}	0.37±0.04^{a}	14.82±0.10^{c2}	14.04±0.03^{c23}	11.04±0.03^{b2}	10.30±0.01^{b2}	0.000
40 mg L^{-1}	0.47±0.06^{a}	15.96±0.07^{c2}	14.68±0.02^{c3}	11.57±0.09^{b23}	10.49±0.11^{b2}	0.000
60 mg L^{-1}	0.43±0.05^{a}	17.42±0.06^{e3}	15.68±0.02^{d3}	12.59±0.02^{c3}	11.13±0.06^{b2}	0.000
80 mg L^{-1}	0.40±0.03^{a}	15.79±0.03^{d2}	12.59±0.06^{c2}	11.97±0.06^{bc23}	10.16±0.06 ^{b2}	0.000
P value (Time*Dose)	0.741	0.000	0.000	0.000	0.000	

In each row, mean values with different alphabet superscripts differ significantly at p<0.05, where each hormone dose was tested at different time intervals (dose*time).

In each column, mean values with different numerical superscripts differ significantly at p<0.05, where different hormone doses were tested against each time interval (time*dose).

and did not find any significant difference in fatty acid content when they enriched for longer duration, up to 24 hrs (Kotani *et al.*, 2010). Similarly, rotifer kept for 8 hrs in higher concentration of enrichment medium did not produce significantly higher quantity of fatty acid (Kotani *et al.*, 2010). At 6 hrs, there is no statistical difference in assimilation of 17 α-MT in the tissues of rotifers kept in 20 mg L^{-1}, 40 mg L^{-1} and 80 mg L^{-1} treatment groups 9 (Table 2). Generally, in rotifer, it has been suggested to increase the duration of enrichment rather than quantity of enrichment medium to obtain more efficient enrichment (Rodriguez *et al.*, 1996; Yahyavi and Farzane, 2009). Interestingly, the present study found significantly higher assimilation of 17 α-MT in shorter enrichment period (in 6 hrs) which in turn may help to reduce cost associated with live feed production (Southgate and Lou, 1995).

Conclusion

The harmful residual effect of hormones, released from the hormone incorporated diets, to the natural environment is alarming. As an alternate, supplementation of hormone *via* live feed, using enrichment techniques, could be a viable option which improves the larval ingestion rate, survival rate, growth rate and reduces hormone residual effects. The study found higher assimilation of 17 α-MT by *Artemia* (at 24 hours in 80 mg L^{-1}) and rotifer (at 6 hours in 60 mg L^{-1}) which could be further explored for mono-sex seed production in a more sustainable way.

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Conflict of interest

All authors declared that there is no conflict of interest.

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