ABSTRACT

This study evaluated the use of daidzein, chrysin, and caffeic acid as potential sex reversal agents in Nile tilapia by dietary administration or immersion treatments. Three feeding experiments were conducted at two locations: Aquaculture Laboratory at the Ohio State University (Experiment 1 and 2) and the Aquaculture laboratory at the Universidad Juarez Autonoma de Tabasco UJAT-Mexico (Experiment 3). An immersion treatment experiment took place at the Aquaculture Laboratory of The Ohio State University (Experiment 4). The dietary administration experiments were as follows: for Experiments 1 and 3, diets control, chrysin (500 mg/kg), daidzein (500 mg/kg) and caffeic acid (500 mg/kg) along with the steroidal compounds spironolactone (500 mg/kg), 1,4,6-androstratriene-3-17-dione ATD (150 mg/kg) and 17α-methyltestosterone (MT) (60 mg/kg) were fed for 8 weeks. For Experiment 3, the diets were control, chrysin (500 mg/kg), daidzein (500 mg/kg) and caffeic acid (500 mg/kg) along with the steroidal compounds spironolactone (500 mg/kg) were offered for 6 weeks. In all cases, semi-purified casein gelatin diets were used to avoid contamination with external sources of either phytochemicals or steroid-like compounds. For the immersion experiment (Experiment 4), four immersion trials were carried out at 10, 17, 21, and 28 day post-hatching on the following chemicals and concentrations, vehicle DMSO (1 ml/l), daidzein 400 (mg/l), chrysin (20 mg/l), caffeic acid (40 mg/l), spironolactone (5 mg/l), ATD (1.2 mg/l) and MT (400 µg/l). In all experiments, final sex ratio was determined by gonad squash; in feeding trials, the final individual body weight and survival were evaluated. Results of experiments conducted at The Ohio State University indicate that the presence of the tested phytochemicals in food or in immersion baths has no effect on the final sex ratio of tilapia or growth performance. In Experiment 1, ATD and MT had a significant effect on final sex ratio (50% and 100% masculinization, respectively); in Experiment 3 (UJAT-Mexico) MT and ATD along with SPIRO affected the male ratio significantly. No effects were observed in Experiment 2 (97±3% males) or Experiment 4 (60-40% male:female ratio) across experimental groups.

INTRODUCTION

The increased use of synthetic steroid hormones to produce monosex populations of tilapia for intensive productive systems may lead to environmental and public health concerns. Such a situation is derived from the fact that female organism of tilapine species have a high fecundity, generally reproducing at a small size and exhibiting stunted somatic growth at higher densities, while male tilapias exhibit faster growth rates and are often preferred gender for monosex aquaculture (Hines and Watts 1995). The efficacy of MT is dependent on many factors such as dose, timing, and duration of treatment (Mirza and Shelton, 1988), providing another disadvantageous characteristic on the use of hormones in tilapia aquaculture.
et al., 2000) as well as steroidal and non steroidal chemical compounds (Brodie et al., 1999, Smith, 1999, Seralini and Moslemi, 2001) that have achieved a certain degree of success in sex inversion in fish. Consequently it can be considered that inhibition of aromatase action by physical and chemical factors could mimic the sex-reversal effects of androgen treatments in some fish species (Kwon et al., 2001).

Chemical compounds classified as phytochemicals (isoflavonoids, flavonoids and ligands) are natural steroid-like compounds derived from soy, tea, fruits and vegetables with a reported aromatase inhibition ability that could be able to suppress estrogen biosynthesis in cells (Geahlen et al., 1989; Pelissero et al., 1996; Eng et al., 2001). Such action is facilitated given the stable structure and low molecular weights of phytochemicals that can pass through cell membranes (Oosaki and Kenelly, 2003). In general, the action of phytochemicals when administered to fish is a recent topic of study, the possible effect of flavonoids on fish aromatase caught attention in a nutritional study with female sturgeons, the hormonal profile in those fish fed with a high dietary inclusion of soybean meal; led to a decrease of plasma estrogen levels (Pelissero et al 1996).

Many studies indicate that aromatase inhibition capacity of phytochemicals is closely related to their chemical structure (Le Bail et al., 1998, Jeong et al., 1999, Saa-rinen et al., 2001). The degree of hydroxylolation on the molecule increases their enzymatic inhibitory ability, therefore a higher number of hydroxyl radicals increases inhibitory activity (Krazeisen et al., 2002). The flavone 7-hydroxyflavone and apigenin used in microsomes of the human placenta after normal full-term delivery were the most effective aromatase and 17β-hydroxysteroid dehydrogenase inhibitors; this experiment showed that flavonoids with 7-methoxy or 8-hydroxyl groups in the ring A showed an important anti-aromatase activity, thus there is an implied structure-activity relation (Le Bail et al., 1998). However it is very important to consider that aromatase affinity for flavonoids in generally lower, than it is for steroidal derivatives (Seralini and Moslemi 2001).

A second approach on the use of phytochemicals as endocrine disruptors is their interaction with estrogen nuclear receptors. Isoflavonoids such as genistein act as estrogen agonists via estrogen receptors in cultured cells and also manifest estrogen-like effects in the female reproductive system (Miksicek, 1995; Santell et al., 1997). Flavonoids have been found to act as phytoestrogens since these compounds have structures that are recognized as estrogen mimics for the estrogen receptor. They can compete with endogenous estrogens for binding to the estrogen receptor; therefore, they can act as antiestrogens or weak estrogens (Miyahara et al., 2003). The ability of non-steroidal compounds such as phytoestrogens to bind to the estrogen receptor is partially determined by the distance between the two extreme hydroxyl groups. Phytoestrogens are usually weak estrogens due to their lower affinity for the estrogen receptors. Whether phytoestrogens act as estrogens depends on the presence and relative concentration of stronger steroidal estrogens (Rickard and Thompson, 1995).

There is a strong variation of the results obtained after using pure phytochemicals and their action in vitro in the inhibition of estrogen synthesis (Joshi et al., 1998). Most of the information available in fish is related to this activity in vitro using gonad cells and measuring the coefficients of inhibitions of synthesis of estrogens when flavonoids are present at several concentrations. There is not an established knowledge of how flavonoids with steroidal activity are absorbed and metabolized by animals, thus their in vivo effect remains mostly unknown. The present research work is focused on the use of three different phytochemicals to evaluate their potential action in vivo on sex inversion in tilapia larvae as non steroidal endocrine disruptors, simultaneously with other steroidal endocrine disruptor chemicals.

**Methods and Materials**

**Study:** Evaluation of potential action of potential phytochemicals on sex differentiation of tilapia by dietary administration.

A series of experiments were conducted at the Ohio State University (OSU) aquaculture laboratory and UJAT-Mexico. In these experiments the purified phytochemicals daidzein, chrysin, and caffeic acid (ICN® Aurora, OH) were supplemented to semi-purified casein gelatin-based diets at a continuous concentration of 500 mg/kg of diet (see Table 1). Also the steroidal compounds 17α-methyltestosterone (MT) (Sigma® St Louis, MO), 1,4,6-androstatrien-3-17-dione or androstatrienedione (ATD) (Steraloids Inc® Newport, RI) and spironolactone (ICN®) for comparison among non-steroidal and steroidal sex reversal agents. For each experiment different genotypes were used to evaluate the potential effect of the mentioned phytochemicals, each experiment by genotype and location is described as follows:

**Experiment 1: All-female tilapia experiment OSU**

The experiment was conducted on first feeding tilapia Oreochromis niloticus genetically all-female (Phil-FishGen, Nueva Ecija Philippines) Fish were randomly distributed into glass aquaria (35 l) in a recirculation system at a constant temperature of 26±2°C, and a density of 60 fish per tank with three replicates per treatment. Experimental casein-gelatin based diets were formulated (Table 1) and designated as follows: control (CON), 17α-methyltestosterone (MT) 60 mg/kg, androstatrienedione (ATD)150 mg/kg, daidzein (DAID) 500 mg/kg, chrysin (CHR) 500 mg/kg, caffeic acid (CAF) 500 mg/kg and spironolactone (SPIRO) 500 mg/kg, for the MT and ATD.
diets, in both cases the chemicals were dissolved in ethyl ethanol and incorporated to the control diet. Fish were fed at 10-15% body weight ratio for 8 weeks. Growth performance was evaluated in terms of the final individual mean body weight, survival (%), specific growth rate (SGR, %) and weight gain (%). The final sex was determined by microscopic analysis of gonad squashes at the end of experiment (Guerrero and Shelton, 1974). Partial samples per tank (5 fish) took place at 2, 4, 6, and 8 weeks to establish phytochemicals absorption.

**Experiment 2: All-male tilapia experiment OSU**

The experiment was conducted on first feeding genetically all-male tilapia (Til-Tech, Robert, LA). Fish were randomly distributed into glass aquaria (35 l) in a recirculation system at a constant temperature of 26±2 °C, and a density of 100 fish per tank with three replicates per treatment. Experimental casein-gelatin based diets were used as follows: control (CON), daidzein (DAID) 500 mg/kg, chrysins (CHR) 500 mg/kg, caffeic acid (CAFF) 500 mg/kg and spironolactone (SPIRO) 500 mg/kg. Fish were fed at 10-15% body weight ratio for 6 weeks. Growth performance was evaluated in terms of the final individual body weight, survival (%), specific growth rate (SGR, %) and weight gain (%). The final sex was determined by microscopic analysis of gonad squashes at the end of experiment (Guerrero and Shelton, 1974). Fish samples (10 fish) per tank took place at weeks 4 and 6 to establish phytochemicals absorption.

**Experiment 3: Mixed sex tilapia UJAT-Mexico**

The experiment was conducted on a mixed sex group of first feeding tilapia. Fish were randomly distributed into plastic containers (20 l) with daily water exchanges at a temperature 21.5±2 °C, and density of 150 fish per aquarium with three replicates per treatment. The same experimental casein-gelatin based diets that were used for the all-female OSU experiment were used in this experiment as follows: control (CON), 17α-methyltestosterone (MT), androstatrienedione (ATD), daidzein (DAID), chrysin (CHR), caffeic acid (CAFF) and spironolactone (SPIRO). Fish were fed at 10-15% body weight ratio for 4 weeks. Growth performance was evaluated in terms of the final individual mean body weight and survival (%). The gender was determined by microscopic analysis of gonad squashes at the end of the experiment (Guerrero and Shelton, 1974).

**Study:** High pressure liquid chromatography (HPLC) determination of phytochemical concentration in fish tissue.

An analysis on the bioaccumulation of the 3 phytochemicals tested in the experiments using HPLC was conducted. In all cases an acid hydrolysis with 1M HCl in acidified methanol (100:5 v:v methanol:acetic acid) was used as extraction solution for concentration of phytochemicals from matrix (whole body tissue). The extraction procedure consisted of homogenization of individual fish in the extraction solution for 30 sec at 5000 rpm, later incubation at 37 °C for 16 h (overnight); acidity was neutralized with 10N NaOH (equivalent volume to sample weight) to achieve a total dilution rate 1:10. Samples were either injected immediately to the HPLC or frozen at -80 °C for further analysis. Recovery rate was estimated on the range of 95% for all three phy-
tochemicals. The HPLC system consists of a Beckman® 110B pump, 166 system gold detection module and a 406 system gold analog interface module; a Peaksimple® chromatography data system was used for chromatogram analysis.

The detection by HPLC for chrysin was performed using a modified procedure from Shahnrzad and Bitsch, (1996). Mobile phase composition was 1M acetic acid in 80% methanol with a flow rate of 0.8 ml/min; wavelength for detection was 280 nm. A Synergi hydro 250X4.6 mm 4m (Phenomenex®) was used for the analysis. The detection limit was 50 ng/ml.

The detection by HPLC for daidzein was conducted using a modification of the method described by Hutabarat et al., (1998). Mobile phase composition was 33% acetonitrile in water: acetic acid (99:1, v:v) at a flow rate of 1.0 ml/min; wavelength for detection was 260 nm. An Ultrasphere ODS 150X4.6 mm 5m (Beckman®) was used for the analysis. The detection limit was 45 ng/ml.

The detection by HPLC for caffeic acid was carried out using a modification of the method described by Walle et al., (1999). Mobile phase composition was water: ethylacetate:acetic acid (95.6:4.1:0.3 %) at a flow rate of 1.0 ml/min; wavelength for detection was 320 nm. An Ultrasphere ODS 150X4.6 mm 5m (Beckman®) was used for the analysis. The detection limit was 40 ng/ml.

Statistical Analysis

Mean final weight and survival were analyzed by one-way analysis of variance analysis, where significant differences were found, a Tukey test was performed (Zar, 1990), and for final sex ratio chi-square contingency tables were used. All statistical analysis was performed using the SAS version 8.02 (SAS Institute, Inc. Cary, NC USA).

RESULTS

Experiment 1

No significant differences were observed on survival rate after 8 weeks, across all treatments (87.9±6.8 %) (Table 3). Growth rate results indicate a differential growth observed at the different points of evaluation (2, 4, 6, and 8 weeks) (Figure 1), being more evident at week 8 (Table 2) where in general CON showed a significant higher final mean weight (P<0.05) than MT and ATD. Fish fed with MT and ATD were significantly smaller (P>0.05) compared to fish fed with the different phytochemicals. The final sex ratio of the experimental fish was not affected by the dietary administration of the tested phytochemicals; only the steroidal compounds ATD and MT achieved 50 and 100% masculinization rates, respectively when compared with the control treatment (Figure 2).

Experiment 2

When compared with the control treatment (Figure 2), achieved 50 and 100% masculinization rates, respectively chemicals; only the steroidal compounds ATD and MT infected by the dietary administration of the tested phytochemicals. The final sex ratio of the experimental fish was not affected by the dietary administration of the phytochemicals; the observed mean concentrations in mg/g are displayed in Figure 7. Sample chromatograms of detection peaks from CON and CHR groups and standards can be observed in Figure 10.

Experiment 3

Several setbacks were observed in this experiment. There was a very high variation in survival rates across treatments; 11.7 to 55% (Table 3). In two cases, for CHR and CAFF only one replicate could be evaluated towards the end of the experiment for sex ratio determination. On week 6 of the experiment a few significant differences in growth rate were established; however, towards the end of the experiment individual mean weight was not different among dietary treatments (Figure 4). SPRO, MT and ATD treatments had a significant higher male percentage (P<0.01), (Figure 5) than the control group.

Experiment 4

The survival rate in this experiment was very variable. Despite the fact that for the no-effect concentrations of phytochemicals were establish over a 24h period immersion, for CHR there was 100% mortality at the second immersion at day 17. Similar responses were observed in other treatments, where fish mortality increased throughout the experiments (Table 3). Sex ratio was not skewed towards a higher male presence when compared to the CON group (65% males) for all treatments (Figure 6).

HPLC phytochemical concentration analysis

The detection in fish tissue of the phytochemicals chrysin and daidzein was achieved by HPLC techniques. The extraction technique was successfully adapted for fish tissue and detection conditions were modified to fit our specific laboratory. Although detection conditions were successfully established in our HPLC detection system, no peak was observed for caffeic acid (Figure 11), either in Experiments 1 or 3 fish samples at the different sampling times for each experiment. For Daidzein in Experiment 1, detection was variable throughout the different sampling times; at week 2, daidzein was only detected in 40% of the samples, at week 4 in 66%, at week 6 in 93%, and at week 8 in 100% of the samples, for Experiment 3 at both week 4 and 6 the chemical was detected; the observed mean concentrations in mg/g are displayed in Figure 7. Sample chromatograms of detection peaks from CON and CHR groups and standards can be observed in Figure 10.

For chrysin, the phytochemical was detected in all analyzed samples in Experiments 1 and 3 (100% detection); the observed mean concentrations are displayed in Figure 8. Sample chromatograms of detection peaks and standards can be observed in Figure 11.
DISCUSSION

This study addresses the possibility of using phytochemicals such as chrysin, daidzein, and caffeic acid to induce the required hormonal imbalance needed to observe an effect on sex differentiation in tilapia (Howell et al., 1994). Experimental conditions in vitro have shown that several phytochemicals block the biosynthesis and action of estrogens by (1) inhibition of aromatase activity and other steroid metabolism related enzymes, or (2) by competition for the estrogentic nuclear receptors (α and β ER), that could possibly mimic the sex-reversal effects of androgen treatments in fish (Collins et al., 1997, Le Bail et al., 1998, Jeong et al., 1999).

The first premise is based on the aromatase affinity for flavonoids. This enzyme is the only known enzyme able to catalyze the irreversible conversion of androstenione and testosterone into estrone and estradiol respectively; therefore aromatase is a good target for selective inhibition because estrogen production is the last step in the biosynthetic sequence of steroid synthesis (Brodie et al., 1999). Given that the majority of information that supports this theory is based on studies on in vitro conditions (Collins-Burrows et al., 2000, Le Bail et al., 1998, Jeong et al., 1999), when extrapolated to a live organism, the results obtained are diverse. Saarinen et al. (2001), showed that chrysin and 7-hydroxyflavone inhibit the formation of 3H-17α-estradiol from 3H-androstenione in human choriocarcinoma JEG-3 and human embryonic kidney HEK 293 cells, but when administered at a dosage of 50 mg/kg to immature rats to evaluate the in vivo response, it failed to promote a reduced growth response in estrogen-dependent uterine enlargement. The lack of an in vivo response may be due to their relatively poor absorption and/or bioavailability and they state that the in vivo effects of flavonoids in aromatase inhibition cannot be predicted on the basis of in vitro results. In our study, given the lack of evidence of an active response on sex differentiation after phytochemical administration by diet and immersion treatments, we can speculate that phytochemicals are not fully effective in affecting sex differentiation in tilapia.

The bioactivity of phytochemicals upon consumption is controversial. The evidence of high concentrations of phytochemicals in urine indicates that most of the chemical is eliminated by this means by humans and other vertebrates (Pelissero et al., 1996). In general, phytochemical absorption involves a series of conjugation and deconjugation steps facilitated by the gut bacterial flora and the liver. (Patisaul and Whitten 1999, Hollman and Arts 2000, Miyahara et al., 2003). Differences in results between OSU and UJAT suggest two possibilities: First there is a chance that all these steps are not fully accomplished given the degree of development of the digestive tract of the larval fish used for our experiments; second, there are possibilities of interspecies differences on the degree on the efficiency of phytochemical absorption due to variation in the release of many different digestive enzymes by microflora involved in food metabolism processes (Bairagi et al., 2002) or specific rearing conditions that can influence digestive tract development and the specificity of the present bacterial flora in tilapia (LeaMaster et al., 1997). A possible indication of such a relationship between the degree of absorption and metabolism of phytochemicals and the degree of gut development and present microflora and their effect on sex differentiation could be the observed differences between Experiments 1 and 3 given the origin and rearing conditions. In Experiment 3, the chrysin final male percentage was higher (but not significant different) than the control group (Figure 5), and a similar occurrence was observed on the treatments spironolactone and androstatrienedione that had a significant effect on final sex ratio, contrary the results observed in Experiment 1, where only MT and ATD induce a sex inversion effect.

Thus, to establish to what extent larval fish absorb and metabolize phytochemicals could be an important factor to determine the possible in vivo bioactivity of flavonoids as endocrine disruptors. Future studies could focus on evaluating chrysin tissue concentrations of fish from the two locations, and contrast that if in both cases, the same trend as in Experiment 1 tissue concentrations of chrysin and daidzein is observed (Figures 7 and 8). For both phytochemicals, a diminution on tissue concentration throughout the feeding trial was observed in Experiments 1 and 3 in both genotypes (all-male and all-female), which could be related to an increased metabolism by enzymes produced by the digestive tract and microflora. Also the proper chemical identification of the free and sulfate-conjugated phytochemicals produced after enzymatic reactions, which have a reported higher in vivo endocrine disruptor activity (Miyahara et al., 2003), is warranted due to a potential “activation” of some phytochemicals after metabolism that facilitates their absorption by intestinal membranes and transportation to the liver, re-metabolization, and delivery to target tissues (Kinjo et al., 2004).

Research on the bioavailability of flavonols, flavones, and flavanols has to be expanded. Attention must be given to the identification and quantification of their metabolites in body fluids and tissues, and sensitive and selective analytical methods will have to be developed (Hollman and Arts 2000). The HPLC detection methods described in this provide preliminary information on the absorption of phytochemicals in fish. Although several modifications were made from the original papers that describe the analytical conditions, such as type of column, flow rate, and wavelength for detection, the chromatograms from Figures 9 and 10 confirm the presence of the parent compound in fish whole body tissue, although the free and conjugated metabolites need to be identified. For example, daidzein is metabolized
into dihydrodaizein, then equol, and finally O-demethyl-yangolensin (O-DMA) in humans. This has unknown estrogenic properties (Patisaul and Whitten, 1999). Once consumed, caffeic acid is transformed into a series of derivatives, mostly ferulic, isoferulic, and dihydroferulic acid that can be used as specific biomarkers for the bioavailability and metabolism of this phytochemical. However there is no information of the relevance of such derivatives as possible endocrine-disruptors chemicals (Rechner et al., 2001). Chrysin has apparent favorable membrane transport properties through cell membranes; however its absorption may still be seriously limited by a highly efficient conjugation metabolism by glucuronidation and sulfation by intestinal epithelial cells (Walle et al., 1999). Therefore, pharmacokinetic studies involved in the absorption of phytochemicals in fish need to be conducted.

Whether phytochemicals exert an anti-estrogenic effect once they bind to the steroid receptors is yet another point of controversy. It is well known that estrogen influences the growth, development, behavior, and regulation of reproductive tissues in all vertebrates. Many of the effects of estrogens are mediated through binding to the estrogen receptors (ERs) (Cole and Robinson, 1992, McLachlan et al., 1992, Rickard and Thompson 1995, Matthews et al., 2000). Isoflavones such as daidzein bind to estrogen receptors with a lower affinity than 17α-estradiol (between 1 X 10⁻⁴ and 1 X 10⁻² lower) on a molar basis. But still there are differences when it comes to different estrogen receptors sub-units given that binding affinity can vary 5 to 20 times more efficiently to the ERα than the ERβ receptor (Messina et al., 2001). Chrysin binding affinity is 100,000 fold or more less than E₂, and has very low or no agonistic activity in the ER’s (Kuiper et al., 1998). Ligand-binding specificities could influence the actions of phytochemicals as endocrine-disrupting agents at different target tissues (Thomas, 2000).

Another consideration when evaluating possible effects of phytochemicals on sex differentiation in juvenile fish is their effect on growth. In all feeding trials (Experiments 1, 2, and 3), no significant differences on final individual mean weight were observed on the phytochemical fed fish after 6 or 8 weeks of feeding, when compared with the control group in all three experiments. No growth data was obtained during immersion experiments given the low carrying capacity of the biofiltration unit was limited the total amount of food provided per day (5%). Higher mortality rates were observed relative to Experiments 1 and 2 (Table 3). The suboptimal rearing conditions may have reduced the potency of treatments used (phytochemicals and steroidal compounds) or altering sex differentiation in tilapia.

A number of studies have focused on the use of alternative steroidal compounds for sex inversion in fish. In this case, spironolactone was selected because of its inhibitory effect on steroid synthesizing enzymes and androgen receptor affinity (Steelman et al., 1969, Howell et al., 1994, Canosa and Ceballos 2001). Although spironolactone has promoted “paradoxical masculinization” effects in juvenile mosquito fish (Gambusia affinis), the responses observed were an expression of secondary male sex characteristics (Howell et al., 1994). The results observed in Experiment 4 (Figure 5) provide a promising expectation for future research on the use of this steroidal compounds. Relative to the use of steroidal aromatase inhibitors such as ATD, distinct results have been reported, given that fadrozole (an steroidal aromatase inhibitor) can present estrogenic activity on the long run by competing with estrogen receptors and inducing an estrogen-like effect on DNA expression (Serallini and Moslemi 2001). Bertolla-Afonso et al., (2001) conducted another study with fadrozole that when administered on food for 15 or 30 days can influence sex determination in tilapia. The results indicate that treatment at dosages of 75 and 100 mg/kg achieve a 100% masculinization rate when fed for 30 days. However fadrozole is a more potent aromatase inhibitor than ATD, where a dosage of 150 mg/kg for 30 days only induces a 75% masculinization rate (Guiguen et al., 1999); in our study ATD only promoted a 50% masculinization effect in Experiment 1 (Figure 2), but a higher response of 97% in Experiment 4 (Figure 5), indicating a probable variability in genotype responses to steroidal aromatase inhibitors.

In conclusion, this study provides another resource in the evaluation of the effect in vivo of phytochemicals on sex differentiation in Nile tilapia. Although no significant effect on final masculinization percentage was observed using chrysins, daidzein, and caffeic acid, the validation of the presence of the parent compound with HPLC techniques in fish tissue encourages future research on the use of these chemicals for induced sex reversal. Also, the evidence supporting the possibility of alternative steroidal compounds, such as spironolactone, for sex inversion provides a future line of research.

**Literature Cited**


The given text contains references to various studies and publications related to the effects of phytoestrogens, flavonoids, and other substances on the production and reversal of sex in silver carp. The text also includes references to the structure of flavonoids and their role in inducing gynogenesis and sex reversal in silver carp. Additionally, the text mentions the effects of dietary phytoestrogens on the reproductive systems of male and female animals and the role of receptor binding in the production of sex in fishes. The text concludes with references to the effects of phytoestrogens and lignans on reproduction and chronic disease, the transport of flavonoids in human intestinal cells, and the role of phytochemicals in food.

References:


Table 1. Diet formulation used for Experiments 1, 2, and 3. Values in percent of inclusion / 100g.

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<th>Chrysin (500 mg/kg)</th>
<th>Spironolactone (500 mg/kg)</th>
<th>Daidzein (500 mg/kg)</th>
<th>Caffeic acid (500 mg/kg)</th>
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Table 2. Summary of mean final individual weight (± SD), for experiments 1, 2 and 3. Different letters indicate significant differences (P > 0.05) among groups by experiment.

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<th>Treatment (Diet)</th>
<th>Final Ind. Weight (g) by Experiment</th>
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<td>Daidzein</td>
<td>0.67±0.1</td>
</tr>
<tr>
<td>Caffeic Acid</td>
<td>0.64±0.1</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>0.63±0.1</td>
</tr>
<tr>
<td>17α-Methyltestosterone</td>
<td></td>
</tr>
<tr>
<td>1,4,6-androstatrien-3-17-dione</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Summary of final survival rates (% ± SD) for experiments 1, 2, 3 and 4.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>All-Male OSU (Exp 1)</th>
<th>All-Female OSU (Exp 2)</th>
<th>Mixed Sex UJAT (Exp 3)</th>
<th>Mixed Sex OSU (Exp 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>97.6±2.0</td>
<td>88.3±5.0</td>
<td>55.5</td>
<td>47.2±12.0</td>
</tr>
<tr>
<td>Chrysin</td>
<td>97.0±1.0</td>
<td>87.7±2.5</td>
<td>11.7</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Daidzein</td>
<td>98.6±1.5</td>
<td>88.9±5.3</td>
<td>30.2</td>
<td>25.0±1.7</td>
</tr>
<tr>
<td>Caffeic Acid</td>
<td>97.6±2.3</td>
<td>77.8±10.5</td>
<td>11.7</td>
<td>47.7±7.0</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>97.6±1.5</td>
<td>85.0±1.6</td>
<td>46.0</td>
<td>31.6±23.3</td>
</tr>
<tr>
<td>17α-Methyltestosterone</td>
<td>97.6±1.5</td>
<td>92.8±2.5</td>
<td>28.4</td>
<td>37.2±21.8</td>
</tr>
<tr>
<td>1,4,6-androstatrien-3-17-dione</td>
<td>95.0±1.6</td>
<td>45.7</td>
<td></td>
<td>47.7±3.9</td>
</tr>
</tbody>
</table>

Figure 1. Mean weights (± std error) of all female tilapia (*O. niloticus*) by dietary treatment from Experiment 1, reared at the Aquaculture Laboratory of The Ohio State University. Control (CON), chrysin (CHR), daidzein (DAID), spironolactone (SPIRO), caffeic acid (CAFF), 17α-methyltestosterone (MT) and androstatrienedione (ATD).
Figure 2. Final percent of males (± std error) of all female tilapia (*O. niloticus*) by treatment from Experiment 1 at the Aquaculture Laboratory of The Ohio State University. Control (CON), chrysin (CHR), daidzein (DAID), spironolactone (SPIRO), caffeic acid (CAFF), 17α-methyltestosterone (MT) and androstatrienedione (ATD). ** indicates significant differences (P > 0.05) form CON group.

Figure 3. Final individual mean weights (± std error) of all male tilapia (*O. niloticus*) by dietary treatment from Experiment 2, reared at the Aquaculture Laboratory of The Ohio State University. Control (CON), chrysin (CHR), daidzein (DAID), spironolactone (SPIRO) and caffeic acid (CAFF).
Figure 4. Final individual mean weights (± std error) of mixed sex tilapia (*O. niloticus*) by dietary treatment from Experiment 4, reared at the Aquaculture Laboratory of UJAT-Mexico. Control (CON), chrysin (CHR), daidzein (DAID), spironolactone (SPIRO), caffeic acid (CAFF), 17α-methyltestosterone (MT) and androstatrienedione (ATD).

Figure 5. Final percent of males (± std error) of mixed sex tilapia (*O. niloticus*) by treatment from Experiment 3 at the Aquaculture Laboratory UJAT-Mexico. Control (CON), chrysin (CHR), daidzein (DAID), spironolactone (SPIRO), caffeic acid (CAFF), 17α-methyltestosterone (MT) and 1,4,6- androstatrienedione (ATD). ** indicates significant differences (P > 0.05) from CON group.
Figure 6. Final male ratio (± std error) of mixed sex tilapia (*O. niloticus*) by treatment from Experiment 4 at the Aquaculture Laboratory of The Ohio State University. Control (CON), chrysin (CHR), daidzein (DAID), spironolactone (SPIRO), caffeic acid (CAFF), 17α-methyltestosterone (MT) and androstatrienedione (ATD).

![Bar graph showing male ratio by treatment](image)

Figure 7. Mean daidzein concentrations (± std error) in tilapia whole body from Experiment 1 (2, 4, 6, and 8 weeks) and Experiment 2 (4 and 6 weeks) reared at the Aquaculture Laboratory of The Ohio State University. n=15 fish per week.

![Bar graph showing daidzein concentration by time](image)
Figure 8. Mean chrysin concentrations (± std error) in tilapia whole body from Experiment 1 (2, 4, 6, and 8 weeks) and Experiment 2 (4 and 6 weeks) reared at the Aquaculture Laboratory of The Ohio State University. n=15 fish per week.
Figure 9. Chrysin chromatograms of whole body tissue, a) Experiment 3 week 6 control group sample, b) Experiment 3 week 6 sample, c) 1.5 µg/ml std.
Figure 10. Daidzein chromatograms of whole body tissue, a) Experiment 3 week 4 control group sample, b) Experiment 3 week 6 sample, c) 1.5 µg/mL std.
Figure 11. Caffeic acid chromatograms of whole body tissue, a) Experiment 1 week 6 sample, b) Experiment 1 week 6 sample + spike (0.50 µg/ml caffeic acid), c) 1.17 µg/ml std