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RESEARCH ARTICLE **ARAŞTIRMA MAKALESİ**

Effects of synthetic and natural steroids on the growth, sex reversal and gonadal development of rainbow trout, *Oncorhynchus mykiss* (Walbaum)

Doğal ve sentetik steroidlerin Gökkuşağı alabalığında, *Oncorhynchus mykiss* (Walbaum) gonadal gelişim, cinsiyet dönüşümü ve büyüme üzerine etkileri

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Özet: Tamamı dişi salmonid stokların üretiminde sentetik cinsiyet hormonlarının kullanımına dayanan erkekleştirme uygulamaları gibi dolaylı yöntemler yaygın bir şekilde kullanılmaktadır. Ancak söz konusu sentetik hormonların toksik etkiler ve yüksek maliyetler gibi dezavantajları bulunmaktadır. Bu bakımdan mevcut araştırmada, doğal steroidlerin kullanımı yolu ile tamamı dişi alabalık populasyonlarının üretimi için dolaylı yöntemlerin geliştirilmesi amaçlanmıştır. Bu çalışmada 7000 prelarval gökkuşağı alabalığı yedi tanka eşit olarak bölünmüş ve bunlar sentetik hormonlar (3 ve 6 mg metiltestosteron -MT ve testosteron -T kg^{-1}yem) ve doğal hormonlar (50 ve 100 mg *Tribulus terrestris* -TT kg^{-1}) içeren ticari alabalık yavru yemleri (Skretting, France) ile keseli dönemden sonraki 90 gün boyunca beslenmiştir. 90 günlük ilk besleme periyodunun sonunda balıklar cinsiyetlerin makroskopik ve histolojik yöntemlerle belirlenebildiği döneme kadar hormon içermeyen ticari alabalık yemi ile beslenmiştir. Araştırma sonunda 50 ve100 mg/ kg *Tribulus terrestris (*TT*)* içeren yemle beslenen balıkların sıra ile %55 ve 63'ü erkek iken, 3 ve 6 mg/ kg testosteron (T) içeren yemle beslenen balıkların sıra ile %51 ve 53 oranında erkek olduğu bulunmuştur. Bu durum *Tribulus terrestris*'in testosterona göre alabalıkların doğrudan erkekleştirilmesinde daha etkin olduğunu fakat gruplar arasında fark bulunmamıştır. Ayrıca bu araştırma, *Tribulus terrestris* içeren (100 mg/kg mg/ kg yemde) yemlerle beslenen alabalıkların diğer deneme gruplarındaki balıklara kıyasla daha yüksek canlı ağırlığa ulaştığını bildiren ilk çalışmadır. Kontrol ve deneme grupları arasında farklılaşmamış gonad yapıları bakımından önemli bir farklılık gözlenmemiştir. Sonuç olarak araştırmada, daha yüksek cinsiyet dönüşüm ve büyüme oranlarına ulaşmak için *Tribulus terrestris*'in daha yüksek konsantrasyonlarının (150, 200, 250 mg kg^{-1}) etkilerini araştırılmalıdır. Ayrıca bu konsantrasyonların gökkuşağı alabalığında, diğer salmonid ve ticari türlerde kan testosteron seviyelerinin yanısıra gonad yapısına olan etkileri de belirlenmelidir.

***Anahtar kelimeler*:** Testosterone, *Tribulus terrestris*, rainbow trout, histoloji, erkekleştirme, büyüme.

Abstract: Indirect methods such as masculinizing treatments by synthetic sex steroids has widely been used to produce all-female stocks in salmonids, but they have some disadvantage such as toxicity and high cost. Therefore, the main objective of this research was to develop indirect methods for the production of all-female populations of *Oncorhynchus mykiss* through the use of natural steroids.In this study seven thousand prelarval rainbow trout were divided evenly among seven tanks and fed a commercial starter trout diet supplemented with synthetic (3, 6 mg testosterone and methyltestosterone kg^{-1} diet) and natural steroids (50 and 100 mg *Tribulus terrestris* kg^{-1} diet) during 90 days after hatching. Fish were then further reared on a commercial trout diet until sex ratios could be determined through histological and macroscopic observations. The *Tribulus terrestris* (50 and100 mg/ kg) treatments yielded 55–63% males, while the testosterone (3 and 6 mg/ kg) treatment yielded 51–53% male population. This demonstrates that *Tribulus terrestris* was effective in the direct masculinization of rainbow trout compared to testosterone, but no significant difference was observed between groups. Also, this study was the first attempt that yielding higher growth of *Oncorhynchus mykiss* fed with diets containing TT (100 mg kg^{-1} diet) compared to other experimental groups. There were no marked differences in the structure of the undifferentiated gonads between the control group and all treatment groups. In conclusion, the effects of higher concentrations (150, 200, 250 mg kg^{-1}) of TT should be investigated to reach the higher sex reversal and growth rates, and also determined the blood testosterone levels, gonadal structure of rainbow trout and/or other salmonids, and cultivable fish species.

***Keywords*:** Testosterone, *Tribulus terrestris*, rainbow trout, histology, masculinization, growth.

INTRODUCTION

Ingestion of synthetic steroid residues in sex reversed fish may be potentially hazardous to human consumers. According to the EU Directive 96/22/CE (29th April 1996), only the "indirect feminization" approach is authorized in European countries. This methodology requires steroid-induced functional masculinization of genetic females into so called "neomales" and their crossing with normal females to produce all-female progenies. The synthetic steroid, especially methyltestosterone, has potential use in finfish culture due to its anabolic and also androgenic actions. Many previous efforts on the use of methyltestosterone have focused both on the effectiveness of its growth-promoting properties in various fish species (Higgs *et al*., 1977; Yu *et al*., 1979; Kuwaye *et al*., 1980; Higgs *et al*., 1982; Boney *et al*., 1984; Degani and Dosoretz, 1986; Manzoor Ali and SatyanarayanaRao, 1989), and also on its ability to produce sex reversal when

administered to fish larvae (Nagy *et al.*, 1981; Owuso-Frimpong and Nijjhar, 1981; Rothbard *et al.*, 1983; Solar *et al.*, 1984; Schmelzing and Gall, 1991; Pandian and Sheela, 1995). Although it is apparent that methyltestosterone is a good argument for sex reversal in aquaculture, it's human food safety has been an unresolved issue. It has been well known that higher dose exposures of methyltestosterone can cause adverse health effects such as hepatotoxicity (Lucey, 1987; Hartleb and Nowak, 1990) and fetotoxicity in humans (Wilkins *et al.*, 1958; Grumbach and Ducharme, 1960; Wilkins, 1960; Schardein, 1980). Due to all these reasons the indirect method is preferred to direct feminization in trout culture because fish destined for market are not directly exposed to steroids. Therefore, the resulting sex reversed male broodfish must be only used for propagation and are not allowed to be sold for human consumption.

Despite indirect protocols to produce all-female populations using methyltestosterone (MT) having been published for most salmonids (Pandian and Sheela, 1995; Devlin and Nagahama, 2002), However, there is no record on the masculinisation effect of *Tribulus terrestris* (TT) in indirect sex reversal of rainbow trout instead of methyltestosterone. Gokshura, *Tribulus terrestris* (TT), is an herb that is widely distributed in China, Japan, Korea, the western part of Asia, the southern part of Europe and Africa. TT contains a number of different substances known as steroidal saponins. Protodioscin, the most dominant saponin in TT, is thought to be main substance responsible for increasing testosterone production (Ganzera *et al.*, 2001). Protodioscin has also been found to increase the levels of non-hormonal phyto-dehydroepiandrosterone (DHEA), (Adimoelja *et al.*, 2005) dihydrotestosterone and dehydroepiandrosteronesulphate (Gauthaman *et al.*, 2000). It has been shown to raise testosterone levels safely and naturally in humans and is rumoured to be the secret behind the success of many top Bulgarian weightlifters (Bucci, 2000). The administration of TT to humans and animals improves the libido and spermatogenesis (Tomova *et al.*, 1981). In humans, it has been used to treat impotence and has been found to increase testosterone levels and improve athletic performance (Adimoelja and Adaikan, 1997; Adimoelja, 2000; Adaikan *et al.*,2000; Bucci, 2000; Gauthaman *et al.*, 2002). In fish, Çek *et al.* (2007a, b) and Turan and Çek (2007) reported the effects of TT on sex reversal, survival rate and growth performance of different fish species (*Clarias gariepinus, Cichlasoma nigrofasciatum and Poecilia reticulate*). They found successful sex reversal, spermatogenesis and better growth rates than untreated progenies.

The objective of this research was to develop an alternative method by the using TT in order to produce all-male monosex populations for the safer trout farming.

MATERIALS AND METHODS

Farming conditions

The experiments were implemented at the Tekir Trout Farm, a commercial hatchery (Kahramanmaraş, Turkey). Water was supplied from the spring of Ceyhan River, which undergoes very limited variations in water temperature between winter (10.5 °C) and summer (12.5 °C). Newly hatched seven thousands larvae used in the sex reversion trials were obtained from the commercial production involving a large number of parents (>30 females and 20 males) (Bromage et al., 1992). Broodstock and experimental fish (alevin, fry, sub adult, broodstock) were fed with commercial trout feeds during the experiments (Le Gouessant, Skretting). Broodstock reproduced naturally by hand stripping under a natural photoperiod regime without hormonal stimulation. Fries were grown in fiber glass tanks until the fry stage and were then transferred to concrete ponds for further growth. A natural spring water was continuously used to provide 80% oxygen saturation at least. Density during grow-out period did not exceed 50 kg m^{-3}.Under the farm conditions, eggs began to hatch at 300 degree *x* days (°d) after fertilization and this was considered as the reference for the time of starting hormonal treatments.

Preparation of herbal extracts and experimental diets

The TT extract (Origin: Bulgaria) was purchased from Dietharmonie Corp. (France). The aqueous extracts of TT were prepared by boiling 50 mg of the pure and finely ground extract of TT in 250 ml distilled water for 10 minutes and 100 mg of TT in 500 ml distilled water for 20 minutes and then filtering it through a Whatman paper filter twice (Gauthaman and Adaikan, 2005; Çek *et al.*, 2007a). The solutions were freshly prepared each time when the experimental diets finished. Diets were sprayed and mixed continuously by each concentration of the TT solution. The diets were dried at room temperature (25 ºC) and stored frozen (-4ºC) until their use. Methyltestosterone (MT) and testosterone (T) (Sigma, St. Louis, MO, USA) were dissolved in ethyl alcohol and sprayed into the starter trout diets at a dose 0 (control diet) or 3, 6 mg methyltestosterone and testosterone kg^{-1} of feed. The ethyl alcohol was allowed to completely evaporate at room temperature and the diets were stored at -4°C.

Experimental design

Experiment was designed to evaluate the efficiency of oral treatments of TT compared to other synthetic hormones. Mixed sex (XX and XY) larvae were subdivided into seven groups; a control group (Group A) and six oral-treated groups receiving a diet of 3 mg testosterone kg^{-1} diet (Group B), 6 mg T kg^{-1} (Group C), 3 mg 17α-methyltestosterone kg^{-1} (Group D), 6 mg MT kg^{-1} (Group E), 50 mg *Tribulus terrestris* kg^{-1} (Group F) and 100 mg (TT) kg^{-1} (Group G) delivered during 90 days from the first feeding. After the hormonal treatment, fish in all experimental groups were fed the same commercial grow-out trout feeds up to 9 months of age. At 9 months of age, 51 to 64 individuals per group were weighed and sacrificed using a lethal dose of anesthetic (2-phenoxy ethanol). The gonads were dissected and sex was determined

by visual observation of both gonads. At the termination of the experiment the number of males and females were recorded for each treatment. Gonadosomatic (GSI) and Hepatosomatic indexes (HSI) were calculated according to (Devlaminget *al*., 1982; Delahunty and Devlaming 1980) as follows: GSI = (L+R)x100/BW; HSI = LWx100/BW; where, LW= liver weight (g); L = left ovary weight (g); R = right ovary weight (g); BW = body weight (g). Non-parametric tests were used to determine whether the sex ratios observed were significantly different from the expected sex ratios of the rainbow trout. χ^2 tests were performed to analyze the statistical differences in the sex ratios among groups at levels of P<0.05 or P<0.001. The sex ratio of each experimental group was compared to a theoretical equilibrated sex ratio (50:50). Individual body weight was recorded at the time of sexing (±0.1 g). Differences in growth within sexes among treatments were evaluated by ANOVA (Zar, 1996).

Histological procedures

One hundred forty eight specimens were sacrificed using an overdose of 2-phenoxy ethanol. After using benzocaine the artery of each fish was cut to prevent any bleeding in the body cavity. Ovaries and testes from specimens were then taken monthly during 90 days. Gonads were also dissected at the termination of the experiment and they were directly fixed in 10% neutral buffered formalin. After being, preserved in formalin for about one week, transverse sections of the central portion of the gonad samples were dehydrated in graded ethanol, embedded in paraffin, sectioned at 4-5 μm and stained with haematoxylin and eosin (MERCK) for histological examination (Çek and Yılmaz, 2009). Samples from small larvae were not decalcified but large samples were placed face down in a decalcifying for at least one hour. After histological work, all slides were examined under a light microscope (CH-2 Olympus-Japan). Sexual differentiation and developmental stages of female and male gamete cells were identified according to descriptions given by Bromage and Cumaranatunga (1988), and Çek*et al*., (2001). The stages of oocytes and spermatozoa development were classified on the basis of observations of changes in the nucleus, nucleoli and cytoplasm.

RESULTS

Effects of synthetic and natural steroids on the growth and sex ratio of *Oncorhynchus mykiss*

The current study was the first attempt that yielding higher male growth of *Oncorhynchus mykiss* fed diets containing TT (Group G, 100 mg kg^{-1} diet) compared to other experimental groups, although females in control group had the highest weight at the end of the study (P<0.05) (Table 1). Higher concentration of TT (Group G) increased the gonadosomatic index (GSI) in females. Also, higher concentrations of MT (Group E, 6 mg MT kg^{-1} diet) and TT (Group G) elevated the HSI values of males significantly. In a similar manner, high doses of testosterone (Group C, 6 mg T kg^{-1} diet) and TT (Group G) treatments increased HSI ratios in female rainbow trouts (P<0.05) (Table 2).

The sex ratio observed in 100 fish in the control group of the experiment was nearly the expected sex ratio of 1:1 (male: female). TT treatments yielded 55–63% males, while the T treatment yielded a 51–53% male population. The sex ratio observed in the second series (Groups B and C) of the experiment was almost equal to the expected sex ratio of 1:1 (male: female). In these groups, the difference between male and female sex ratio were not statistically significant (p>0.001). In the third series of the experiment, we achieved 100 and 92.59% masculinization in *O. mykiss* by oral treatment for 90 days in diet containing 6 and 3 (MT) mg kg^{-1}, respectively. Fish groups (Group F and G) treated with 50 and 100 mg kg^{-1} doses of TT showed less masculinizing effects during the 90-day experimental period. At the termination of the experiments, MT had resulted in a statistical significant difference in the sex ratio (p<0.001) compared to the other groups. MT was the most effective in terms of masculinization, resulting in a maximum male ratio of 100%. Increased concentration of TT caused an increase in the number of males produced. Testosterone was not found to be effective in producing male populations (Table 3).

Table1. Effects of synthetic and natural steroids on the growth (mean ± standard error; weight, W, g; length, L, cm) of rainbow trout (*Oncorhynchusmykiss*) at the end of the experiment*.

Groups	Male		Female	
	W	L	W	L
A	98.80±10.74	19.02±0.75	98.80±5.86 ^{b}	19.83±0.49 ^{b}
B	105.45±7.74	19.57±0.47	72.45±7.54 ^{a}	17.55±0.54 ^{a}
C	91.64±6.40	19.42±0.59	96.27±6.07 ^{b}	20.05±0.63 ^{b}
D	103.76±3.91	19.74±0.19	**	
E	100.81±7.55	19.25±0.52	**	
F	95.06±7.45	19.67±0.54	81.24±4.79^{ab}	19.04±0.40^{ab}
G	117.09±13.37	20.54±0.73	92.14±9.49^{ab}	19.71±0.72 ^{b}

A-controlgroup, B- lowdose of testosterone (3 mg kg^{-1}diet), C-highdose of of testosterone (6 mg kg^{1}diet), D- lowdose of methyltestosterone (3 mg kg^{-1}diet), E-highdose of methyltestosterone (6 mg kg^{-1}diet), F- lowdose of *Tribulus terrestris* (50 mg kg^{-1}diet), G- highdose of *Tribulus terrestris* (100 mg kg^{-1}diet)
*Different letters in the same columns indicate the statistical differences between groups (P<0.05)
** No females were recorded

Table 2. Effects of synthetic and natural steroids, and their different doses on the gonadosomatic (GSI) and hepatosomatic (HSI) indexes (%) of rainbow trout (*Oncorhynchusmykiss*) at the end of 90 d*.

Groups	Male		Female	
	GSI	HSI	GSI	HSI
A	1.09±0.72	1.15±0.11^{ab}	0.05±0.01^{a}	0.97±0.06^{b}
B	1.62±0.98	1.16±0.08^{ab}	0.06±0.02^{a}	0.72±0.09^{a}
C	1.78±0.84	1.06±0.06^{ab}	0.06±0.02^{a}	0.99±0.03^{b}
D	0.03±0.01	1.03±0.05^{b}	**	
E	0.02±0.01	1.26±0.08^{a}	**	
F	0.15±0.07	0.93±0.07^{ab}	0.07±0.01^{ab}	0.88±0.02^{ab}
G	0.48±0.40	1.23±0.13^{b}	0.08±0.01^{b}	1.04±0.07^{b}

A-controlgroup, B- lowdose of testosterone (3 mg kg^{-1}diet), C-highdose of of testosterone (6 mg kg^{-1}diet), D- lowdose of methyltestosterone (3 mg kg^{-1}diet), E-highdose of methyltestosterone (6 mg kg^{-1}diet), F- lowdose of *Tribulus terrestris* (50 mg kg^{-1}diet), G- highdose of *Tribulus terrestris* (100 mg kg^{-1}diet)
*Different letters in the same columns indicate the statistical differences between groups (P<0.05)
** No females were recorded

Table 3. Masculinizationeffect of thesynthetic (testosterone and 17a- methyl testosterone) and natural (*Tribulus terrestris*) steroids on the of rainbow trout, *Oncorhynchus mykiss**

Sex	Treatment						
	A	B	C	D	E	F	G
Male (%)	48.00	53.70	51.85	92.59	100.00	55.55	63.46
Female (%)	52.00	46.30	48.15	7.41	0.00	44.45	36.54
χ^2	0.667	0.296	0.740	39.185*	50.074*	0.667	3.769
Asymp Sig.	0.414	0.586	0.785	0.000	0.000	0.414	0.052
df	1	1	1	1	1	1	1

A-controlgroup, B- lowdose of testosterone (3 mg kg^{-1}diet), C-highdose of of testosterone (6 mg kg^{-1}diet), D- lowdose of methyltestosterone (3 mg kg^{-1}diet), E-highdose of methyltestosterone (6 mg kg^{-1}diet), F- lowdose of *Tribulus terrestris* (50 mg kg^{-1}diet), G- highdose of *Tribulus terrestris* (100 mg kg^{-1}diet)
*Indicate the statistical differences from the other groups (P<0.001)

Effects of synthetic and natural steroids on sexual differentiation and gonadal development of *Oncorhynchusmykiss*

There were no marked differences in the structure of the undifferentiated gonads among the control group and all treatment groups (synthetic and TT-treated groups) (Figure 1A). Sexual differentiation first recorded in the Group E (6 mg MT kg^{-1} diet treated group) (Figure 1B). Latest sexual differentiation was recorded in the TT-treated groups (Figure 1C). Gonadal differentiation in the control group were similar to that of TT-treated groups. It was clearly indicated that, in all treatment groups including control, the differentiation of the primordial gonad into ovary took place at an earlier stage than that of the testes (Figure 1B and C). Differentiation in the male gonads detected just before the maturation stage. The slowest testes development was observed in the control groups (Figure 2A). In Group E, 6 mg kg^{-1} MT- treated groups, testis has an increased number of sperm ducts and only a few spermatogenetic cysts (Figure 2B). These testis' showed degeneration in seminiferous tubules which were free from spermatocytes and spermatids. (Figure 2B). Histological examination of the TT-treated testes revealed no damage to the testicular structure (Figure 2C and F). All spermatogenesis stages were present in T, MT and TT-treated male testes. However, spermatogenesis was more advanced among the TT-treated groups (Figure 2C and F) of *O. mykiss* compared to the testosterone (Group B) and methyltestosterone treated groups (Groups D and E).

It was clearly detected that the histological response of the testis particularly in Group G (100 mg kg^{-1} TT treatment group) included an increased number of spermatogenic cysts and an abundance of the late stages of spermatogenesis (Figure 2C and F). These testes contained a preponderance of spermatozoa in the lobular lumen. All stages of spermatogenesis, including ruptured spermatozoa were detected in the sperm ducts. Lobules containing numerous spermatocytes from early stages (spermatogonia) to complete spermatogenesis (spermatocytes, spermatids and spermatozoa) were observed. In comparison in the MT and testosterone- treated groups, free spermatozoa were only occasionally recorded, and the testis contained mostly spermatogonia and spermatocytes (Figure 2B, E, D). Ovarian development in the Group F (50 mg TT kg^{-1} diet-treated group) was similar to that of the control and Group B (3mg T kg^{-1} diet) (Figure 3A, B, C). In the Group E (6 mg MT kg^{-1} diet -treated group), no female ovaries were detected. All samples were belong to the males, however in the Group D (3 mg MT kg^{-1} diet) and Group C (6 mg T kg^{-1} diet), the development of the ovaries were defective (Figure 3D, E). Pre-vitellogenic

oocytes were commonly affected by atresia. The first sign of atresia in the previtellogenic oocytes was the shrinkage of oocytes, which was closely accompanied by the development of clear spaces in the peripheral ooplasm (Figure 3D, E). The nucleoli that were generally arranged to form a regular layer in the nuclear envelope of normal growing previtellogenic oocytes from control, T and TT- treated groups (Figure 3B, C and F) were distributed irregularly in the nucleoplasm which was sampled from MT-and T- treated groups (Figure 3D, E). Alpha (α) stages of atretic oocytes were clearly detectable as well. On the basis of histological examination of the TT-treated ovaries, it was concluded that TT revealed no damage to the ovarian structure (Figure 3B, C and F). Up to three stages were present in control, T and TT-treated female ovaries. However oogenesis was more advanced among the TT-treated groups (Figure 3B, C and F).

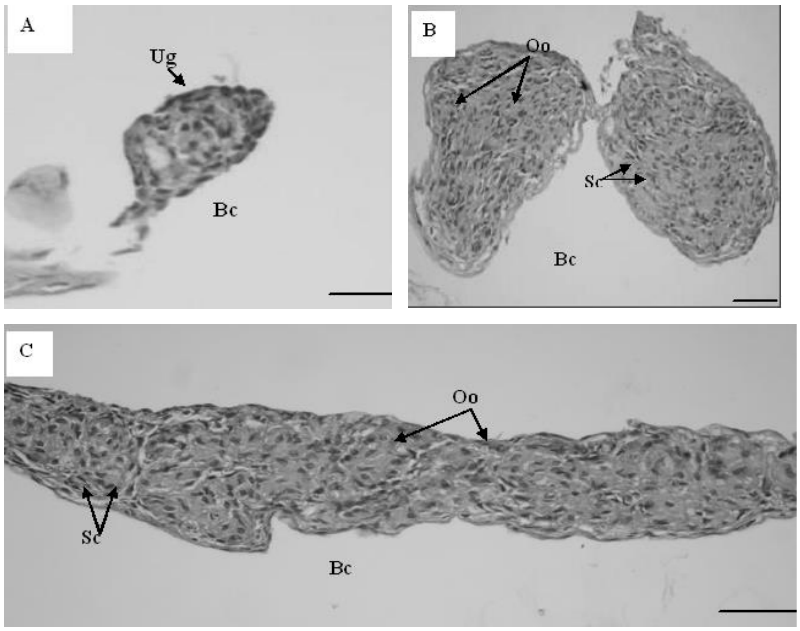

Figure 1. Transverse section of larvae, showing differentiation of the gonads in different-treatment groups. A) Transverse section of larvae, showing undifferentiated gonad, 40 day after hatching in the T- treated group, B) Transverse section of the trunk region of a 60 day old fish, taken from MT-treated group, showing the differentiated ovary within the peritoneal body cavity, C) Differentiated ovary 60 day after hatching, taken from the TT-treated group. Stained with Haematoxylin and Eosin (H&E).Oo, Oogonia, Bc, body cavity, Ug, undifferentiated gonad, Sc, somatic cells. Scale bars, A, 250µm, B, 50 µm, C, 25 µm.

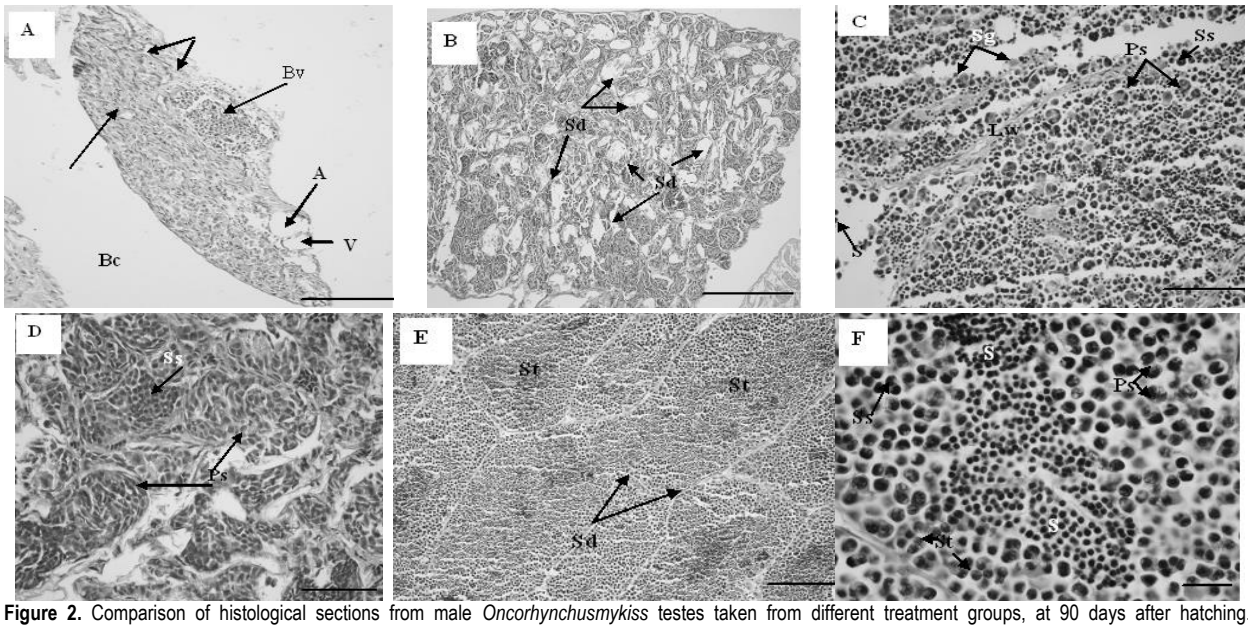

Figure 2. Comparison of histological sections from male *Oncorhynchusmykiss* testes taken from different treatment groups, at 90 days after hatching. A)Histological cross-section through a single testicular lobe that were taken from control group, B) Histological cross-section through a single testicular lobe, taken 6 mg kg^{-1}MT-treated group, arrow shows increased number of sperm ducts.C) 50 mg kg^{-1} TT- treated group, showing different developmental stages of spermatogenesis D)6 mg kg^{-1} T-treated group, showing primary and secondary spermatocytes E) 3 mg kg^{-1}MT-treated

group, showing sperm ducts and spermatids. F) 100 mg kg^{-1} TT- treated group, showing free sperm inside the lumen. (Stained with H&E), Bc, body cavity; Bv, blood vessel; A, artery; V, vein; S, spermatozoa; Sg, spermatogonia; Ps, primary spermatocytes; Ss, secondary spermatocytes; Lw, lobular wall; Sd, sperm duct. Scale bars, a, 250µm; b, 200 µm; d,e, 50 µm; c,f, 25 µm.

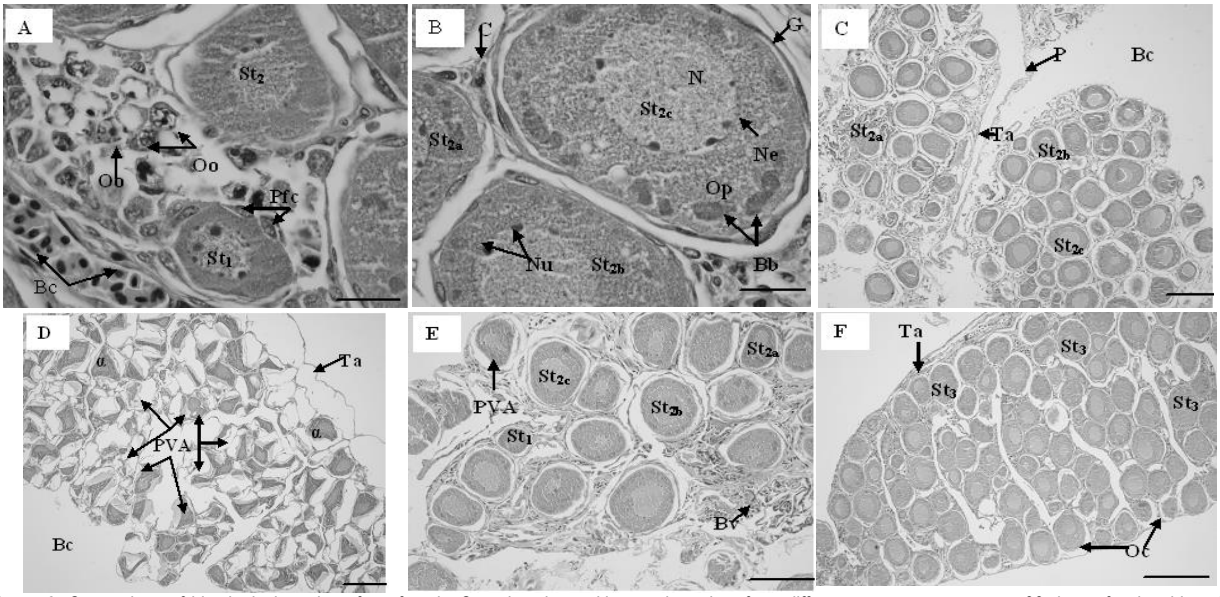

Figure 3. Comparison of histological sections from female *Oncorhynchusmykiss* ovaries taken from different treatment groups, at 90 days after hatching. A) Arrows show oogonia taken from the control group. B and C) Samples taken from 50 mg kg^{-1}*Tribulus terrestris* (TT) treated groups. D) Sections of atreticprevitellogenic follicles that were taken from 3mg kg^{-1} 17α-methyltestosterone-treated groups, showing their shrinkage and disorganization as evidenced from the presence of white spaces (arrows between ooplasm and follicle wall E) Sections of atreticprevitellogenic follicle that were taken from 6 mg kg^{-1} T -treated groups. F) Sections of normal developed ovary, showing oocytes at stage three. Sections were taken from 100 mg kg^{-1} TT-treated groups. (Stained with H&E), PFC, pre-follicle cells; Oo, oogonia; Bv, blood vessel; St_1, stage 1 oocytes; St_{2a}, stage 2a oocytes; St_{2b}, stage 2b oocytes; St_{2c}, stage 2c oocytes; St_3, stage 3 oocytes; C, connective tissue; G, granulosa; N, nucleus; Ne, nuclear envelope; Nu, nucleoli; Op, ooplasm; Bb, balbiani bodies (yolk nuclei); Th, theca; P, peritoneum (mesovarium), Bc, body cavity; Ta, tunica albugenia, PVA, pre-vitellogenic atresia; Oc, ovarian cavity Scale bars, a,b= 25µm, c=200 µm, d,e,f= 250 µm.

DISCUSSION

The main objective of the present investigation was to improve the indirect feminization methods in rainbow trout through the use of a natural steroid (*Tribulus terrestris,* TT). TT treatment is a more effective method than the administration of synthetic hormone in terms of being more environmental friendly. In addition, the persistence and fate of synthetic hormones and hormone metabolites in fish, water and sediment may represent potential environmentally and health risks that have to be considered when using hormonal sex control technology (Contreras-Sanchez *et al.*, 2001). Therefore fish offered to the consumer will not be treated with synthetic hormones, and producers may have an alternative method for producing monosex populations based on natural products.

To this end, we used *Tribulus terrestris* (TT) and find out that TT extract alone was effective at higher dose level (100 mg kg^{-1}) in increasing the proportion of males in the population, advancing spermatogenesis and improving the growth performance among male individuals. In respect to anabolic effect, the current study was the first and only attempt reporting the anabolic performance of TT on growth for *O. mykiss* (Table 1). In addition, the anabolic effects of synthetic MT on growth had also been reported in rainbow trout before (Ostrowski and Garling 1987). Growth promoting effects of synthetic androgen treatments have previously been reported in teleosts, possibly due to increased appetite and enhanced food utilization and protein synthesis (Donaldson *et al.*, 1979; Mukhopadhyay *et al.*,1986; Jayaprakas and Sindhu, 1996; Fagerlund and Dye, 1979; Grau, 1993). In the present study, the growth rate of fish treated with higher concentration of *Tribulus terrestris* (TT) was found to be faster than that of the controls, testosterone (T) and methyltestosterone (MT)-treated groups (Table 1). The effects of the TT extract on body weight have been studied by Çek *et al.* (2007a) in *Cichlasoma nigrofasciatum* and in *Poecilia reticulate* (Çek *et al.* 2007b) and by Turan and Çek (2007) in *Clarias gariepinus*. Çek *et al.*, (2007) found an increase in body weight, sexual differentiation and spermatogenesis. In addition, they concluded that TT had no negative effect on the survival rate of *C. gariepinus*. In addition, in our study, TT-treated fish exhibited high survival (almost no mortality), improved spermatogenesis and masculinization. While these findings are not contradictory with the present results, there is a lack of information in the literature on the effects of a plant extract on sex-reversal, gonad differentiation and/or development, and growth performance in *O. mykiss*. We demonstrate here, for the first time, that TT- extracts are potent and able to induce a higher rate of growth in male *O. mykiss* compared to other experimental groups.

Survival rates at the termination of the present experiment, in the control were found similar to those observed in the TT-treated groups, and were almost 100%. TT was not found to be toxic to humans by Adimoelja (2000) and to rabbits by Adaikan *et al.* (2000). Tapia *et al.* (1994); Waller and Yamasaki (1996); and Aslani *et al.*, (2004) studied the toxicity of TT in livestock and concluded that the consumption of TT by livestock led to the photosensitization syndrome known as yellow thick head. In these studies, TT was not purified and it was fed to the animals ad libitum for at least 2 months. Photosensitization has not been observed in humans and is highly unlikely in fish at the recommended dosage (personal communication from A. Adimolja). Kavitha and Jagadessan (2002, 2003) studied the role of the TT extract on mercury-intoxicated mice, *Mus musculus*. In their study, a lethal dose of mercuric chloride was administrated through the drinking water to female mice every day for 45 days. Its toxicity altered the histo-architecture of the large intestine. During the recovery period, the mice were dosed with a TT extract of different solvent fractions for 15 days; these mice showed a complete regeneration of the large intestine from the toxic effect of the mercury. In the present study photosensitization was not observed, and the survival ratios of the TT-treated groups of *O. mykiss* were not different than that of the control groups. However, the toxicity of TT in fish (if present) needs to be studied. We also determined in this study that TT was not harmful by inhalation and that absorption through the skin did not cause irritation. Synthetic androgens are harmful when inhaled, ingested and/or absorbed through the skin and can cause irritation.

There are few study concentrates on the masculinizing effect of *Tribulus terrestris* in fish and the other experimental animals. A previous attempt by Gauthaman *et al.* (2002) to determine the aphrodisiac properties of TT in normal and castrated rats yielded successful results. They concluded that the TT extract increases testosterone levels in rats. Also, in earlier studies, the masculinization effect of TT was found significant in two aquarium species, convict cichlid (*Cichlasoma nigrofasciatum*) and guppy (*Poecilia reticulate*) by Çek *et.al.* (2007a and 2007b) and in African catfish (*Clarias gariepinus)* by Turan and Çek (2007), respectively. Although the present research provides evidence that higher concentration of TT treatment (Group G) result in a high rate of masculinization comparing to the lower TT treated group (Group F), whether this potency is caused by increases in androgens or testosterone cannot be deduced from the present results, as we did not measure plasma testosterone level during the experiment. When compared with the other studies, masculinization rate was lower (63.46%) in the present study, this was interpreted as the level of TT was lower and/or inadequate level in the diet. However, to the best of our knowledge prior to the present research, the potency of TT/or a plant extract as a masculinizing agent in *O. mykiss* has not been reported before. Therefore, it could be advised to investigate the testosterone-releasing property and optimum concentration of TT promoting the higher sex reversal of rainbow trout, *O. mykiss.* Future researches aiming to measure the amount of testosterone levels after TT treatment in *O. mykiss* may provide more conclusive evidence in terms of the effects of TT on the sex ratio and whether it can be successfully used as an agent and/or a method in *O. mykiss* culture. Further investigations are also necessary to determine the effects of TT on the other cultivable fish species. In conclusion, the effects of higher concentrations (150, 200, 250 mg kg^{-1}) of *Tribulus terrestris* should be investigated to reach the higher sex reversal and growth rates, and also determined the blood testosterone levels, gonadal structure of rainbow trout and other salmonids, and/or cultivable fish species.

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