

EFFECTS OF 17 α -METHYLTESTOSTERONE AND *TRIBULUS TERRESTRIS* EXTRACT ON SEX RATIO AND GONADS HISTOLOGY OF RED TILAPIA HYBRID

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SUMMARY

Tribulus terrestris extract and 17 α -methyltestosterone were added into the diets of red tilapia fry with an initial average weight of 0.02 ± 0.003 g and an average length of 0.6 ± 0.012 cm to study their effects on sex reversal and gonads histology. Two *T. terrestris* extract treatments (1 and 2g/kg diet); three hormonal treatments of 17 α -methyltestosterone (30, 60 and 100 mg/kg diet) were used in this study and a control group, the study lasted 112 days. Results showed that *T. terrestris* extract and 17 α -MT has a potential effect on directing gonadal sex differentiation of red tilapia towards males depending on the dose applied. The highest male percentage 90% was obtained by 60 mg 17 α -MT /kg diet followed by 2g *T. terrestris* / kg diet (84.40%). Histological examination of the gonads showed a clear deterioration in their structure as a result of hormonal therapy, the intensity of which increased with an increase in the hormonal dose. The effect from using a high dose of *T. terrestris* was slight. It can be concluded that the addition of *T. terrestris* extract and 17 α -MT to the ration of red tilapia hybrid fry directs the gonads of red tilapia towards males and negatively affects the structure of the gonads.

Keywords: Red tilapia, gonads histology, 17 α -methyltestosterone, *Tribulus terrestris*, sex reversal

INTRODUCTION

Red tilapia has a high popularity worldwide due to its characteristics (high growth rates, attractive skin color, tolerance to high salinity, and relatively high price) (Thodesen *et al.*, 2013). *T. terrestris*, also called gokshura, is a medicinal plant used in many countries to treat sexual deficiencies (Bucci, 2000; Gauthaman *et al.*, 2002). The inclusion of *T. terrestris* extract in the diets of African catfish resulted in the production of high male populations (Turan and Cek, 2007). The treatment of newborn guppies with *T. terrestris* resulted in sex reversal of the fish, underwent spermatogenesis (Cek *et al.*, 2007). Moreover, many of the phytochemicals reported to stimulate testosterone secretion such as ginseng extract (Tsai *et al.*, 2003). *Tribulus terrestris* extract (TTE) causes increased testosterone level and improved sexual status (Gauthaman and Ganesan, 2008).

17 α -Methyltestosterone (17 α -MT) is a synthetic androgen that has an anabolic effect as it promotes muscle growth and the development of the sexual characters in males. Rao and Rao (1983) obtained high percentages of male and sterile gonads in *C. carpio* after oral administration of 220 ppm 17 α -MT during 131 days after hatching.

The objective of this study was to evaluate the effects of varying dietary levels of *T. terrestris* and 17 α -MT on sex reversal and histological structure of gonads of red tilapia.

MATERIALS AND METHODS

Fish and experimental design

Red tilapia fry (Florida strain) aged two days were obtained from Kilo 21 hatchery belongs to the General Authority of Fish Resources Development (GAFRD), Alexandria, Egypt. Initial mean weight and length of experimental fish were 0.02 ± 0.003 g and 0.6 ± 0.012 cm, respectively. Eighteen circular plastic tanks (60 liter each) were stocked with red tilapia fry at 6 fry/l. The tanks were used as static bath with manual water change and were aerated with air stones. De-chlorinated tap water represents the fresh water source in the experiment and for water management at the beginning of the experiment; saline water (42 ppt) were obtained from Suez Gulf and transferred in plastic pails into the lab. Fish fry were acclimated from water salinity of 10 ppt to 0.5 ppt within 5 days. The experiment was conducted at aquaculture lab in the Faculty of Fish Resources, Suez University, Suez, Egypt. The experiment lasted for 112 days.

The experiment included 6 treatments, control group (T₁), two treatments of *Tribulus terrestris* extract at two concentrations 1 and 2g/kg diet (T₂ and T₃); three doses of 17 α -MT treatments 30, 60 and 100mg/kg diet (T₄, T₅ and T₆) were applied; with three replicates for each treatment.

Experimental management

The addition of 17 α -MT and *T. terrestris* extract into the diets were conducted according to Navarro-Martín *et al.* (2009). The control group fed a basal diet treated with ethanol only. Feeding for all experimental groups with 17 α -MT and gokshura extract lasted for a period of 28 days and then all experimental fish were reared for an additional period of 14 weeks feeding on the basal diet only. A fine commercial powder feed containing 40% crude protein (Skritting Egypt[®]) was used for the first 28 days of the experiment, while pelleted (1.2 mm) commercial fish feed containing 30% crude protein (Grand Aqua[®]) were used for the rest of the experiment. Fish were fed five times a day at 15% of live body weight for 2 weeks, then the fish were fed three times daily at 10% to the end of the experiment. Samples of fish were weighed biweekly to adjust feed quantity. Water quality parameters were measured daily to ensure proper water quality for the experimental fish. The sex ratio was calculated by visual examination of the gonads in samples of 60 fish for each treatment. At the end of the trial period, the histology of the gonads was performed.

Histological examination

Specimens from gonads of fish (three fish from each replicate) were fixed in 10% formalin. Tissues were dehydrated in an ascending series of ethyl alcohol (70%, 80%, 90%, 95% and 100%), then cleared in xylol overnight and embedded in paraffin wax blocked and sectioned at 5-6 μ m. The gonads sections were stained with Haematoxylin-Eosin (H&E) and examined by "Zeiss" microscope. Three sections of each specimen were examined and photographed according to Drury and Wallington (1980).

Statistical analysis

Data were analyzed using Statistical Package for Social Science (SPSS) version 22 (2014). One-way ANOVA was applied to analyze the data (Basavaraja & Raghavendra, 2017). Duncan's test was used to determine the significance ($p \leq 0.05$) of differences among experimental treatments.

RESULTS AND DISCUSSION

Sex ratio

The highest male percentage ($p < 0.05$) was recorded for T₅ (60 mg 17 α -MT/kg diet) recording 90% (Fig. 1) followed by T₃ (2g *T. terrestris*/kg diet) recording (84.4%). The significantly lowest male percentage ($p < 0.05$) was observed in the control group (T₁) recording only (46.7%). In the present study administration of 60 mg/kg diet of 17 α -MT produced high percentage of male. These results are in

agreement with the results obtained by Beaven and Muposhi (2012) and Ajiboye *et al.* (2015) who reported a 90 % male at 60 mg 17 α -MT/kg diet. However, Sreenivasa and Prabhadevi (2018) reported a slightly lower male percentage of 86.66 in Nile tilapia fry compared to higher male percentages (93% to 100%) reported in Nile tilapia fry fed diet containing 60 mg/kg of 17 α -MT (Ali *et al.*, 2011; Celik *et al.*, 2011 and El-Greisy and El-Gamal, 2012).

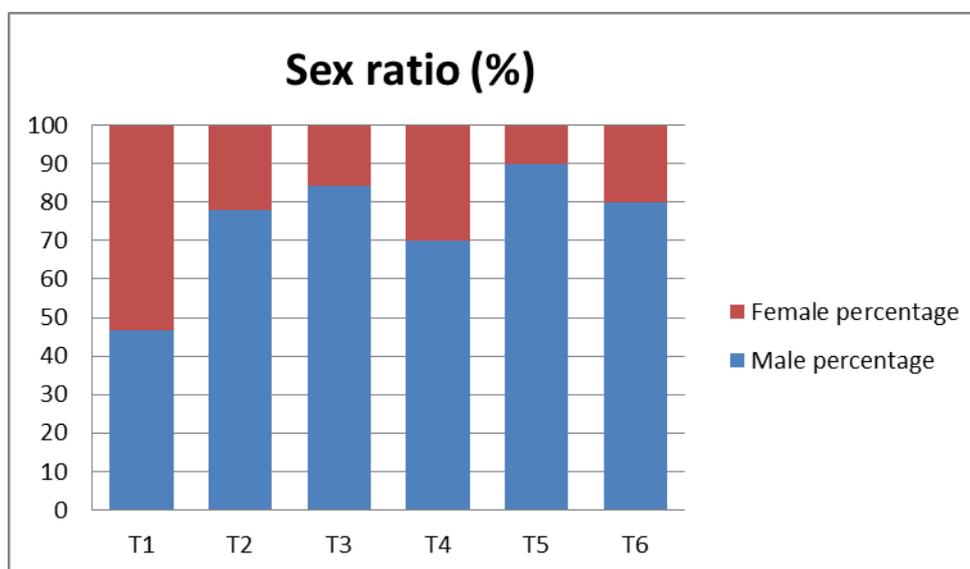


Figure (1). Percentages of males and females of red tilapia treated with different doses of 17 α -MT or *T. terrestris* extract for 28 days then reared for 112 days as a trial period.

Administering high dose (100 mg 17 α -MT/kg diet) reduced male percentage (80 %) , which is in agreement with the results of Marjani *et al.* (2009) and Basavaraja and Raghavendra (2017) , who reported lower male percentage of red tilapia fry exposed to 100 mg of 17 α -MT/kg feed compared to other lower doses. Wolf *et al.* (2004) reported that administration of 17 α -MT in fry fish diets suppresses the oogenesis process. Goudie *et al.* (1983) reported that high concentrations of 17 α -MT results in sterility, however, sub-optimal doses cause intersexes (Popma and Green, 1990).

Red tilapia fry treated with *T. terrestris* extract (2g/kg diet) recorded (84.4%) male percentage compared to (77.8%) in-group fed *T. terrestris* at (1g/kg diet), which is in agreement with the results of Kavitha and Subramanian (2011) and Ghosal *et al.* (2015). Higher male percentage (91:97%) was achieved in tilapia fry fed diet containing 2g/kg of *T. terrestris* extract (Noor El Deen *et al.*, 2020; Ghosal and Chakraborty, 2020). The high male percentages obtained from the inclusion of *T. terrestris* extract in the sexually undifferentiated gonads of tilapia fry may be attributed to some phytochemical compounds in the plant extract. The phytochemical compounds such as steroidal saponin protodioscin may be regarded as androgenic bioactive phyto-constituent that inhibit the biological synthesis of estrogen by its action as aromatase inhibitor and antagonist to nuclear estrogen receptor in gonad germ cells (Rempel and Schlenk, 2008).

The higher effect of hormone on male percentage (90.0%) in 60 mg 17 α -MT/ kg diets compared to male percentages of (84.4 and 77.8%) in fish groups treated with *T. terrestris* at (2 and 1 g, respectively) is agreed with the study of Omar *et al.* (2014) who recorded a significantly higher male conversion rate (92.30%) in the 60 mg 17 α -MT/kg feed treatment compared to other *T. terrestris* extract treatments and the control group.

Pandian and Varadaraj (1990) and Phelps and Pompa (2000) stated that male conversion ratio influenced by many genetic and environmental factors including water temperature, degree of hormone solubility in the solvent, feeding protocol, salinity, photoperiod, stocking density, conditions at the

storage of the hormone or the plant extract and also storage conditions of treated feed, and others). Consequently, all the previous factors should be considered to optimize sex-reversal to produce almost all male population of red tilapia, preferably over 98% male.

Histological examination of the gonads

Ovaries

Ovarian histology of red tilapia hybrid treated with different sex reversal treatments for 28 days then reared for 112 days is shown in Figure 2.

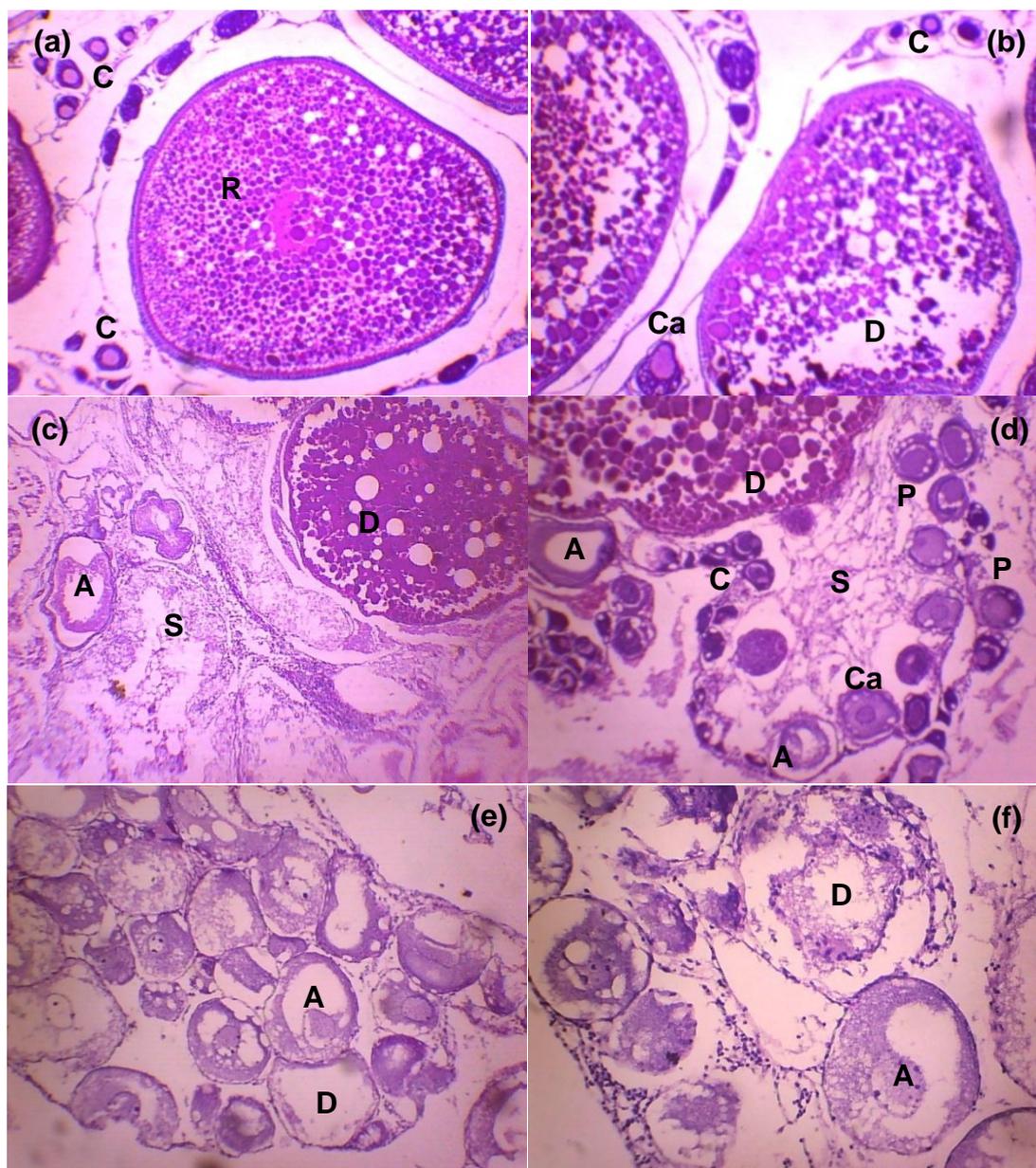


Figure (2): H&E stained ovarian sections of red tilapia treated for 28 days with *T. terrestris* and 17α -MT after the whole experimental period of 112 days, X40. (a) control group; (b) 1g *T. terrestris* /kg diet; (c) 2g *T. terrestris* /kg diet; (d) 30 mg 17α -MT/kg diet; (e) 60 mg 17α -MT/kg diet; (f) 100 mg 17α -MT/kg diet. R, ripe oocyte; C, chromatin nucleolar oocyte; Ca, cortical alveolar oocyte; De, degeneration of ripe oocyte; P, perinucleolar oocyte; A, atretic oocyte; S, stroma.

The histological examination of the ovary in the control group (Fig. 2a) showed high number of matured oocyte, some chromatin nucleolar oocytes and some cortical alveolar oocytes. The ovary of fish fed 1 g *T. terrestris* extract (Fig. 2b) showed degeneration of ripe oocyte; the ovary in fish group treated with 2g *T. terrestris* extract (Fig. 2c) showed degenerated ripe oocyte and atretic oocytes with large area of stroma. The ovary of fish treated with 30 mg 17 α -MT (Fig. 2d) showed degenerated ripe oocytes and atretic follicles. Females treated with 60 mg 17 α -MT and with 100 mg 17 α -MT (Fig. 2e, f) showed almost atretic oocytes in different stages.

Ovarian histological examination of fish treated with *T. terrestris* showed degenerations in oocytes at both doses (1 and 2 g *T. terrestris* / kg feed), these results agree with Ugonna *et al.* (2018) as they observed the effect of pawpaw seed meals (PSM) on gonad histology of *O. niloticus* at doses of (0, 2, 4, 6 and 8g/kg) and found various forms of deformities in female gonads of the treated groups. Similar results were observed by Abdelhak *et al.* (2013), when PSM were incorporated in the diets of Nile tilapia at 120g/ kg diet. Different gonads deformities were also reported by Jegede (2010) and Jegede (2011), when *Hibiscus rosasinesis* leaves (at 3.0 g/kg) and *Aloevera* latex (2.0 ml/kg) were incorporated in the diet of Nile tilapia, respectively.

On contrast, Turan and Cek (2007) reported that there was no effect of *T. terrestris* extract on gonads of *C. gariepinus* treated by immersing the sexually undifferentiated fry in water containing the aqueous extract of *T. terrestris* at a dose of 0, 3, 6, or 9 g/30 L water for one month, where gonads of the treated fish were histologically similar to fish gonads in the control groups. The degeneration in oocytes resulted from the plant extract treatment may be attributed to the presence of oleanolic glycoside in some plant seeds (Das, 1980).

The histological observations of fish ovaries treated with 17 α -MT hormone showed some degenerations in oocytes, these results are in agreement with Hirose and Hibiya (1968 a; b) in goldfish and rainbow trout, where they reported that using 17 α -MT at a dose of 2.5 mg/kg diet caused degenerations in the fish ovaries and testes. Moreover, Higgs *et al.* (1977) observed signs of gonads degeneration in coho salmon treated with 17 α -MT causing fish sterility. Also, Komen *et al.* (1989) reported that oral administration of 17 α -MT in diets at 50 and 100 mg/L to common carp fry resulted in high percentages of sterile fish and caused severe malformations of fish. Sacobie and Benfey (2005) obtained similar observations when treated the diets of tilapia mossambica with 17 α -MT at 50 mg/kg diet, as the histological examination showed some germ cells in the gonads of possible genetic females underwent oogenesis even after the effect of androgen, but were observed to be degenerated eventually.

Testis

Testicular histological sections of red tilapia hybrid treated with different sex reversal treatments for 28 days then reared for 112 days are shown in Figure 3. The histological examination of the testis in fish of the control group (Fig. 3a) showed the presence of spermatocytes, spermatids and spermatozoa in the lumen of seminiferous tubules. Testis in fish fed 1g and 2g *T. terrestris* extract (Fig. 3b) showed normal spermatogenic stages, while fish fed diet containing 2g *T. terrestris* extract (Fig. 3c) showed testicular deterioration and germ cell degeneration. Fish treated with 30 mg 17 α -MT (Fig. 3d) showed the presence of spermatids and spermatozoa in seminiferous tubules and testicular lumens; while in males treated with 60 mg 17 α -MT (Fig. 3e) the testis showed mild reduction in all spermatogenic stages and severe reduction in all spermatogenic stages was observed in fish fed diet containing 100 mg 17 α -MT (Fig. 3f).

As the histological examination of testis in fish treated with *T. terrestris* extract showed normal spermatogenic stages, these observations agrees with Hassona *et al.* (2020), who recently observed that testes of males treated with 750 mg/kg diet *T. terrestris* for 45 days showed normal dilated seminiferous tubule filled with large amount of free spermatozoa in the lumen.

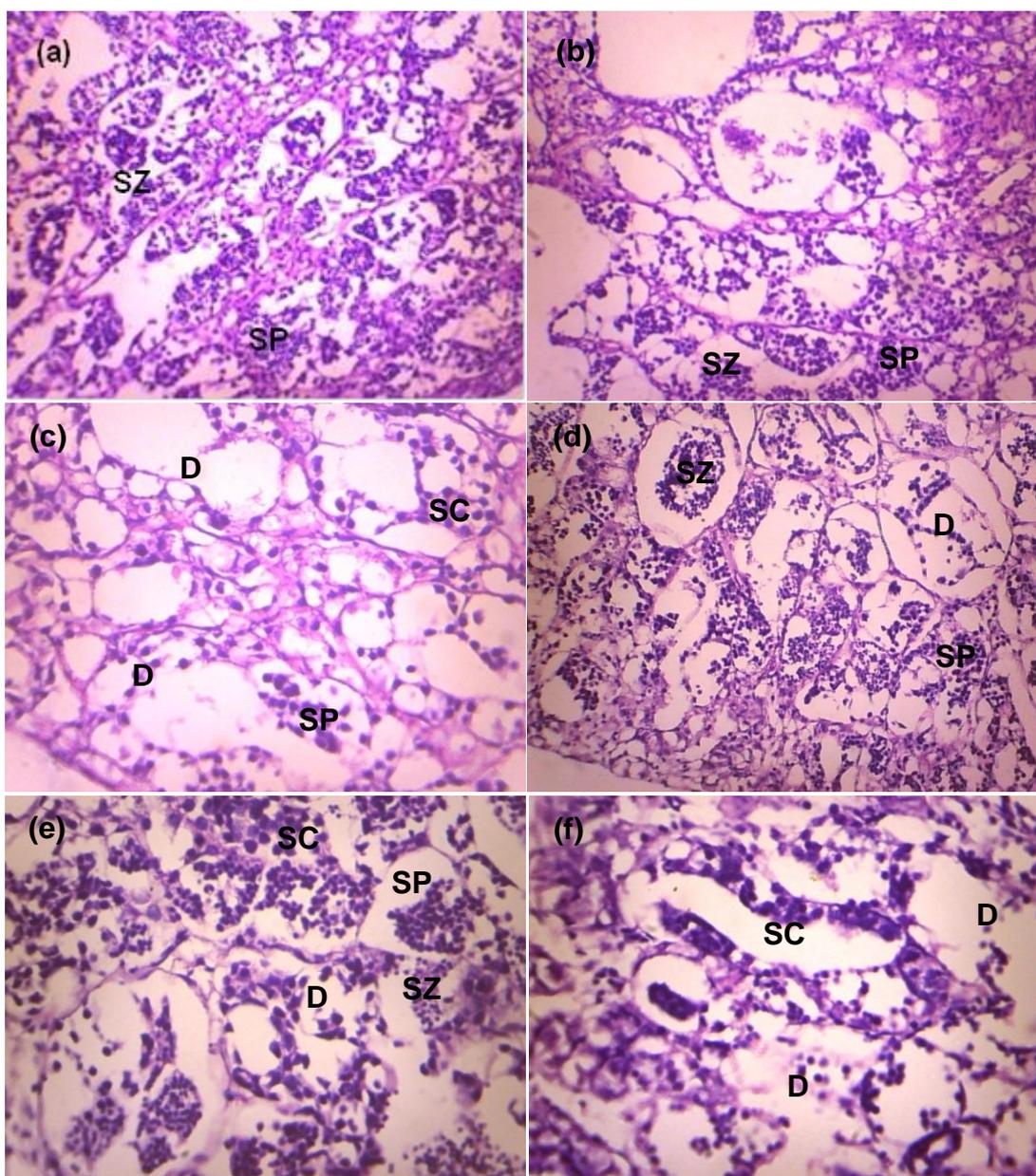


Figure (3): H&E stained testicular sections of red tilapia treated for 28 days with *T. terrestris* and 17α -MT after the whole experimental period of 112 days, X 40. (a) control group; (b) 1g *T. terrestris* /kg diet; (c) 2g *T. terrestris* /kg diet; (d) 30mg 17α -MT /kg diet; (e) 60mg 17α -MT /kg diet; (f) 100mg 17α -MT /kg diet. SC, spermatocytes; SP, spermatids and SZ, spermatozoa in the lumen of seminiferous tubules. Note the mild reduction (D) in all spermatogenic stages in 60mg 17α -MT treated fish (e) and severe reduction in all spermatogenic stages in 100mg 17α -MT treated fish (f).

On contrast, Ugonna *et al.* (2018) evaluated the effect of PSM on gonad histology of *O. niloticus* at doses of (0, 2, 4, 6 and 8g/kg diet) for 28 days and observed significant effect of PSM on the gonads structure. Degenerative stroma was found in the fish fed with PSM at a dose of (2g/kg). However, histological examination of testis of fish treated with 17α -MT showed reduction in different spermatogenic stages at high doses (60 and 100mg/kg diet), similar results were obtained by Hirose and Hibiya (1968 a; b) in goldfish and rainbow trout, who reported that using 17α -MT at a dose of 2.5mg/kg diet caused degenerations in the fish ovaries and testes. Moreover, Macintosh *et al.* (1987) reported that the dose of 60mg 17α -MT /Kg diet caused degeneration in fish testes, which lowered the value of GSI and severe degeneration of testicular tissue of *O. niloticus* was observed by El-Nahal *et al.* (2019). Also,

El-Greisy and El-Gamal (2012) observed that *O. niloticus* fry testes of fish group treated with 40mg of 17 α -MT/kg diet for 28 days showed slow development.

The presence of few oocytes in testicular sections of 30mg 17 α -MT treated fish indicating the existence of intersex, this agrees with Popma and Green (1990), who reported that sub-optimal doses of MT causes intersexes, however, high concentrations of the hormone results in sterility (Goudie *et al.* 1983).

The results of the present study clearly confirms that the inclusion of *T. terrestris* extract at 1g and 2g/kg of red tilapia fry diets and the oral administration of 17 α -MT at 60mg/kg diet can achieve high masculinization percentage in tilapia population and negatively affected gonads structures at high doses. These results may help in optimizing seed production practices in red tilapia hatcheries in Egypt.

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تأثير استخدام مستخلص نبات الحسك *Tribulus Terrestris* وهرمون 17 ألفا ميثيل تستوستيرون على النسبة الجنسية وتطور أنسجة الغدد التناسلية في هجن البلطي الأحمر

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تهدف الدراسة الحالية إلى إنتاج البلطي الأحمر وحيد الجنس (ذكور فقط) عن طريق إضافة مستويات مختلفة من هرمون الميثيل تستوستيرون ومستخلص نبات الحسك إلى عليقة زريعة البلطي الأحمر، ودراسة أثر هذه الإضافات على النسبة الجنسية والفحص النسيجي للغدد الجنسية للأسماك. تمت إضافة مستخلص نبات الحسك و17 ألفا ميثيل تستوستيرون إلى النظام الغذائي لزريعة البلطي الأحمر بمتوسط وزن أولي يبلغ 0.003 ± 0.02 جم ومتوسط طول 0.6 ± 0.012 سم. استخدمت للدراسة مستويان من مستخلص نبات الحسك (1 و2 جم/كجم علف) وثلاثة تركيزات هرمونية من $\alpha 17$ -ميثيل تستوستيرون (30، 60 و100 ملجم/كجم) ومجموعة مقارنة بدون إضافات، واستمرت الدراسة لمدة 112 يوماً. أوضحت النتائج وجود تأثير لكل من مستخلص نبات الحسك وMT- $\alpha 17$ على توجيه الجنس في البلطي الأحمر تجاه الذكور وفقاً للجرعة. تم الحصول على أعلى نسبة ذكور 90% في معاملة 60 ملجم MT- $\alpha 17$ /كجم علف يليها 2 جم مستخلص الحسك/كجم عليقة (84.40%). أظهر الفحص النسيجي للغدد الجنسية تدهور واضح في تركيبها نتيجة المعاملة الهرمونية تشتت حدته بزيادة مستوى الهرمون وكان التأثير السلبي الناتج عند استخدام جرعة عالية من نبات الحسك طفيفاً. يمكن الاستنتاج أن إضافة مستخلص نبات الحسك أو MT- $\alpha 17$ إلى علائق يرقات هجن البلطي الأحمر يوجه مناسل البلطي الأحمر إلى الذكور ولكن يحدث تأثيرات سلبية على بنية الغدد الجنسية للأسماك المعاملة خصوصاً عند استخدام جرعات عالية من الهرمون.