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The Effects of Gokshura, *Tribulus terrestris* on Sex Reversal of Guppy, *Poecilia reticulata*

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Abstract: This study examined the effects of *Tribulus terrestris* (TT) on sex reversal in guppy, *Poecilia reticulata.* The objective of this study was to introduce a new environmentally friendly method for masculinization in *P. reticulata.* Since male guppy has higher commercial value than female. TT is a natural, non-toxic herb which helps enhance testosterone levels in human and animals. It was prepared in a laboratory in France. Different concentration (0.0, 0.05, 0.1 and 0.15 g L^{-1}) of TT was investigated for sex reversal in the *Poecilia reticulata.* TT extract was administered by immersion of newly born offspring once weekly for two months. Among the dosages used in the present study 0.15 g L^{-1} TT was the most effective dosage that ensured maximum male ratio (80%, $p<0.01$). Although, sex ratios of 0.05 and 0.1 g L^{-1} TT were not significantly different from the expected 1:1 ratio, in these two groups treatment with TT also result in higher number of males (58.25 and 59.77%, respectively), than control ($p>0.05$). Total survival rates in all treatments and control were uniformly high ranging from 83 to 87% ($p>0.05$). It is concluded that TT has no negative effect on survival rate of *P. reticulata.* All groups of TT-treated fish exhibited successful growth acceleration comparing to the control group, but only TT treatment at the concentration of 0.15 and 0.1 g L^{-1} TT significantly improved growth rate of *P. reticulata* ($p<0.01$). Histological examinations revealed that testes of fish treated with TT-extract contained all stages of spermatogenesis. Sex reversal in *P. reticulata* demonstrated that TT treated new-born progenies showed successful sex reversal, spermatogenesis and better growth rate than untreated progenies.

Key words: Plant extracts, masculinization, aquarium fish, growth, histology

INTRODUCTION

Gokshura, *Tribulus terrestris* L. (Zygophyllaceae) is an herb that is widely distributed in China, Japan, Korea, the western part of Asia, the southern part of Europe and Africa. It has been shown to raise testosterone levels safely and naturally and is rumoured to be the secret behind the success of many top Bulgarian weightlifters (Bucci, 2000). Administration of TT to human and animals improved libido and spermatogenesis (Tomova *et al.*, 1981). It has been used in the treatment of impotence and has been found to increase testosterone levels and improve athletic performance (Bucci, 2000; Adaikan *et al.*, 2000; Adimoelja, 2000; Gauthaman, 2002). Testosterone is a sex hormone and its role in sexuality is unequivocal. Embryonic differentiation of the larvae into a male and its subsequent growth along this line is essentially due to the presence of physiological amounts of testosterone in the body. Testosterone is the biosynthetic precursor to both androgens and estrogens in teleosts (Baroiller *et al.*, 1999; Devlin and Nagahama, 2002). However, in addition to testosterone, 11-oxygenated androgens are commonly found at high concentrations in the plasma and tissues of male fish and are generally considered to be the most important physiological androgens in males (Borg, 1994).

TT has been found to increase the levels of testosterone (Adaikan *et al.*, 2000; Adimoelja, 2000; Gauthaman *et al.*, 2000). TT contains a number of different substances known as steroidal saponins. The saponin in TT thought to be responsible for it is effect on testosterone levels is known as protodioscin (Adimoelja and Adaikan, 1997; Ganzera *et al.*, 2001; Joshi *et al.*, 2005).

The live-bearing guppy, *Poecilia reticulata*, is a popular aquarium fish. Aquarists use them for obtaining new morphs with variable colours. The males of this species are more attractive than the females, therefore maintenance and breeding of male populations has generated a great amount of commercial interest. Masculinization of *P. reticulata* can be produced by direct synthetic hormonal treatment that is efficient and straightforward (Takahashi, 1975; Kavumpurath and Pandian, 1992, 1993). However synthetic hormones are

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more expensive than plant extracts and their fate in water and sediment is providing information on the potential risks of using synthetic hormones (Contreras-Sanchez *et al*., 2001). An alternate technique for commercial production of all male production is perhaps to use plant extracts. Therefore the objective of the present study is to investigate the effect of TT on sex reversal in *P. reticulata*.

MATERIALS AND METHODS

Plant material and herbal extracts preparation: TT extract (origin: Bulgaria) were purchased from Dietharmonie Medicinal Plants (Valence, France). The aqueous extracts of TT was prepared by boiling 18 g pure and fine extract of TT in 1500 mL distilled water for 30 min and then filtering it with a whatman filter paper twice (Kavumpurath and Pandian, 1993; Gauthaman and Adaikan, 2005). This solution was prepared 8 times (weekly for 60 days, each aquaria was 20 L), in another words, 3 replicates for the TT treatment 18 g of TT was used per immersion and the larvae were exposed 8 times.

A stock of guppy (85 fish), procured from a local ornamental fish dealer, (the mean weight and length for males were 0.135±0.15 g, 2.21±1.71 cm (mean±SE), respectively and for females were 0.399±0.24 g, 3.25±0.95 cm (mean±SE), respectively. Twenty five male and Twenty five female from this stock in an aquarium containing recirculating water (26±1 °C) exposed to 12:12 h light/dark cycle. The brood stock aquaria were observed at least 10 times daily (particularly in the early mornings) for the presence of offspring. When female had given birth, the new-born fry were immediately removed from the aquarium, counted, measured and placed in 12 small glass aquarium, each containing 20L of water which was continuously aerated with a 4 cm length air stone. All fry were identical age. The fry were fed three times a day with commercial dry flake food (Inve, Aquamaks, Türkiye) and the dry food was supplemented with freshly hatched *Artemia salina* (Subreme Bay Brand INC., San Franciso, USA) and tubifex. Fish were randomly assigned to four different treatment groups that each one received one of the four doses of TT tested: 0 (control), 0.05, 0.1 and 0.15 g L^{-1}. Treatment started 0 days post-hatching (dph) and continued until 60 dph. The four treatments, each consisted of three replicates, were done simultaneously. The aquarium system was static bath with changing water manually. Each aquaria comprised 35 fry. Thus 12 batches, including control batch, were used. For the experiment a total of 420 fry were used.

Sampling and histological procedures: At the end of the experiment, 60 days old fish were anaesthetized in 2-phenoxethanol (Sigma Chem. Co., Dorset, U.K) at a concentration of 1: 20000 in water and counted with the aim of assessing the survival rate and whole body weights and lengths were recorded. The head and tail of the fry were cut and the body was fixed in 10 % neutral buffered formalin, dehydrated, embedded in paraffin, sectioned at 5 μm and stained with haematoxylin and eosin for histological examination (Çek *et al*., 2001; Çek, 2006). The stage of testes development was determined for each fish. Classification was based on the histological criteria adapted from Grier (1981). Oogenesis was not observed.

Statistical analysis: Differences in mortality and differences in body weight and length between groups were tested with the Kruskal-Wallis one way analysis of variance by ranks (SPSS 10,0 for windows) followed by the Duncan non-parametric multiple comparison procedure. Differences between groups in sex ratio of the offspring-based on secondary sex characteristics (In males: gonopodium, veil-like caudal fin, more body pigmentation and small body size) and on gonad histology the Chi-Square (χ^2) test was used (Zar, 1984).

RESULTS

Effect of *Tribulus terrestris* on the sex ratio of *P. reticulata*: In the present investigation, we achieved 80% masculinization by immersing 0 day old fry for 60 days in water containing 0.15 g TT per litre. All groups of TT-treated fish exhibited more male number than female number comparing to the control group, but only TT treatment at the concentration of 0.15 g L^{-1} significantly changed sex ratio in the *P. reticulata* ($p<0.001$; Table 1). The sex ratio observed for the *P. reticulata* was nearly the expected ratio of 1:1 (male: female) in the first series of the experiments (control groups) (Table, 1). The sex ratio observed in 91 fish taken from the second series of experiment (0. 05 g L^{-1} TT-treatment groups) was 53:38 (male:female); this difference was not statistically significant. In the third series of experiments (0.1 g L^{-1} TT-treatment groups), sex ratio was 52:35 (male:female); this difference was also not statistically significant. The sex ratio observed in 90 fish taken from the last series of experiment (0. 15 g L^{-1} TT- treatment groups) was 72:18 (male:female); this difference was statistically significant at the $p<0.001$ level (Table 1). In the present study, no inter-sex fish were recorded.

Survival and growth of the *Tribulus terrestris* administered fish: Table 2 shows the survival rate and growth rates in total length and body weight of the *P. reticulata*. Total survival rates in all treatments and controls were uniformly high ranging from 83 to 87% ($p>0.05$). All groups of TT-treated fish exhibited successful growth acceleration comparing to the control

Table 1: Effect of TT on sex ratio of *Poecilia reticulata*

	Dose ($g\ L^{-1}$)	Treatment duration (day)	Sex ratio (Male: Female) ♂ : ♀	Sex ratio (%) ♂ : ♀	χ^2
TT	0	60	36 : 51	41.38 : 58.62	_
	0.05	60	53 : 38	58.24 : 41.76	2.473
	0.1	60	52 : 35	59.77 : 40.23	3.322
	0.15	60	72 : 18	80.00 : 20.00	32.400***

***Sex ratio significantly different from expected 1 M: 1F ($p<0.001$, χ^2 test), n = 100. TT, *Tribulus terrestris*

Table 2: Effect of TT on growth and survival rate of *Poecilia reticulata*

	Dose ($g\ L^{-1}$)	Survival rate (%)	Total length (cm)*	Body weight (g)*
TT	0	82.85±1.65^a	2.29±0.09^a	0.051±0.01^a
	0.05	86.66±0.95^a	2.44±0.03^a	0.086±0.020^a
	0.1	82.85±1.65^a	2.80±0.08^b	0.227±0.028^b
	0.15	85.71±3.30^a	2.96±0.15^b	0.226±0.036b

*Values superscripted by different alphabets within the same column are significantly ($p<0.01$) different. TT, *Tribulus terrestris*. Results are expressed as means±SE

group, but only TT treatment at the concentration of 0.15 and 0.1 $g\ L^{-1}$ significantly improved growth rate of *P. reticulata* ($p<0.01$). Body length and weight (2.96±0.15 cm; 0.226±0.036 g) of fish treated with 0.15 $g\ L^{-1}$ TT group were significantly increased compared to control (2.29±0.09 cm; 0.051±0.01 g).

Gonad histology: The testis of control and TT-treated group was enclosed by thin capsule of collagenous tissues and was divided into two lobes by the connective tissue of the capsule and it was located in the posterior region of the body cavity where they were suspended from the dorsal wall by the mesorchia (Fig. 1a and b). The testis of control groups was smaller than the testis of TT-treated groups. In control groups, each testicular lobe contained numerous spermatocytes that were round in

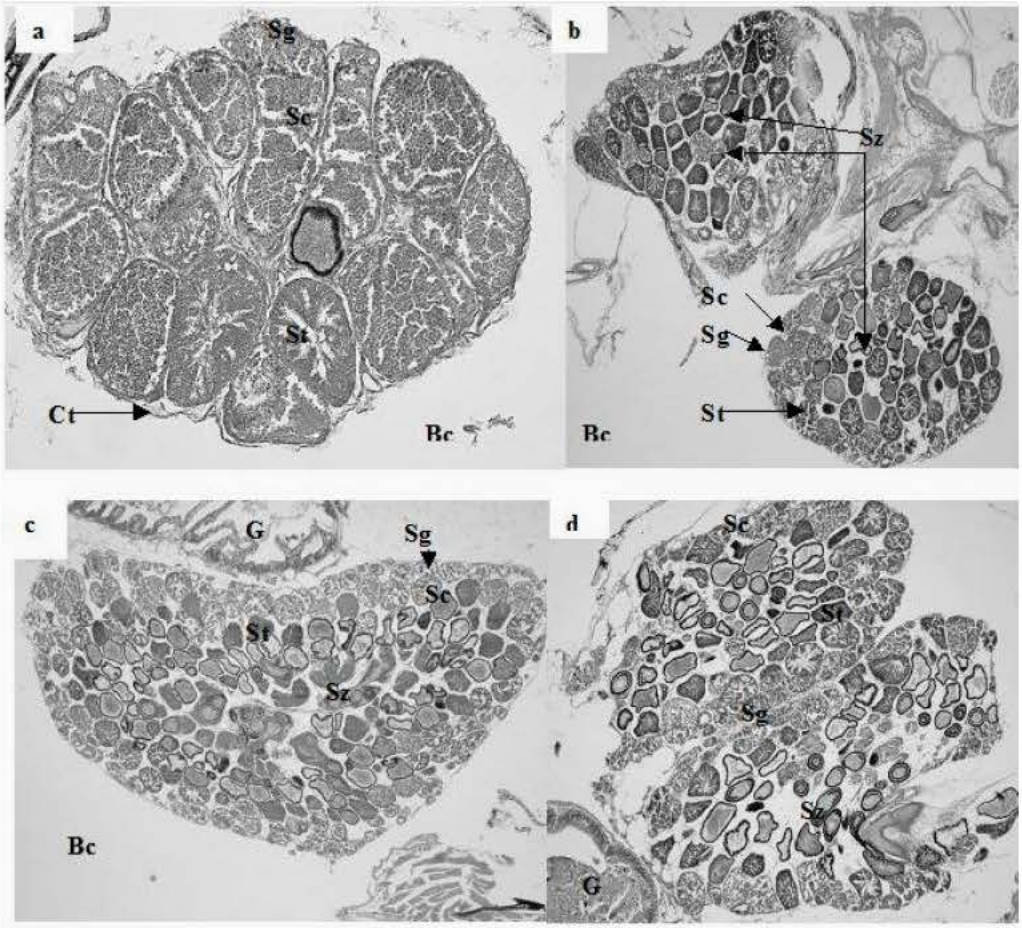

Fig. 1: Light micrographs, each depicting testis structure from the guppy *Poecilia reticulata*. (a) Testis of control. The testis contains spermatogonia, spermatocytes and spermatids,HEx400 µm; (b) Fish treated with 0.05 $g\ L^{-1}$ *Tribulus terrestris*. The testis contains all stages of spermatogenesis, Hex200 µm; (c) Fish exposed to 0.1 $g\ L^{-1}$ *Tribulus terrestris*. The testis has an increased number of spermatozeugmata and was larger than control and 0.05 $g\ L^{-1}$ TT-treated fish, HEx250 µm; (d) Fish exposed to 0.15 $g\ L^{-1}$ TT. The testis has an increased number of spermatozeugmata, Hex250 µm. Sg, spermatogonia; Sc, spermatocytes; St, spermatids; Sz, spermatozeugmata; Bc, body cavity; G, gut; Ct, collagenous tissue

shape, spermatozeugmata and free spermatozoa were not detected. The testis contained mostly spermatogonia, primary, secondary spermatocytes and spermatids (Fig. 1a). In control and TT-treated groups secondary spermatocytes were smaller in size than primary spermatocytes but were considerably larger than spermatids (Fig. 1a-c).

The histological response of the testis in all TT-treatment groups included an increase number of spermatogenetic cysts and abundance of the late stages of spermatogenesis. These testis contained cysts of all spermatogenetic stages in tubules (Fig. 1b-d). During early stages of development, spermatozoa were organized in loose bundles, but they eventually become tightly bound (Fig. 2b and c). Finally the spermatocytes containing sperm opened to the intra testicular duct. However, the testis of control fish did not contain all stages of spermatogenesis. No intact spermatozeugmata and free spermatozoa were observed in the sperm ducts and they did not show a zonate arrangement (Fig. 1a). A zonate arrangement was observed in cross sections of the lobules in 0.05, 0.1 and 0.15 g L^{-1} TT-treated groups. Each zone consisted of spermatocytes that contained germ cells at the same stage of development (Fig. 1c and Fig. 2c). The zonation was dorsoventrally oriented therefore, a cross section of a testicular lobe displayed a developmental succession in which the dorsal spermatocytes were the least and the ventral ones were the most advanced. Because new spermatocytes were being formed and added continually, older spermatocytes were moved ventrally where they formed in sequential order, spermatogonial, spermatocytes, spermatid,

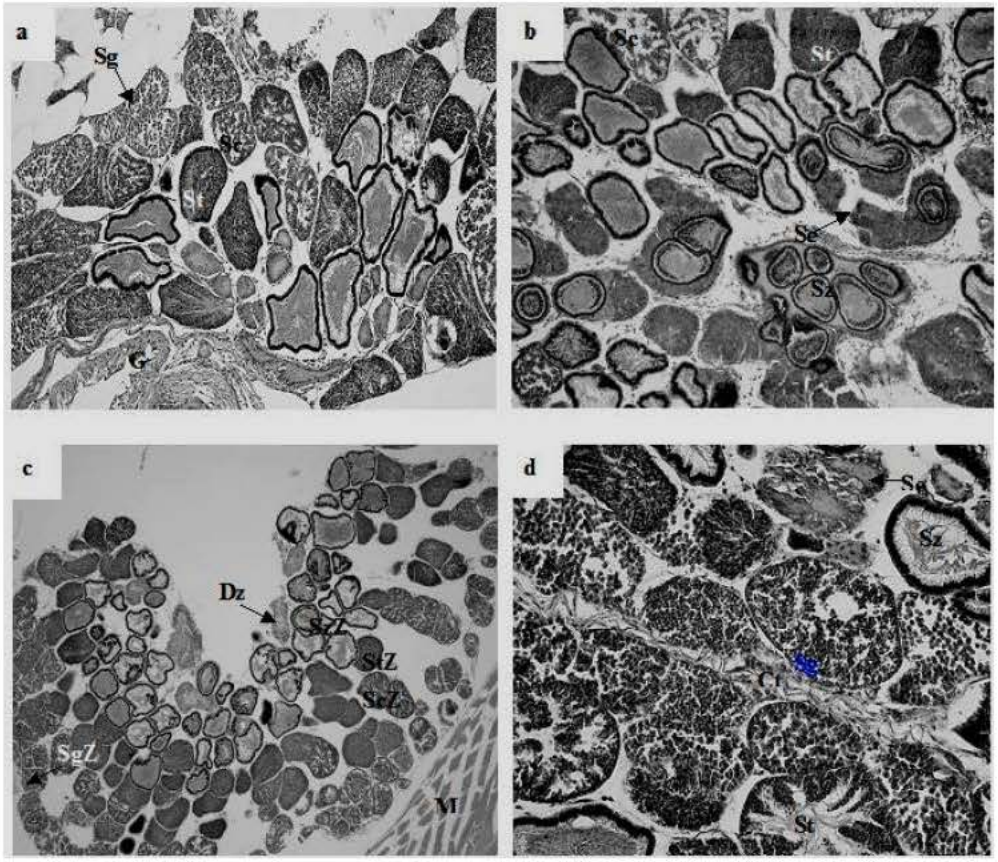

Fig. 2: Light micrographs, each depicting one lobe of the bilobed testis from the TT-treated guppy *Poecilia reticulata*. Administration of TT to *P. reticulata* clearly improved spermatogenesis and spermiation. (a) fish exposed to 0.05 g L^{-1} TT, HEx250 μm; (b) fish exposed to 0.1 g L^{-1} TT, HEx250 μm; (c) fish exposed to 0.15 g L^{-1} TT, showing zonated arrangement of spermatocytes. Zonate formation was not detected in control group. Hex200 μm; (d) 0.05 g L^{-1} TT- treated fish, showing spermatogonia associated with connective tissue. Hex600 μm. Sg, spermatogonia; Sc, spermatocytes; St, spermatids; Sz, spermatozeugmata; G, gut; Ct, connective tissue; Se, hypertrophied sertoli cells; M, muscle. SgZ, spermatogonial zone; ScZ, spermatocytes zone; StZ, spermatid zone; SzZ, spermatozoal zone; Dz, degenerate zone

spermatozoal and degenerate zones (Fig. 1c and 2c). However in control group, the formation of zones were not observed (Fig 1a). It was evident that spermatogenesis increased in all TT- treated groups. Moreover, the testes of these groups were clearly larger than the control group. The testes of the TT- treated fish had a dose dependent increase in the number of spermatogonia, primary and secondary spermatocytes, spermatids, concomitant with an increase in the number of spermatozeugmata (Fig. 1b-d). Of the fish treated with the TT-extract had testes filled with all stages of spermatogenesis and free spermatozoa in enlarged ducts (Fig. 1b-d). In all TT- treated groups, the spermatocytes become small and flattened while undergoing degeneration and resorption. Degeneration of spermatocytes in all TT-treated groups were detected. However, in 0.15 g L^{-1} TT-treated groups, these degenerated spermatocytes were most abundant (Fig. 2c). In the control groups, spermatids were not undergoing spermiogenesis, bundles of spermatozoa and degenerated spermatocytes were not detected. The two main internal sperm ducts and its branches the efferent ducts were lined by cubiodal or columnar epithelium. Tubules, contained cysts with the different spermatogenetic stages (Fig. 2c), radiate from the main ducts toward the periphery of the testis. In these tubules, spermatogonia were located at the periphery of the testis, where they were associated with Sertoli cells (Fig. 3a). These reorganize to form cysts by the time spermatogonia transform into primary spermatocytes (Fig. 3a). As spermatogenesis proceeds, the cysts migrate along the tubule toward the efferent ducts. The secondary spermatocytes in the cysts transform into spermatids which differentiated into spermatozoa (Fig. 3b and c). The heads of the spermatozoa become attached to the inner margin of the Sertoli cells lining the cysts (Fig. 3d). In this way spermatozeugmata, bundles of spermatozoa with heads pointing outward tails in the centre were formed (Fig. 3d). The Sertoli cells surrounding the mature spermatozegmata fuse with the wall of the efferent duct. The head of the

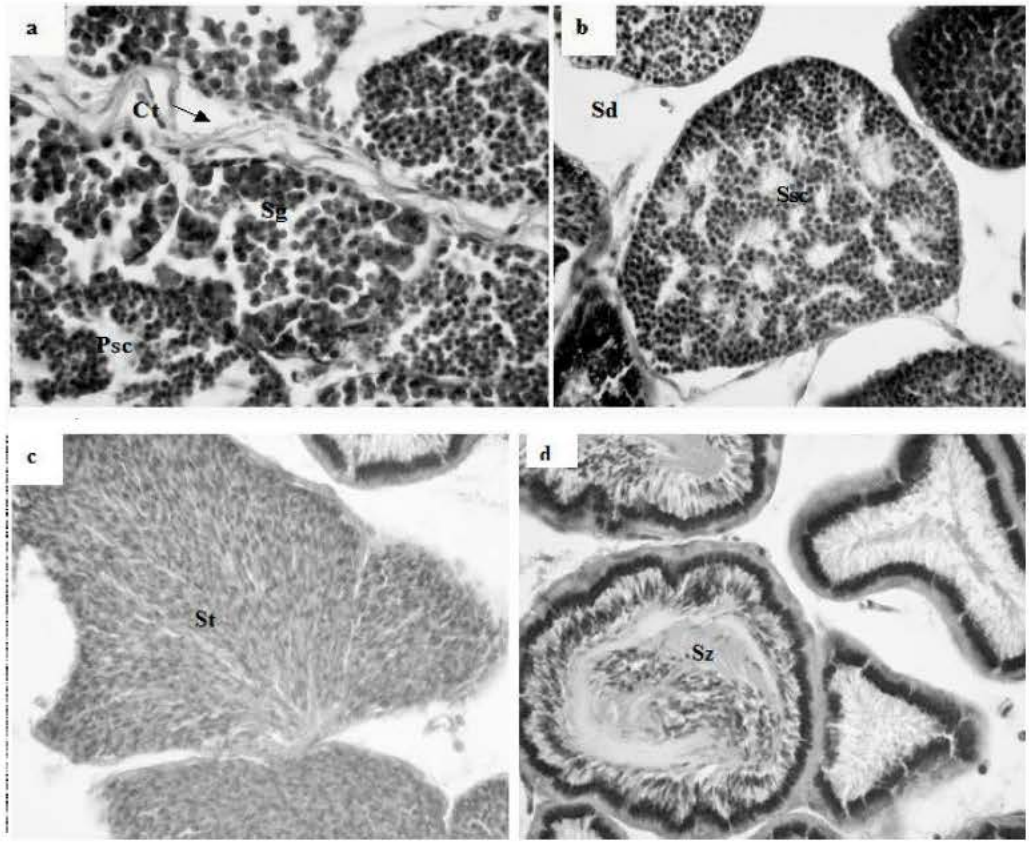

Fig. 3: Cross sections of testicular lobe of *P. reticulata* (0.05 g L^{-1} TT- treated). (a) Primary spermatocytes were most dominated in control group. (b)Secondary spermatocytes. (c) Spermatids. (d) Bundles of late spermatids showing nuclear spiralization formation. This stage was not detected in control fish. HEx800 μm. Sg, spermatogonia; Psc, spermatocytes; Ssc, spermatocytes St, spermatids; Sz, spermatozeugmata; Ct, connective tissue; Sd, sperm ducts

spermatozoa withdraw from the cysts Sertoli cells, the cyst opened and the bundle of spermatozoa (spermatozeugmata) passed into the lumen of the efferent duct. The cysts Sertoli cells hypertrophy and transform into efferent duct cells (Fig. 2d and Fig. 3d).

Testes of fish treated with the 0.15 g L^{-1} TT-extract had changes similar to those observed in the testis of fish exposed to the 0.05 and 0.1 g L^{-1} TT-extract. However in the 0.15 g L^{-1} TT-extract-treated fish the effects were even more pronounced and the testis of 0.15 g L^{-1} TT-treated groups were clearly larger than 0.05 and 0.1 g L^{-1} TT-treated groups (Fig. 1b-d).

Beside differences in sex ratio, growth rate and spermatogenesis, marked differences in the external appearance of the fish were found between control and TT-treated groups. Males have enhanced expression of secondary sexual characteristics (Personal observation).

DISCUSSION

The goal of the present study was to find a cheap, environmentally friendly, easily obtainable and more effective masculization method for sex reversal and growth performance in fish culture by using the *P. reticulata* as a model fish. We found that TT-extract alone was effective, only at one dose, in producing male populations, increasing spermatogenesis and improved growth performance in *P. reticulata*. Before the present study, TT had been shown, in our laboratory, to produce male population in *Cichlasoma nigrofasciatum* with similar results (unpublished data). To our knowledge, this study documents the first reported investigation to evaluate TT as a potent masculinizing agent in *P. reticulata*. Survival ratios at the termination of the experiment in controls were similar to those observed in the TT-treated groups, where no significant dose-related inter-group differences noted. Although the present research provides evidence that TT treatment resulted in a high rate of masculalinazation, whether this potency is caused by androgen or testosterone increase cannot be deduced from the present results, as we did not measure plasma testosterone level during the experiment. However, the treatment of the newly-born progenies using TT 's extract significantly ($p<0.01$) increased the percentage of males to 80%. Yet, TT extract was ineffective in producing 100% males. Therefore, usage of a higher dose may lead to the production of all-male *P. reticulata* population. In the present study, 80% masculinization was achieved in *P. reticulata* by treatment with TT extract. Most authors have reported similar observation for other fish species, treated with synthetic androgens (Baker *et al.*, 1988; Ali and Rao, 1989; Guerrero, 1993).

On the basis of histological studies the development of spermatozoa was normal and healthy in all TT-treated groups. This founding was similar to those of Grier (1981), Kinnberg *et al.* (2003), Kinnberg and Toft (2003). The histological observation that in testes of *P. reticulata* there were significantly improved spermatogenesis in all TT treatment groups than in control was similar to that found by Seth (1974) and Protich *et al.* (1983) in humans; Georgiev *et al.* (1988) in lambs and Dimitrov *et al.* (1987) in rats. A previous attempt by Gautman *et al.* (2002) to search the aphrodisiac properties of TT, in normal and castrated rats yielded successful result. They concluded that TT extract increases testosterone levels in rats. In the present study growth rate of fish treated with TT was found to be faster than that of the control. This indicates that TT has no negative effect on survival rate of *P. reticulata* and it has the ability to increase total body weight and length at the tested concentrations. The effects of TT-extract on body weight have been studied by Georgiev *et al.* (1988) in immature sheep and by Gauthaman *et al.* (2002) in rats. Both authors have found out an increase in body weight and sexual activity. These findings are not contradictory with the present results. However no previous author has studied the effect of a plant extract on sex-reversal, spermatogenesis and growth performance in a fish species. The present study has demonstrated, for the first time that TT extracts was potent and induced 80% sex-reversal and accelerated growth rate in *P. reticulata*.

TT treatment is a better method than the synthetic hormonal treatment and is environmental friendly. Since synthetic hormones and hormone metabolites persistence and their fate in fish, water and sediment will provide information on the potential risks of using hormonal sex control technology (Contreras-Sanchez *et al.*, 2001) It can be applied with ease to a large number of individuals simultaneously. The use of TT as an alternative method to produce all-male populations of *P. reticulata* may address environmental safety issues. 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.

Therefore the use of TT extract is recommended in attempts to achieve masculinization, as well as growth performance. The findings from this study add further support to the TT 's effect on growth and its testosterone releasing property. Further studies to measure the amount of testosterone levels, after TT-treatment in *P. reticulata* may provide more conclusive evidence as regards to the effects of TT on sex ratio and it can be successfully used as an agent in fish culture and further investigations can be carried out to find the effects of TT on other cultivable fish species.

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