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Effect of *Tribulus terrestris* on monosex production in *Poecilia latipinna*

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***Tribulus terrestris* (*Tt*) is a traditionally known, non-toxic aphrodisiac herb for effecting maleness. It would help develop an eco-friendly method to masculinize the fish, *Poecilia latipinna*, because males have higher commercial value. A dose-dependent masculinization occurs on administration of *Tt*, which improved male ratio. All groups of *Tt*-treated fish also exhibited notable growth acceleration.**

Keywords: Masculinization, *Poecilia latipinna*, sex reversal, *Tribulus terrestris* hormones.

TRIBULUS TERRESTRIS (*Tt*; Zygophyllaceae) is a ground-spreading herb, widely distributed in China, Japan, Korea, the western part of Asia, the southern part of Europe, and Africa. It is a common plant known to elevate the testosterone levels in humans and animals. In humans, it has been used to treat impotence and has been found to increase testosterone levels and improve athletic performance as well[1–6]. It contains a number of different substances, including steroidal saponins. Protodioscin, the most dominant saponin in *Tt*, is considered to be the main substance responsible for increasing testosterone production[7].

The sex ratio of *Poecilia* fish has great significance in aquaculture because uncontrolled reproduction of this group in production ponds is one of the most serious limitations in *Poecilia* culture. Males grow faster than females[8,9]. Therefore, the maintenance and breeding of male populations have generated interest in terms of commercial applications. *Poecilia* fish can be masculinized by direct synthetic hormonal treatment that is efficient and straightforward[10–12]. However, synthetic hormones are more expensive than local plant extracts; also their administration in fish is time-consuming and labour-intensive and requires expertise. Further, synthetic hormones accumulate in the sediment water and aquatic biota[13,14]. There is no report on the accumulation of protodioscin in the sediment water or on the toxicity of *Tt* in fish. An alternate technique for commercially producing all-male fish populations would be to use plant extracts. Therefore, the present study was undertaken to investigate the effect of *Tt* on sex reversal to produce all-male population and enhance growth rate in *Poecilia latipinna*.

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Tt collected from natural habitats during flowering were air-dried. After evaporation of the solvent, the residue was powdered (250 g) and extracted with 500 ml 70% ethanol[15]. The prepared crude extract was used for the entire experiment as a stock solution. Sexually mature male and female fishes were obtained from a local ornamental fish dealer and maintained in the aquarium separately. They were reared under laboratory temperature at 28 ± 2°C and normal illumination (approx. 12 h light and 12 h dark) with the ration of pieces of earthworm twice a day. The newly born young ones were separated from their respective mothers and maintained in a 20 litre tank. A total of 540 fry were separated into six equal treatment groups (30 fry/aquarium, three aquaria/treatment). For each dose triplicates of experimental groups were maintained. The young ones were fed with commercial flake food throughout the experimental period of 2 months. As *Tt* is insoluble in water, the immersion treatment was used as an alternative to the more common oral/diet application method. The immersion method also ensures synergic induction, cheaper than the dietary treatment and requires almost no skill[10]. The effect of different concentrations [0 (control); 10, 15, 20, 25 and 30 ppm (experimental)] of *Tt* on the sex ratio and growth rate was studied in *P. latipinna*. The aquarium system was static and bathing medium was changed once a week with the same concentration of *Tt*.

After the experiment, two-month-old fish were counted to assess the whole body weight and length. Exactly 2 g of fish tissue from each fish sample was separately prepared, extracted and cleaned according to Verdeke[16]. *P. latipinna* representing all the treated groups and control fishes was analysed using chemiluminescent immunoassay analytical instrument (courtesy, Thyrocare Analytical Laboratory, Mumbai). Testosterone (ng/dl), estrogen (pg/ml), progesterone (ng/ml), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (mIU/ml) were measured[17].

Differences between groups in terms of sex ratio of the offspring were determined on the basis of secondary sex characteristics and analyses by the chi-square (χ^2) test[18]. Differences in body weight, length and hormone levels were analysed by one-way analysis of variance (ANOVA) using the Statistical Package for Social Sciences (SPSS) software package for Windows (version 16.0). Posthoc testing was performed for inter-group comparisons using the least significant difference (LSD) test. $P < 0.05$ was considered statistically significant.

Figures 1 and 2 show the growth in terms of total body length and body weight of both control and *Tt*-treated *P. latipinna*. No mortality was observed in both treatment and control groups. All groups of *Tt*-treated fishes exhibited a successful growth acceleration compared to the control group, but only the 30 ppm *Tt* treatment significantly increased the growth rate of male and female *P. latipinna*. In the last series (30 ppm) of the experiment, total body length and weight (male 3.28 ± 2.48 cm, female 2.88 ± 1.72 cm; and male 4.69 ± 4.94 g; female 5.24 ± 5.91 g respectively) were significantly increased compared to the controls (male 1.76 ± 0.37 cm, female 1.70 ± 0.89 cm; and male 1.49 ± 1.62 g, female 2.14 ± 3.45 g respectively). This indicates that *Tt* has no negative effect on the survival rate of *P. latipinna*, but it has the ability to increase total body weight and length at the treated concentrations.

In the present study, 97% masculinization was achieved in *P. latipinna* by immersing 0-day-old fry for 60 days in water containing 50 ppm *Tt* per litre. All the fish groups treated with various doses of *Tt* showed marked masculinizing effects during the 60-day experimental period. At the termination of the experiment, this effect had resulted in a statistical difference in the sex ratio compared to the sex ratio of fry in the first series of the experiment. In the control group, sex ratio was 40 : 60 (male : female) in the 10 ppm exposed group; 40 : 60 in the 15 ppm exposed group; 60 : 40 in the 20 ppm exposed group; 65 : 35 in the 25 ppm exposed group; 70 : 30 and 80 : 20 in the 30 ppm exposed group; 85 : 15 in the 40 ppm exposed group, and 97 : 3 in the 50 ppm exposed group respectively (Figure 3). Thus it is discernible that increasing concentrations of *Tt* caused an increase in the number of males produced.

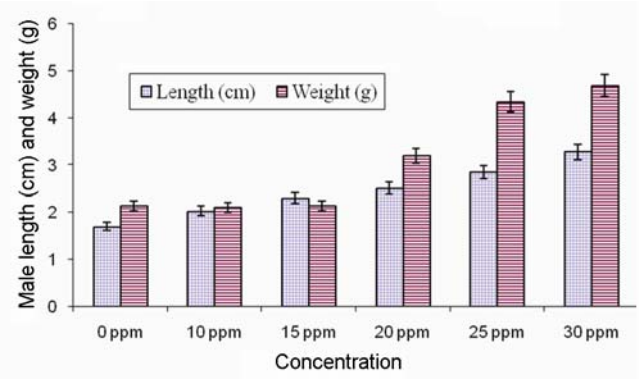

Figure 1. Length and weight relationship of *Tribulus terrestris*-treated male *Poecilia latipinna* after 60 days. Values are expressed as mean ± SE. Significant at $P < 0.05$.

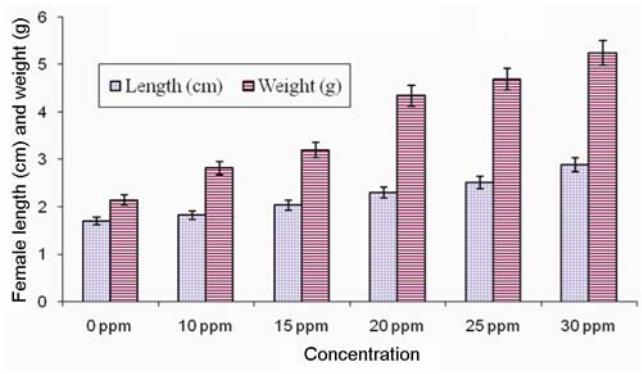

Figure 2. Length and weight relationship of *Tt*-treated female *P. latipinna* after 60 days. Values are expressed as mean ± SE. Significant at $P < 0.05$.

The relevance of sex ratio was observed by performing hormonal assay. Different concentrations of *Tt* extract were found to alter the hormone level in *P. latipinna*.

In control male and female fishes, the testosterone level after 60 days was 5.640 ± 5.943 and 4.845 ± 1.360 ng/dl respectively, whereas 10 ppm *Tt* extract-treated fish showed testosterone level of 5.656 ± 0.145 and 2.022 ± 0.006 ng/dl respectively; for 15 ppm it was 6.806 ± 0.082 and 4.093 ± 0.268 ng/dl; for 20 ppm, 7.848 ± 0.147 and 5.710 ± 8.954 ng/dl; for 25 ppm, 9.720 ± 0.115 and 9.240 ± 0.145 ng/dl, and for 30 ppm, 4.434 ± 4.900 and 2.022 ± 0.006 ng/dl respectively. These results show that the level of testosterone was high in herbal extract-treated fish compared to the control (Figure 4). However, beyond the optimum level (25 ppm), it showed a decline (e.g. for 30 ppm).

In control male and female fishes, the estrogen level after 60 days was 1.269 ± 0.021 and 6.492 ± 0.036 pg/ml respectively, whereas in 10 ppm *Tt* extract-treated fish it was 1.493 ± 0.075 and 2.988 ± 0.064 pg/ml respectively; for 15 ppm, it was 7.376 ± 0.029 and 8.285 ± 0.071 pg/ml; for 20 ppm, 7.935 ± 0.129 and 9.890 ± 0.069 pg/ml; for 25 ppm, 8.080 ± 0.053 and 9.768 ± 0.039 pg/ml, and for 30 ppm, 1.417 ± 0.184 and 2.527 ± 0.098 pg/ml respectively. These results show that the level of estrogen was higher in the herbal extract-treated fish compared to the control (Figure 5). Estrogen level elevation was also dose-dependent until it reached an optimum (25 ppm); then it declined (30 ppm).

In control male and female fishes, the progesterone level after 60 days was 2.792 ± 0.009 and 2.916 ± 0.021 ng/ml respectively, whereas in 10 ppm *Tt* extract-treated fish it was 0.124 ± 0.008 and 2.694 ± 0.005 ng/ml respectively; for 15 ppm it was 0.063 ± 0.006 and 2.196 ± 0.054 ng/ml; for 20 ppm, 0.055 ± 0.008 and 1.046 ± 0.006 ng/dl; for 25 ppm, 0.055 ± 0.008 and 0.075 ± 0.008 ng/ml, and for 30 ppm, 0.027 ± 0.003 and 0.055 ± 0.008 ng/ml respectively. These results show that the level of progesterone was lower in herbal extract-treated fish compared to the control (Figure 6).

In control male and female fishes, the FSH level after 60 days was 3.860 ± 0.021 and 6.490 ± 0.120 mIU/ml

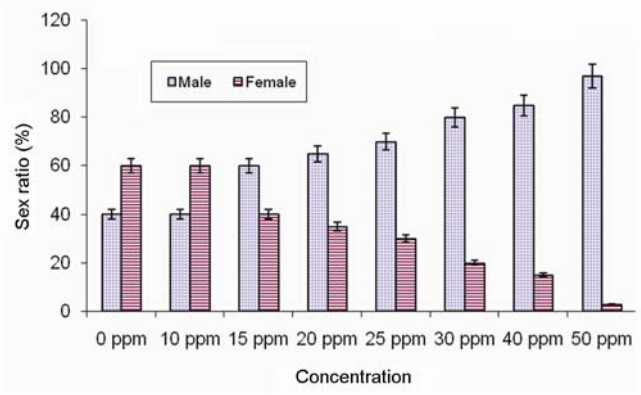

Figure 3. Sex ratio significantly different from the expected 1 male : 1 female ($P < 0.001$, χ^2 test), *Tt*-treated *P. latipinna* (after 60 days).

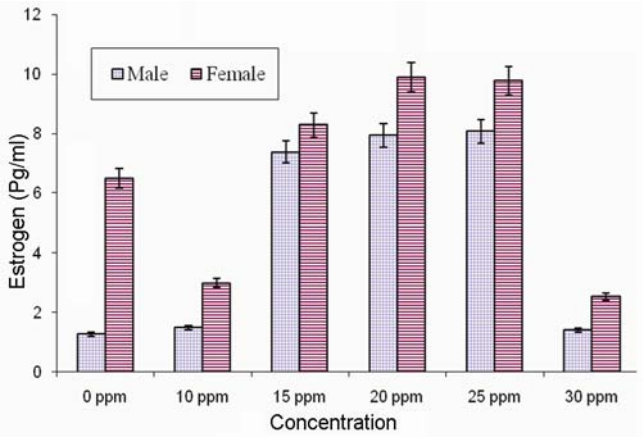

Figure 5. Estrogen level for different concentrations of *Tt*-treated *P. latipinna* after 60 days. Values are expressed as mean ± SE. Significant at $P < 0.05$.

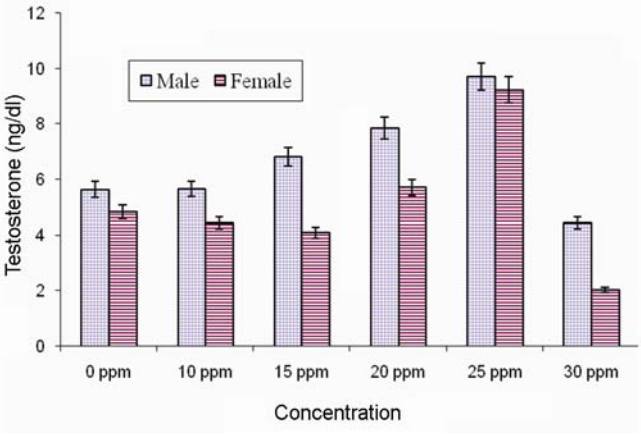

Figure 4. Testosterone level for different concentrations of *Tt*-treated *P. latipinna* after 60 days. Values are expressed as mean ± SE. Significant at $P < 0.05$.

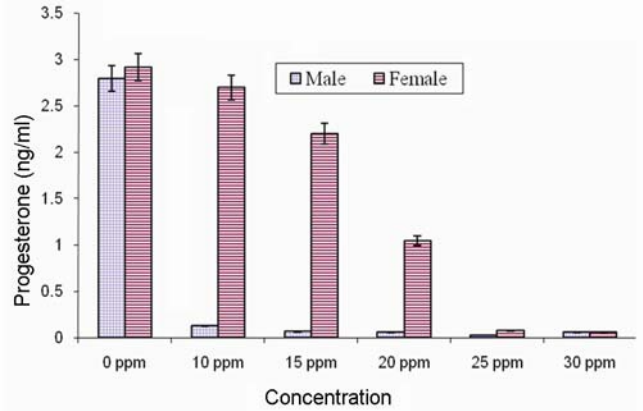

Figure 6. Progesterone level for different concentrations of *Tt*-treated *P. latipinna* after 60 days. Values are expressed as mean ± SE. Significant at $P < 0.05$.

respectively, whereas in 10 ppm *Tt* extract-treated fish it was 2.540 ± 0.038 and 2.926 ± 0.101 mIU/ml respectively; for 15 ppm it was 3.388 ± 0.026 and 8.782 ± 0.029 mIU/ml; for 20 ppm, 1.936 ± 0.020 and 8.692 ± 0.097 mIU/ml; for 25 ppm, 1.430 ± 0.049 and 1.602 ± 0.095 mIU/ml, and for 30 ppm, 1.380 ± 0.104 and 1.620 ± 0.021 mIU/ml respectively. These results show that the level of FSH was lower in herbal extract-treated male compared to the control male (Figure 7). On the other hand, FSH increased until 20 ppm and then declined in herbal extract-treated female fish when compared to the control female.

In control male and female fishes, the LH level after 60 days was 0.174 ± 0.005 and 0.346 ± 0.013 mIU/ml respectively, whereas in 10 ppm *Tt* extract-treated fish it was 0.220 ± 0.017 and 0.512 ± 0.026 mIU/ml respectively; for 15 ppm it was 1.587 ± 0.925 and 1.302 ± 0.456 mIU/ml; for 20 ppm, 3.864 ± 0.005 and 0.122 ± 0.017 mIU/ml; for 25 ppm, 0.280 ± 0.003 and 0.452 ± 0.009 mIU/ml, and for 30 ppm, 0.122 ± 0.017 and 0.300 ± 0.018 mIU/ml respectively. These results show that the level of LH was higher in herbal extract-treated fish compared to the control (Figure 8).

The aim of the present study was to find an effective masculinization method for sex reversal and growth performance in fancy fish culture. *Tt* has been found as an effective tool in gonadal development, maturation and spawning of *P. latipinna*. *Tt* extract was effective at various dose levels in increasing the proportion of males in the population, advancing hormonal level and improving growth performance in *P. latipinna*. Development of testes in the control group was normal to its age and size, whereas the *Tt*-treated male fish showed relatively larger gonads compared to the control group fish. In contrast, groups treated with various doses of *Tt* showed increased masculinizing effect, and their testes showed advance development. The improved growth and male sex population have been previously noticed in *Poeciliata reticulata* and cichlid *Cichlasoma nigrofasciatum*[14].

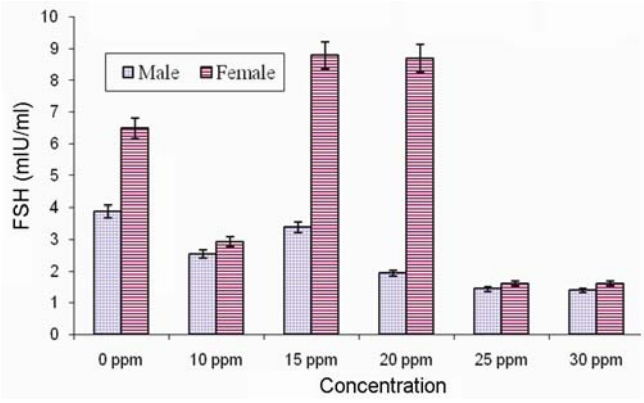

Figure 7. Follicle-stimulating hormone level for different concentrations of *Tt*-treated *P. latipinna* after 60 days. Values are expressed as mean ± SE. Significant at $P < 0.05$.

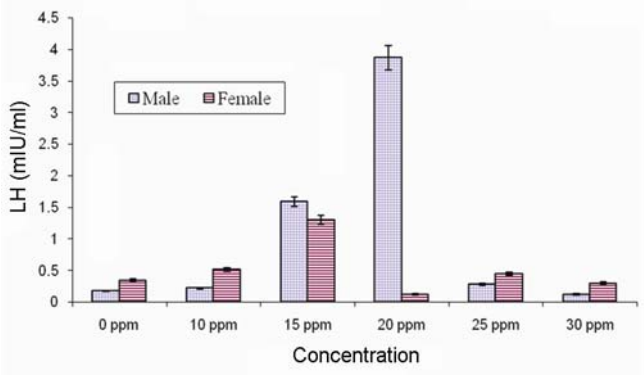

Figure 8. Luteinizing hormone level for different concentrations of *Tt*-treated *P. latipinna* after 60 days. Values are expressed as mean ± SE. Significant at $P < 0.05$.

Adimoelja[2] and Adaikan *et al.*[5] have presented evidence to show that *Tt* is non-toxic to humans and rabbits respectively. Kavitha and Jagadessan[19,20] studied the role of *Tt* extract on mercury-intoxicated mice, *Mus musculus*. A lethal dose of mercuric chloride was administered through drinking water to female mice every day for 45 days. Its toxicity altered the histoarchitecture of the large intestine. During the recovery period, the mice were dosed with a *Tt* extract of different solvent fractions for 15 days and a complete regeneration of the large intestine from the toxic effect of the mercury was found[19,20]. The present study also provides evidence that *Tt* treatment results in a higher rate of masculinization. In order to ensure the root cause for this potency, the levels of reproductive hormones such as testosterone, estrogen and LH were quantified and it was found that all these hormone levels were higher in herbal extract-treated fish compared to the control.

Although the present study provides evidence that *Tt* treatment results in high rate of masculinization, whether this potency is caused by increase in testosterone hormone levels is not clear. However, the present results are consistent with those reported for fish species such as *Onchorynchus tshawtscha*, *Cyprinus carpio* and *C. nigrofasciatum*, all of which were treated with synthetic hormone[11,21,22].

Gauthaman *et al.*[6] demonstrated the aphrodisiac properties of *Tt* in normal and castrated rats. They showed that the *Tt* extract increases testosterone levels in rats. In the present study, the growth rate of fish treated with *Tt* was faster than that of the control. Similar effect of *Tt* extract on body weight gain of immature sheep was reported by Georgiev *et al.*[23], and in rat by Gauthaman *et al.*[6]. Both research groups reported an increase in body weight, sexual activity and hormone level. Although these findings are not contradictory to the present results, there is a lack of information in the literature on the effects of a plant extract on sex reversal, gonad development and growth performance in a fish species. The *Tt* extract was ineffective in producing 100% males in the given dose. Therefore, higher dose may lead to production of all-male *P. latipinna* population. In the present

study, 80% male population was achieved in *P. latipinna* by treatment with *Tt* extract. *Tt* treatment is a more effective method than the administration of synthetic hormones in terms of effecting sex reversal; it is more environment-friendly and economically viable. In addition, the persistence and fate of any synthetic hormones and hormone metabolites in fish, water and sediment may represent potential environmental and health risks that have to be considered when using hormonal sex control technology[13,14]. *Tt* can be applied with ease to a large number of individuals simultaneously.

In the present study, *Tt* administered at various concentrations was found to enhance the hormone levels. *Tt* induced hormone (testosterone, estrogen and LH) production, which could possibly be due to the presence of steroidal glycosides, among them is protodioscin (PTN), one of the major active principles in *Tt*. It may act either by binding to hormone receptors or to enzymes that metabolize hormones. Most biological actions of plant-derived compounds function by the above mechanisms[24].

Tt has been traditionally used as an aphrodisiac and enhancer of sperm production and more recently, as an alternative to hormone replacement therapy in ageing men and women[25]. The different concentrations of *Tt* extract altered the hormone levels. LH secreted by the basophil cells of the anterior pituitary controls testosterone production from Leydig cells through negative feedback of the hypothalamic–pituitary–adrenal loop. Therefore, the *Tt* steroid saponins influence androgen biosynthetic pathways, directly or indirectly, increasing LH concentration[17].

Tt has been used as an alternative to produce all/more male population of *P. latipinna.* Edible fishes treated with synthetic hormones are usually rejected, and an alternative method for higher growth as well as producing monosex populations is required. Thus *Tt* would be a good choice.

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