Dietary Administration of Daidzein, Chrysin, Caffeic Acid and Spironolactone on Growth, Sex Ratio and Bioaccumulation in Genetically All-Male and All-Female Nile Tilapia (Oreochromis niloticus)

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Abstract

Aromatase inhibitors can produce monosex populations of fish by blocking estrogen induced ovarian differentiation. Phytochemicals such as flavonoids and other phenolic compounds can exhibit aromatase inhibitor-like characteristics as reported for many of these compounds. Two experiments were conducted with genetically all-female or genetically all-male first feeding Nile tilapia to evaluate the potential in vivo aromatase inhibitory activity of three selected phytochemicals in parallel with synthetic steroidal compound treatments. Experimental diets were the following: control, 17α-methyltestosterone (MT); 1,4,6-androstatrien-3-17-dione (ATD); spironolactone (SPIRO); daidzein (DAID); chrysin (CHR) and caffeic acid (CAFF) at different inclusion levels. Fish were fed for 6 weeks (all-male) and 8 weeks (all-female). Survival, final individual body weight and specific growth rate and final sex ratios were recorded. All phytochemicals were effectively detected using HPLC analyses. No differences were observed in survival, final mean weight, SGR between treatments in all-male tilapia. For all-female tilapia, MT and ATD groups showed significantly smaller final mean weights (p<0.05); still, survival or SGR were not significantly different. Final sex ratios were as follows: for all-male juveniles no effect was observed in the final sex ratio for any of the phytochemicals or spironolactone. The sex ratio of genetically all-female tilapia was not affected by the inclusion of tested phytochemicals and spironolactone, while MT and ATD male ratios of 100% and 50%, respectively. Thus, selected dietary inclusion levels of phytochemicals did not exert an in vivo effect on sex differentiation in Nile tilapia.

Keywords: Tilapia, sex reversion, chrysin, caffeic acid, daidzein, methyltestosterone, spironolactone.

INTRODUCTION

At this moment, commercial tilapia aquaculture is performed using all-male stocks. Such fish are producing by means of dietary administration of synthetic steroid hormones, such as 17α-Methyltestosterone (MT); however, some environmental and public health concerns are always present; thus the development of alternative methods of tilapia masculinization is desirable. The study of the mechanisms of hormone biosynthesis and action on the gonadal sex differentiation process could provide an insight on alternative methods for producing monosex populations of fish. Among these mechanisms, the activity of the enzyme aromatase is of particular interest in the sexual differentiation in fish and many other organisms. This enzyme is a cytochrome P-450 hemoprotein that catalyzes the conversion of
androgens to estrogens (Brodie et al. 1999; Le Bail et al. 1998), a critical stage that affects the sex differentiation process in vertebrates. There is evidence of aromatase inhibition by physical factors, such as temperature (D’cotta et al. 2001) as well as steroidal and non-steroidal chemical compounds (Smith 1999; Seralini and Moslemi 2001). With both approaches a certain degree of success has been achieved in sex reversion in fish. Consequently it can be considered that inhibition of aromatase action by physical and/or chemical factors could mimic the sex-reversal effects of androgen treatments in some fish species (Kwon et al. 2001).

Specific studies in several species provide valuable insight on the feasibility to produce high male sex ratios by means of aromatase inhibitors in both gonochoristic and hermaphroditic fish. Fadrozole, a synthetic non-steroidal aromatase inhibitor induced 100% masculinization in Nile tilapia (Kwon et al. 2000; Bertolla-Afonso et al. 2001), Japanese flounder, Paralichthys olivaceus (Temminck and Schlegel, 1846) and in the protogynus fish honeycomb grouper, Epinephelus merra (Bloch) (Bhandari et al. 2003). Another compound, 1,4,6-androstatrien-3-17-dione (ATD), a synthetic steroidal aromatase inhibitor, induced 54% masculinization in all-female Atlantic salmon, Salmo salar L. (Lee et al. 2003), and 75% and 100% masculinization in Nile tilapia and rainbow trout Oncorhynchus mykiss (Walbaum), respectively (Guigen et al. 1999). Spironolactone, although it was considered primarily as an anti-androgenic compound due to aldosterone antagonism (Jankowski et al.1996); it also has a reported aromatase inhibitory activity (Carr, 1986), as well as causing paradoxical masculinization in mosquito fish, Gambusia affinis after external-bath exposure (Howell et al. 1994).

Phytochemicals, specific flavonoids and other phenolic compounds, can be considered an alternative to the use of steroidal and other potentially toxic synthetic inhibitors. Relevant in vitro studies dealing with aromatase inhibition coefficients, have observed that chrysin, has a potential as aromatase inhibitor when compared to other compounds such as aminoglutethimide (Chen et al. 1997; Le Bail et al. 1998; Jeong et al. 1999, Saarinen et al. 2001). Among the most effective molecules reported as in-vitro aromatase inhibitors are chrysin, apigenin and naringenin (Ohno et al., 2004). Also, promising results have been obtained with synthetically modified flavonoids, with attached functional groups to the flavonoid structure (Pouget et al. 2002). However, only a few studies with higher vertebrates have been able to provide the conclusive evidence of phytochemicals as aromatase inhibitors. This has been achieved by means of extraction of flavonoids by activity guided fractionation (Eng et al. 2001). Nevertheless, flavonoids may also act as estrogenic agents, possibly by stimulating cellular estrogen receptors (Santell et al. 1997), therefore estrogenic properties must be elucidated for different molecules.

There is a considerable variation of the results obtained after using pure phytochemicals and their in vitro effects on 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 inhibition of synthesis of estrogens when flavonoids are presented at several concentrations (Pelissero et al. 1996). There is no information how flavonoids with steroidal activity are absorbed and metabolized by animals, in particular by fish. Thus, the flavonoid in vivo effect remains mostly unknown. The present work is focused on the use of three different phytochemicals as non-steroidal aromatase inhibitors to evaluate their potential in vivo impact on sex inversion in tilapia larvae when contrasted with a androgenic hormone such MT, and two steroidal synthetic aromatase inhibitors such as 1,4,6-androstatrien-3-17-dione and spironolactone. Simultaneously, we aim to contribute towards the understanding of phytochemicals absorption and metabolization in fish, especially in Nile tilapia.

MATERIALS AND METHODS

Reagents

The following compounds, were obtained: daidzein, (4’,7-dihydroxyflavone) from Indofine Chemical Co. (Hillsborough NJ, USA), chrysin (5,7-dihydroxyflavone), caffeic acid (3,4-dihydroxycinnamic acid) and spironolactone from MP Biomedicals® (Aurora OH, USA), 17α-methyltestosterone from Sigma® (St Louis, MO, USA) and 1,4,6-androstatrien-3-17-dione from Steraloids Inc® (Newport RI, USA). Mobile phase HPLC grade components were purchased form Fisher Scientific Inc. (Pittsburgh, PA, USA).

Diet formulation

Seven casein-gelatin based diets were prepared as follows: control (CON), chrysin 500 mgkg-1 (CHR), caffeic acid 500 mgkg-1 (CAFF), daidzein 500 mgkg-1 (DAID), spironolactone 500 mgkg-1 (SPIRO), 1,4,6-androstatrien-3-17-dione 150 mgkg-1 (ATD) and 17α-methyltestosterone 60 mgkg-1 (MT). Semi-purified diets were formulated to avoid contamination with natural steroids (Feist and Shreck, 1990). For CHR, DAID, CAFF and SPIRO diets, the compounds were diluted in 10 ml of DMSO and then incorporated to the diet prior to final mixing and pelletizing. For MT and ATD diets, the steroid
Table 1. Composition of the control and experimental diets supplemented with active compounds for first feeding tilapia larvae (expressed as percentage of dry matter per 100 g).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>CON</th>
<th>CHR</th>
<th>DAID</th>
<th>CAFF</th>
<th>SPIRO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (vitamin free)</td>
<td>36</td>
<td>36</td>
<td>36</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td>Gelatin</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Dextrin</td>
<td>32.6</td>
<td>32.6</td>
<td>32.6</td>
<td>32.6</td>
<td>32.6</td>
</tr>
<tr>
<td>Fish protein hydrolyzate&lt;sup&gt;1&lt;/sup&gt;</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Cod liver oil</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Vitamin mix&lt;sup&gt;2&lt;/sup&gt;</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Mineral mix&lt;sup&gt;3&lt;/sup&gt;</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Carboxymethylcellulose</td>
<td>2</td>
<td>2</td>
<td>2</td>
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<td>2</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
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</tr>
<tr>
<td>L-Methionine</td>
<td>0.4</td>
<td>0.4</td>
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<tr>
<td>L-Lysine</td>
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<tr>
<td>Choline chloride</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Phospitan C&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
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<td>0.04</td>
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<tr>
<td>Chrysin</td>
<td>0</td>
<td>0.05</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>Daidzein</td>
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<td>0</td>
<td>0.05</td>
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<td>0</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.05</td>
<td>0</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.05</td>
</tr>
<tr>
<td>Cellulose</td>
<td>0.66</td>
<td>0.61</td>
<td>0.61</td>
<td>0.61</td>
<td>0.61</td>
</tr>
</tbody>
</table>

All ingredients purchased from Sigma Chemical Company (St. Louis, MO) or MP Biomedicals (Aurora, OH), except where noted.

<sup>1</sup> Concentrate of fish soluble protein (CPSP: crude protein 82-84%; crude lipid 9-13% WW), Sopropreche SA, Boulogne-sur-Mer, France).

<sup>2</sup> Roche Performance Premix (Hoffman-La Roche, Nutley, NJ).

<sup>3</sup> Bernaht Tomarelli salt mixture (5 g Na<sub>2</sub>Se-Se/kg mixture; ICN Pharmaceuticals, Aurora, OH).

<sup>4</sup> Mg-L-ascorbyl-2-phosphate (Showa Denko, Tokyo, Japan).

was dissolved in ethanol as vehicle and mixed thoroughly in the control diet; later ethanol was allowed to evaporate overnight at room temperature later diets were stored at -20°C. Diets compositions are listed in Table 1.

Feeding trials

The experiment was conducted on genetically all-female tilapia (Phil-FishGen, Nueva Ecija, Philippines) first time feeding, initial weight 10±0.3 mg. Fish were randomly distributed into glass aquaria (35 L) in a recirculation system at an average temperature of 26±2 °C. Starting fish density was 60 larvae per tank with three replicates per treatment. The following experimental diets were used: CON, or diets supplemented with DAID, CHR, CAFF, SPIRO, MT and ATD. Fish were fed at a decreasing rate beginning at 20 until 10% body weight ratio for 8 weeks, adjusted every week. Dietary group performance was evaluated in terms of the final individual mean body weight, survival (%) and SGR (%/day). The sex was determined by microscopic analyses of gonad squashes (Guerrero and Shelton, 1974) at the end of experiment (65 days). Samples of fish (5 fish per tank) were also taken at 2, 4, 6 and 8 weeks in order to establish concentration of phytochemicals in fish bodies.

Simultaneously an experiment was conducted on genetically all-male tilapia (Til-Tech, Robert, LA, USA) feeding for the first time, initial weight 10±0.2 mg to evaluate potential estrogenic responses due to the inclusion of selected phytochemicals or anti-androgenic effects by spironolactone. 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. The following experimental diets were used: CON, DAID, CHR, CAFF and SPIRO. Fish were fed at a
decreasing rate from 20 to 10% body weight ratio for 6 weeks, adjusted weekly. Fish performance was evaluated in terms of the final individual body weight, survival (%) and specific growth rate (SGR% day⁻¹). The sex of fish was determined in the final samples (42nd day) by microscopic analyses of gonad squashes at the end of experiment (Guerrero and Shelton 1974). Fish samples were also taken (10 fish per tank) at week 4 and 6 to establish phytochemicals absorption. The whole fish were frozen (-80°C) for further analyses.

**Determination of phytochemical concentrations in fish tissue by HPLC analysis**

The analyses of the concentrations for the three phytochemicals tested in the experiments were conducted using HPLC. In all cases, acid hydrolysis with 1M HCL in acidified methanol (100:5 v:v methanol:acetic acid) was used as the extraction solution to examine concentrations of phytochemicals in whole body tissues. The extraction procedure included homogenization (GLH-115 homogenizer Omni Int. Marietta GA, USA) of individual fish in the extraction solution for 30 sec at 5000 rpm, followed by incubation at 37°C for 16 h (overnight), and subsequently, centrifugation of the extracts for 10 min at 10,000 rpm at 4 °C. Prior to injection into HPLC, acidity was neutralized with 10N NaOH (equivalent volume to sample weight) to achieve a 1:10 total dilution ratio. Samples were filtered through 0.45 µm disk filters and either injected immediately to the HPLC or frozen at -80 ºC for further analysis. Recovery rate was estimated using internal standards, and were found to be approximately 95% for all three phytochemicals. The HPLC system consisted 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 Shanhrzad and Bitsch (1996). Mobile phase composition was 1M acetic acid in 80% methanol and the flow rate was established at 0.8 ml/min; wavelength for detection was 280 nm. A Synergi hydro 250X4.6 mm 4µ (Phenomenex®) column was used. The detection limit (LOL) was 50 ng ml⁻¹.

The measurement of daidzein was performed using a modified HLPC procedure of Hutabarat et al. (1998). Mobile phase composition was: 33% acetonitrile in water; acetic acid (99:1, v:v) and flushed at a flow rate of 1.0 ml/min. The detection wavelength was 260 nm. A Synergi hydro 250X4.6 mm 4µ (Phenomenex®) column was utilized. The detection limit was 45 ng ml⁻¹.

The estimation of caffeic acid was carried out using a modification of the HPLC method described by Walle et al. (1999). Mobile phase composition was as follows: water:ethylacetate:acetic acid (95.6:4.1:0.3 %) and provided at a flow rate of 1.0 ml/min. Detection wavelength was 320 nm, coupled to a BAS® (West Lafayette, IN, USA) electrochemical detector equipped with a CC-5 flow cell and a LC-44 detector, using a 0.08 to 8.0 mV output. An Ultrasphere ODS 150X4.6 mm 5µ (Beckman®) column was used. The detection limit was 25 ng ml⁻¹.

**Statistical Analysis**

Mean individual final weight, SGR (%/day) and survival were analyzed by one-way ANOVA. If significant differences were found, Fisher protected test for least square means multiple comparison was used to establish dietary treatment significant differences. Differences in final sex ratios were tested using chi-square contingency tables. All statistical analysis was performed using SAS version 8.02 software (SAS Institute, Inc. Cary, NC USA) at a significance level of α=0.05.

**RESULTS**

**All-male tilapia**

After 6 weeks of feeding with the experimental diets, final mean weight (0.69±0.1 g) (Figure 1, Table 2) and survival rate (97.7±2%) (Table 2) of all-male tilapia were not significantly different across treatments. The final phenotypic sex ratio was not altered from 100% in genetically all-male groups by the inclusion of CHR, DAID, CAFF or SPIRO as observed in the control group.

**All-female tilapia**

No significant differences were observed in survival rates after 8 weeks across all treatments (87.9±6.8 %) (Table 2). Growth rate results indicate a differential growth at different points of evaluation (2, 4, 6 and 8 weeks). The most significant differences in weight gain became evident at week 6 to 8, where, in general, CON group showed a significantly higher mean weight (p<0.05) than other groups. Fish fed with MT and ATD were significantly smaller.
Figure 1. Male ratio of genetically all-female tilapia observed in each diet treatment group (CON=control, CHR=chrysin, DAID=daidzein, CAFF=caffeic acid, SPIRO=spironolactone, MT=17α-methyltestosterone, ATD=1,4,6-androstatrien-3-17-dione) at the conclusion of experiment. Data for each treatment is presented as a mean ± SD of 20 fish, three replicates per treatment. Fish age at sexing was 65 days post-hatching, mean weight 2.3±0.7 g.

Table 2. Final mean weight, daily specific growth rate (%/day) and survival observed in both experiments per dietary treatment (mean ± SEM, n=3).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Final mean weight (g)</th>
<th>Daily SGR (%)</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All-male</td>
<td>All-female</td>
<td>All-male</td>
</tr>
<tr>
<td>CON</td>
<td>0.75±0.09</td>
<td>2.2±0.4^a</td>
<td>10±0.1</td>
</tr>
<tr>
<td>CHR</td>
<td>0.63±0.10</td>
<td>1.7±0.3^a</td>
<td>9.6±0.4</td>
</tr>
<tr>
<td>DAID</td>
<td>0.66±0.07</td>
<td>1.9±0.4^a</td>
<td>9.7±0.2</td>
</tr>
<tr>
<td>CAFF</td>
<td>0.67±0.09</td>
<td>1.7±0.2^a</td>
<td>9.8±0.3</td>
</tr>
<tr>
<td>SPIRO</td>
<td>0.65±0.04</td>
<td>1.4±0.5^bc</td>
<td>9.7±0.1</td>
</tr>
<tr>
<td>MT</td>
<td>1.1±0.1^b</td>
<td>8.4±0.2</td>
<td>92.8±2.5</td>
</tr>
<tr>
<td>ATD</td>
<td>1.1±0.0^c</td>
<td>8.4±0.1</td>
<td>95.0±1.6</td>
</tr>
</tbody>
</table>

Different letters indicate significant differences (P< 0.05) in columns.

(p>0.05) compared to fish fed with the different phytochemicals (Table 2). The final sex ratio of the experimental fish was not affected by the dietary administration of the tested phytochemicals. The only noticeable differences were effects of the steroidal compounds ATD and MT that resulted in 50 and 100% masculinization rates, respectively (Figure 1).

Concentrations of phytochemicals

The detection of the phytochemicals chrysin, daidzein and caffeic acid in fish tissue was accomplished. Extraction was successfully customized for fish tissue and HPLC detection conditions were modified to fit our specific laboratory equipment and conditions. For chrysin, in all-male tilapia at week 4, the parent compound was detected in 73% of the samples, and for week 6 in 93% of the analyzed fish. In experiment 2 the phytochemical was detected in all analyzed samples (100% detection) with a range of concentrations from 0.2 to 105.6 µg g⁻¹ whole body tissue; the with a range of concentrations from 0.2 to 105.6 µg g⁻¹ whole body tissue; the x whole body tissue concentrations are presented in table 3.
For daidzein in all-male tilapia at both, week 4 and 6, the chemical was detected, and the observed mean concentrations in µg g⁻¹ (Table 3). In all-female tilapia, detection was variable across 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. Detected values were between 0.2 and 146 µg g⁻¹ (Table 3).

For caffeic acid, a preliminary sample analysis by UV detection proved to lack the sensitivity required to quantify the observed low concentrations in whole body tissue in both all-male and all-female tilapia (Table 3). Once a the electrochemical detector was coupled to the HPLC system, we were able to detect the parent compound in 100% of the analyzed samples in all-male fish, both at 4 and 6 weeks; for all-female tilapia there was 100% detection at week 2, 80% at weeks 4 and 6, but only 20% at week 8 samples. Detected values were in the range of 0.1 to 3.4 µg g⁻¹ (Table 3).

It is apparent that the concentrations of chrysin, daidzein and caffeic acid diminish in a measurable manner throughout the sampling period for both feeding trials (All-male and all-male fish). Calculation of residual proportions of the presence of the parent compound throughout both feeding trials indicates that for all-male tilapia, whole body tissue concentrations dropped from week 4 to week 6 to 10, 22 and 27% for caffeic acid, daidzein and chrysin, respectively. For all-female tilapia, after 8 weeks only 0.5 to 1.5% of the observed initial concentrations at week 2 can be detected for all three phytochemicals at the end of the feeding trial (Table 3).

**DISCUSSION**

The inclusion at selected levels of phytochemicals as potential *in vivo* aromatase inhibitors in the diet of sexually undifferentiated tilapia did not exert a sex reversal effect in genetically all-female Nile tilapia in the present study. It was assumed that given the similarities on chemical structures to natural hormones, they could induce differential hormonal responses, due to the inhibition of hormone metabolizing enzymes (Brodie et al. 1999; Miyahara et al. 2003) and to achieve the required hormonal shift needed to observe an effect in sex differentiation of undifferentiated gonads in tilapia (Howell et al. 1994). The present study was based primarily on the reported aromatase affinity for flavonoids; therefore aromatase is a good target for selective inhibition of estrogen production and is the last step in the biosynthetic sequence of steroid synthesis (Brodie et al. 1999). Given that the majority of evidence that supports this pathway is based on *in vitro* studies (Collins et al. 1997; Le Bail et al. 1998; Jeong et al. 1999), when extrapolated to live organisms the results may become quite diverse as observed in our experiment. It was decided to include tested phytochemicals in an excess of at least 3 times the results may become quite diverse as observed in our experiment. It was decided to include tested phytochemicals in an excess of at least 3 times such concentration. Nevertheless, such level of inclusion for chrysin, daidzein and caffeic acid was not sufficient to induce a sex reversion in all-female tilapia (Figure 1).

The selected levels of inclusion for tested phytochemicals were mostly based on the assumption that their aromatase inhibition affinities are ranges from 2 to 5 times lower than other steroidal aromatase inhibitors (Jeong et al. 1999; Saarinen et al. 2001; Ohno et al. 2004; Monteiro et al. 2006); therefore, if inclusion level of ATD as steroidal aromatase inhibitor was on the level of 150 mg kg⁻¹, we decided to include tested phytochemicals in an excess of at least 3 times such concentration. Nevertheless, such level of inclusion for chrysin, daidzein and caffeic acid was not sufficient to induce a sex reversion in all-female tilapia (Figure 1).

There are several contrasting studies on the *in vivo* effect of decreased estrogen production due to aromatase inhibition by phytochemicals (Eng et al. 2001). A reduction of uterine weight in rats after consumption of extracts of different red wine varieties, with an identified high phytochemical concentration, was observed and it was a similar response to rats treated with a synthetic aromatase inhibitor (Letrozole). However, these results are quite different from those obtained by Saarinen et al. (2001), where chrysin and 7-hydroxyxavone inhibited the formation of ³H-17β-estradiol from ³H-androstenedione in human choriocarcinoma and embryonic kidney cells. When administered at a dosage of 50 mg/kg to immature rats, it failed to promote a reduced growth response in estrogen-dependent uterine enlargement. In conclusion, these effects may have been due to their relatively poor absorption and/or bioavailability. The authors stated...
that the in vivo effects of flavonoids on aromatase inhibition cannot be predicted on the basis of in vitro results. As an example, only chrysin fed group (Figure 1) had a slightly higher male ratio (24%) compared to the control group (18%); thus, results were not significant; therefore, we concur to such asseveration. Still, several phytochemicals had a 2-5 fold increased aromatase inhibition capacity, as demonstrated in KGN human ovary granulosa-like carcinoma cell preparations when compared with aminoglutethimide, a first generation aromatase inhibitor (Ohno et al. 2004).

The degree of absorption and metabolization of phytochemicals after ingestion could provide strong evidence of their potential bioactivity. Therefore, the next step was to validate the rate of retention of tested phytochemicals. The evidence of high concentrations in the urine, indicate high elimination rates in humans and other vertebrates (Pelissero et al. 1996).

In general, absorption of phytochemicals proceeds through 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). There is a possibility that not all these steps are fully accomplished given the degree of development of the digestive tract of the larval/juvenile fish used in present experiments. Another possibility is interspecies differences in the release of hydrolytic enzymes by microflora that are involved in food metabolism processes (Bairagi et al., 2002). Also, specific rearing conditions of the fish larvae (i.e. use of water sterilization unit) can influence digestive tract development and the specificity of the bacterial flora present in tilapia intestine (LeaMaster et al. 1997). This could have a negative impact on the absorption and metabolization of many food components in larval fish (Rawls et al., 2004), perhaps including phytochemicals. A possible indication of such relationship between the degree of absorption and metabolization of phytochemicals and the degree of gut development and present microflora could be associated with a significant decrease of concentration of phytochemicals in larger (older) fish (Table 3).

It is noticeable that the decline in concentrations in whole body for all three phytochemicals observed in both genotypes could be related to the increased intestinal length and metabolism by enzymes produced by the digestive tract and microflora, facilitating further metabolization and perhaps excretion. However, to validate such asseveration, studies are required on the proper chemical identification of phytochemicals metabolites. Thus, to establish what extent larval fish absorb and metabolize phytochemicals could be an important factor to determine the possible in vivo bioactivity of flavonoids as aromatase inhibitors. As stated, special 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).

In relation to the reduction of detection levels of the phytochemical parent compound by HPLC, studies in mammals have elucidated metabolic pathways for the tested phytochemicals. For example, daidzein is metabolized into dihydroadizein, then to equol, and finally to O-demethylandolensis (O-DMA), which has unknown estrogenic properties in humans (Patisaul and Whitten 1999). Caffeic acid is transformed into a series of derivatives, mostly ferulic, isofurolic and dihydroferulic acid; however, there is no information of the relevance of such derivatives as potential in vivo aromatase inhibitors (Rechner et al. 2001). Chrysin has an apparent favorable membrane transport properties through cell membranes. However, its absorption may still be seriously limited by a highly efficient conjugation reaction (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 performed in a greater detail to validate the dose and duration of treatment for possible activity. In a preliminary conclusion, if such transformation rates can be established, the decline of parent compound observed in table 3 throughout feeding, could be related to a direct dose-dependent lack of response as in vivo aromatase inhibitors of chrysin, daidzein and caffeic acid at selected levels of inclusion.

The described identification and detection methods by HPLC in this work provide a starting point in this area of research with fish. To the best of our knowledge, there is currently little or no information available on the absorption of phytochemicals in fish (D’Souza et al. 2005). Although several modifications were made from the original papers that described the analytical conditions, such as type of column, flow rate and wavelength for detection, here we confirm the presence of the parent compound in fish whole body tissue. In addition, the proper identification of the possible free and conjugated metabolites needs to be addressed.

Related to the possible estrogenic effects of the selected phytochemicals in present experiments with tilapia, we decided to conduct the experiment with both genotypes. As previously mentioned, the sex ratio did not change for any of the experimental groups in all-male tilapia. Such results could be related to specific features of the molecular structure of phytochemicals and receptor-binding affinity and the presence of multiple receptors sub-types (Thomas 2000). According to Benassayag et al. (2002) phytochemicals could overcome their rather weak binding affinity (10^2 – to 10^3 – fold) unless high concentrations in body fluids and tissues are attained.

Another consideration made on the present study was the effect on growth after selected phytochemical supplementation on the diet. In both feeding trials significant differences in the final individual mean weights were observed in the phytochemical fed treatments after 6 or 8 weeks of feeding, when compared with the control group (Table 2). A negative effect was also observed, in groups fed MT and ATD (Table 2). A negative effect was also x supplementation on the diet. In both feeding trials significant differences in the final individual mean weights were
observed in the phytochemical fed treatments after 6 or 8 weeks of feeding, when compared with the control group (Table 2). A negative effect was also observed, in groups fed MT and ATD (Table 2). A negative effect was also observed, in groups fed MT and ATD (Table 2). The feasibility of the continuation of this research by the inclusion of a higher percentage as diet ingredients, at the levels these phytochemicals are naturally occurring, such as in soybean meal (Hollman and Arts 2000; Pietta, 2000), could require special considerations.

In conclusion, this study provides original, new data in the evaluation of the effect in vivo of phytochemicals on sex differentiation in Nile tilapia as aromatase inhibitors. No significant effects on final masculinization percentage were observed using chrysin, daidzein and caffeic acid for all-female tilapia; thus, no in vivo response was observed. Also, although no significant response on masculinization was observed, we expect to continue the research on the use of spironolactone at higher inclusion rates to conclude with harder evidence whether or not could induce a sex reversal effect in fish as a potential aromatase inhibitor. Our result on the validation of the presence of the parent compound with HPLC techniques in fish tissue encourages future research on the degree of absorption and metabolism of these phytochemicals after dietary consumption.

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