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Effects of immersion based Proanthocyanidin treatment on the masculinization of betta fish (*Betta splendens*)

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Abstract
Betta splendens, commonly known as the Siamese fighting fish, is a highly esteemed ornamental species noted for its vibrant coloration and gentle disposition. Given rising concerns regarding the ethical and environmental implications of synthetic hormones for sex reversal, this study explored the use of an eco-friendly, plant-derived aromatase inhibitor Proanthocyanidin, extracted from grape seeds for sex manipulation via immersion techniques. Fish underwent discrete immersion treatments at concentrations of 250, 500, and 750 µg/l for three hours daily on the third, fifth and eighth day post-hatching, while 17α-methyltestosterone (17α-MT at 40 mg/kg) was used as a positive control throughout both experimental phases. Results indicated that the 750 µg/l Proanthocyanidin treatment yielded the highest male conversion rate (70.37%) along with an impressive post-treatment survival rate of 90.00%, compared to a survival rate of 76.66% in the 17α-MT control group. Histological analyses further revealed a suppression of atretic oocyte formation and an increased presence of immature oocytes in both the 17α-MT and high-dose Proanthocyanidin (750 µg/l) groups.

Keywords: Siamese fighting fish, Proanthocyanidin

Introduction
The ornamental fish market reached a valuation of USD 5.4 billion in 2021 and is projected to grow at a compound annual growth rate (CAGR) of 8.5% from 2022 to 2030 (Shraborni *et al.*, 2024) [41]. In this market, tropical freshwater fish held the largest revenue share at 51.7% in 2021, with species such as guppy (*Poecilia reticulata*), molly (*Poecilia sphenops*) and zebra fish (*Danio rerio*) being particularly popular among beginners due to their low cost typically ranging from $1 to $6. Marine ornamental species, noted for their vivid colors and engaging behaviors, account for nearly 15% of all traded fish and are predominantly harvested from natural habitats. Combined with the appeal of aquarium photography, the practice of keeping ornamental fish in home aquaria or garden ponds has evolved into a globally cherished hobby, earning these species the label "living jewels" because of their diverse coloration, structure, and behavior, and contributing to a rapid increase in their international trade; it is estimated that over 20 million marine ornamental fish are collected annually and sold to more than 2 million aquarium enthusiasts worldwide (Duffy, 2018; Hoseinifar *et al.*, 2023) [12, 16].

Sex manipulation in fish involves artificially altering their sexual characteristics through techniques such as hormonal treatments or genetic modifications. This approach is significant because it enables the production of all-male or all-female populations, which can substantially enhance productivity and profitability in aquaculture and fisheries management. Although a range of steroids has been effectively used to manipulate fish sex, factors such as the treatment method, dosage, and the developmental stage of the fish critically influence the outcome. For instance, initiating sex differentiation in larvae when they reach approximately 9 mm in size and maintaining a treatment period of about six weeks is generally recommended (Widyawati *et al.*, 2021) [47]. Moreover, the efficacy of sex-reversal procedures is contingent on hormone dosage, mode of administration, treatment duration,

and the species under study. In addition to hormonal interventions, adjustments in photoperiod and temperature can be employed to either postpone or encourage maturation, thereby playing an essential role in reproductive control. Throughout these processes, observation and histological studies are crucial to evaluate changes in sex ratios and a variety of strategies including sex reversal, manual sexing, hybridization, and the production of super males are utilized to achieve the desired sex composition (Katare *et al*., 2017) [22]. Notably, a Letrozole immersion treatment at 500 µg/l has previously achieved 100% masculinization in Siamese fighting fish (*Betta splendens*) (Katare *et al*., 2015) [21].

The Siamese fighting fish, commonly referred to as bettas, are indigenous to Southeast Asia and have become a staple of the aquarium trade owing to their brilliant coloration and distinctive aggressive behavior among males. The species, designated as *Betta splendens*, is celebrated not only for its aesthetic appeal but also for the high market value of its males in ornamental breeding. There is a growing trend toward breeding these colorful males for both local sales and export, with key traits such as color intensity, pattern uniformity, scale iridescence, body conformation and fin size being highly prized by enthusiasts. Shifting the focus from traditional fighting fish to ornamental bettas is not only ethically favorable but also commercially advantageous, given that the latter commands higher profit margins in international markets. In the context of mass export, optimal strategies—including maintaining water quality in breeding environments, providing high-quality feed, implementing robust disease prevention measures, improving transport conditions and securing certification are imperative. While wild-caught varieties are in high demand as breeding stock for fighting bettas, it is the captive-bred individuals that contribute significantly to the diversity of ornamental traits found in commercial bettas (Mazur, 2005; Sermwatanakul, 2019) [29, 40].

Aromatase inhibitors (AIs) offer an alternative approach to conventional steroid treatments by impeding the conversion of naturally occurring androgens into estrogens, thereby promoting male differentiation without the introduction of external steroids. This mechanism generally results in higher levels of male development compared to treatments with steroidal compounds such as 17α-methyltestosterone (17α-MT) (Kwon *et al*., 2000) [27]. Beyond aquaculture, AIs have been effectively employed in the treatment of breast cancer in postmenopausal women (Howell *et al*., 2005) [50] and have similarly induced male phenotypes in birds (Elbrecht and Smith, 1992; Hudson *et al*., 2005) [13, 17] as well as reptiles (Wibbels and Crews, 1994; Belaid *et al*., 2001) [46, 7]. In several teleost species including tilapia (Kwon *et al*., 2000; Afonso *et al*., 2001; Das *et al*., 2012) [27, 1, 10], Salmon (Piferrer *et al*., 1994), Japanese flounder (Kitano *et al*., 2000) [25], Zebra fish (Fenske and Segner, 2004) [14] and Guppy (Basavaraja *et al*., 2012) [6] successful production of all-male or male-biased populations has been documented using AIs.

Several phytochemical dietary supplements, such as soya isoflavones, tea seed cake, white button mushroom and grape seed extract, have been identified as potential aromatase inhibitors (Kaur *et al*., 2009) [23]. Proanthocyanidin, the principal bioactive compound in grape seeds and skins, is primarily responsible for this anti-aromatase activity (Hur *et al*., 2005; Palomares *et al*., 2006) [19, 33]. In mice, grape seed extract has been shown to inhibit aromatase activity in a dose-dependent fashion, and its high procyanidin B dimer content further supports its role as an aromatase inhibitor in humans (Kijima *et al*., 2006; Palomares *et al*., 2006) [24, 33]. Additionally, plant-derived aromatase inhibitors such as Proanthocyanidin have been reported to delay gonadal maturation in rohu (Charan *et al*., 2014) [9] and to induce masculinization in GIFT tilapia (Unnikrishnan, 2018) [44]. Therefore, incorporating cost-effective grape seed extract into aquaculture practices could significantly boost profitability and add value when producing monosex ornamental fish populations.

Materials and Methods

The present study was carried out in Fisheries Research and Information Centre (Inland), KVAFSU, Hesaraghatta, Bengaluru. Siamese fighting fish (Betta splendens) were maintained at Fisheries Research and Information Centre (Inland), Hesaraghatta, Bengaluru. The fishes were fed with high protein diet to raise them to broodstock in the 4m2 pre fertilized pond. The gravid female and male (identified based on their external characteristics such as body colour and shape) were selected for breeding and fry production (Ogden, 2020) [32]. The brood fish were released into the aquaria in the morning and spawning started a few hours later. The eggs released by the female were immediately fertilized by the male. Eggs that strayed away were collected by the male and placed in the nest and this process continued until the hatching was completed. The spawning session lasted for two to four hours. Upon completion of spawning process, the female was transferred to a separate tank and male continues to guard the eggs until they hatch. The eggs hatched in about 24-28 hours. The fry remained in the bubble nest. In about 3 days, the fry became free swimming and left the nest.

Immersion Treatment

The yolk-sac absorbed fry were removed from the breeding aquaria tank, counted and transferred to immersion tanks, Treatment tanks were kept inside the field laboratory to avoid temperature fluctuation. A total of 270 randomly selected Siamese fighting fish fry were equally divided into 3 groups (30 fry/treatment) with each triplicate groups. The fry was subjected to discrete immersion treatment with a natural AI, Proanthocyanidin for 3 hrs daily on third, fifth and eight days after hatching.

The experiment was carried out in three treatment, control and positive control in triplicates. 30 Nos of betta fry were given a discrete immersion treatment of proanthocyanidin with control and positive control (Table 1). The experimental glass aquarium tanks were prepared with three doses of proanthocyanidin at 250 µg/l (IT_1), 500 µg/l (IT_2), 750 µg/l (IT_3) of water with control (CT_0) and positive control by using 17α-Methyl Testosterone at a concentration of 1000 µg/l (PT_0) (Akhtar, 2022). After completion of every immersion treatment duration, the fry were transferred to separate aquaria and fed with infusoria, for five times a day. Similarly, on fifth and eight day of fry treatment, fry were transferred from aquaria to plastic bowls and transferred back to aquaria after 3 h of treatment. After the end of immersion treatment, the fry were transferred to FRP tanks for the growth study for period of 58 days. During this period fishes were fed with commercial diet. Temperature was recorded (25-27 °C) during treatment period and no

feed was given during treatment period and after that the fry were transferred to green water for growth and survival study.

Table 1: Dose of proanthocynidin used in the immersion treatment

Treatments	Plant extraction	Incorporation level (µg/l)
CT_0 (control)	Basal Diet	-
PT_0 (Positive Control)	17α-Methyl Testosterone	1000
IT_1	Proanthocyanidin	250
IT_2	Proanthocyanidin	500
IT_3	Proanthocyanidin	750

Assessment of sex and treatment evaluation

After 90 days post-hatching, Siamese fighting fish (*Betta splendens*) fry (Full Moon Betta) were sexually matured, all the surviving fish were harvested and the proportion of males and females was determined. Mean length, weight and survival of the fish in each treatment were recorded. The phenotypic sex of the treated and control fish was determined based on secondary sexual characters such as the shape of the belly, body colouration and finnage (Katare, 2015) [21]. Fishes were euthanized with an anesthetizing agent, quinaldine (80-100 mg/l) for further studies of histology of gonads. A total of five fish (3 and 2 from each replicate) were randomly selected from each treatment of *Betta splendens*. Fish with recognizable ovarian and testicular portion were classified as female and male respectively and the gonads having both testicular and ovarian tissue were scored as inter-sex (Wang, 2018) [45].

Growth and survival

After completion of the experiment, total number of survived fishes were recorded and FCR, PER and SGR were analyzed. Water quality is monitored throughout the study period**.** The survival rate (%) after the completion of the experiment was recorded. The growth performances were calculated using standard formulae as follows.

$$\text{SGR}(\%) = \frac{[\text{Ln}(\text{Final weight}) - \text{Ln}(\text{Initial weight})]}{\text{No. of days}} \times 100$$

$$\text{FCR} = \frac{\text{Dry weight of the feed given}}{\text{Gain in wet weight of fish}}$$

$$\text{PER} = \frac{\text{Gain in body}}{\text{Protein intake}}$$

Histology

To study the impact of Proanthocyanidin and 17α-MT on the gonads of the fish of both the strains, gonads were collected, fixed in 10% neutral buffered formalin followed by cassetting, processing, embedding, sectioning (sections of 5 µ were cut using a microtome and fixed on slides), staining (Hematoxylin and Eosin), microscopy and photography (Gray,1964).

Statistical analysis

Survival and GSI of fishes were analysed statistically using one way ANOVA. Chi-square test was used to analyse the significance of any deviations from a 1:1 sex ratio. For comparison of different treatment means, Duncan's multiple range test was used (SPSS windows 21.0).

Results

The effect of Proanthocyanidin on sex ratio is presented in Table 2. The sex ratio of negative control deviated (1:1.30) from the expected 1:1 ratio. In the treated group, highest male percentage was recorded in the treatment IT_3 (70.37%) followed by IT_2 (54.03%) and IT_1 (36.62%) and it was found that percentage of males increased with increase in the dose of Proanthocyanidin. In contrast, 17α-MT at 1000 µg/l induced higher masculinization effect of 78.26% males (p<0.05). The average percentage of females decreased (63.37, 45.92 and 29.62%), with an increase in dosage of Proanthocyanidin at 250, 500 and 750 µg/l, respectively, barring 500 µg/l where it was similar with that of control. The minimum proportion of females was observed in 17α-MT group. Average survival of fry of Siamese fighting fish (*B. splendens*), during treatment (after each treatment on 3rd, 5th and 8th day) period is presented in Table 3 and depicted in Fig. 1. Average survival percentage during treatment period was maximum (100, 97.76 and 92.20% in the 3rd, 5th and 8th dph respectively) in the treatment IT_3 (750 µg/l) and minimum (100, 91.10 and 82.23% in the 3rd, 5th and 8th dph respectively) in the 17α-MT (1000 µg/l). Survival of Siamese fighting fish (*B. splendens*) during post-treatment (60 dph) presented in Table 6 and depicted in Fig. 2. Average survival percentage of *B. splendens* was highest (90.00%) in IT_3 and lowest (76.66%) in PT_0. Among the Proanthocyanidin treatments, lowest survival (78.90%) was obtained in IT_1 Proanthocyanidin treatment group, compared to 87.76% survival in control group.

Table 2: Effect of Proanthocyanidin on sex composition in *Betta splendens*

Treatment	Avg No of fish recovered	Males	Females	Sex ratio (M: F)	Chi-square value
CT_0 (Control)	26.33±0.3 (87.76)	12.33±0.32 (46.82)	14.00±0.58 (53.17)	1:1.13	0.105
PT_0 (Positive Control)	23.00±0.58 (76.66)	18.00±0.58 (78.26)	5.00±0.57 (21.73)	1:0.27	7.347
IT_1	23.67±0.88 (78.90)	8.67±0.33 (36.62)	15.00±0.57 (63.37)	1:1.73	1.692
IT_2	24.67±0.88 (82.23)	13.33±0.68 (54.03)	11.33±1.20 (45.92)	1:0.84	0.162
IT_3	27.00±0.58 (90.00)	19.00±0.56 (70.37)	8.00±1.00 (29.62)	1:0.42	4.481

Table 3: Average survival of fish, *Betta splendens* during the treatment period

Treatment	Initial	3dph	5dph	8dph
CT_0 (control)	30	30±0.0 (100)	28.67±0.3 (95.56)	27.67±0.3 (92.23)
PT_0 (Positive Control)	30	30±0.0 (100)	27.33±0.3 (91.10)	24.67±0.3 (82.23)
IT_1	30	30±0.0 (100)	28.67±0.3 (95.56)	26.33±0.3 (87.76)
IT_2	30	30±0.0 (100)	27.33±0.3 (91.10)	25.00±0.5 (83.30)
IT_3	30	30±0.0 (100)	29.33±0.3 (97.76)	27.67±0.3 (92.2)

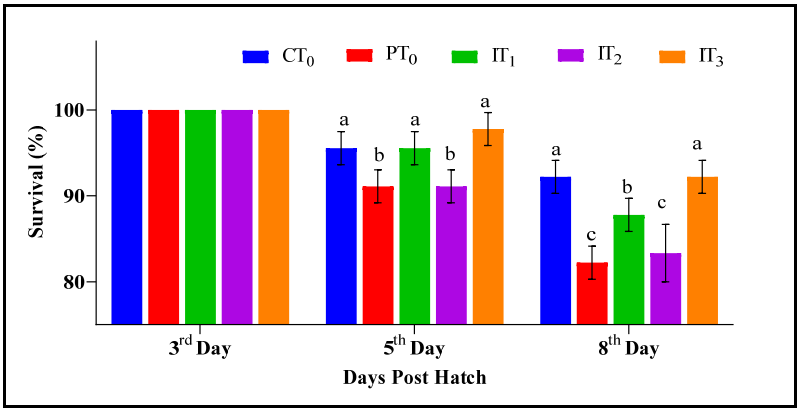

Fig 1: Survival (%) of *Betta splendens* fry during treatment period

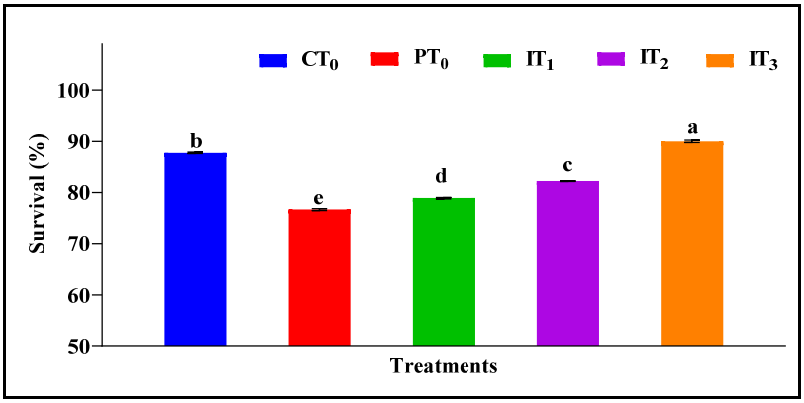

Fig 2: Average post-treatment percentage (%) survival in *Betta splendens*

The growth performance were depicted in table 3, and treatment IT_1 (2.66%) showed highest specific growth rate, followed by IT_3 (2.59%), PT_0 (2.44%), T_0 (2.42%) and IT_2 (2.35%). Feed conversion ratios was ranged from 1.71 to 1.89. Treatment IT_1 showed highest FCR (1.89) followed by PT_0 (1.85%), IT_3 (1.84%), IT_2 (1.73%) and T_0 (1.71%). Treatment IT_1 (4.58%) showed high protein efficiency ratio followed by IT_3 (4.44%), PT_0 (4.18%), T_0 (4.15%) and IT_2 (4.02%).

Table 3: Growth performance of *Betta splendens* recorded in different treatments and control group.

Parameters	Treatments				
	CT0	PT0	IT_1	IT_2	IT_3
SGR (% per day)	2.42±0.092	2.44±0.139	2.66±0.047	2.35±0.095	2.59±0.072
FCR	1.71±0.072	1.85±0.084	1.89±0.118	1.73±0.14	1.84±0.121
PER	4.15±0.156	4.18±0.234	4.58±0.075	4.02±0.163	4.44±0.126

The results of histological examination of ovary of Siamese fighting fish (*Betta splendens*). In *Betta splendens*, the ovary of control group showed different stages of oogenesis having more mature oocytes, advanced oocytes consisting of large number of yolk granules and a germinal vesicle, mature oocytes showed a substantial yolk deposition. In case of 17α-MT (1000 µg/l) treatment group and 750 µg/l Proanthocyanidin treatment group, few mature oocytes and more immature oocytes, perinucleolar stage, early vitellogenic and atretic oocytes were observed in Fig 3 and 4.

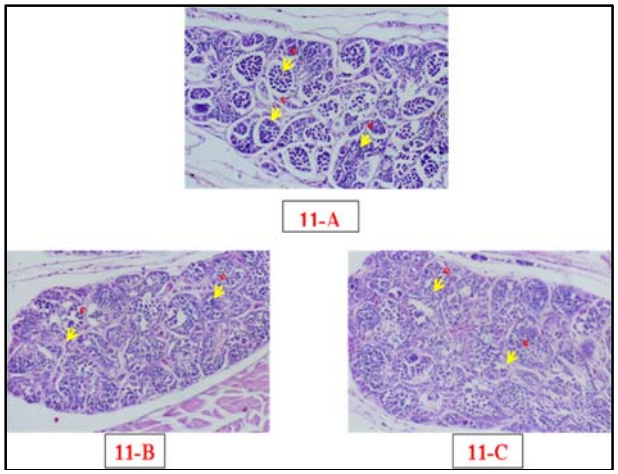

Fig 3: Photomicrograph of *Betta splendens* testis during (Immersion treatment): Control (11-A), 17α-MT (11-B), and PROANT-750 µg/l (11-C) treated fish showing different stages of spermatogenesis like; spermatogonia (Sg), spermatids (St), spermatocytes (Sc) and spermatozoa (Sz).

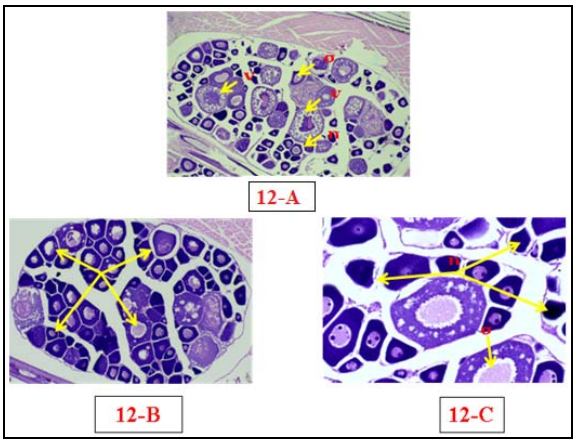

Fig 4: Photomicrograph of *Betta splendens* ovary during (Immersion treatment): Control (12-A), 17α-MT (12-B) and PROANT-750 µg/l (12-C) treated fish showing different stages of oogenesis; mature oocyte (Mo), yolk granule (Yg), yolk vesicle (Yv), perinuclear stage (Pn), atretic oocytes (Ao), previtollogenic stage (Pv) and germinal vesicle (Gv).

Discussion

One of the common and widely used approach to produce an all-male population of fish is the use of synthetic male hormone i.e 17α-MT. The impact of proanthocyanidin treatment on sex reversal in fish was aimed to determine if these compounds can influence the hormonal pathways that control sex differentiation and development. In the present study, 17α-MT at 1000 µg/l (used as a positive control) following discrete immersion treatment on third, fifth and eight days after hatching induced significantly higher percentage of males than negative control and Proanthocyanidin treatment groups. In *Betta splendens*, 78.26% male progeny was obtained in positive control. The discrete immersion treatment of 17α-MT at 1000 µg/l for constant duration (3 h/day) on third, fifth and eight days after hatching induced 84.29% male population without any body and fin abnormalities in *Betta splendens* (Katare *et al.*, 2017) [22]. Proanthocyanidins, due to their antioxidant and potential endocrine-modulating effects, have been proposed as potential candidates for inducing sex reversal in fish. The compounds may interact with the estrogenic and androgenic pathways, influencing the expression of genes involved in sex differentiation and gonadal development (Paniagua-Chavez *et al.*, 2020) [36]. Hormonal treatments like methyltestosterone are effective in inducing sex reversal, but they may have significant side effects, including skewing the sex ratio beyond what is desired, potential impacts on the fish's reproductive health, and environmental concerns if they leach into aquatic ecosystems (Scholz, S and Klüver, N, 2009) [39]. Proanthocyanidin treatment represents a novel, potentially safer and more sustainable approach to sex reversal in fish compared to traditional hormonal treatments. While its mechanisms of action are not yet fully understood, PACs offer promise as a less invasive alternative to inducing sex change, especially for species where sex ratio manipulation is important for aquaculture or conservation purposes. However, the efficacy and practicality of PACs for sex reversal still require further investigation to optimize treatment conditions and confirm their long-term viability (Xu *et al.*, 2017) [48].

The present study is the first of its kind on sex manipulation in *Betta splendens* with a natural aromatase inhibitor, Proanthocyanidin (from grape seed extract), reporting the use of natural non-steroidal aromatase inhibitor for sex manipulation. In general, the optimum dose required to induce complete sex reversal is species-specific and in some species, strain-specific (*Cyprinus carpio:* European strain-Nagy *et al.*, 1981; Asian strain of common carp-Rao and Rao, 1983) [31, 38]. Increase in the percentage proportion of male clearly indicates that the higher treatment dose is necessary for effective masculinisation in *Betta splendens*. Proanthocyanidin at concentration 200, 300 and 400 ppm skewed significantly higher male (p<0.05) when fed for 30 days and Proanthocyanidin fed for 40 days induced significantly higher male sex ratio at 100, 200, 300 and 400 ppm in *O. niloticus* (Unnikrishnan, 2018) [44]. Cek *et al.* (2007) [8] achieved 80% masculinization by immersing in the water containing 0.15 g/l of the extract of *Tribulus terristralis* for 60 days in *Poecilia reticulata* (guppy) fish and 0.30 g/L for 60 days in *Cichlasoma nigrofasciatum*. Sex reversal depends not only on the concentration of hormone incorporated in the diet, but on the age of the fish, duration of the hormone treatment, rearing temperature, food and the species used *etc*. Wrong choice of treatment time/duration or setting of wrong parameters have also led to numerous unsuccessful attempts (Yamamoto, 1969) [49]. Pandian and Sheela (1995) [34] reported that the immersion alone has not ensured 100% sex reversal in most salmonids. In the present study, Proanthocyanidin treatment did not induce 100% masculinisation which may be attributed to lower dosage and duration of treatment. Other factors like potency of natural extract of grape seed may be less, compared to synthetic steroid hormone. Although a wide range of natural and synthetic steroids have been used successfully to induce sex manipulation, their individual biological activities vary. In general, the synthetic androgens were more potent than the natural androgens and synthetic steroids have greater effects due to longer activity *in vivo* (Hunter and Donoldson, 1983) [18].

In the present investigation, 17α-MT at 1000 µg/l showed significantly lower (p<0.05) survival (100, 91.10, and 82.23%) during discrete immersion treatment period on the third, fifth and eight day after hatching, respectively,

compared to control and proanthocyanidin treated groups in *B. splendens*. The present results clearly indicate negative effect of synthetic 17α-MT at higher dose on survival when compared to natural proanthocyanidin. Katare *et al.* (2017) [22] observed significantly lower survival (65%) compared to control group (81.66%) when immersion treatment of 17α-MT at 1000 µg/l was given to *B. splendens*. Basavaraja and Raghavendra (2017) [5] reported that significantly lower survival (63%) during treatment period compared to control group (99%), when 17α-MT was fed at 100 ppm for 30 days to 6 day old red tilapia. This clearly indicate negative impact on survival during treatment when steroidal 17α-MT is used for sex inversion.

Immersion treated groups recorded significantly higher survival in a dose dependent manner in *B. splendens*, the highest survival of 100, 97.76 and 92.20% at 750 µg/l (IT_3) compared to the control which had 100, 95.56 and 92.23% survival during treatment on 3rd, 5th and 8th day of treatment period respectively. Unnikrishnan (2018) [44] reported a dose dependent increase in average survival during treatment period when proanthocyanidin was employed at 100, 200, 300 and 400 ppm for 30 and 40 days in GIFT strain Nile tilapia and recorded highest survival of 81.40 and 92.5% at 400 ppm compared to control (71.45 and 84.15%) after 30 and 40 days of treatment period respectively. In the present study, in immersion treatment, the higher values of average weight, SGR and PER of *B. splendens* was recorded in the treatment IT_1 (0.24 gm) compared to other treatments and control groups whereas the FCR value was higher in IT_1 compared to other treatments and control groups. Comparable GSPE-containing meals produced outcomes in rainbow trout (*O. mykiss*) and *O. niloticus* (Arslan *et al.*, 2018; Mousavi *et al.*, 2020) [3, 30].

Sexual development is a complex, often species-specific process in fish. Therefore, technological innovations that successfully control sex in one species are certainly not guaranteed to work in another. Treatment should be initiated prior to the onset of normal sex differentiation and continued until the time of morphological differentiation, failure to comply with this criterion results in ineffectual treatment (Baroiller *et al.*, 1999) [4]. The timing and duration of an effective treatment regime plays an important role for effective masculinisation (Dean *et al.*, 2012) [11]. The paucity of studies and a general lack of understanding of the mechanisms involved in sex differentiation has resulted in considerable difficulty in establishing criteria suitable for the identification of the initiation of sex differentiation. Histological examination of fish gonads will provide visual guides to the initiation and completion of sex differentiation, so that the timing and duration of an effective treatment regime can be established (Johnson *et al.*, 2009) [20].

In the present study, the histology of ovary of control fish showed normal oocyte development comprising of maturing, matured oocytes, yolk vesicle, vitellogenic and post vitellogenic stage oocytes. Advanced oocytes contained a large number of yolk granules. Early sign of sex differentiation of gonads in some cichlids was reported by Youichi and Makito (2012), who observed sex differentiation of gonads appear to begin by day 25 post hatch. In tilapia, the first histological signs of sexual differentiation are apparent at around 15-17 days post hatch (Kwon *et al.*, 2000) [27]. Pandian and Varadaraj (1987) [35] found sex differentiation to take place between 11 and 19 days in *O. mossambicus*, where in golden rabbit fish (*Siganus guttatus*) its 30 dph onwards (Komatsu *et al.*, 2006) [26] and zebra fish from 15-40 dph (Uchida *et al.*, 2004) [43]. Mahadevi (2016) [28] observed similar observations in control (GIFT strain) ovary which consisted of developing oocyte in different stages with immature oocyte, yolk vesicle stage and mature oocytes, while advanced oocytes composed of a large number of yolk granules and migrating/migrated germinal vesicle. In the present study, ovaries of proanthocyanidin (750 µg/l, IT_3) group largely contained immature and perinuclear stage oocytes, confirming the delay or disruption in commencement of oocyte maturation in *Betta splendens*. In immersion treatment proanthocyanidin at 750 µg/l and 17α-MT at 1000 µg/l lead to slight increase in testicular volume and more spermatids and spermatozoa compared to control group. The testis of 17α-MT treated fish consisted of smaller seminiferous lobule with germ cell in different stages of development with mature testis of control fish showing mostly the spermatids and spermatogonia and transformation of spermatocytes to spermatids. Unnikrishnan (2018) [44] observed that the histological sections of testis did not show much change in testicular cell structures between control and Proanthocyanidin treated groups. The testis consisted of well-developed seminiferous tubules, lined with spermatocytes and filled with spermatozoa in fadrozole treated Blackeye goby.

Conclusion

This study demonstrates that immersion-based treatment with proanthocyanidin offers an effective and eco-friendly approach for inducing masculinization in *Betta splendens*. The treatment induced a clear dose-dependent shift towards male differentiation while maintaining high survival rates, suggesting that proanthocyanidin can serve as a sustainable alternative to conventional synthetic hormones. These findings not only provide valuable insights into the potential mechanisms of sex manipulation in ornamental fish but also pave the way for more environmentally responsible practices in aquaculture.

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