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Effects of phytoestrogens on sex reversal of Nile tilapia (*Oreochromis niloticus*) larvae fed diets treated with 17α-Methyltestosterone

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ABSTRACT

Two consecutive experiments were carried out to investigate the effects of phytoestrogens on sex reversal of Nile tilapia (*Oreochromis niloticus*) larvae. Experiment 1 investigated the effects of phytoestrogen contents of soybean meal (SBM) on sex ratio. Nile tilapia larvae (0.01 g/fish) were fed isonitrogenous (35% crude protein) and isocaloric (19 MJ/kg) diets containing fishmeal (FM) or SBM as protein sources, each with or without supplementation of 17α-methyltestosterone (MT) at 60 mg/kg diet, for 28 days. The control, SBM-based diet produced significantly higher percentage of females (77%) than the FM-based diet (52%). The addition of MT to SBM resulted in a significant reduction in percentage of females (31%), however, this percentage was significantly higher than that produced by FM-based diet treated with MT (only 3% females).
The second experiment was carried out to verify the results of the first experiment by investigating the effects of different concentrations of pure, crystalline genistein and daidzein (the major phytoestrogens present in soybean) on sex reversal of Nile tilapia larvae fed on a FM-based diet treated with MT. The same FM-based diet used in experiment 1 was used in experiment 2, and treated with MT, and then divided into equal portions treated with genistein or daidzein at 0, 10, 20 and 30 mg/kg diet. The diets were fed to Nile tilapia larvae for 28 days. The control, hormone-free diet produced almost equal ratio of females and males (0.96:1). The addition of MT significantly increased the percentage of males. The percent of males was sharply reduced with increasing daidzein and genistein concentrations in the diets. These results revealed that phytoestrogen contents of SBM have estrogenic effects on Nile tilapia larvae. Therefore, soybean meal and other plant protein sources containing high levels of phytoestrogens should be avoided as protein sources for Nile tilapia larvae during sex reversal treatments.

1. Introduction

Tilapia culture has sharply expanded throughout the world during the past two decades. Production systems of tilapia have also witnessed a rapid industrialization during this period. As a result, the global production of farmed tilapia has increased from 383,654 metric tons (mt) in 1990 representing 4.5% of total farmed fish production to 3,497,391 mt in 2010, representing 8.9% of total farmed fish production, with an average annual growth of 13.5% (FAO, 2012). This trend has created a gap between seed supply and farmer's demand. Therefore, the major challenge facing tilapia culture industry is the production of sufficient amounts of quality seed.

The production of monosex (all-male) tilapia played a significant role in facing this challenge, due to their fast growth rates, tolerance of a wide range of environmental conditions, resistance to stress and diseases, higher energy conservation, reduced aggressiveness and greater uniformity of size at harvest (El-Sayed, 2006). Therefore, extensive attention has been given to monosex culture of tilapia during the past two decades. Oral administration of 17α-methyltestosterone (MT) hormone is the most common and successful method used for producing all-male tilapia (Beardmore et al., 2001; El-Sayed, 2006; Penman and McAndrew, 2000). The hormone is generally incorporated into starter larval feed at 30–60 mg MT/kg feed, during the critical period of sex differentiation.

Soybean meal (SBM) has been widely used as a major protein source for farmed fish, due to its good protein content and essential amino acid (EAA) profile (El-Sayed, 1999, 2006; Robaina et al., 1995). The inclusion of SBM in fish diets depends on SBM source and processing methods, fish species and size and culture systems employed. However, SBM is limiting in sulfur containing AA-Met, Lys and Cys. It also contains many endogenous antinutrients including protease (trypsin) inhibitor, phytohaemagglutinin and anti-vitamins. Many of these factors can be destroyed or inactivated during thermal processing (Francis et al., 2001).

Phytoestrogens are another antinutrient found in SBM, which may affect reproductive development and sex differentiation in fish. They are plant-derived substances with estrogenic activity (Francis et al., 2001). The major phytoestrogens contained in soybeans are genistein and daidzein (Pelissero et al., 1991a). These phytoestrogens may

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either have the same effects as estrogen or block estrogen's effects (Bennetau-Pelissero et al., 2001; Green and Kelly, 2009; Monteiro et al., 2000), depending on the ratio of phytoestrogens to endogenous estrogens, aromatase activity, animal species and reproductive status, length of the exposure and method of administration (Andersen et al., 2003; Trant et al., 2001; Tsai et al., 2000).

Little information is available on the effects of phytoestrogens on fish, with varying results. Tzchori et al. (2004) found that genistein-treated diets increased the proportion of females in juvenile eel. Similarly, Bennetau-Pelissero et al. (2001) reported that rainbow trout fed on a diet treated with genistein showed a decrease in 11-ketotestosterone (11-KT) and testosterone levels before and during spawning. In support, Zhang et al. (2002) found that when Japanese rice fish *Oryzias latipes* was injected with genistein, testosterone production decreased, while estradiol production increased. The feminization effect of phytoestrogens on sex differentiation of African catfish *(Clarias gariepinus)* has also been reported by Yılmaz et al. (2009). Genistein, daidzein, and soybean-based diets also increased plasma vitellogenin concentrations in male and female rainbow trout (Bennetau-Pelissero et al., 2001), Siberian sturgeon (*Acipenser baeri*) (Pelissero et al., 1991b), juvenile striped bass (*Morone saxatilis*) (Pollack and Ottinger, 2003) and Mozambique tilapia (*Oreochromis mossambicus*) (Davis et al., 2009).

On the contrary of the above results, Kaushik et al. (1995) found that the replacement of fish meal by soy protein concentrate in juvenile rainbow trout diets did not result in any estrogenic effect. However, Ng et al. (2006) reported that genistein and other isoflavones found in soybeans inhibit hepatic and renal estrogen metabolism in Atlantic salmon (*Salmo salar*), and lake trout (*Salvelinus namaycush*). Davis et al. (2010) found also that plasma levels of vitellogenin were significantly reduced in Mozambique tilapia (*O. mossambicus*) fed SBM-based diets, with or without 11-KT treatment, compared to those fed FM diet.

These studies suggest that the estrogenic effect of phytoestrogens on fish is probably species specific, and these substances can act as estrogen agonists or antagonists to endogenous estrogens. However, more research is needed to support this assumption. Therefore, the present study was carried out to investigate the effects of phytoestrogen contents of soybean meal on sex reversal of Nile tilapia (*Oreochromis niloticus*) larvae.

2. Materials and methods

Two successive experiments were carried out in this study. The first experiment investigated the effect of phytoestrogens contained in SBM on sex reversal of Nile tilapia (*O. niloticus*) fry that were fed on a SBM-based diet treated with 17α–methyltestosterone (MT). The second experiment was conducted to verify the results of the first experiment, and evaluate the effect of different concentrations of pure, crystalline genistein and daidzein (the major phytoestrogens in soy beans) on sex reversal of Nile tilapia fry fed a FM-based diet treated with MT.

2.1. Fish and culture facility

The first feeding Nile tilapia larvae (average weight 0.01 g/fish) used in the first experiment were produced from Nile tilapia broodstock kept in captivity in Aquaculture Laboratory, Oceanography department, Faculty of Science, Alexandria University, Alexandria, Egypt. Nile tilapia larvae (0.01 g /fish) used in the second experiment were obtained from Maryut Fish Farming Company, Alexandria, Egypt. Both experiments were carried out in 70 L rearing glass aquaria. The fish were stocked into the aquaria at a density of two fish per liter. The aquaria were filled with dechlorinated tap water, and provided with continuous aeration through an air compressor. Each aquarium was supplied with an air-lifting filter. Water temperature was maintained at 25 ± 1 °C throughout the study, using 60–80 W heaters. The aquaria were cleaned every morning, before the first feeding, and excreta was siphoned, and about half of the water was replaced with fresh, dechlorinated water of similar temperature. Lighting in culture unit was set at 12:12 light:dark cycle using fluorescent lamps. Water quality parameters including, dissolved oxygen (DO), Ammonia (NH_4-N), Nitrates (NO_3-N), Nitrites (NO_2-N) and pH were monitored weekly, using HACH test kit (Loveland, Colorado, USA).

2.2. Experiment 1

2.2.1. Experimental diets and hormonal treatments

Two isonitrogenous (35% crude protein) and isocaloric (19 MJ/kg) diets were prepared, as described by El-Sayed (1990), with fish meal (FM) or soybean meal (SBM) as protein sources (Table 1). Each diet was mashed, sieved through a 200–800 micron sieve, to get 0.2-0.8 mm pellets, and divided into two portions; one portion was treated with 17α-methyltestosterone (MT) (obtained from ARGENT laboratories Inc. Philippines) at a concentration of 60 mg/kg, while the other portion was left without hormonal treatment, and served as a control. The hormone was incorporated in the diets as follows: the hormone was weighed by a 4-digital balance and dissolved in 100 ml of Ethanol solution 95%. The diet was spread on a plastic tray covered with aluminum foil sheet. The alcohol solution was sprayed over the diet, and then the diet was left to dry at room temperature, with ventilation by an electric fan, for 12 hours. Each diet was stored into dry, clean plastic jar, labeled and stored in the freezer at −20 °C until used.

The test diets were fed to duplicate groups of first feeding Nile tilapia larvae, to satiation, 3 times per day (09:00, 13:00 and 17:00 hr) for 28 days. The test diets were then withdrawn and replaced with a hormone-free diet (25% cp, 7.5% lipids, 5.4% fiber, 3.9% ash and 58.2% nitrogen free extract (NFE)), which was fed to the experimental fish for another 8 weeks, until their gonads can be identified, removed and examined for sex differentiation.

At the end of the feeding trial, all fish in each aquarium were netted, counted and killed by cold shock. The fish were dissected and

Table 1
The formulation and composition (percentage dry weight) of the test diets used in Experiment 1.

Ingredients (%)	Fishmeal diet	Soybean diet
Fishmeal[a]	46	5
Soybean meal[b]	0	56
Corn flour	45	30
Fish oil	4	2
Corn oil	0	2
Vitamin and minerals mixture[c]	3	3
CMC[d]	2	2
Total	100	100
Crude protein	34.94	32.75
Ether extract	9.03	7.62
Crude fiber	4.31	6.72
Ash	5.47	4.49
NFE[e]	45.75	48.42
GE[f]	19.79	19.08

[a] Herring origin (crude protein 72%).
[b] De-hulled (crude protein 45%).
[c] Vitamin and minerals mixture contains (mg/kg or IU/kg of dry vitamins & minerals powder): Vit. A 2.200.000 IU., Vit. D_3 1.100.000 I.U., Vit. E 1.500 I.U., Vit. K 800 mg, Vit. B_1 1,100 mg, Vit. B_2 200 mg, Vit. B_6 2.000 mg, Vit. H 15 mg, Vit. B_{12} 4 mg, Vit. C 3.000 mg, Iron 160 mg, Magnesium 334 mg, Copper 21.6 mg, Zink 21.6 mg, Selenium 25 mg, Cobalt 2.38 mg.
[d] Carboxy methyl cellulose, as a binder.
[e] Nitrogen free extract, determined by difference.
[f] Gross energy (MJ/kg), calculated based on 0.17, 0.237 and 0.398 MJ/g, for carbohydrates, proteins and lipids, respectively.

their gonads removed using a forceps, placed on a clean glass slide and covered with another glass slide. The gonads were squashed between the two slides and then examined under a binocular microscope using magnifications of 40×. The numbers of males, females and intersex fish were calculated as a % of the total gonads tested in each aquarium.

2.3. Experiment 2

2.3.1. Fish and culture facilities

Nile tilapia fry used in the second experiment were reared in the same culture system and under the same culture conditions used in experiment 1, and also for the same period of time (12 weeks).

2.3.2. Experimental diets and hormonal treatment

The same FM-based diet used in experiment 1 was used in experiment 2 (Table 1). The diet was divided into 8 portions, and allocated to the dietary treatments as follows. One portion was left without any hormonal treatment and served as control. Another portion was treated with MT alone at 60 mg/kg diet, as described in experiment 1; three portions were treated with MT and genistein (4′, 5, 7-trihydroxyisoflavone) at three concentrations (10, 20 and 30 mg/kg diet) (FM-MT-G10, G20 and G30) and three portions were treated with MT and daidzein (7,4′-dihydroxyisoflavone) (10, 20 and 30 mg/kg diet) (FM-MT-D10, D20 and D30). Genistein and daidzein were obtained from Sigma Chemicals Company (Saint Louis, Missouri, USA).

The test diets were fed to duplicate groups of first feeding Nile tilapia larvae (0.01 g average weight) stocked into the culture aquaria at a density of two fish per liter. Feeding protocols, sampling, analyses and sex determination were carried out as described in experiment 1.

2.4. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). The results of experiment 1 were statistically analyzed by One-Way Analysis of Variance (ANOVA) to test the effects of phytoestrogen content of soybean meal on sex reversal of Nile tilapia larvae, and differences between means were compared using Duncan's Multiple Range Test. A two-way ANOVA was used to test the effects of phytoestrogen source (genistein and daidzein) and concentration level (10, 20 and 30 mg/kg) on sex reversal of Nile tilapia larvae in the second experiment. Student's t-test was used to compare means at $P = 0.05$.

3. Results

3.1. Experiment 1

The effects of MT on sex reversal of Nile tilapia fry fed the test diets in experiment 1 are summarized in Table 2. Nile tilapia fry fed a FM, MT-free diet had almost equal numbers of males and female (male: female ratio = 1.08:1). The inclusion of MT to FM-based diet significantly increased male percentage to 97% ($P<0.05$). No intersex was detected in Nile tilapia fry fed FM-based diet treated with MT. Intersex appeared only in fish fed SBM-based diet treated with MT. Fish survival was significantly affected by dietary treatments ($P<0.05$), but showed irregular patterns.

The MT-free SBM diet resulted in 23% males and 77% females (male: female ratio = 0.3:1). The inclusion of MT to the SBM diet significantly increased the males percentage from 23% to 65.6% (male: female ratio = 2.12:1), whereas the intersex fish represented only 3%. MT-treated SBM diet has also led to a significant decrease in the number of developing oocytes.

Table 2
The percentage of males, females and intersex of Nile tilapia fry fed test diets in Experiment 1. Values in the same column with different letters are significantly different at $P = 0.05$.

The diet	% Males	% Females	% Intersex	Survival rate (%)
FM (MT-free)	48.0 ± 6.0^{a}	52.0 ± 4.0^{a}	0.0	89.5 ± 5.5^{a}
FM-MT	97.0 ± 1.0^{b}	3.0 ± 0.5^{b}	0.0	77.5 ± 3.0^{b}
SBM (MT-free)	23.0 ± 8.2^{c}	77.0 ± 5.5^{c}	0.0	74.0 ± 2.0^{b}
SBM-MT	65.5 ± 1.5^{d}	31.0 ± 2.0^{d}	3.0 ± 0.5	82.0 ± 4.0^{ba}

3.2. Experiment 2

The results of experiment 2 indicated that the MT-free diet (control) produced almost equal numbers of males and females (male: female ratio = 0.96:1). The inclusion of MT to FM-based diet significantly increased male percentage to 97% ($P<0.05$). The inclusion of genistein and daidzein in MT-treated FM diet fed to Nile tilapia larvae significantly increased the female percentage ($P<0.05$) compared to the control group (Table 3). Increasing genistein and daidzein concentrations from 10 to 30 mg/kg diet has increased female percentage from 24.5 to 47.8% and from 17.5 to 42.5%, respectively. No intersex individuals were detected in fish fed the control diet (free of genistein and daidzein) or diets containing low concentrations (G10 and D10) of these phytoestrogens. Intersex gonads appeared only at medium and high concentrations of genistein (G20 and G30) and medium daidzein (D20). However, intersex proportion was relatively low (2-4%), and not significantly different among treatments ($P>0.05$). Fish survival was significantly affected by treatments ($P<0.05$) in experiment 2. The survival rate decreased with increasing genistein and daidzein levels in the diets.

4. Discussion

Varying and sometimes contradictory results have been reported on the estrogenic effects of phytoestrogens on fish. The present results indicated that MT-free Soybean meal (SBM) diet produced higher percentage of Nile tilapia females than did the fish meal diet. The feminizing effects of SBM-based diet may have been due to the phytoestrogen contents of the diet, especially genistein and daidzein, which, in turn, may have led to the exhibition of estrogenic activity and inhibition of masculinization effect of MT in fish fed MT-treated diets. Many studies have indicated that soy bean meal contains varying levels of genistein (2.6-21.4 mg/100 g) and daidzein (0.8-20.0 mg/100 g), depending on the environment and soy bean variety (Chen and Wei, 2008; Eldridge and Kwolek, 1983; Xiao et al., 2011).

Commercial fish feeds also contain a wide range of phytoestrogens, especially genistein and daidzein, depending on their protein and

Table 3
The percentage of males, females and intersex of Nile tilapia fry fed test diets in Experiment 2. Values in the same column with different letters are significantly different at $P = 0.05$.

The diet	Number of fish examined	Male %	Female %	Intersex %	Survival rate %
FM (control)	125	49.0 ± 6.0^{a}	51.0 ± 4.0^{f}	0.0	90.4 ± 4.0^{e}
FM-MT	105	97.0 ± 1.0^{a}	3.0 ± 1.0^{a}	0.0	77.0 ± 1.5^{c}
FM-MT-G10	120	75.5 ± 3.0^{b}	24.5 ± 3.0^{b}	0.0	88.0 ± 3.7^{e}
FM-MT-G20	110	64.5 ± 4.5^{cb}	33.5 ± 3.0^{cb}	2.0 ± 2.0^{a}	82.0 ± 2.0^{d}
FM-MT-G30	112	48.2 ± 3.0^{c}	47.8 ± 1.0^{c}	4.0 ± 2.0^{a}	80.0 ± 4.5^{cd}
FM-MT-D10	105	82.5 ± 2.5^{b}	17.5 ± 2.5^{b}	0.0	75.0 ± 4.0^{c}
FM-MT-D20	91	65.0 ± 4.5^{cb}	32.5 ± 3.5^{cb}	2.5 ± 1.0^{a}	56.5 ± 2.5^{b}
FM-MT-D30	70	57.5 ± 3.5^{c}	42.5 ± 4.0^{c}	0.0	51.0 ± 1.5^{a}

energy sources and ingredients processing methods. For example, genistein and daidzein contents ranged from 6.76 to 23.71 mg/100 g and 5.02 to 17.58 mg/100 g, respectively, in carp diet, 4.16 to 5.02 mg/100 g and 2.05 to 3.80 mg/100 g, respectively, in trout diets, and 0.93 to 5.85 mg/100 g and 3.32 to 3.73 mg/100 g, respectively, in Japanese rice fish *O. latipes* diets (Kobayashi et al., 2006; Miyahara et al., 2003). Matsuoka et al. (2005) also detected genistein (concentration ranging from 3.97 to 11.89 mg/100 g) and daidzein (concentrations ranging from 2.87 to 8.13 mg/100 g) in fish diets from Japan, United Kingdom and Korea.

The genistein and daidzein levels tested in the present study were within the potential ranges reported for SBM and commercial fish feeds. It is most likely, therefore, that the estrogenic effects of the tested diets were mainly due to their daidzein and genistein contents.

Miyahara et al. (2003) tested the estrogenic activity of a number of commercial animal diets, including fish diets. They found that a carp diet containing defatted SBM contained the highest levels of genistein and daidzein and showed the highest estrogenic activity, and highest relative estrogenic activity levels for human estrogen receptors α (hER-α) and -β (hER-β), compared to other animal diets which contain lower levels of phytoestrogens. The authors also found a positive correlation between the estradiol-17 β (β-E2) conversion from the phytoestrogen contents and the estrogenic activity of β-E2 conversion, using the yeast two-hybrid assay. Similar results have been reported by Matsuoka et al. (2005). These findings suggest that phytoestrogens (daidzein and genistein) are the main substances contributing to the estrogenic activity of the diets.

The estrogenic effects of phytoestrogens have also been reported on European eel (*Anguilla Anguilla*) juveniles (Tzchori et al., 2004) and African catfish *(Clarias gariepinus)* (Yılmaz et al., 2009). These hormones have also been reported to inhibit masculinization hormone production and increase feminization hormone production. For example, the production of 11-ketotestosterone and testosterone decreased in rainbow trout fed on a diet treated with genistein (Bennetau-Pelissero et al., 2001). When Japanese rice fish was injected with genistein, testosterone production decreased in the testis, while estradiol production increased in the ovaries (Zhang et al., 2002).

On the contrary of the above results, Kaushik et al. (1995) found that phytoestrogens found in soy protein concentrate did not result in any estrogenic effect on juvenile rainbow trout. Ng et al. (2006) reported that genistein and other isoflavones found in soybeans inhibit hepatic and renal estrogen metabolism in Atlantic salmon (*Salmo salar*), and lake trout (*Salvelinus namaycush*). Dietary genistein did not also induce vitellogenesis in yellow perch (*Perca flavescens*) (Ko et al., 1999).

It is clear from the above discussion that phytoestrogens can act as weak estrogen agonists, partial agonists, or antagonists to endogenous estrogens and xenoestrogens, at estrogen receptors (ERs). In other words, phytoestrogens may either have the same effects as estrogen or block estrogen's effects (Green and Kelly, 2009; Ng et al., 2006).

These phytoestrogens have high binding affinity for the estrogen receptors (Miyahara et al., 2003). By inhibiting the synthesis and activity of certain enzymes involved in estrogen metabolism, phytoestrogens may alter the biological activity of endogenous estrogens and androgens (Latonnelle et al., 1999). For example, Mueller et al. (2004) reported that phytoestrogens at low concentrations work as an agonistic estrogen, where they bind to the ERs and induce the transcription factors for genes involved in cell growth, proliferation and differentiation. This mode of action was confirmed by Tzchori et al. (2004) who found that genistein at low concentrations elevated estrogen receptor transactivation.

At higher concentrations, phytoestrogen may act as antagonists to endogenous estrogens through the inhibition of aromatase. Aromatase is the enzyme responsible for the production of 17β-estradiol (E2), and therefore, correlates with the process of ovarian differentiation in fish (Nakamura et al., 2003). There are reports that phytoestrogens (genistein and daidzein) have an aromatase inhibitory effect in fish (Monteiro et al., 2000; Noaksson et al., 2003) by blocking ERs and decreasing estradiol synthesis.

The antagonistic effect of phytoestrogens on estrogen receptors through the inhibition of gonadal aromatase is controversial. Gonadal aromatase, which is implicated in ovarian differentiation, lacks estrogen-responsive elements (EREs) in its promoter, and thus, may not be directly regulated by estrogens. On the contrary, brain aromatase has EREs in its promoter and is known to be directly regulated by these estrogens (Tsai et al., 2000). However, it has been found that phytoestrogens are able not only to bind to respective receptors, but also to directly interact with aromatase CYP19, leading to possible inhibition of this enzyme (Cheshenko et al., 2008). Phytoestrogens can also inhibit aromatase activity on the protein level in fish (Ankley et al., 2002; Monod et al., 1993; Monteiro et al., 2000; Noaksson et al., 2003) through competing with the androstenedione substrate for binding to the active site of the enzyme. In addition, the inhibitory effect of estrogens on the activity of ovarian or brain aromatase is species-specific, depending on fish species and developmental stages, tissue context, sex and exposure times. For example, Tsai et al. (2000) reported that E2 administration in Nile tilapia larvae decreased aromatase activity in the brain 7–10 days post fertilization (dpf), while further exposure for 20–30 days strongly induced aromatase activity. Similarly, MT treatment caused the upregulation of cyp19b gene expression in the heads of zebrafish juveniles (Trant et al., 2001), but did not significantly affect aromatase activity in the brain of adult fish (Andersen et al., 2003). It is clear from this discussion that more work is needed to verify the effects of phytoestrogens on estrogen activities in fish.

Several studies have been carried out on the effects of phytoestrogens on sexual phenotype of many fish species. These studies indicated that sex differentiation depends on fish species and size, hormonal dose, timing and duration of treatment and environmental conditions, including temperature (Abucay et al., 1999), pH (Rubin, 1985), pollution (Ashfield et al., 1998; Gimeno et al., 1998; Jobling et al., 1996) and social conditions (Toguyeni et al., 1997). For example, Green and Kelly (2009) determined the effects of increasing dietary levels of genistein on sex differentiation of channel catfish (*Ictalurus punctatus*). They also examined the effect of timing and feed duration on sex determination. They found that phenotypic sex was significantly dependent on dietary genistein concentration. Chronic dietary exposure to genistein at high concentrations (4 and 8 mg/g) resulted in greater proportions of phenotypically males and intersex. The authors suggested that the increased proportions of male and intersex individuals may have resulted from the dual role of genistein as not only an estrogen agonist, but also as an antagonist blocking estrogen's action, presumably due to the inhibition of aromatase activity. Similar intersex gonads in the Nile tilapia *O. niloticus* (Afonso et al., 2001) and rabbitfish *S. guttatus* that received 50 mg/ kg aromatase inhibitor (AI) for 30 days (Komatsu et al., 2006) were reported.

It was also reported that high doses or prolonged treatment of MT can induce gonadal intersexuality and paradoxical feminization, due to the conversion of androgens by aromatase to estrogens, rather than to the inhibition of *in vivo* synthesis of androgens (Blazquez et al., 1995; Papoulias et al., 2000; Rinchard et al., 1999). However, treatment of medaka embryos with non-aromatizable androgen (MT) resulted in sex reversal of genotypic males to females without increasing the content of estrogens (Iwamatsu et al., 2006). Feminization of channel catfish with non-aromatizable androgen dihydrotestosterone has also been reported (Davis et al., 1992). These results suggest that conversion of androgens to estrogens may not always be the cause for induction of paradoxical sex reversal caused by androgen exposure.

In the present study, no intersex individuals were detected in fish fed diets free of phytoestrogens or diets containing low concentrations of these phytoestrogens. Intersex gonads appeared only in fish fed SBM-MT diet and also at medium and high concentrations of genistein (G20 and G30) and medium daidzein (D20). However, the increased proportion of phenotypically females with increasing genistein and

daidzein in fish fed diets treated with MT in the present study suggests that these phytoestrogens act as estrogen agonist. The short dietary exposure to these phytoestrogen, together with the relatively low concentration may also exclude the "estrogen antagonism" assumption. In addition, the stability of water quality parameters throughout the study suggests that they did not affect sexual phenotype of tilapia larvae. The presence of intersex gonads in fish fed medium and high genistein (G20 and G30) or medium daidzein (D20) may have been due to other factors, including chemical pollution.

Nile tilapia larvae used in the present study may have been produced from broodstock exposed to water pollutants, and in turn, were more viable to phytoestrogen effects. In support, it was reported that surfactants present in sewage and industrial effluents (e.g. 4-nonylphenol) and bisphenol-A, have estrogenic activity in fish, and can bind to estrogen receptors and induce vitellogenin, and impair gonadal development (Ashfield et al., 1998; Gimeno et al., 1998; Jobling et al., 1995, 1996). Some estrogenic endocrine-disrupting compounds can also act as androgen agonists or antagonists (Sohoni and Sumpter, 1998).

The estrogenic effect on sex ratio of fish can be permanent or reversible, depending on fish species, life stage, developmental pattern, dose, and exposure time. For example, Maack and Segner (2004) reported that the exposure of zebrafish to ethinylestradiol (EE2) during the sex differentiation period led to 100% female population. Subsequent rearing of these fish in clean water resulted in normal sex ratios, indicating that the feminizing action of this estrogen was not permanent. On the other hand, estrogen treatment of fathead minnow during sensitive period in differentiated gonochorists leads to irreversible effects (Lange et al., 2001). Modulation of CYP19 activity in developing zebrafish by exposure to aromatase inhibitor results also in irreversible alterations of gonadal differentiation (Fenske and Segner, 2004).

In the present study, Nile tilapia fry were fed the test diets for 28 days. It is not known whether the estrogenic effect of genistein and daidzein is permanent, and whether longer dietary exposure to these phytoestrogens would have resulted in paradoxical sex reversal, producing more phenotypic males. However, since the production of all-male tilapia is an ultimate goal of many tilapia farmers, such paradoxical sex reversal would further increase the proportion of tilapia males, leading to better performance and more profits. It is also not known whether the effect of genistein and daidzein on sex proportion of sex-reversed individuals in the present study is permanent. More research is needed to answer this question.

The effect of phytoestrogen on fish survival is controversial. For example, survival in experiment 2 in the present study decreased with increasing dietary genistein and daidzein. This agreed with the results of Ingham et al. (2004) who found that exposing fathead minnows to genistein decreased fish survival. This contradicts the results of de Oca (2005) where genistein did not affect tilapia growth and survival. Aromatase inhibitor did not also affect the survival of bluegill sunfish *Lepomis macrochirus* (Gao et al., 2010). Therefore, more work is needed to resolve this contradiction.

In conclusion, the present study indicated that phytoestrogens genistein and daidzein have a significant estrogenic effect on sexual differentiation of Nile tilapia larvae, and in turn, impair the masculinization effect of methyltestosterone. The estrogenic effect increased with increasing genistein and daidzein concentration. The results also suggest that soybean meal and other phytoestrogen-containing plant protein sources should not be used as protein sources for Nile tilapia larvae during sex reversal treatments.

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