EVALUATION OF DIETARY PHYTOCHEMICALS ON SEX DIFFERENTIATION AND GROWTH IN NILE TILAPIA (*Oreochromis niloticus*)

DISSERTATION

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Monosex fish stocks are desirable in aquaculture in order to control reproduction and select the gender that displays faster growth characteristics in a particular species. Synthetic steroids are commonly used to sex-reverse tilapia but because of their potential hazards; the use of new chemicals is a potential alternative to be explored. Phytochemicals present in many plants have many reported biological properties. This study explores the possible utilization of selected phytochemicals as potential in vivo enzymatic inhibitors of aromatase and of nuclear estrogen receptors antagonist in gonad germ cells. Such response could modulate the sex differentiation process of the gonad in sexually undifferentiated Nile tilapia.

In a first trial, experimental diets were supplemented with genistein (500 mg/kg) and quercetin (10 g/kg) along with the androgenic synthetic hormone 17α-methyltestosterone (MT) (60 mg/kg) and we evaluated the in vivo response to the dietary administration of these phytochemicals towards the masculinization of first feeding genetically all-female Nile tilapia (Oreochromis niloticus). In a second trail, experimental diets with caffeic acid (500 mg/kg), chrysin (500 mg/kg), daidzein (500 mg/kg) including MT (60 mg/kg), along with the steroidal aromatase inhibitor 1,4,6-androstatrien-3-17-dione (ATD) (150 mg/kg), and a
second steroidal compound spironolactone (500 mg/kg), were administered to
first feeding tilapia in order to assess the response in final phenotypic sex of the
gonad in experimental fish. In this second trial, phytochemicals were also
administered to genetically all-male tilapia. Growth response to the administration
of all phytochemicals and steroidal compounds was evaluated for both trials. Our
results indicate that the phenotypic sex of experimental fish is not affected by the
inclusion of phytochemicals at supplemented levels in the diets. MT and ATD
induced masculinization both feeding trials, final sex ratio for MT was 86 and
100% for experiments 1 and 2 respectively, ATD induced a 50% masculinization
rate. Spironolactone did not affect the sex ratio on either all-female or all-male
tilapia. Survival and growth as final mean weight and daily growth rate was not
different across treatments in experiment 1 and all-male experiment 2. In all-
female experiment 2, treatment groups for MT and ATD were significantly smaller
(p<0.05). Phytochemical absorption rates were validated with HPLC methods,
after adaptation of extraction procedures and chromatographic conditions that
allowed estimating concentrations in whole body after administration for either 6
or 8 weeks. Antioxidant biological activity of phytochemicals (quercetin) was also
under study; here we explored the possible interactions with ascorbic acid and
their biological effect on the experimental fish after acute UV-irradiation exposure
in order to reduce ascorbic acid depletion in skin tissue.
To my wife Leticia; thanks for your support and understanding while I achieved another of my crazy endless personal and professional goals.

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vi
# TABLE OF CONTENTS

Abstract ................................................................................................................................. ii
Dedication ............................................................................................................................. iv
Acknowledgments ............................................................................................................... v
Vita ........................................................................................................................................ vi
List of Tables ...................................................................................................................... ix
List of Figures .................................................................................................................... xi

**CHAPTERS:**

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Introduction ........................................................................................................... 1</td>
</tr>
</tbody>
</table>

2. Evaluation of two phytochemicals, genistein and quercetin by dietary administration as possible sex differentiation-affecting agents in all-female Nile tilapia ........................................................................................................ 16

| Abstract ......................................................................................................................... 16 |
| Introduction .................................................................................................................. 17 |
| Materials and Methods ............................................................................................... 20 |
| Results .......................................................................................................................... 24 |
| Discussion ................................................................................................................. 25 |

3. Effect of the dietary administration of daidzein, chrysin, caffeic acid and spironolactone on sex differentiation and growth of all-male and all-female Nile tilapia ........................................................................................................ 41

vii
LIST OF TABLES

Table…………………………………………………………………………………………………. Page

2.1 Composition of experimental diets for first feeding tilapia expressed as percentage of dry matter per 100 g.................................................................34

2.2 Summary of the final mean weight (WG), daily specific growth rate (SGR) and mean survival (±SD) of tilapia from each dietary treatment (n=3). Diets used were: control (CON), or supplemented with 17α-methyltestosterone (MT), quercetin (QUER) and genistein (GEN).................................................................35

3.1 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)........................................61

3.2 Final mean weight, daily specific growth rate (%/day) and survival observed in both experiments per dietary treatment (n=3). Experiment 1 (Exp 1), all-male tilapia; experiment 2 (Exp 2) all-female tilapia.................................................................62

4.1 Chromatographic conditions for quercetin detection in tilapia whole body tissues.................................................................75

4.2 Chromatographic conditions for genistein and daidzein detection in tilapia whole body tissue.................................................................75

4.3 Chromatographic conditions for chrysin detection in tilapia body ........76

4.4 Chromatographic conditions for caffeic acid detection in tilapia whole body tissues.................................................................76

4.5 Standard curve equations, correlation coefficients, detection limits (standard) and retention times of selected phytochemicals in accordance to described chromatographic conditions........................................88
5.1 Composition of experimental diets for first feeding tilapia expressed as percentage of dry matter per 100 g. 100 mg/kg AA (C-Q-), 100 mg/kg AA + quercetin 10 g/kg (C-Q+), 1000 mg/kg AA (C+Q-), 1000 mg/kg AA + quercetin 10 g/kg (C+Q+). .................................................................114

5.2 Observed performance in Nile tilapia (Orechromis niloticus) after 19 weeks feeding with experimental diets. Final individual weight and survival values (mean±SD) (n=3). Diets: 100 mg/kg AA (C-Q-), 100 mg/kg AA + quercetin 10 g/kg (C-Q+), 1000 mg/kg AA (C+Q-), 1000 mg/kg AA + quercetin 10 g/kg (C+Q+). .................................................................115

5.3 Proximate and mineral composition of tilapia juveniles body after 19 weeks feeding with experimental diets. Values are indicated as mean±SD (n=3). Diets: 100 mg/kg AA (C-Q-), 100 mg/kg AA + quercetin 10 g/kg (C-Q+), 1000 mg/kg AA (C+Q-), 1000 mg/kg AA + quercetin 10 g/kg (C+Q+). .................................................................116

5.4 Observed concentrations of dehydroascorbic acid (DHA) in skin tissue prior and 24h and 7 days after the second UV acute exposure. Values are indicated as mean±SD (n=6). Diets: 100 mg/kg AA (C-Q-), 100 mg/kg AA + quercetin 10 g/kg (C-Q+), 1000 mg/kg AA (C+Q-), 1000 mg/kg AA + quercetin 10 g/kg (C+Q+). .................................................................118

5.5 Observed reduced ascorbic acid concentrations in skin tissue prior to and after 24h and 7 days of second UV acute exposure. Values are indicated as mean±SD (n=6). Diets: 100 mg/kg AA (C-Q-), 100 mg/kg AA + quercetin 10 g/kg (C-Q+), 1000 mg/kg AA (C+Q-), 1000 mg/kg AA + quercetin 10 g/kg (C+Q+). .................................................................119
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Molecular structures of phytochemicals: quercetin (QUER), genistein (GEN) and steroid hormones: 17α-methyltestosterone (MT) and estradiol (E2).</td>
<td>33</td>
</tr>
<tr>
<td>2.2</td>
<td>Progress in individual mean fish weights observed during experimental feeding trial at 2, 4, 6 and 8 weeks. Values are mean ± SEM of triplicate groups (n=3). Diets: control (CON), 17α-methyltestosterone (MT), quercetin (QUER) and genistein (GEN).</td>
<td>36</td>
</tr>
<tr>
<td>2.3</td>
<td>Morphological appearance of male (A) and female (B) gonads. Gonadal squashes with aceto-carmine were analyzed by microscopic observation under 200X magnification.</td>
<td>37</td>
</tr>
<tr>
<td>2.4</td>
<td>Morphological appearance of female fish from control group (A) and male from MT group (B) gonad. Week 4 samples (0.15±0.08 g). Individual fish were analyzed by histological microscopic observations of abdominal sections (5-7µm) with eosin-hematoxilin staining.</td>
<td>38</td>
</tr>
<tr>
<td>2.5</td>
<td>Final male percentage observed in different diet treatment. Diets control (CON) (n=64), 17α-methyltestosterone (MT) (n=53), quercetin (QUER) (n=66), and genistein (GEN) (n=68). Fish age at sexing was 95 days, mean weight 8.3±3.2g.</td>
<td>39</td>
</tr>
<tr>
<td>2.6</td>
<td>Mean concentrations in whole body tissue (µg/g, mean ± SEM) of quercetin (QUER) and genistein (GEN) diets fed fish at 4 and 6 weeks (n=30). No phytochemicals were detected in the control group fish (CON).</td>
<td>40</td>
</tr>
<tr>
<td>3.1</td>
<td>Molecular structures of tested chemicals: 17α-methyltestosterone (MT), 1,4,6-androstatrien-3-17-dione (ATD), spironolactone (SPIRO), chrysirin (CHR), daidzein (DAID) and caffeic acid (CAFF).</td>
<td>60</td>
</tr>
<tr>
<td>3.2</td>
<td>Progress in individual mean fish weights observed in experiment 1 (all-male tilapia) at 2, 4, and 6 weeks. Values are mean ± SEM (n=3) groups.</td>
<td></td>
</tr>
</tbody>
</table>
Diets: control (CON), chrysin (CHR), daidzein (DAID), spironolactone (SPIRO) and caffeic acid (CAFF)…………………………………………………………………………..63

3.3 Progress in individual mean fish weights in experiment 2 (all-female tilapia) at 2, 4, 6 and 8 weeks. Values are mean ± SD of triplicate groups (n=3). Diets: control (CON), chrysin (CHR), daidzein (DAID), spironolactone (SPIRO) and caffeic acid (CAFF), 17α-methyltestosterone (MT), and 1,4,6-androstatrien-3-17-dione (ATD)………………………..……64

3.4 Final sex ratio (%) in experiment 2 observed (mean ± SD) per treatment (n=20 per replicate, three replicates per treatment). Diets: control (CON), chrysin (CHR), daidzein (DAID), spironolactone (SPIRO) and caffeic acid (CAFF), 17α-methyltestosterone (MT), and 1,4,6-androstatrien-3-17-dione (ATD)…………………………………………………………………………..65

3.5 Mean concentrations of chrysin (CHR) in whole body tissue (µg/g, mean ± SEM). Experiment 1, weeks 4 and 6 (n=15). Experiment 2, weeks 2, 4, 6 and 8 (n=10). No detection was observed in control group (CON)……..……………………………………………………….……………66

3.6 Daidzein (DAID) concentrations observed in whole body tissue (µg/g, mean ± SEM). Experiment 1, weeks 4 and 6 (n=15). Experiment 2, weeks 2, 4, 6 and 8 (n=10). No detection was observed in control group (CON)…………………………………………………………………………..67

3.7 Caffeic acid (CAFF) observed concentrations in whole body tissue (mean ± SEM). Experiment 1, weeks 4 and 6 (n=15). Experiment 2, weeks 2, 4, 6 and 8 (n=10). No detection was observed in control group (CON)……..…………………………………………………………………………..68

4.1 Molecular structures of selected experimental phytochemicals. Isoflavonoids: genistein (GEN) and daidzein (DAID), flavonol: quercetin (QUER), flavone: chrysin (CHR) and phenolic acid: caffeic acid (CAFF)…………………………………………………………………………..87

4.2 Caffeic acid chromatograms with electrochemical detection. a) standard 0.5 µg/ml. b) whole body tilapia sample. Retention time: 8.7 min………89

4.3 Chrysin chromatograms with UV detection. a) standard 0.5 µg/ml. b) whole body tilapia sample. Retention time: 9.4 min…………………………90

4.4 Daidzein chromatograms with UV detection. a) standard 0.5 µg/ml. b) whole body tilapia sample. Retention time: 9.3 min………………………91

xii
4.5 Quercetin chromatograms with electrochemical detection. a) standard 0.5 µg/ml. b) whole body tilapia sample. Retention time: 5.5 min…………….92

4.6 Genistein chromatograms with UV detection. a) Standard 1.2 µg/ml. b) whole body tilapia sample. Retention time: 16.1 min………………………93

5.1 Molecular structures of L-ascorbic acid (AA) and quercetin (QUER)…..113

5.2 Progress of individual mean body weight of Nile tilapia fed with experimental diets for 19 weeks. Values are mean ± SEM of triplicate groups (n=3). Diets: 100 mg/kg AA (C-Q-), 100 mg/kg AA + quercetin 10 g/kg (C-Q+), 1000 mg/kg AA (C+Q-), 1000 mg/kg AA + quercetin 10 g/kg (C+Q+)……………………………………………………………………….117

5.3 Total ascorbic acid concentrations in skin tissue (µg g⁻¹ wet tissue) observed prior (A), 24 h control (B) and UV (C) and 7 days control (D) and UV (E) after 2⁰ UV irradiation acute exposure (0.9 J/cm²). Data are means ± SD (n=6) for total ascorbic acid concentrations (µg/g). Different letters indicate significant differences (P> 0.05) per sampling. Diets: 100 mg/kg AA (C-Q-), 100 mg/kg AA + quercetin 10 g/kg (C-Q+), 1000 mg/kg AA (C+Q-), 1000 mg/kg AA + quercetin 10 g/kg (C+Q+)……………………………………………………………………….121

5.4 Chromatograms for quercetin (A ) in skin tissue samples and (B) liver tissue samples determined with electrochemical detection. Top shows quercetin standard 200 ng/ml, bottom shows tissue chromatograms….122

5.5 Quercetin concentrations in liver tissue observed 24h after 2⁰ UV irradiation acute exposure (0.9 J/cm²). Data are means ± SD (n=6). Means with the same letter are not significantly different (P> 0.05). Diets: 100 mg/kg AA + quercetin 10 g/kg (C-Q+), 1000 mg/kg AA + quercetin 10 g/kg (C+Q+)……………………………………………………………………….123

5.6 Head skin in control (unexposed) fish (a) and lesions and in UV-exposed fish (b, c and d) observed by histological analysis display cell alteration that include hyperplasia, intracellular edema and necrosis (arrows). Hematoxilin-eosin stained 400–1000x. a) Control treatment. b) UV exposed –C-Q. c) UV exposed –C+Q. d) UV exposed +C-Q…………..124
Food production is without a doubt, a very relevant aspect in human life. Among the different food production activities the most important are agriculture, livestock production and fisheries. These three economic activities and more specifically fisheries, both inland and marine fisheries are close to reaching their maximum capacity in term of sustainable production. In order to reduce the pressure on wild stocks of many fish under commercial use, aquaculture is a viable alternative to fisheries throughout the world (Marra, 2005).

In recent statistics, the Food and Agriculture Organization (FAO) of the United Nations in their 2004 report, indicates that while the three main food production activities have a fairly low percentage of increase per year (close to 1%), aquaculture has a steady 10% expansion every year, and that within 5 to 10 years will supply up to 50% of the world demand for fisheries products. However, in order to make aquaculture a highly efficient activity, many considerations should be taken by producers. Although fish farming is deemed as an activity that uses resources which are not useful for other inland food production efforts, it also deals with the fact that the product or fish should be something that has a high economic value, in term of commercial demand and low production costs.
Once a fish species has been identified to be of interest for aquaculture production, specific biological aspects need to be considered to determine the best production technology at high scale for a particular fish. Characteristics such as fast growth rates in captivity and at high densities, resistance to pathogens, acceptance of formulated commercial diets, and controlled reproduction in order to ensure a constant supply of progenies and to control population dynamics in the production units, are extremely important. Nevertheless, in special situations, a particular fish species can exhibit some specific characteristics that could be considered of a possible negative connotation. It is known that in certain species, one gender exhibits faster growth rates than the other, or that they reach sex maturity early in their life span and that uncontrolled reproduction can take place affecting the optimization of the production system. There are many variations across different species, so that it is difficult to make a generalization. Therefore, each case should be treated individually. Manipulation of phenotypic sex in fish farming is generally desirable since one gender, depending on the species, grows faster (Uguz et al., 2003). A lot of research has focused on the understanding of how these factors can be manipulated in order to optimize the production of fish by means of intensification of aquaculture.

Tilapia (Cichlidae) is among the species extremely suitable for aquaculture. Muir et al. (2000), indicates that even considering that the potential of tilapia for intensive production has been realized in general, the species has not had the same immediate appeal that other food fish species. It still provides many desired characteristics. Fitzsimmons (2000) reviews these characteristics
in terms of the unique mix of the tilapia’s physiology, reproductive biology, genetic plasticity, development of domesticated strains, and ready marketability that have put it at the forefront of aquaculture. He also remarks that the future convergence of improved culture techniques, new farms, low cost diets, ecological efficiency and emerging markets will boost tilapia to be the world largest aquaculture crop. As a final remark this author states that tilapia is likely to be the most important of all aquacultured fish in the 21st century.

Despite promising expectations in tilapia aquaculture, there are a series of factors that are considered to be detrimental in the expansion of production. Strong environmental concerns have been outlined by several authors in relation to the ecological burden of tilapia aquaculture. These include the amount of organic matter and wastewater discharged due to the fertilization of ponds and use of supplemental feeding to increase production (Yi et al., 2002; Abdelghany and Ahmad, 2002), and the release of hormones used to produce monosex populations for intensive aquaculture (Green et al., 1997; Abucay and Mair, 1997; Gale et al., 1999).

Among the new approaches to integrate tilapia production and wastewater treatment management are those that prove that tilapia can be a useful species in other aspects, not related to food production. Several studies draw attention to the possibility of using tilapia as ‘biofilters’ based on observations of the ability of tilapia growing exclusively from other species culture system effluents (Appelbaum and Volvich 2000), polyculture with other fish species such as Chinese carps (Papoutsoglou et al., 2001; Abdelghany and Ahmad 2002) and
aquatic macrophytes (Yi et al., 2002). Therefore, an increased production of tilapia and other species, along with an efficient removal of inorganic nitrogen from wastewater, can be observed while tilapia is functioning as a biological component in wastewater treatment/food production ecosystem role (Costa-Pierce, 1998).

In contrast, a major drawback in tilapia aquaculture is that all-male populations are desirable because males demonstrate superior growth characteristics compared to females. In addition, culture of monosex populations prevents reproduction and results in a uniform fish size, given that females reach sexual maturity earlier. With the intention of solving such situation, the synthetic steroid 17α-methyltestosterone (MT), a derivative of testosterone is frequently used to masculinize tilapia juveniles in order to later be used in commercial aquaculture (Green et al., 1997; Abucay and Mair, 1997; Gale et al., 1999).

The success of the utilization of steroid hormones to produce monosex populations of fish is well documented. Gonadal sex differentiation in fish is a complex process, unlike in higher vertebrates. In fish, besides genetic or chromosomal sex determination, there are other factors that can be involved in the outcome of the final process of gonad development and final sex phenotype. The sex determination mechanism is driven by specific genes that control only the “initial decision” of phenotypic gonad gender, but this initial instruction that directs the gonad sex differentiation process can be overruled later on, by numerous intrinsic and extrinsic factors (Hayes, 1998). The fact is that at the moment of hatching in many species of fish, the gonads are not fully
differentiated, and in other species are still in the process of tissue arrangement in the body cavity (Strussmann and Ito, 2005). In gonochoristic species such as tilapia, two major hypothesis of early gonadal development are suggested. In one case the undifferentiated gonad develops directly to ovary or testis, as seen in tilapia. Alternatively all individuals initially develop ovarian tissue, but later gonad masculinization can occur in some individuals. In the second case the gonads are initially intersex prior to differentiation into either testis or ovaries (Devlin and Nagahama, 2002). During the specific critical periods of early gonad development, changes in sex hormone levels can affect the final sex independently of the genetic sex (Andersen et al., 2003). Since the process can take place at different stages in the life span of the individual species (for instance at prehatching in Japanese medaka or at the 2-year in sturgeon), there is a time window where external manipulation of several variables can re-direct the gonad differentiation process. In general, the most sensitive period is just prior to or concomitant with the initial histological differentiation of the primary gonad (Devlin and Nagahama, 2002).

**Synthetic androgens**

One of the main factors involved in directing the gonad differentiation process are sex steroids hormones (Piferrer, 2001). Such information is inferred based on multiple experiments in which administration of exogenous androgens or estrogens has successfully skewed the phenotypic sex ratio to the desired sex (Baroiller and D’cotta, 2001; Strussmann and Nakamura, 2002). It has been reported that the presence of either an androgenic or an estrogenic hormone in
the gonad at higher concentrations, can be correlated to the final response of phenotypic sex of the gonad. Research has been focused on whether the manipulation of biological synthesis of steroids can have an effect similar to the administration of exogenous steroid hormones. Experiments with inhibitors of steroid synthesizing enzymes, such as aromatase, have proven to be valuable in establishing the involvement of estrogens in ovary differentiation (Barollier et al., 1999). Given that estrogens are converted from androgens, research has focused on the elucidation of how specific enzymes that are responsible for the metabolic transformation of androgens to estrogens within the gonad, can be inhibited, potentially inducing a partial or complete masculinization of the gonad as a biological response (Baroiller and D’cotta, 2001).

However, the utilization of androgenic hormones to produce all-male population is not free of concerns. In the particular case of tilapia, the efficacy of the hormone of choice (MT) depends on various factors, such as dose, timing and duration of treatment, and mode of administration (Mirza and Shelton, 1988). When used at high doses or in prolonged treatment, MT can induce gonadal intersexuality and paradoxical feminization (Goudie et al., 1983; Solar et al., 1984; Van den Hurk et al., 1989; Blasquez et al., 1995; Rinchard et al., 1999; Papoulias et al., 2000). Piferrer and Donaldson (1989) suggested that paradoxical feminization might be due more to the transformation of MT by aromatase to 17α-methylestradiol, than to inhibition of in vivo synthesis of androgens. This fact was recently reported in fathead minnow (Pimephales
promelas), where MT exposed adult males expressed secondary female characteristics after 7-day waterborne exposure (Hornung et al., 2004).

In particular the proclivity towards the use $17\alpha$-alkyl anabolic synthetic steroids, such as MT is due to the fact that alkylation at the 17-position prevents the rapid oral inactivation that occurs with other anabolic steroids, and consequently eliminates the need to inject the drug (Stanley et al., 1997, Gonzalo-Lumbreras and Izquierdo-Hornillos, 2003). Several studies have addressed the dynamics of the uptake and depletion of MT in different teleost fish, including cichlids (Fagerlund and Dye, 1979; Johnstone et al., 1983, Goudie et al., 1986; Curtis et al., 1991; Cravedi et al., 1993, Rinchard et al., 1999). As MT administrated orally is readily metabolized, research on the fate of MT and on its metabolites needs to address the human and environmental safety perspective. Chronic exposure of humans to MT can cause adverse health effects such as hepatotoxicity; therefore, ingestion of MT residue in treated fish may be a potential hazard to human consumers. The quantity of MT residue in fish tissue will depend on its dosing history and its pharmacokinetics characteristics (Vick and Hayton, 2001). Use of synthetic steroids in fish culture is associated with potential release to the environment and contamination of the biota. This situation causes public concerns as to the safety of the product. Therefore, alternative methods and new, safe chemicals to produce monosex populations should be considered.

The direct study of the mechanisms of hormone biosynthesis and action on the gonadal sex differentiation process could also provide alternative methods
for producing monosex populations of fish. Among these mechanisms, the enzyme aromatase is of particular interest in 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, Strauss et al., 1998, Griffiths et al., 1999, Eng et al., 2001), 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, Kitano et al., 2000) as well as by steroidal and non-steroidal chemical compounds (Brodie et al., 1999, Smith, 1999, Seralini and Moslemi, 2001) that have achieved a relative degree of success in sex inversion in fish. Thus, hypothetically the inhibition of aromatase by physical and/or chemical factors could mimic the sex-reversal effects of androgen treatments in some fish species (Kwon et al., 2001), although not on the same magnitude as the efficiency reported for MT and other androgenic steroids.

Phytochemicals

Chemical compounds classified as phytochemicals (isoflavonoids, flavonoids and lignans among others) are natural steroid-like compounds derived from soy, tea, fruits and vegetables with a reported aromatase inhibition ability that are able to suppress estrogen biosynthesis in cells (Geahlen et al., 1989; Pelissero et al., 1996; Eng et al., 2001). Such action is facilitated by the stable structure and low molecular weights of phytochemicals that can pass through cell membranes (Ososki and Kenelly, 2003). In general, the action of phytochemicals when administered to fish is a recent subject of study. The possible effect of
flavonoids on fish aromatase was suggested by a nutritional study with female sturgeons. The hormonal profile was monitored in fish fed with a high dietary inclusion of soybean meal; these analyses showed a decrease of plasma estrogen levels (Pelissero et al., 1996).

Many studies indicate that the capacity of phytochemicals to inhibit aromatase is closely related to their chemical structure (Le Bail et al., 1998; Jeong et al., 1999; Saarinen et al., 2001). The degree of hydroxylation of the molecule increases their ability to inhibit enzymatic activity. Therefore, higher number of hydroxyl radicals increases anti-estrogenicity (Krazeisen et al., 2002). A study where the flavone 7-hydroxyflavone and apigenin were used in microsomes of human placenta showed that these two specific flavonoids were very effective aromatase and 17ß-hydroxysteroid dehydrogenase inhibitors. In the same experiment, it was observed that flavonoids with 7-methoxy or 8-hydroxyl groups in the A ring 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 is generally lower than it is for steroidal derivatives (Seralini and Moslemi, 2001).

A second approach in the use of phytochemicals as endocrine disruptors involves 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.
by the estrogen receptor. They can compete with endogenous estrogens for binding sites 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 natural steroidal estrogens (Rickard and Thompson, 1995).

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 need to be developed (Hollman and Arts, 2000). Once flavonoids and other phytochemicals are absorbed, they undergo metabolic transformations, which in most cases have unknown biological properties as estrogenic or anti-estrogenic compounds (Patisaul and Whitten, 1999). Once polyphenols are transformed into a series of derivatives, they can be used as specific biomarkers for the bioavailability and metabolism of a phytochemical. It will then remain to clarify the relevance of such derivatives as possible endocrine-modulating chemicals (Rechner et al., 2001). Therefore, comprehensive pharmacokinetic studies involved in the absorption of phytochemicals in fish, including aglycones and glucosides need to be conducted (D'Souza et al., 2005).

There is a significant variation on the results obtained after using pure phytochemicals, and in observations of their action in vitro in the inhibition of
estrogen synthesis (Joshi et al., 1998). Most of the information available in fish is related to the activity on in vitro essays using gonad cells and measuring the ratio of inhibitions of synthesis of estrogens when flavonoids are presents at different concentrations (Pelissero et al., 1996). There is only limited understanding of how flavonoids with steroidal activity are absorbed and metabolized by fish. Some information is available on absorption of genistein aglycone in Siberian sturgeon (Acipenser baeri) and rainbow trout (Onchorhynchus mikiss) (Gontier-Latonelle, 2001). The in vivo effects in tilapia remain mostly unknown.

Another important aspect related to phytochemicals and in particular to flavonoids and other phenolic compounds is their reported association with nutritional factors. A series of reviews in the literature (Knight and Eden, 1995; Manach et al., 1996; Pietta, 2000; Dixon, 2004) suggest that among the additional reported biological activities for these compounds are strong antioxidant capacity, along with the ability to interact with protein phosphorylation, iron chelation, and a series of enzymatic reactions (Bonina et al., 1996; Boyle et al., 2000). The most relevant biological property of phytochemicals is their ability to act as antioxidants. The antioxidant capacity in general, is conferred by a high number of hydroxyl substitutions which has a direct effect on the donating ability of hydrogen (Pietta, 2000; Kim, 2001). In addition, flavonoids may regenerate other antioxidants such as tocopherol by donating a hydrogen atom to the tocopheryl radical (Boyle et al., 2000). In the public health domain, consumption of phytochemicals is associated with a decreased risk of cardiovascular disease by protecting against oxidative cell
damage (Reiterer et al., 2004). Therefore, special attention should be also given to this subject in respect to the use of phytochemicals in fish nutrition or as endocrine mimics of steroid hormones.

The present research is focused on the use of different phytochemicals, to evaluate their potential action in vivo on sex inversion in Nile tilapia larvae as non-steroidal endocrine disruptors, in parallel with other endocrine action modulating chemicals, such as steroids or aromatase inhibitors. New approaches are required to explore the possible use of phytochemicals as sex inversion agents in tilapia, in order to ensure that they are as effective as the chemicals commonly used to produce monosex populations, steroid hormones such as MT. We hypothesize that feeding fish with diet containing these natural substances at appropriate dietary levels and at the time of ontogenesis (sex differentiation), will affect the sex ratio of the tilapia populations.

Chapter 2 addresses the evaluation of quercetin and genistein as possible in vivo enzymatic inhibitors of aromatase and as potential antagonists of nuclear estrogen receptors in cells that could modulate the response of sex differentiation on the gonad of sexually undifferentiated tilapia. Experimental diets supplemented with quercetin 10 g/kg (QUER) or genistein 500 mg/kg (GEN) were compared with a control diet (CON) (free of hormones and phytochemicals) and a diet containing 60 mg/kg of 17α-MT (MT), evaluated on first feeding all-female tilapia. Final sex ratio, mean individual weight (g), SGR (%/day) and survival (%) were recorded. Results indicate that the presence of genistein and quercetin does not affect negatively either growth or survival. However, no effect
was observed towards masculinization of gonads in all-female tilapia, as opposed to the MT treatment were the final male ratio was 86%. Quercetin and genistein absorption in treated fish was analyzed by HPLC; despite the observed parent compound concentration in whole body, no response in sex differentiation towards masculinization in all-female tilapia was observed in the genistein and quercetin groups.

Chapter 3 is focused on the evaluation of chrysin, caffeic acid and daidzein, given their reported increased aromatase inhibition capabilities. Their effects were contrasted with the administration of MT, or with two other steroidal compounds. Two feeding experiments were conducted on first feeding Nile tilapia (*Oreochromis niloticus*), genetically all-female and genetically all-male, to evaluate the response on sex inversion. For all-male tilapia (experiment 1) control, daidzein, chrysin, caffeic acid and spironolactone were compared. For all-female tilapia (experiment 2), control, MT, 1,4,6-androstatrien-3-17-dione (ATD), daidzein, chrysin, caffeic acid and spironolactone were used. Fish were fed for 6 weeks (Exp. 1) or 8 weeks (Exp. 2). Results indicate that the sex ratio of genetically all-female tilapia is not affected by the tested phytochemicals. MT and ATD produced sex reversion ratios up to 100% and 50% respectively; spironolactone did not induce a sex inversion response in all-female tilapia. For all-male juveniles no effect was observed in male sex ratio for any of the phytochemicals or spironolactone. No differences were observed in survival, final mean weight, or SGR between treatments in experiment 1; for experiment 2, MT and ATD groups were significantly smaller, but survival and SGR were not
different. Accumulation rates for the three phytochemicals are described.

Chapter 4 describes the elucidation of the analytical conditions to detect phytochemicals in fish tissue. Most of the reported methodologies for determination of absorption and metabolization have been conducted by means of HPLC detection techniques, and are based in the analysis of biological samples either from their natural sources (soy products, fruits, etc), and from body fluids (plasma, urine) in major vertebrates (mostly mammals). We focused on the analysis of caffeic acid, chrysin, daidzein, genistein and quercetin in fish using Nile tilapia (*Oreochromis niloticus*) as a model. Here, we contrast two extraction methods and describe the specific chromatographic conditions with respect to selected analytical column, mobile phase composition and type of detector for each compound. All phytochemicals were successfully detected in fish samples of juvenile Nile tilapia.

Chapter 5 portrays the potential action of quercetin to minimize depletion of ascorbic acid in skin tissue after ultraviolet light exposure. Both quercetin and ascorbic acid (AA) exhibit a potent antioxidant activity. Four semi-purified casein-gelatin based diets with various levels of AA and quercetin were prepared. Experimental organisms were fed for 19 weeks (initial weight 9.6±1 mg). After 19 weeks, 25 fish per replica per diet were divided into 2 groups control and UV treatments, and exposed to UV radiation. Fish were exposed twice with a 7 day waiting period between exposures. Prior to each exposure and 24 hours and 7 days after the 2nd treatment liver and skin were sampled for analysis of quercetin and AA. Twenty-four hours after 2nd exposure, head sections were fixed for
histological analysis of skin damage in both groups. Results on concentration of AA and quercetin in selected tissues indicate that depletion of ascorbic acid was not significantly minimized by the presence of quercetin in the diet. Quercetin concentration in skin was below the limits of detection, but quercetin in liver was reduced after acute UV exposure. Skin damage was of similar magnitude across all dietary treatments. The inclusion of quercetin in the diet did not affect the growth and survival of the experimental fish.
CHAPTER 2

EVALUATION OF TWO PHYTOCHEMICALS, GENISTEIN AND QUERCETIN
BY DIETARY ADMINISTRATION AS POSSIBLE SEX DIFFERENTIATION-
AFFECTING AGENTS IN ALL-FEMALE NILE TILAPIA.

ABSTRACT

All-male tilapia stocks are desirable in aquaculture in order to control
reproduction and superior growth characteristics compared to females. The
synthetic steroid, 17α-methyltestosterone (MT) is commonly used to sex-reverse
tilapia but, because of its latent health and environmental hazards, the use of
alternative chemicals in aquaculture should be explored. Phytochemicals are
molecules naturally produced by plants and have a wide number of reported
biological properties. In this work we evaluate quercetin and genistein as
possible in vivo enzymatic inhibitors of aromatase and as potential antagonists of
nuclear estrogen receptors in cells that could modulate the sex differentiation
response in the gonad of sexually undifferentiated tilapia. Experimental diets
supplemented with quercetin 10 g/kg (QUER) or genistein 500 mg/kg (GEN)
along with a control diet (CON) (free of hormones and phytochemicals) and a diet
Results indicate that the presence of dietary genistein and quercetin does not affect negatively either growth or survival, and no effect was observed with respect to masculinization of gonads in all-female tilapia. To the contrary, we observed that the MT treatment was effective in increasing final male ratio to 86%. Quercetin and genistein absorption in treated fish was analyzed by HPLC; despite detected concentrations of parent compound in whole fish body, no response in sex differentiation towards masculinization in all-female tilapia was seen in the genistein and quercetin treated groups.

INTRODUCTION

Sex plasticity is a well documented phenomenon in teleost fish, given that gonadal sex differentiation can be re-directed by many other factors, besides genetic sex determination mechanisms, such as environmental conditions and exogenous endocrine-active chemicals (Blazquez et al., 1995; Kitano et al., 2000; Bertolla-Afonso et al., 2001; Galbreath et al., 2003). Effects of environmental parameters such as temperature (Baroiller and D’cotta, 2001; Devlin and Nagahama, 2002; Nakamura et al., 2003) and pH (Zelennikov, 1997) are well documented. This issue is of considerable relevance for species of interest for aquaculture. Sex inversion is used to minimize reproduction in tilapia, given that when this species is cultured in mixed sex populations it begins to
reproduce before reaching marketable size (Teichert-Coddington et al., 2000). The synthetic steroid, 17α-methyltestosterone is a derivative of a male specific hormone commonly used to masculinize tilapia juveniles (Abucay and Mair, 1997; Gale et al., 1999). This can lead to criticism that aquatic systems are acting as a sink for many persistent compounds of MT with characteristics that potentially disrupt endocrine systems of man, livestock and wild-living animals when exposed incidentally (Lagana et al., 2001). Therefore, concerns regarding chronic exposure of humans to MT as a cause of adverse health effects such as hepatotoxicity (Vick and Hayton, 2001) favors research on alternative chemicals to produce monosex populations in tilapia and other fish.

A possible alternative approach to the use of steroid hormones for sex reversal in tilapia may involve the use of phytochemicals such as isoflavonoids, flavonoids and saponins, which are natural compounds characterized by estrogenic/androgenic activity. They may be derived from soy (Nguyenle et al., 1995), tea (Fillip and Ferraro, 2003), fruits (Tomas Barberan et al., 2001) and vegetables (Boue et al., 2003). Among the known actions of phytochemicals that suggest estrogenic/androgenic activity, are the inhibition of several steroid metabolizing enzymes such as aromatase, and phytochemical ability to interact with nuclear estrogen receptors and consequently the modulation of the genomic response to estrogenic hormones (Thomas, 2000; Eng et al., 2001; Saarinen et al., 2001). Therefore, these phytochemicals may direct the sex differentiation of the ovary in gonochoristic fish species towards testis (Piferrer, 2001).
Aromatase, a P450 cytochrome enzyme catalyzes the conversion of androstenedione and testosterone to estrogenic sex hormones (Brodie et al., 1999; Griffiths et al., 1999; Eng et al., 2001). Aromatase is an enzyme of particular interest in sexual differentiation in fish since inhibition of aromatase action mimics the sex-reversal effects of androgen treatments in some fish species (Bhandari et al., 2003; Lee et al., 2003). Given that estrogen is the product of aromatase, it is not unexpected that compounds with aromatase inhibition properties can suppress estrogen biosynthesis in cells and specific tissues (Geahlen et al., 1989; Pelissero et al., 1996).

There are a limited number of studies on the biological effects of phytochemicals in aquatic species. Studies in Japanese medaka (Oryzias latipes), have showed that quercetin administration can cause endocrine disruption in females (Weber et al., 2002). Genistein induced increased levels of endogenous testosterone as indication of aromatase inhibition in rainbow trout (Bennetau-Pelissero et al., 2001). Only a few studies can be used as valid references on the potential utilization of genistein and quercetin to induce a sex inversion effect in fish. A recent study in European eel (Anguilla anguilla), where genistein induced a feminization effect after dietary administration (Tzchori et al., 2004) is of uncertain value, as the control group possessed only 5% of females. Several studies had involved the use of both genistein and quercetin in-vitro to evaluate both aromatase inhibition and estrogen receptor binding affinity in various tissues of rainbow trout (Oncorhynchus mykiss) and other species (Pelissero et al., 1996; Latonelle et al., 2002; Tollefsen et al., 2002). Although the
results are not completely in agreement these studies still provide a valuable insight for this study. Here we provide further insights concerning steroid-free methods for producing all-male populations of tilapia using specific phytochemicals, such as genistein and quercetin (Fig. 2.1), as potential masculinizing agents. As genistein and equol were observed to act as estrogen antagonist and delayed oocyte maturation in previtellogenic Japanese medaka (Kiparissis et al., 2003), we hypothesize that feeding fish with diets containing these phytochemicals, could affect the sex ratio in all-female tilapia groups.

MATERIALS AND METHODS

Reagents

All chemicals used for diet formulations and used for HPLC analyses standards were of analytical grade; genistein (4',5,7-trihydroxyisoflavone), quercetin (3,5,7,3',4' pentahydroxyflavone) and 17α-methyltestosterone (MT) were purchased from Sigma (St. Louis MO, USA). Mobile phase HPLC grade components were purchased form Fisher Scientific Inc. (Pittsburgh, PA; USA).

Feeding experiments

The experiment was conducted on the first feeding, genetically all-female tilapia Oreochromis niloticus (Phil-FishGen, Nueva Ecija, Philippines), initial individual weight 10 mg. Fish were randomly distributed into 12 glass aquaria (35 L) in a semi-closed recirculation system with constant temperature (26±1 ºC). An initial density of 150 fish per aquarium was used with three replicates per
treatment. Outflow from MT treatment tanks was diverted from the recirculation setup to avoid contamination by MT of other experimental units.

Four casein-gelatin based diets were prepared as follows: control (CON), 60 mg of 17α-methyltestosterone/kg (MT), quercetin 10 g/kg (QUER) and genistein 500 mg/kg (GEN). Semi-purified diets were formulated to avoid contamination with natural steroids commonly provided when fish meal based products are used (Feist and Schreck, 1990). For MT diet, the steroid was dissolved in ethanol as vehicle and mixed thoroughly in the control diet after pelleting; later, ethanol was allowed to evaporate overnight at room temperature prior to feeding trials. Diet compositions are detailed in Table 2.1. Fish were fed at a decreasing rate for 20 to 10% body weight ratio for 8 weeks, adjusted weekly. MT diet was given to the fish only during the first 4 weeks; after MT withdrawal fish were fed with the control diets for the remaining 4 weeks of the experimental trial. Performance was evaluated in terms of the individual body weight (every two weeks), the final survival (%) and daily specific growth rate (%/day).

Sex ratio analysis

After 8 weeks of feeding with experimental diets, fish were fed with a tilapia commercial diet until they reached a minimum size of 5 cm total length, to facilitate gonad removal from abdominal cavity. Sex was determined in individual fish (mean weight 8.3±3.8 g) by microscopic analysis of gonadal squashes (Guerrero and Shelton, 1974).
A few fish samples from each treatment (n=10) at weeks 4 and 6, were processed for histological analysis of the gonad. Abdominal sections were embedded in paraffin and stained using Eosin-hematoxilin technique.

**Phytochemical analysis**

An analysis on the bioaccumulation of the two phytochemicals tested in the experiment was conducted by HPLC techniques. Fish samples (n=10) were withdrawn at 4 and 6 weeks of feeding trial, fish were not fed 24 h prior to sampling. 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 consisted of homogenization (Omni Int. GLH-115 homogenizer) of individual fish in the extraction solution for 30 sec at 5000 rpm, and later incubation at 37°C for 16 h (overnight). Prior to injection into HPLC, acidity was neutralized with 10N NaOH (equivalent volume to sample weight) to achieve a total dilution rate 1:10. Samples were filtered through 0.45 µm disk filters and either injected immediately to the HPLC or frozen at -80 °C for further analysis. Recovery rate was estimated with internal standards to be in the range of 95% for both 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.

Quercetin analysis in tissues by HPLC was performed using the methodology from Park et al., (in press). Mobile phase composition was 27%
acetonitrile in water: acetic acid (99.5:0.5 v:v); flow rate was 1.0 ml/min; detection was achieved by means of an BAS® electrochemical detector equipped with a CC-5 flow cell and a LC-44 detector, set at 0.08 to 8.0 mV output. The analytical column used was an ODS Beckman® 150X4.6 mm 5 µ reverse-phase column. Detection limit of standard was 20 ng/ml.

The detection of genistein in whole body tissue was conducted using a modified procedure from Hutabarat et al. (1998). Mobile phase composition was 33% acetonitrile in water: acetic acid (99:1, v:v) at a flow rate of 1.2 ml/min; wavelength for UV detection was 260 nm. A reverse phase Synergi hydro 250X4.6 mm 4µ (Phenomenex®) column was used for the analysis. The detection limit of standard was 45 ng/ml.

**Statistical analysis**

Mean individual final weight and survival were analyzed by one-way ANOVA, if significant differences were found, a Tukey test was performed (Zar, 1990). 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**

Evaluation on growth performance did not show any significant differences on the evaluated parameters (Table 2.2). The MT group showed a lower final mean individual weight (0.7±0.3 g) (Figure 2.2) but the difference was not
statically significant. The total weight gain (aprox. 1 g) and SGR (8% per day) were similar in all groups. Cumulative survival did differ significantly across dietary treatments (Table 2.2). Observed overall mean survival rate in experimental fish was close to 65% in the experiment. No variations were observed in food consumption among experimental groups. Growth rates during the experiment were similar for the first 4 weeks across all treatments (Figure 2.2). A slight drop in weight gain was observed in the MT group after withdrawal of steroid supplement from the feed; still, such difference was not significant ($p>0.05$).

Results indicate that the sex ratio of genetically all-female tilapia gonads was not affected by the inclusion of genistein or quercetin in the diets. In other words, the final female/male ratio was equal to that observed in the CON group (97%). A small percentage of males were observed in CON, GEN and QUER groups (3%). To the contrary MT group exhibited a male dominance up to 86% (Figure 2.5). No intersex gonads (presence of both testis and ovary tissue) were observed in GEN and QUER groups, and only one case of intersex gonads was observed in the MT group out of all fish observed by microscopic analyses. Histological appearance of the gonad was also observed for a limited number of samples (Figure 2.4). General morphological structures of the gonad were observed in order to have a closer approximation of the possibility of intersex gonads in the experimental groups. However, similarly to the freshly stained gonad observations, no intersex gonads were observed (Figure 2.3).
Detection was achieved for both phytochemicals by HPLC. Quercetin was detected in 100% of the analyzed samples (n=30 per sampling time) in fish sampled at 4 and 6 weeks. Genistein was detected in 98% of the samples (n=30 per sampling time). Mean whole body tissue concentrations observed after HPLC analyses are presented in Figure 2.6. Quercetin accumulation in whole body tissue was observed to uphold similar concentrations; for both sampling points the mean concentration at 4 and 6 weeks was close to 40 µg/g of tissue. Genistein, however, had shown an apparent reduction in whole body tissue concentrations of the parent compound from week 4 to week 6. The mean concentration declined by 90%, from 11 µg/g to 1.3 µg/g (Figure 2.6).

**DISCUSSION**

Genistein and quercetin were selected for this study due their relative abundances in nature. It has been reported that in their natural sources, quercetin is present in up to 400 mg per 100 g in capers (*Capparis spinosa*) (Inocencio *et al.*, 2000) and 100 mg per kg in onions (De Vries *et al.*, 1998). Although quercetin concentration in the experimental diets exceeded by far, the above concentrations, the level of inclusion of 1% per kilogram of the diet was proposed based on relatively low affinities of to aromatase inhibitors (EC$_{50}$ 139 µM) compared with apignin (EC$_{50}$ 84 µM) (Pelissero *et al.*, 1996), or compared with chrysin (EC$_{50}$ 0.5 µM) (Saarinen *et al.*, 2001), as reported in the literature. For genistein, the content in soybean products, which are also used as
ingredients in manufacturing for aquaculture feeds, the reported concentrations were up to 900 mg per kg of soybean flour (Liggins et al., 2002).

The two phytochemicals used for this experiment have received special attention by many authors given their potential capability to interfere with endogenous estrogen biosynthesis by aromatase inhibition. All these experiments were, however, performed in \textit{in vitro} conditions (Campbell and Kurser, 1993; Pelissero et al., 1996; Chen et al., 1997; Joshi et al., 1998; Kao et al., 1998; Jeong et al., 1999; Eng et al., 2001). Our study attempted to corroborate those findings in an \textit{in vivo} essay with sexually undifferentiated tilapia. Our observations indicate that dietary administration of genistein and quercetin did not have a significant effect ($p>0.05$) on a deviation from expected gonad phenotypic sex ratio in genetically all-female tilapia. The male ratio observed in these two experimental groups was equal to that observed in the control group (3%) while in the MT group 86\% masculinization rate was achieved.

To our knowledge, there is no information available on the possible response to the dietary administration of phytochemicals as pure compounds to sexually undifferentiated fish and their biological effects. Thus our study provides a preliminary insight on the lack of an \textit{in vivo} response to pure phytochemicals when dietary administration was used in sexually undifferentiated fish. One of the few references available in the recent literature is the study conducted by Tzchori et al., (2004) where genistein as an aglycone (pure compound) was administered in the diet at 2 and 20 mg/kg to the European eel. The group at the lower dose
had a significantly higher female ratio (55%) than the control group (5%); therefore the author concluded in this case genistein had an estrogenic effect. Similarly, in the positive control group, where 17β-estradiol was used, feminization was also observed. In a study in goldfish (Carassius auratus), a commercial ornamental fish, diet with soybean meal as a major component, with a mean concentration of genistein close to 400 mg/kg of diet, did induce a response manifested by an increase in plasma vitellogenin levels in male adult goldfish (Ishibashi et al., 2002). Therefore, it can be considered that even the presence of genistein glycosides in the diet is capable to exert some endocrine responses in fish.

It has been emphasized in the literature that the structural molecular characteristics of phytochemicals are shared with steroid hormones. The main aim of this study was to compare the masculinization response in genetically all-female tilapia to the presence of phytochemicals of reported aromatase inhibition capacity. It has been reported that other chemicals also classified as aromatase inhibitors such as tamoxifen and ATD (Hines and Watts, 1995; Guigen et al., 1999) can successfully reverse the gonad sex in tilapia, however, there is no evidence if phytochemicals share the same biological activity that these aromatase inhibitors in sexually undifferentiated tilapia. In our experiment no response was observed at the inclusion levels in diet of both phytochemicals. Both quercetin and genistein show a molecular structure in which the rings -A and –C mimic rings –D and –C of the steroid substrate, and this structure provides the molecular basis for aromatase inhibition by phytochemicals (Eng et
Genistein in particular posses a conformational structure in the phenolic ring and the positioning of the hydroxyl radicals in the positions 4’ and 7’ that emulates 17β-estradiol structure to a high degree (Dixon and Ferreira, 2002). However, these characteristics apparently are not sufficient to wield an *in vivo* response in sexually undifferentiated tilapia.

There are several experiments focused on the evaluation of the effect of phytochemicals such as flavonoids and other compounds present in fish feed formulations, mostly thought *in vitro* essays. The results indicate that in fact phytochemicals may result in endocrine modulating effect, given their capacity to interact with estrogen receptors (Matsumoto *et al*., 2004). These activities are mostly resulting from the presence of soybean ingredients. Therefore present results with tilapia give some indication of the actual possibility of bioactivity of phytochemicals present in the diet (Miyahara *et al*., 2003), although aglycone/glycoside derivatives of isoflavonoids need to be specifically addressed.

A second possible response to the presence of phytochemicals is the regulation interaction with estrogen receptors activity by binding competition. This is of crucial relevance given that estrogen actions in target cells are mediated through binding to the estrogen receptors (ER) (Matthews *et al*., 2000). Although phytoestrogens are capable to compete for binding sites and displace estradiol from estrogen receptors, and consequently modulating gene expression, they activity is weak compared to estrogens (Morito *et al*., 2001). Phytoestrogens can also exert some agonistic responses, such as specific cell mediated growth. In a
study with MCF-7 cells, genistein induced growth of this cell line in in vitro conditions (Morito et al., 2001); thought, the genistein concentrations required to induce an active response in estrogen mediated formation of specific cell products (β-galactosidase) were in the order of 4-5 fold higher than steroids (Morito et al., 2002). In respect to quercetin, a study in roosters fed with several flavonoids, including quercetin up to 1 mmol/kg (0.338 mg/kg), had an antiestrogenic effect on the expression of estrogen regulated mRNA stabilizing factor (Ratna and Simonelli, 2002). Thus, this evidence of regulation of estrogen metabolism although it could not be entirely be extrapolated to fish, it requires further research.

As presented in Table 2.2 and Figure 2.2, the addition of quercetin and genistein to the experimental diets, did not have a detrimental effect on growth in juvenile tilapia. Several studies where genistein was evaluated to assess possible changes in reproductive performance in fish, also indicated that genistein, in general is not detrimental to growth. Dietary administration of 500 and 1000 mg/kg in the diet of rainbow trout for over a year, did not affect growth, but caused a delay in oogenesis (Bennetau-Pelissero et al., 2002). Similar no-effect on growth was reported for juvenile stripped bass (Morone saxatilis) fed with 2, 4 and 8 mg/kg of genistein. These genistein induced an increased vitellogenin expression in experimental fish at both, 2 and 8 mg/kg (Pollack et al., 2003). Yellow perch (Perca flavescens) fed with experimental diets with a genistein content of 0.75 and 7.5 mg/kg, did not show weight differences gain in males, but females had significant lower weight gain on the 7.5 mg/kg. The
authors suggested that genistein may have a positive effect on growth in this fish, but no apparent estrogenic effect on gonadosomatic indices in both sexes was observed (Ko et al., 1999).

There is not an established mechanism of how flavonoids with potential steroidal activity are absorbed and metabolized by fish especially in tilapia, thus their potential \textit{in vivo} effect remains unknown. The major question here is to what extent flavonoids are absorbed from the gastrointestinal tract and which factors affect their absorption. Studies on the absorption of pure aglycone compounds indicate only limited absorption, since up to 60 to 70\% of flavonoid aglycones such as quercetin, are excreted in urine and feces in humans after administration (Hollman and Katan, 1997). It is established that in general, after phytochemical intake and absorption, the main metabolites that can be identified are those with substitutions in the B and/or C ring, being sugars, methyl and sulfate radicals those attached to the molecule (Doerge et al., 2001). Therefore in order to positively correlate absorption with biological response, the proper identification of phytochemical metabolites in tilapia should be considered a noteworthy continuation for this kind of studies.

A possible explanation for the observed results can be related to the degree of bio-absorption and metabolization of these two phytochemicals in juvenile tilapia. The numbers of studies on phytochemical metabolization in fish are limited, but it is known that different species have different absorption and metabolization of a particular compound. Such case is reported by Gontier-Latonelle \textit{et al.} (2001) where rainbow trout was described to produce mostly
metabolites with a carbohydrate addition, and Siberian sturgeon (*Acipenser baeri*) mostly sulfate conjugates that facilitate bioavailability. These authors also indicated given that excretion rates were lower in sturgeon than in rainbow trout. This study permits to draw preliminary conclusions in regards to the possible low absorption and metabolization rates of phytochemicals in juvenile tilapia. It has been earlier described that both intestine enzymatic activity and gut flora activity play a mayor role in other higher vertebrates in facilitating phytochemical metabolic transformation in order to facilitate bio-assimilation (Brienholt *et al.*, 2000). Consequently, it could be assumed that marked differences in biotransformation and assimilation of genistein and other phytochemicals in different species of fish will be found.

The analysis of whole body tissue provides an interesting insight of the fate of the parental compound. While quercetin supplementation of the diet was 20 times higher, the observed concentrations in tissues were only three times higher than the observed genistein concentrations after 4 weeks of feeding. A considerable difference is observed at week 6, were quercetin concentration is 40 times higher. The decrease in the observed concentrations of genistein, that dropped by 10-fold from week 4 to week 6, could be explained by a higher metabolic transformation to more easily excreted metabolites, such as glucoronides, as the digestive tract matures and a more active microbial digestion is taking place (Wisseman, 1999). For quercetin, a methylated conjugate isorhamnetin (Azuma *et al.*, 2003), was identified in some samples (results not showed). However, it was not possible to estimate an accurate
concentration rate of transformation of this metabolite. No metabolites for
genistein were identified in the analyzed fish whole body tissue samples; it is
known that genistein is metabolically transformed to dihydrogenistein and P-
ethylphenol, but in both cases there is no reported biological activity of either
metabolite (Wisseman, 1999).

In conclusion, to validate the possibility of the use of phytochemicals as
sex reversal agents in tilapia it is imperative to consider that the complexity and
quantity of possible number of molecular mechanism for phytochemical action in
target cells, such as the interaction with molecules, proteins, enzymes, cells
membranes and estrogen receptors is quite considerable (Benassayag et al.,
2002). Present study demonstrates that considerable effort is required in order to
ensure that there are new and effective techniques proposed to produce
monosex populations that involve the use of phytochemicals.
Figure 2.1 Molecular structures of phytochemicals: quercetin (QUER), genistein (GEN) and steroid hormones: 17α-methyltestosterone (MT) and estradiol (E2).
## Experimental Diet

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Control</th>
<th>Genistein</th>
<th>Quercetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (vitamin free)</td>
<td>36.00</td>
<td>36.00</td>
<td>36.00</td>
</tr>
<tr>
<td>Gelatin</td>
<td>6.00</td>
<td>6.00</td>
<td>6.00</td>
</tr>
<tr>
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<tr>
<td>Cellulose</td>
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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>Bernahrt 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).

Table 2.1 Composition of experimental diets for first feeding tilapia expressed as percentage of dry matter per 100 g.
<table>
<thead>
<tr>
<th>Treatment (Diet)</th>
<th>Final weight (g)</th>
<th>SGR (%/day)</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>1.1±0.2</td>
<td>8.5±0.10</td>
<td>67.4±14</td>
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<tr>
<td>MT</td>
<td>0.7±0.3</td>
<td>7.7±0.06</td>
<td>62.2±8.7</td>
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<tr>
<td>QUER</td>
<td>1.1±0.1</td>
<td>8.5±0.01</td>
<td>71.1±11</td>
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<tr>
<td>GEN</td>
<td>1.2±0.3</td>
<td>8.6±0.50</td>
<td>69.9±9.7</td>
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</table>

Table 2.2 Summary of the final mean weight (WG), daily specific growth rate (SGR) and mean survival (±SD) of tilapia from each dietary treatment (n=3). Diets used were: control (CON), or supplemented with 17α-methyltestosterone (MT), quercetin (QUER) and genistein (GEN).
Figure 2.2 Progress in individual mean fish weights observed during experimental feeding trial at 2, 4, 6 and 8 weeks. Values are mean ± SEM of triplicate groups (n=3). Diets: control (CON), 17α-methyltestosterone (MT), quercetin (QUER) and genistein (GEN).
Figure 2.3. Morphological appearance of male (A) and female (B) gonads. Gonadal squashes with aceto-carmine were analyzed by microscopic observation under 200X magnification.
Figure 2.4. Morphological appearance of female fish from control group (A) and male from MT group (B) gonad. Week 4 samples (0.15±0.08 g). Individual fish were analyzed by histological microscopic observations of abdominal sections (5-7µm) with eosin-hematoxilin staining.
Figure 2.5 Final male percentage observed in different diet treatment. Diets control (CON) (n=64), 17α-methyltestosterone (MT) (n=53), quercetin (QUER) (n=66), and genistein (GEN) (n=68). Fish age at sexing was 95 days, mean weight 8.3±3.2g.
Figure 2.6 Mean concentrations in whole body tissue (µg/g, mean ± SEM) of quercetin (QUER) and genistein (GEN) diets fed fish at 4 and 6 weeks (n= 30). No phytochemicals were detected in the control group fish (CON).
CHAPTER 3

EFFECT OF THE DIETARY ADMINISTRATION OF DAIDZEIN, CHRY SIN, CAFFEIC ACID AND SPIRONOLACTONE ON SEX DIFFERENTIATION AND GROWTH OF ALL-MALE AND ALL-FEMALE NILE TILAPIA.

ABSTRACT

Aromatase inhibitors have been used to produce all-male populations of fish. These compounds inhibit the expression of female genotype by blocking estrogen induced ovarian differentiation, reversing the phenotypic sex of the fish. Phytochemicals such as flavonoids and other phenolic compounds have been reported to posses aromatase inhibitor characteristics, both in *in vitro* and *in vivo* conditions. Two feeding experiments were conducted with first feeding Nile tilapia (*Oreochromis niloticus*) genetically all-female or genetically all-male, to evaluate the potential biological endocrine modulating activity of three selected phytochemicals towards sex inversion in sexually undifferentiated tilapia in parallel with steroidal compound treatments. Experimental casein-gelatin based diets were prepared as follows: for all-male tilapia (experiment 1): control, daidzein 500 mg/kg, chrysin 500 mg/kg, caffeic acid 500 mg/kg and
spironolactone 500 mg/kg. For all-female (experiment 2): control, MT 60 mg/kg, 1,4,6-androstatrien-3-17-dione (ATD) 150 mg/kg, daidzein 500 mg/kg, chrysin 500 mg/kg, caffeic acid 500 mg/kg and spironolactone 500 mg/kg. Fish were fed for 6 weeks (Exp. 1) and 8 weeks (Exp. 2). Survival (%) and growth performance as final individual body weight, specific growth rate (%/day) was evaluated. Final sex ratios were determined by microscopic analysis of gonadal squashes at the end of each experiment (65 Days). HPLC analyses of body tissues were conducted to estimate the concentrations of phytochemicals accumulated in whole body tissues. All phytochemicals were detected, for chrysin concentrations levels were from 0.4 to 37.8 µg/g, for daidzein were in the range of 0.3 to 19.2 µg/g, and for caffeic acid were 0.01 to 1.7 µg/g. Results indicate that the sex ratio of genetically all-female tilapia evaluated by gonad squashes was not affected by the inclusion of the tested phytochemicals (final male ratio 18±5 %.), while MT and ATD exhibited a sex reversal of females to 100% and 50% males, respectively. Spironolactone did not induce a sex inversion in all-female tilapia. For all-male juveniles no effect was observed in final sex ratio for any of the phytochemicals or spironolactone. No differences were observed in survival, final mean weight, SGR between treatments for experiment 1. For experiment 2, MT and ATD groups showed a significantly minor final mean weight (p<0.05); still, survival or SGR were not significantly different.
INTRODUCTION

The direct study of the mechanisms of hormone biosynthesis and action on the gonad 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 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; Strauss et al., 1998; Griffiths et al., 1999; Eng et al., 2001), 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; Kitano et al., 2000) as well as steroidal and non steroidal chemical compounds (Brodie et al., 1999; Smith, 1999; Seralini and Moslemi, 2001). With both approaches a certain degree of success has been achieved in sex inversion 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)
and in the protogynus fish honeycomb grouper (*Epinephelus merra*) (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*) (Lee *et al*., 2003), and 75% and 100% masculinization in Nile tilapia and rainbow trout (*Onchorhynchus mykiss*), respectively (Guigen *et al*., 1999). Spironolactone, although it is considered primarily as anti-androgenic compound due to aldosterone antagonism (Jankowski *et al*., 1996; Garthwaite and McMahon, 2004), it has been reported as causing a 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 proven its 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). Also, promising results have been obtained with synthetically modified flavonoids, with attached functional groups to the flavonoid structure (Pouget *et al*., 2002; 2002b; 2002c). 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). More experimentation is required.
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 present 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. Simultaneously, we aim to establish how exogenous endocrine modulating compounds will impact gonad differentiation and improve 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). Molecular structures are
depicted in Figure 3.1. Mobile phase HPLC grade components were purchased from Fisher Scientific Inc. (Pittsburgh, PA, USA).

**Diet formulation**

Seven casein-gelatin based diets were prepared as follows: control (CON), chrysin 500 mg/kg (CHR), caffeic acid 500 mg/kg (CAFF), daidzein 500 mg/kg (DAID), spironolactone 500 mg/kg (SPIRO), 1,4,6-androstatrien-3-17-dione 150 mg/kg (ATD) and 17α-methyltestosterone 60 mg/kg (MT). Semi-purified diets were formulated to avoid contamination with natural steroids (Feist and Shreck, 1990). For CHR, DAID, CAFF and SPIRO diets, the compound 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 was dissolved in ethanol as vehicle and mixed thoroughly in the control diet; later ethanol was allowed to evaporate overnight at room temperature and diets stored at -20°C. Diets compositions are listed in Table 3.1.

**Experiment 1: All-male tilapia experiment**

The experiment was conducted on first feeding genetically all-male tilapia (Til-Tech, Robert, LA, USA). 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: control (CON), daidzein (DAID), chrysin (CHR), caffeic acid (CAFF) and spironolactone (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). The sex of fish was determined in the final samples (42\textsuperscript{nd} 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.

**Experiment 2: All-female tilapia experiment**

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 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: control (CON), or diets supplemented with daidzein (DAID), chrysin (CHR), caffeic acid (CAFF), spironolactone (SPIRO), 17α-methyltestosterone (MT) and 1,4,6-androstatrien-3-17-dione (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.
Determination of phytochemical concentrations in fish tissue by HPLC analysis

The analyses of the concentrations of the 3 phytochemicals tested in the experiments were conducted using HPLC. In all cases, extraction and acid hydrolysis with carried out using a solution of 1M HCL in acidified methanol (100:5 v:v methanol:acetic acid. The extraction procedure consisted of homogenization of individual fish in the acidified methanol for 30 sec at 5000 rpm (Omni Int. GLH-115 homogenizer). The hydrolysis was followed by incubation at 37 °C for 16 h (overnight). Acidity was then 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 analyses. Recovery rate was estimated with internal standards and was in the range of 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 analyses.

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 of the standard was 50 ng/ml
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 of the standard was 45 ng/ml.

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 of the standard was 25 ng/ml.

**Statistical Analysis**

Mean individual final weight, SGR (%/day) and survival were analyzed by one-way analysis of variance. When significant differences were found, a Tukey test was performed (Zar, 1990). For final sex ratio data, chi-square contingency tables were used. All statistical analysis was performed using SAS version 8.02 (SAS Institute, Inc. Cary, NC USA) at a significance level of α=0.05.
RESULTS

Experiment 1

After 6 weeks of feeding with the experimental diets, final mean weight (0.69±0.1 g) (Figure 3.2, Table 3.2) and survival rate (97.7±2%) (Table 3.2) 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 as observed in the control group, given to the inclusion of CHR, DAID, CAFF or SPIRO supplemented diet groups (results not shown).

Experiment 2

No significant differences were observed in survival rates after 8 weeks across all treatments (87.9±6.8 %) (Table 3.2). Growth rate results indicate a differential growth at different points of evaluation (2, 4, 6 and 8 weeks) (Figure 3.3). 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 (p>0.05) compared to fish fed with the different phytochemicals (Figure 3.3).

The final sex ratio of the experimental fish was not affected by the dietary administration of the tested phytochemicals. The only noticeable differences was the effect of the steroidal compounds ATD and MT where 50 and 100% masculinization rates were observed, respectively (Figure 3.4).
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 experiment 1 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 whole body tissue; the observed mean whole body tissue concentrations are presented in Figure 3.5.

For daidzein in experiment 1 (all-male) at both, week 4 and 6, the chemical was detected, and the observed mean concentrations in µg/g are displayed in Figure 3.6. In experiment 2 (all-female), 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.

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 experiment 1 and 2 (Figure 3.7). 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 fish from for experiment 1 both at 4 and 6 weeks, and in experiment 2. There was 100% detection at week 2, 80%
at weeks 4 and 6, but only 20% at week 8 samples. Detected values were on the range of 0.1 to 3.4 µg/g (Figure 3.7).

It is evident that for all phytochemicals the concentrations in both genders diminish in a considerable manner throughout the sampling period. Calculation of residual proportions of the presence of the parent compound throughout both feeding trials indicates that in experiment 1 (all-male), whole body tissue concentrations dropped from week 4 to week 6 to 10, 22 and 27% for caffeic acid, daidzein and chrysin, respectively. In experiment 2 (all-female), 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.

DISCUSSION

In the recent studies that have been performed concerning the influence of endocrine-modulating activity chemicals on humans and wildlife, it is assumed that these chemicals have similar structures to natural and synthetic hormones, and they can induce abnormal hormonal responses, and potentially cause changes in metabolic systems due to the inhibition of enzymatic activity of drug-metabolizing enzymes (Brodie et al., 1999; Miyahara et al., 2003). Such is the premise of this research work, and particularly in this study we addressed the possibility of using phytochemicals such as chrysin, daidzein and caffeic acid to achieve the required hormonal shift needed to observe an effect in sex differentiation of undifferentiated gonads in tilapia (Howell et al., 1994). In
experimental conditions in vitro it has been 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 estrogenic 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 it 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 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.

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 on uterine weight in rats after consumption of different red wine varieties extracts 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 their experiments showed that chrysin and 7-hydroxyflavone inhibited the formation of $^3$H-17β-estradiol from $^3$H-androstenione in human choriocarcinoma JEG-3 and human embryonic kidney
HEK 293 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, and in conclusion, may have been due to their relatively poor absorption and/or bioavailability. The authors stated that the \textit{in vivo} effects of flavonoids on aromatase inhibition cannot be predicted on the basis of \textit{in vitro} results.

In our study, although chrysin supplement fed group had a slightly higher male ratio (24%) compared to the control group (18%), given the lack of more conclusive evidence, we can imply as provisional conclusion that this phytochemical could not fully acquire the definition of sex differentiation disruptor in tilapia, until further research is conducted.

Whether phytochemicals are bioactive once they are consumed is a point of controversy. Given that little is known on plasma and body tissues concentrations once the phytochemicals are absorbed, the evidence of high concentrations in the urine, indicate that most of the chemicals are eliminated in humans and other vertebrates (Pelissero \textit{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 \textit{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 fish used in present experiments. Another possibility is interspecies differences in the degree on the efficiency of absorption of phytochemicals due to variation in the release of many different hydrolytic
enzymes by microflora that are involved in food metabolization 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 phytochemicals (Rawls et al., 2004). 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 significant decrease of concentration of phytochemicals in larger (older) fish (Fig. 3.5, 3.6 and 3.7).

It is noticeable that the decline in concentrations in whole body for all three phytochemicals observed in experiment 1 and 2 (both genotypes all-male and all-female) could be related to the increased intestinal length and metabolization by enzymes produced by the digestive tract and microflora, facilitating further metabolization and perhaps excretion. The proper chemical identification of the free and sulfated conjugated phytochemicals produced after enzymatic reactions that have a reported higher in vivo endocrine disruptor activity (Miyahara et al., 2003), is particularly relevant to preliminary conclusions we would like to draw. By predicting “an activation” of some phytochemicals after metabolization, that facilitates their absorption by intestinal membranes and transportation to the liver, we would expect transformation and delivery to target tissues (Kinjo et al., 2004). 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.

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 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.

Studies in mammals have elucidated metabolic pathways for the tested phytochemicals. For example, daidzein is metabolized into dihydrodaizein, then to equol, and finally to O-demethylandolensis (O-DMA), which has unknown estrogenic properties in humans (Patisaul and Whitten 1999). Caffeic acid is a hydroxycinnamate (phenolic compound) of unstable characteristics, being subject to degradation by non-enzymatic autooxidative phenolic browning reaction, which is temperature and pH dependent (Tsai et al., 1999). Once this substance is consumed it 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 an apparent favorable membrane transport properties through cell membranes. However, its absorption may still be seriously limited by a highly efficient conjugation metabolism by glucoronidation 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.

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). Matthews et al. (2000) conducted a study were Isoflavones such as daidzein bind to estrogen receptors with a lower affinity than 17β-estradiol (between 1 X 10^{-4} and 1 X 10^{-2} lower) on a molar basis when evaluated in a series of recombinant ER’s from human, rat, chicken, green anole and trout. 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 ERβ than the ERα receptor (Messina et al., 2001). Chrysin binding affinity is 100,000 fold or more less than estradiol and has very low or no agonistic activity in human recombinant ER’s (Kuiper et al., 1998).
Certain features of the molecular structure of steroids can affect the activities of these chemicals, including species differences in receptor-binding affinity and the presence of multiple receptor sub-types (Thomas, 2000). According to Benassayag et al. (2002) phytochemicals could overcome their rather weak binding affinity \((10^{-2} \text{ to } 10^{-3} \text{-fold})\) if very high concentrations in body fluids and tissues are attained and therefore account for the many biological effects attributed to them. Consequently, in order to have an insight on 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 experiment 1 (all-male).

In general, the \textit{in vitro} results obtained as indication of the interaction of several chemicals, including phytochemicals and estrogen receptors in cells, are difficult to extrapolate to the \textit{in vivo} effect. There is no endocrine system on the different strains of genetically modified yeasts, where recombinant human ER and other vertebrates ERs are expressed (Nishikawa et al., 1999). In addition, yeast do not contain endogenous steroid or thyroid hormone receptors or related proteins such as aromatase that could interfere with phytochemical action (Collins et al., 1997). Hence, the observed response to estrogen receptor antagonism, assuming this particular mechanism, in the present studies with tilapia should be carefully extrapolated.

Another consideration when evaluating possible the potential effect of phytochemicals on sex differentiation on larval fish is their effect on growth. 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. A negative effect was observed, however, in groups fed MT and ATD. The feasibility of the continuation of this research by the inclusion of a higher percentage of diet ingredients, where phytochemicals are naturally occurring, such as soybean meal (Hollman and Arts, 2000; Pietta, 2000), would 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. Although no significant effects on final masculinization percentage were observed using chrysin, 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. Alternatively, the evidence on the possibility of other steroidal compounds, such as spironolactone, for sex inversion provides a future line of research.
Figure 3.1 Molecular structures of tested chemicals: 17α-methyltestosterone (MT), 1,4,6-androstatrien-3-17-dione (ATD), spironolactone (SPIRO), chrysin (CHR), daidzein (DAID) and caffeic acid (CAFF).
<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>
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<tr>
<td>Dextrin</td>
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<td>32.6</td>
<td>32.6</td>
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<tr>
<td>Fish protein hydrolyzate(^1)</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>
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<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(^2)</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Mineral mix(^3)</td>
<td>4</td>
<td>4</td>
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<td>4</td>
</tr>
<tr>
<td>Carboxymethylcellulose</td>
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<td>2</td>
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<tr>
<td>L-Arginine</td>
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<td>0.5</td>
<td>0.5</td>
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</tr>
<tr>
<td>L-Methionine</td>
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<td>0.4</td>
<td>0.4</td>
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<td>0.4</td>
</tr>
<tr>
<td>L-Lysine</td>
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<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Phospitan C(^4)</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>Chrysin</td>
<td>0</td>
<td>0.05</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Daidzein</td>
<td>0</td>
<td>0</td>
<td>0.05</td>
<td>0</td>
<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>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0.05</td>
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<tr>
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<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.

\(^1\)Concentrate of fish soluble protein (CPSP: crude protein 82-84%; crude lipid 9-13% WW), Soproprierche SA, Boulogne-sur-Mer, France).

\(^2\)Roche Performance Premix (Hoffman-La Roche, Nutley, NJ).

\(^3\)Bernahrt Tomarelli salt mixture (5 g Na\(_2\)Se-Se/kg mixture; ICN Pharmaceuticals, Aurora, OH).

\(^4\)Mg-L-ascorbyl-2-phosphate (Showa Denko, Tokyo, Japan).

Table 3.1 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 3.2

<table>
<thead>
<tr>
<th>Treatment (Diet)</th>
<th>Final mean weight (g)</th>
<th>Daily SGR (%)</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp 1</td>
<td>Exp 2</td>
<td>Exp 1</td>
</tr>
<tr>
<td>CON</td>
<td>0.75±0.09</td>
<td>2.2±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10±0.1</td>
</tr>
<tr>
<td>CHR</td>
<td>0.63±0.10</td>
<td>1.7±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.6±0.4</td>
</tr>
<tr>
<td>DAID</td>
<td>0.66±0.07</td>
<td>1.9±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.7±0.2</td>
</tr>
<tr>
<td>CAFF</td>
<td>0.67±0.09</td>
<td>1.7±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.8±0.3</td>
</tr>
<tr>
<td>SPIRO</td>
<td>0.65±0.04</td>
<td>1.4±0.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>9.7±0.1</td>
</tr>
<tr>
<td>MT</td>
<td>1.1±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>8.4±0.2</td>
</tr>
<tr>
<td>ATD</td>
<td>1.1±0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>8.4±0.1</td>
</tr>
</tbody>
</table>

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

Table 3.2 Final mean weight, daily specific growth rate (%/day) and survival observed in both experiments per dietary treatment (n=3). Experiment 1 (Exp 1), all-male tilapia; experiment 2 (Exp 2) all-female tilapia.
Figure 3.2 Progress in individual mean fish weights observed in experiment 1 (all-male tilapia) at 2, 4, and 6 weeks. Values are mean ± SEM (n=3). Diets: control (CON), chrysin (CHR), daidzein (DAID), spironolactone (SPIRO) and caffeic acid (CAFF).
Figure 3.3 Progress in individual mean fish weights in experiment 2 (all-female tilapia) at 2, 4, 6 and 8 weeks. Values are mean ± SD of triplicate groups (n=3). Diets: control (CON), chrysin (CHR), daidzein (DAID), spironolactone (SPIRO) and caffeic acid (CAFF), 17α-methyltestosterone (MT), and 1,4,6-androstatrien-3-17-dione (ATD).
Figure 3.4 Final sex ratio (%) in experiment 2 observed (mean ± SD) per treatment (n=20 per replicate, three replicates per treatment). Diets: control (CON), chrysin (CHR), daidzein (DAID), spironolactone (SPIRO) and caffeic acid (CAFF), 17α-methyltestosterone (MT), and 1,4,6-androstatrien-3-17-dione (ATD).
Figure 3.5 Mean concentrations of chrysins (CHR) in whole body tissue (µg/g, mean ± SEM). Experiment 1, weeks 4 and 6 (n=15). Experiment 2, weeks 2, 4, 6 and 8 (n=10). No detection was observed in control group (CON).
Figure 3.6 Daidzein (DAID) concentrations observed in whole body tissue (µg/g, mean ± SEM). Experiment 1, weeks 4 and 6 (n=15). Experiment 2, weeks 2, 4, 6 and 8 (n=10). No detection was observed in control group (CON).
Figure 3.7 Caffeic acid (CAFF) observed concentrations in whole body tissue (mean ± SEM). Experiment 1, weeks 4 and 6 (n=15). Experiment 2, weeks 2, 4, 6 and 8 (n=10). No detection was observed in control group (CON).
CHAPTER 4

DETERMINATION OF ABSORPTION OF SELECTED PHYTOCHEMICALS IN FISH TISSUE WITH HPLC DETECTION.

ABSTRACT

Phytochemicals such as flavonoids and other phenolic compounds are under scrutiny in order to validate their numerous potential biological properties in many organisms. Most of the reported methods for determination of absorption and metabolization of flavonoids have been conducted by means of HPLC detection techniques, these methods allow to analyze biological samples either from plants, natural sources of these chemicals (soy products, fruits, etc), or body fluids (plasma, urine) in major vertebrates (mostly mammals) as vegetables and other plants consumers. Our research is focused on the evaluation of caffeic acid, chrysin, daidzein, genistein and quercetin in fish, using Nile tilapia (*Oreochromis niloticus*) as a model, and their impact as exogenous endocrine modulators/disruptors. This research is focused on the description of the proper analytical detection conditions with HPLC of these phytochemicals as parent compounds in the whole body of fish after dietary administration. Here, we
compare two extraction methods and describe the specific chromatographic conditions, such as selection of column, mobile phase composition and type of detector for each specific compound. All phytochemicals were successfully detected and quantified in tissue samples of juvenile Nile tilapia.

INTRODUCTION

Phytochemicals such as flavonoids are receiving considerable attention because their many biological properties reported in the literature. This information is of relevance to the aquaculture industry given that soybean and other legume seed products are important components of fish commercial feed formulations, and phytochemicals are present even after processing by extrusion at high temperatures (Liggins et al., 2002; Pollack et al., 2003). A series of experiments provided relevant information on the evaluation of specific biological responses in fish after flavonoid administration. Studies on evaluation of reproductive performance in stripped bass (Pollack et al., 2003) and sex determination in European eel (Tzchori et al., 2004) indicate that in fact fish showed an in vivo response to phytochemicals provided in the diet. However, no information was available in relation to the extent of the absorption of the tested flavonoid in experimental fish.

The measurement of flavonoids in biological samples is an important tool to assess their absorption, metabolization, and mechanism(s) of action. To this moment, high pressure liquid chromatography (HPLC) is considered the
preferred analytical technique to determine phytochemicals such as phenolic compounds (flavonoids, phenolic acids, etc.) in their natural sources (Nguyenle et al., 1995) and other biological samples such as animal tissues, plasma and urine (Azuma et al., 2003). Maubach et al. (2003) indicates that the prevalence of this technique coupled with UV detection facilitates sample processing and offers the option to quantify several compounds simultaneously or in similar chromatographic conditions. Wilkinson et al. (2002) offered a complete review of analytical procedures for extraction, HPLC separation conditions and detection devices for phytochemicals in foods and human fluids.

The determination of specific flavonoids and their derivatives after metabolization involves the extraction of the compound from its matrix. Given the non polar characteristic of flavonoids, the most widely used extraction procedure involves the use of polar solvents such as methanol (Nguyenle et al., 1995; Bruschi et al., 2003) and acetonitrile (Griffith and Collison, 2001), that could required acidified extraction solvents (Murphy and Suzanne, 2002; Yao et al., 2004). Also the use of enzymatic hydrolysis of samples has been amply suggested for identification of free and conjugated forms in biological samples (Liggins et al., 1998; Walle et al., 2001).

Analytical conditions during determination can be quite variable from one flavonoid to another, but in general similar conditions can allow measuring simultaneously isoflavonoids as genistein and daidzein (Hubarat et al., 1998; Maubach et al., 2003; Peñalvo et al., 2004) or one specific phenolic compound as caffeic acid and its derivatives (Walle et al., 1999). Chrysin have been
successfully identified from sources such as propolis (Tomas-Barberan et al., 2001; Yao et al., 2004) fruit juices (Shanhrzad and Bitsch, 1996) and plasma and urine after plant consumption in humans (Walle et al., 2001) and rats (Kumazawa et al., 2004). Although it is difficult to generalize, the detection of these compounds can be performed both under isocratic or gradient elution, specific for each compound. It is also possible that for a particular polyphenol, UV detection is not sensitive enough, and then electrochemical detection can be coupled to increase sensitivity (Wilkinson et al., 2002).

In the process of conducting a series of experiments that involve the dietary administration of selected phytochemicals to first feeding Nile tilapia (Oreochromis niloticus), we evaluated the response in sex differentiation and growth. However, in order to establish a possible correlation between the biological responses and the concentration of the parent compound in feeds we decided to attempt to elucidate the most appropriate extraction, elution conditions and type of detection for HPLC analyses of the isoflavonoids genistein and daidzein, the flavonol quercetin, the flavone chrysin, and the phenolic acid caffeic acid in whole body tissues of the experimental fish, an early juvenile stages of tilapia.
MATERIALS AND METHODS

Reagents

The following compounds were obtained: daidzein (Indofine Chemical Co. Somerville, NJ, USA); chrysin, caffeic acid, genistein, and quercetin (MP biomedicals® Aurora OH, USA). Mobile phase HPLC grade components were purchased form Fisher Scientific Inc (Pittsburgh, PA. USA).

Phytochemical feeding trials

A series of feeding experiments were conducted on first feeding Nile tilapia (Oreochromis niloticus), where semi-purified, casein-gelatin based diets were formulated and supplemented with chrysin, caffeic acid, daidzein and genistein at concentration of 500 mg/kg; and 10 g/kg for quercetin diet. Fish were fed for 6 to 8 weeks. Samples of 5 to 10 fish were removed from experimental tanks every 2 weeks during feeding experiments.

Fish whole body tissues and diet samples extraction

An analysis of the concentration of the phytochemicals tested in the experiment was conducted with HPLC techniques. In a preliminary approach, body tissue samples were extracted by two methods, by enzymatic and acid hydrolysis (described below). For enzymatic hydrolysis, samples were homogenized in 2 volumes of 0.2 M Na-acetate buffer (pH 5.0). Then samples were incubated with an equal volume of extract in a solution of 0.2 M Na-acetate buffer (pH 5.0) containing 1000 IU of β-glucuronidase (E. coli, Sigma) and 50 UI of sulfatase (H. pomatia, Sigma), for 1 h at 37°C. After comparing both extraction
methods, acid hydrolysis was selected for all phytochemicals, given the observed phytochemical peaks on each specific chromatogram after HPLC analysis.

Whole body tissue were subjected to acid hydrolysis with 1M HCL in acidified methanol (100:5 v:v methanol:acetic acid) as extraction solution of phytochemicals from fish body tissues. The extraction procedure consisted of homogenization of individual fish in the extraction solution for 30 sec at 5000 rpm, followed by incubation at 37 ºC for 16 h (overnight); acidity was neutralized with 10N NaOH (equivalent volume to sample weight) to achieve the final dilution rate 1:10. Samples were filtered through a 0.45 µm micropore disk filter and either injected immediately to the HPLC or frozen at -80 ºC for further analyses. Experimental diets were analyzed using the same procedure described above, in order to verify presence of the phytochemical on its original chemical form. To validate quantity, sub samples of 0.2 g were extracted and injected to the HPLC.

Equipment

The HPLC system consisted of a Beckman® 110B pump, 166 system gold detection module and a 406 system gold analog interface module; coupled to a BAS® electrochemical detector equipped with a CC-5 flow cell and a LC-44 detector, using a 0.08 to 8.0 mV output. A Peaksimple® chromatography data system was used for chromatogram analysis.

HPLC chromatographic conditions

Quercetin concentrations in tissue were analyzed by HPLC and was performed using the methods described by from Park et al. (in press); chromatographic conditions are described in Table 4.1.
Table 4.1 Chromatographic conditions for quercetin detection in tilapia whole body tissues.

Genistein and daidzein detection were conducted using a modified procedure from Hutabarat et al. (1998), and chromatographic conditions are described in Table 4.2.

Table 4.2 Chromatographic conditions for genistein and daidzein detection in tilapia whole body tissue.
Chrysin detection was performed using a modified procedure from Walle et al. (1999); chromatographic conditions are described in Table 4.3.

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>1M acetic acid in 80% methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Synergi Hydro Phenomenex® 250X4.6 mm 4µ C_{18} RP column</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.8 ml/min</td>
</tr>
<tr>
<td>Detection</td>
<td>UV detection at 280 nm</td>
</tr>
</tbody>
</table>

Table 4.3 Chromatographic conditions for chrysin detection in tilapia body.

Caffeic acid detection was carried out using a modification of the method described by Shanhrzad and Bitsch (1996), and chromatographic conditions are described in Table 4.4.

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>Water:ethylacetate:acetic acid (95.6:4.1:0.3 %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>ODS Ultrasphere Beckman® 150X4.6 mm 5 µ C_{18} RP column</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.0 ml/min</td>
</tr>
<tr>
<td>Detection</td>
<td>electrochemical detection (0.08 to 8.0 mV output)</td>
</tr>
</tbody>
</table>

Table 4.4 Chromatographic conditions for caffeic acid detection in tilapia whole body tissue.
Calibration curves preparation

Concentrations of described phytochemicals in fish tissue were determined by means of the external standard method; therefore, a standard curve was calculated for each phytochemical. Stock solutions of each phytochemical were prepared by dissolving 1 mg of the pure compound in 1 ml of DMSO, subsequent dilutions for desired concentrations were attained by dilution in HPLC grade methanol. The range of at least seven concentrations was selected from 0.02 to 5 \( \mu \text{g/ml} \). The detection limit established corresponded to the lowest detectable concentration from standard curve. Linearity was calculated for each standard curve.

Recovery rate estimation

Whole body fish samples from phytochemical unexposed organisms were spiked with known concentrations of each phytochemical, approximately 1 \( \mu \text{g/ml} \), prior to homogenization. The extraction was conducted as described above (acid hydrolysis) and final concentrations in each sample was estimated (\( n=5 \)).

RESULTS

The presence of the selected phytochemicals as parent compounds in tilapia tissues was effectively detected with HPLC. The detection involved several modifications to the referenced methods such as different column, mobile phase modification and variations in wavelength for the UV detector, as well as coupling of alternative detection devices. It should be mentioned that comparison of two
different extraction procedures (enzymatic and acid hydrolysis) was relevant in order to elucidate the most appropriate extraction process for the tested phytochemicals in juvenile tilapia. Given that enzymatic hydrolysis was only effective to determine the presence of the for quercetin parent compound (results not showed), and that caffeic acid, chrysin, daidzein and genistein chromatograms for samples extracted using enzymatic hydrolysis were negative in all four compounds (results not showed), therefore acid hydrolysis with acidified methanol, was the most viable extraction process for all phytochemicals.

Phytochemicals were identified by comparison of retention times versus analytical standards. Same chromatographic conditions allow the simultaneous detection of daidzein (Figure 4.4) and genistein (Figure 4.6), the main modification in this case was the utilization of a column with a different packing material characterized by high affinity to aromatic compounds and change in the detection wavelength from 280 to 260 nm. For chrysin (Figure 4.3), besides alternative column selection, mobile phase was modified from 55% to 80% methanol content to allow a faster elution of the compound; changing its retention time form 16.5 to 9.4 min. Chromatographic conditions for quercetin determination were validated based on our previous results observed in tilapia liver and plasma (Figure 4.5). For caffeic acid (Figure 4.2), analytical column selection and coupling of an electrochemical detector were the main modifications of the original method.

Detection conditions were validated by a series of standard curves that showed a linear relationship between concentrations and their observed peak
areas. The regression equations and correlation coefficients were calculated for specific range of concentrations, their values, as well as detection limits and retention times are listed in Table 4.5. Calculated extraction recovery rates (n=5) for all phytochemicals was very high, close to 95% values in all cases, with no signs of negative impacts after the addition of acidified methanol and later neutralization with highly concentrated sodium hydroxide.

For experimental diets, the presence of the original compound was validated by the described chromatographic conditions. There was no apparent lost of phytochemical during diet mixing, preparation, pelletizing, drying, grinding and storage at -20ºC for several weeks. In all cases, high dilution (1:1000 v:v) of diet extracts were needed in order to adjust concentration of injected fraction to the HPLC to allow an accurate estimation of the concentration in the diet.

DISCUSSION

The presence of selected phytochemicals in fish tissues was determined for all compounds by HPLC coupled with ultraviolet and electrochemical detections. Most references related to phytochemicals are focused on the estimations of concentrations and/or classification and identification of phytochemicals in a great variety of biological samples, such as soybean meal (Liggins et al., 2002), soy products (Nguyenle et al., 1995; Griffith and Collison, 2001), propolis (Bruschi et al., 2003), honey (Tomas-Barberan et al., 2001), and fruits (Shahrzad and Bitsch, 1996). In addition, analyses were carried out in body
tissues after dietary phytochemical administration, such as human breast tissue (Frankie et al., 1998), cell cultures (Walle et al., 1999) and plasma and urine (Boyle et al., 2000; Azuma et al., 2003). To our knowledge, our study provides the first data, on the extraction techniques and chromatographic detection conditions to determine the concentration and of bioaccumulation with time of exposure of caffeic acid, chrysin, daidzein, genistein and quercetin in fish tissues, specifically in tilapia juveniles, after dietary administration.

The analyses of the whole body tissue samples present challenges related the logistics of separation of specific organs or tissues in fish at the sizes reached after feeding with diets supplemented with the selected phytochemicals in present experiments. Fish reached maximum final individual weights between 1 and 2 g in feeding trials (results not showed); and consequently, separation of organs (i.e. liver, intestine, etc.), and extraction of body fluids (i.e. blood, urine) was considered impractical.

The significance of high performance liquid chromatography (HPLC) analytical methods to establish the presence and concentration of phytochemicals is widely reviewed in the literature. Other analytical approaches are also recommended for this purpose, including gas-liquid chromatography and capillary electrophoresis. The latter allow as well the identification and quantification of flavonoids and other phenolic compounds, but when considering specific requirements such as detection limits, complexity of the biological source, and resolution requirements, in most cases HPLC has many advantages (Wang et al., 2002). Other alternative approaches to phytochemical analyses in
biological samples include the use of ELISA techniques, previously used to measure absorption of phytochemicals in human fluid, foods and other sources (Bennetau- Pelissero et al., 2003), but again, this technique might require further validation in samples of fish and other vertebrate tissues and fluids.

It is important to remark that the main objective of this study was to determine the presence of the parent compound in fish tissue, given that most of their biological activities are attributed to these molecular forms (Pelissero et al., 1996; Chen et al., 1997; Joshi et al., 1998; Kao et al, 1998; Jeong et al., 1999; Eng et al., 2001). Although it is quite possible that the same chromatographic setting could allowing to detect and identify conjugated forms, reference standards are not always available for this purpose (Coldham et al., 2002; Merken and Beecher, 2000). The lack of background reference on the metabolic pathways of phytochemicals in fish after dietary intake complicates a proper identification of possible metabolites present in fish after intestinal absorption. The studies on the metabolism of phytochemicals after parent compound administration are undergoing in many organisms (Wilkinson et al., 2002).

HPLC can be coupled with several detectors. In our analyses we focused in the utilization of ultraviolet and electrochemical detectors, given the familiarity of their utilization for the detection of other phytochemicals such as gossypol (Rinchard et al., 2003). In present study the utilization of electrochemical detection increased the sensitivity in order to detect lower concentrations of the chemicals in tilapia whole body for caffeic acid and quercetin (Figures 4.2 and 4.5). HPLC method with electrochemical detection for quercetin has been also
reported by Azuma et al. (2003) for rat plasma. In general, all phytochemicals are detectable by electrochemical sensors given the presence of phenolic structures in their molecule that confers electro-active characteristics to the compound (Wang et al., 2002).

Extraction of phytochemical was efficiently achieved by acid hydrolysis of fish tissue samples. As mentioned before, initially samples were extracted, both including an enzymatic and acid hydrolysis step prior to sample filtration and injection into the HPLC, however, samples treated with β-glucoronidase and sulfatase did not result in increased amounts of the parent compounds for most of the phytochemicals, especially for genistein, daidzein, chrysin and caffeic acid. Day and Williamson (2001) emphasized that the use of enzymes to extract biological samples is critical to allow the deconjugation of glucoside, glucoronide and sulfate metabolites in order to estimate approximated phytochemical absorption.

Enzymatic hydrolysis is mostly recommended to detect and measure phytochemicals conjugated metabolites after consumption as described for detection of genistein and daidzein conjugates in rat plasma by Lamartiniere et al. (2002), for quercetin conjugates in rat plasma by Azuma et al. (2003) and in human plasma by Day et al. (2001). However, hydrolysis of fish samples with these enzymes did not yield noticeable peaks in the chromatograms in our sample analysis. Thus, all samples were extracted following acid hydrolysis with acidified methanol. The utilization of acidified solvents is widely recommended by other authors for HPLC analyses of biological samples. In case of tilapia tissue it
allowed the detection of all tested phytochemicals in the analyzed samples as can be observed in Figures 4.2b-4.6b. Two solvents are commonly used for this purpose, methanol and acetonitrile and there is some controversy regarding which solvent is the most appropriate for flavonoid extraction. Murphy and Suzanne (2002) indicated that no significant differences where observed in extraction efficiencies using either solvent but remarked that acidified conditions gave an apparent improvement in extraction effectiveness, also methanol improves extraction from oily homogenates (Nguyenle et al., 1995). Alternatively, Griffith and Collison (2001) mentioned that substitution of 80% methanol mixture for a 60% acetonitrile solution improves greatly extraction efficiencies of isoflavonoids in soy containing foods. Still, acidified methanol is recommended in the literature as an efficient hydrolyzing solution for extraction of genistein and daidzein from soy flour (Coldham et al., 2002), soybean seeds (Heimler et al., 2004); determination of chrysin and quercetin in honey samples (Yao et al., 2004).

Recovery rate estimation is a crucial point in phytochemical analysis in biological samples. Usually, this calculation is made through the use of internal reference standards added to the sample prior to extraction in order to correct for losses during this step (Wang et al., 2002). The estimated recovery rate for all phytochemicals was close to 90%. Although some concern was raised that daidzein and genistein analyses can be affected by the high protein content in the sample, as experienced in case of human milk by Frankie et al. (1998), our results do not point toward a similar situation in fish tissues.
In general, most authors reported high recovery rates of phytochemicals after extraction, either from their natural sources or biological samples. For instance, Heimler et al. (2004) reported 95% extraction efficiencies from soybean seeds for genistein and daidzein using either acidified methanol or acetonitrile as extraction solutions. Maubach et al. (2003) states that recovery rate of daidzein and genistein from breast tissue is at least 70% and 100% in urine and plasma. Recovery rate for chrysin from propolis can be as high as 95% (Bruschi et al., 2003). Caffeic acid extraction efficiency calculated from fruit juices after basic hydrolysis by Shanhrzad and Bitsch (1996) was close to 100%.

An addition to the use of more selective analytical columns is the use of gradient elution conditions as reported in several studies (Frankie et al., 1998; Mahungu et al., 1999; Heimler et al., 2004). It appears that gradient elution increased considerably samples run time, but also allows a more precise elution of analytes from the sample. These conditions are particularly relevant when identification of conjugated forms is necessary (Coldham et al., 2002). An appropriate column selection can improve resolution under isocratic elution conditions of a series of derivatives of a specific compound, as reported by Kim et al., (2002) for chrysin and other 19 derivatives.

Specific conditions for simultaneous HPLC detection of several phytochemicals are widely reported. For daidzein and genistein these is amply described in the literature (Wang et al., 1990; Nguyenle et al., 1995; Preinerstorfer and Sontag, 2004). Chromatographic conditions for genistein and daidzein modified from after Hubarat et al. (1998) were used successfully to
detect both phytochemicals as parent compounds. This author indicates a setting of 280 nm on the UV detector, however, enhanced peak resolution was observed at 260 nm. This wavelength is similar to that reported by Griffith and Collison (2001) and Heimler et al. (2004).

Several authors described chromatographic conditions for the determination of caffeic acid and chrysin, with minimal variations, such as different wavelengths settings for UV-detector. A gradient elution of the sample to allow an efficient separation was required (Thomas-Barberan et al., 2001; Ahn et al., 2004; Yao et al., 2004). These conditions were not applicable to fish tissues; therefore, the described method is considered the most suitable for these three phytochemicals.

Methanol is an important component of mobile phases in chrysin detection. The original mobile phase composition described by Walle et al. (1999) suggested the use of a 55% methanol in the mixture. However, when analytical column was changed in our study the retention time for the parent compound standard was close to 22 minutes. Hence, methanol inclusion to the mobile phase was modified, to 80%. This resulted in a satisfactory elution and decrease of retention time to only 9.4 minutes. Similar procedures have been described in the literature. For instance, Bruschi et al. (2003) described a series of modification on the mixture of methanol, water and acetonitrile to enhance separation of chrysin and its derivatives in propolis, samples, the most effective being when methanol was at 50%. Thomas-Barberan et al. (2001) and Yao et al. (2004) reported an efficient separation of chrysin using a gradient from 100% to
90% methanol as mobile phase. Other authors used acetonitrile as an alternative solvent and the main component of mobile phase to achieve the separation of chrysin in propolis extracts (Ahn et al., 2004) and rat plasma and urine after propolis intake (Kumazawa et al., 2004).

In conclusion, our study provides new information on the analytical conditions required for the detection of caffeic acid, chrysin, daidzein, genistein and quercetin in juvenile tilapia tissue after dietary administration for 6 to 8 weeks. Our results suggest that appropriate extraction method and modification of already described chromatographic conditions can allow the detection of the parent compounds with HPLC coupled with ultraviolet and/or electrochemical detection.
Figure 4.1 Molecular structures of selected experimental phytochemicals. Isoflavonoids: genistein (GEN) and daidzein (DAID), flavonol: quercetin (QUER), flavone: chrysin (CHR) and phenolic acid: caffeic acid (CAFF).
<table>
<thead>
<tr>
<th>Compound</th>
<th>Equation</th>
<th>Correlation Coefficient</th>
<th>Detection Limit (µg/ml)</th>
<th>Retention Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein</td>
<td>Y = 50.44X-0.6299</td>
<td>$r^2 = 0.9987$</td>
<td>0.04</td>
<td>16.1</td>
</tr>
<tr>
<td>Daidzein</td>
<td>Y = 54.381X-0.7395</td>
<td>$r^2 = 0.9996$</td>
<td>0.05</td>
<td>9.3</td>
</tr>
<tr>
<td>Chrysín</td>
<td>Y = 25.123X-2.2529</td>
<td>$r^2 = 0.9983$</td>
<td>0.02</td>
<td>9.4</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>Y = 478424X-1323.4</td>
<td>$r^2 = 0.9985$</td>
<td>0.004</td>
<td>8.7</td>
</tr>
<tr>
<td>Quercetin</td>
<td>Y = 75.564X-2235.7</td>
<td>$r^2 = 0.9808$</td>
<td>0.02</td>
<td>5.5</td>
</tr>
</tbody>
</table>

Table 4.5 Standard curve equations, correlation coefficients; detection limits (standard) and retention times of selected phytochemicals in accordance with described chromatographic conditions.
Figure 4.2 Caffeic acid chromatograms with electrochemical detection. a) standard 0.5 µg/ml. b) whole body tilapia sample. Retention time: 8.7 min
Figure 4.3 Chrysin chromatograms with UV detection. a) standard 0.5 µg/ml. b) whole body tilapia sample. Retention time: 9.4 min
Figure 4.4 Daidzein chromatograms with UV detection. a) standard 0.5 µg/ml. b) whole body tilapia sample. Retention time: 9.3 min
Figure 4.5 Quercetin chromatograms with electrochemical detection. a) standard 0.5 µg/ml. b) whole body tilapia sample. Retention time: 5.5 min
Figure 4.6 Genistein chromatograms with UV detection. a) Standard 1.2 µg/ml. b) whole body tilapia sample. Retention time: 16.1 min.
CHAPTER 5

INTERACTION OF PHYTOCHEMICALS-QUERCETIN WITH OTHER ANTIOXIDANTS- ASCORBIC ACID. SPARING EFFECT IN TILAPIA AFTER ULTRAVIOLET IRRADIATION EXPOSURE.

ABSTRACT

A consequence of ultraviolet light exposure is skin damage due to the formation of free radicals; administration of antioxidants that scavenge free radicals might minimize such damage. Quercetin, and ascorbic acid (AA), both exhibit a potent antioxidant activity. The possible increased protective effects to UV light exposure by interaction of dietary quercetin and ascorbic acid were examined in all male tilapia. Four semi-purified, casein-gelatin based diets were prepared as follows: control diet 100 mg/kg AA (C-Q-), 100 mg/kg AA + quercetin 10 g/kg (C-Q+), 1000 mg/kg AA (C+Q-), 1000 mg/kg AA + quercetin 10 g/kg (C+Q+), were fed to tilapia for 20 weeks (initial weight 9.6±1 mg). After 19 weeks, 3 X 25 fish per diet were divided into 2 groups, control and UV treatments. Fish were exposed to UV radiation at the rate of 0.33 J/cm² for 3 minutes. Control groups were protected with a MYLAR® polyester film and acrylic glass; UV-irradiated groups were exposed in the container covered with acrylic glass. On the week 20, the same fish were re-exposed to UV radiation (0.99 J/cm² for 1
minute), control groups of fish were protected by a double layer of MYLAR® and the UV treatment radiation groups were exposed with no protection. Prior to each exposure, 24 hours and 7 days after the 2\(^{\text{nd}}\) treatment, liver and skin were sampled for quercetin and AA analysis. Twenty-four hours after the 2\(^{\text{nd}}\) exposure, head sections of fish were fixed in formalin for histological analysis of skin damage in treated and untreated groups. Results indicate no changes in concentrations of AA; however, relative proportion of oxidized ascorbate (DHA) was significantly increased in fish on low dietary vitamins C levels. Quercetin concentrations after exposures were almost negligible in skin and limited to very few samples, liver concentration was significantly different among control (mean 34±10 µg/g) and UV-irradiation exposed fish (mean 11±6 µg/g) for both C-Q+ and C+Q+ groups. The magnitude of the potential interaction between these two dietary antioxidants may change and become more important during increase of UV-irradiation.

**INTRODUCTION**

All living organism are subjected to ultraviolet irradiation (UV). Although UV irradiation is relevant for several biological processes, mostly related to energy production, phagocytosis, regulation of cell growth and intracellular signaling, and synthesis of biologically important compounds (Pietta, 2000), it may also present potential harmful effects (Fidhiany and Winkler, 1999). Exposure to shorter wavelength of UV radiation has been associated with a variety of effects in many organisms ranging from molecular and tissue damage
to population level effects (Armstrong et al., 2002). Among the many probable negative effects of UV irradiation are possible acute adverse effects like sunburn, photosensitivity reactions, or immunological suppression, as well as long-term damage like photo-aging or malignant skin tumors. In many cases these pathologies are mediated at the tissue/cellular levels by produced reactive oxygen species and other compounds (Bonina et al., 1996; Eberlein-Konig et al., 1998; Charron et al., 2000; Moison et al., 2002; Saral et al., 2002). Sunburn is one of the effects of sunlight and UV irradiation in fish (Jokinen et al., 2000). Blazer et al. (1997) remarks that has been recognized as a problem in aquaculture locations where fish are held in relatively shallow water at high altitudes. They also mention that although the damage itself may not be lethal to the fish, that secondary bacterial, parasitic or fungal infections can lead to high mortality following irradiations.

Studies on the effect of UV irradiation in fish suggest that individual species have different responses to this stressor, which vary from behavioral modification in zebra fish (Danio rerio) (Charron et al., 2002), skin histopathological damage in cutthroat trout (Oncorhynchus clarki) (Blazer et al., 1997) and sole (Solea solea) (McFadzen et al., 2000) and selective cell gene expression as defense mechanism in Atlantic cod (Gadus morhua) (Lesser et al., 2001). In other species no visible negative effects were observed in Razorback suckers (Xyrauchen texanus) (Blazer et al., 1997) and turbot (Scophthalmus maximus) (McFadzen et al., 2000).
Most vertebrates have some natural protection against ultraviolet (UV) light induced damage. Many of these effects are minimized by tegument pigmentation and the action of epidermal antioxidants that provide a second line of defense against reactive oxygen species generated after UV light exposure (Charron et al., 2000). The endogenous antioxidant system includes a series of enzymes (Fuchs et al., 1997; Saral et al., 2002) acting in concert with non-enzymatic antioxidants that include glutathione, uric acid, α-lipoic acid (Saral et al., 2002) and α-tocopherol and L-ascorbic acid (Rijnkels et al., 2003). Such defenses are specifically located in different body compartments, but in general, what a great number of fish species have in common is ascorbic acid acting as a UV defense system (Ringvold et al., 2003).

Several authors have addressed the importance of dietary administration of antioxidants as means to increase the endogenous protection against UV irradiation. To this moment the most widely studied dietary antioxidants are ascorbic acid, α-tocopherol and β-carotene and some of these dietary antioxidant have enhanced protection when present in combination in the diet (Offort et al., 2002). Oral supplementations of L-ascorbic acid and α-tocopherol have a synergistic effect in suppressing sunburn reaction in humans (Fuchs and Kern, 1998). Some fatty acids, such as eicosapentaenoic acid (EPA), co-supplemented with ascorbic acid and/or α-tocopherol may result in lowering UV irradiation-induced lipid peroxidation, and may thus preserve or even enhance the beneficial effects of EPA (Moison et al., 2002). It is known that endogenous antioxidants are decreased systematically during UV exposure, thus combined
supplementation of β-carotene, α-tocopherol and ascorbic acid in addition to topical screens may help to lower the risk of skin sunburning (Greul et al., 2002). Therefore, administration of dietary antioxidants that scavenge reactive oxygen species (ROS) might be a promising strategy to decrease UV-induced skin pathologies (Eberlein-Konig et al., 1998).

At this moment, a new group of dietary components are under investigation given their antioxidant capacity. Flavonoids are a group of phenolic compounds widely occurring in the plant kingdom, which exhibit a reported strong antioxidant capacity along with many other biological activities, like capability to interact with protein phosphorylation and iron chelating (Bonina et al., 1996; Boyle et al., 2000). The antioxidant capacity in general, is conferred by a high number of hydroxyl substitutions in each flavonoid molecule, which has a direct effect on the donating ability of hydrogen atoms to scavenge free radicals (Pietta, 2000; Kim, 2001). In addition, flavonoids may regenerate other antioxidants such as tocopherol by donating a hydrogen atom to the tocopheryl radical (Boyle et al., 2000). In a public health domain, consumption of plant phenolics may be associated with a decreased risk of cardiovascular disease by stabilizing and protecting vascular endothelial cells against oxidative and pro-inflammatory insults (Reiterer et al., 2004). In general, several authors consider quercetin as a very effective antioxidant and free radical scavenger given some specific 1.-structural, 2.-molecular and 3.-conformational characteristics such as the presence of a catechol moiety in the B ring, and a 2,3-doble bond in the C ring (Bors et al., 1995; Saija et al., 1995; Kim, 2001).
Despite the increasing evidence of the *in vitro* antioxidant potential of flavonoids, little is known about their efficacy *in vivo* (Pietta, 2000). As mentioned before, fish are frequently exposed to UV irradiation damage, and their sensitivity can be reduced by supplementation with antioxidants. Consequently, in order to validate the possibility of the interaction between ascorbic acid and quercetin in an *in vivo* study in Nile tilapia (*Oreochromis niloticus*) was used as a model to assess growth and development after a dietary administration of quercetin to larvae/juveniles, secondly we aimed to evaluate the 1.-potential, 2.-cumulative, and 3.-protective effects after dietary administration of this two dietary antioxidants after an acute UV irradiation exposure.

MATERIALS AND METHODS

Reagents

The experimental chemicals were obtained from the following sources: quercetin (3,5,7,3’,4’ pentahydroxyflavone) from Sigma, St Louis, MO, USA, and ascorbic acid as Mg-L-ascorbyl-2-phosphate (Phosphitan C) from Showa Denko, Tokyo, Japan. Mobile phase components, acetonitrile, acetic acid and water (HPLC grade) were purchased form Fisher Scientific Inc., Pittsburgh, PA. USA.

Feeding experiment

The interaction of quercetin (Q) and ascorbic acid (AA) was examined using growth response of all-male tilapia (Til-Tech, Baton Rouge LA, USA). Experimental organisms (initial weight 9.6±1 mg) were randomly distributed in 12
aquaria (35 L) in a semi-closed recirculation system with control temperature. An initial density of 150 fish/tank, with three replicates per diet was used. The dietary treatments consisted of four semi-purified casein-gelatin based formulations diets (Table 5.1) designated as follows: control diet 100 mg AA/kg (C-Q-), 100 mg AA/kg + quercetin 10 g/kg (C-Q+), 1000 mg AA /kg (C+Q-), 1000 mg AA /kg + quercetin 10 g/kg (C+Q+). Fish were fed for 19 weeks at the adjusted feeding ratio from an initial 20% to a final 2.5% body weight ratio per day. Progress in weight gain per dietary treatment was assessed at several time points throughout the feeding trial. At the end of feeding trial, individual final weight (g), survival (%), specific growth rate (%/day), and hepatosomatic index (HIS%) were evaluated for each dietary treatment. Body proximal analyses was also determined at end of feeding trial; 10 fish per replica, 3 samples per treatment were pooled after freeze-drying and pulverization for later processing for general proximate analysis (nitrogen, lipids, ash, minerals) following standard procedures (AOAC, 1995).

**Acute UV irradiation procedure for exposure**

After 19 weeks of feeding, the 3 replicates per diet, 25 fish each, were divided into 2 groups: controls and UV treatments. Fish from each group were placed in 2 L glass beakers with 1 L of dechlorinated city water at room temperature and exposed to UV radiation of 0.33 J/cm² (equivalent to 1.85X10⁻³ W/cm²) in a Stratalinker 2400 STRATAGENE®, where control groups were protected with MYLAR® polyester film (0.04mm) and acrylic glass (Plexiglas) (0.2 mm), UV-irradiated groups were exposed only when covered with Plexiglas 0.2
mm for 3 minutes. Seven days later, the same fish groups were re-exposed to UV irradiation at 0.99 J/cm\(^2\) (equivalent to 1.67X10\(^{-2}\) W/cm\(^2\)) for only 1 minute. The time was shortened in order to minimize any increase in water temperature in the glass container where fish were placed in for UV exposure. For the second UV exposure, control groups of fish were protected by a double layer of MYLAR\(^\circledR\) (0.04mm) and the UV radiation treatment groups were exposed with no protection. In order to estimate the extent of physiological UV irradiation damage, histological analyses of dorsal sections of the head were conducted after fixation in 10% buffered formalin. Both groups control and exposed, were analyzed 24 h after the 2\(^{nd}\) exposure. The hematoxilin-eosin staining was used in 5 µm sections of skin. Behavioral modifications were observed for a period of 48 h after second exposure. This included observance of changes on swimming activity and skin pigmentation patterns.

**Antioxidant analysis**

Prior to the first exposure and 24 h, and 7 days after the 2\(^{nd}\) UV-irradiation treatments, skin sections from the lateral portion of the abdominal wall of the experimental fish and livers were sampled (n=6 fish/diet) from both control and UV treatments. Quercetin presence in skin and liver was analyzed after extraction with acidified methanol by an HPLC technique using an isocratic elution with a mobile phase of 27% acetonitrile in water: acetic acid (99.5:0.5 v:v); at a flow rate of 1.0 ml/min. the determination was accomplished by means of a BAS\(^\circledR\) electrochemical detector, set at 0.08 to 8.0 mV output; with an a ODS Beckman\(^\circledR\) 150X4.6 mm 5 µ reverse-phase column analytical column (Park et al.,
Ascorbic acid was analyzed using the dinitrophenylhydrazine (DPNH) method (Dabrowski and Hinterleitner, 1989).

**Statistical analysis**

Tilapia growth, proximate analyses of the whole body and liver quercetin analyses data were subjected to one-way ANOVA test. Ascorbic acid skin concentrations as total, dehydro and reduced forms data were analyzed by two-way ANOVA. Fisher protected test for least square means multiple comparison test (at a p=0.05 significance level) was used to establish treatment and period of exposure significant differences. All statistical was performed by means of Statistical Analysis System software version 8.02 (SAS Institute, Inc., Cary, NC) at a significance level of α=0.05.

**RESULTS**

**Growth, survival and other indices**

After 19 weeks no significant differences (p>0.05) were observed in the evaluated growth parameters (Table 5.2). Final mean individual weight was close to 8 g across treatments. Progress in growth for the initial 19 weeks was uniform for all dietary treatments (Figure 5.2). Overall final survival was 53.3% across all treatments, and other indices, such as hepatosomatic index and daily specific growth rate, were not different among experimental diets (Table 5.2).
Body proximal composition

The experimental diets with different levels of ascorbic acid (100 and 1000 mg/kg) supplementation in the absence or presence of the flavonoid (quercetin, 1%) in the diet, did not induce variations in body tissue components such as water, total protein, total lipids and ash after 19 weeks of feeding with the experimental diets (Table 5.3). Similar results were observed for main minerals components such as phosphorus, potassium, calcium, magnesium and sodium. Trace elements did not show significant variations, except in the case of copper for group C+Q+. The concentration found (mean 321.5 µg/g) was relatively high but not significantly than in the other experimental groups (mean concentration 79.4 µg/g) (Table 5.3).

UV irradiation exposure effects in experimental fish

Fish were exposed on two occasions with different protection materials as cover inside the UV exposure chamber. Following the first exposure, fish did not exhibit any signs on changes in external skin appearance or swimming behavior, in both Mylar+Plexyglas or Plexiglas protected groups. However, after the second UV exposure, fish protected with a double Mylar layer did not show any apparent skin damage or behavioral modification within 48 h following UV exposure. To the contrary, unprotected fish were visibly stressed and in both, skin external appearance and swimming activity have changed. The skin of the head and dorsal portion of the fish was visibly sunburned. Profound changes in increased pigmentation in these areas were observed (white coating). Fish swimming activity was very limited for 24-36 h in UV-exposed groups, and fish
were static at the bottom of the aquaria and regained full mobility only 48 h after UV exposure. Although some mortality was observed in both protected and unprotected groups, especially after the second UV exposure, it only represented less than 1% of UV-exposed fish.

**Ascorbic acid concentrations in skin**

Total ascorbic acid concentrations in skin tissues were significantly different depending on the dietary vitamin C supplementation levels (P<0.0001). Lower level in diet (100 mg/kg) resulted in two-fold lesser skin ascorbic acid concentrations in skin (Figure 5.3a). Also, after UV exposure, both control and UV exposed groups maintained such a trend; 24 h and 7 days following the 2\(^{nd}\) UV exposure (Figures 5.3b, c, d and e).

Stress and possible formation of free radicals after UV exposure did not affect the levels or oxidation of ascorbic acid in diets C-Q- and C-Q+, that is prior and 24 h and 7 d after exposure, levels remained constant (Table 5.4). For diets C+Q- and C+Q+, in control “mylar+plexiglass” protected fish, the levels of oxidation of ascorbic acid was significantly lower 24 h after 2\(^{nd}\) UV exposure than the initial level. In UV-exposed fish skin, tissue concentration of DHA at this time as well as 7 d after exposure for both groups remained unchanged. At any specific time, the same trend was observed for total ascorbic acid and levels corresponded in a dose dependent manner to dietary supplementation. However, these values were not influenced by the presence of quercetin in the diet (P>0.005).
Reduced ascorbic acid values were significantly lower for C-Q- and C-Q+ diet treatments prior to UV exposure (P<0.05). For diet C+Q-, values were more stable independently of time before or after treatment. In diet C+Q+, values remained similar prior to the UV exposure and both protected and unprotected fish 24 h after acute UV treatment, but were lower significantly (P<0.05) 7 days after UV exposure (Table 5.5). Considering the fact that the observed values of ascorbic acid in both, protected and unprotected fish, fed with diets containing a higher level of ascorbyl-phosphate (1000 mg/kg) remained comparable 24 h after acute UV exposure, it can be concluded that increased AA supplementation had in fact an influence in maintaining such higher AA levels (significant at P=0.03). In contrast diets with low level of ascorbic acid, independently of the presence or absence of quercetin supplementation (Table 5.5) had no effect on the presence of reduced AA on skin tissue after UV-irradiation exposure.

**Quercetin concentrations in skin and liver**

Detection of quercetin in skin tissue was very low in a number of samples (>10%) where quercetin was identified in the chromatograms (Figure 5.4). Given this fact, liver tissue analyses were the focus and samples taken 24 h after 2nd UV acute exposure. Examples of chromatograms obtained with coupled electrochemical detection are showed in Figure 5.4. Observed quercetin values in liver tissue, in both diets C-Q+ and C+Q+ showed significantly lower values of quercetin (parent compound) (P<0.05) in UV exposed fish than control (protected) (Figure 5.5). Quercetin concentrations in fish liver tissues were not influenced by dietary AA levels.
Skin histological analysis

Head skin sections were displayed in Figure 5.6. Examples of the degree of lesions observed in UV exposed fish (figure b, c and d), such alterations included cell damage as hyperplasia, intracellular edema and necrosis on the epithelia are demonstrated. These lesions contrasted with the minimal tissue alteration observed in control groups of fish (Figure 5.6a).

DISCUSSION

Tilapia was fed for 19 weeks with experimental diets containing two levels of ascorbic acid as L-ascorbyl-phosphate-Mg, 100 and 1000 mg/kg, and in the absence or presence of quercetin at 10 g/kg of diet. There were no significant differences in the growth and mean final individual weight among treatments. This was not an unexpected finding, given that both concentrations of vitamin C were above the requirement reported for tilapia and other cichlid species. Shiau and Tsu (1999) reported a significantly higher weight gain in hybrid tilapia (O. nilotucus X O. aureus) when fish were fed at the concentration of 23 mg AA/kg in the diet of the same active form of ascorbic acid (phosphate) as used in the present experiment. Al-Amoudi et al. (1992) reported that O. spilurus only show deficiency symptoms when vitamin C inclusion in diet was below 75 mg/kg. Abdelghany (1996) indicated that the best growth rates in O. niloticus were observed at a minimum concentration of 50 mg/kg ascorbate equivalent in the diet.
In relation to the level of quercetin added into the diet (10 g/kg), we suggest that this specific level do not influence negatively the growth and other related parameters in tilapia. Other variables such as survival, hepatosomatic index (HSI), and daily specific growth rate (SGR) was not influenced either by the dietary level of ascorbic acid or the inclusion of the quercetin. Generally, HSI is a useful index to determine possible toxicological effects, as an increase or a decrease in liver weight, after the exposure to specific agents, as reported for medaka (Oryzias latipes) (Ma et al., 2005), and gudgeon (Gobio gobio) (Bervoets and Blust, 1995). Our results showed that fish from all experimental diets had similar HSI values; therefore, no apparent toxicological effects can be suggested at this specific dietary level. Although, there is no literature data on long term quercetin feeding trials in fish, our results suggest that quercetin does not affect normal growth in tilapia at this tested concentration.

Proximal body composition did not show significant variation in the observed values. However, copper concentration was more variable and higher in C+Q+ group, but not significant. It is an important finding given the available information on the interaction of flavonoids such as quercetin and ions of trace elements such as iron and copper (Afsana et al., 2004; Apak et al., 2004). Both quercetin and ascorbic acid can acquire a pro-oxidant activity in a dose dependent manner in the presence of Cu$^{2+}$ ions (Filipe et al., 2004), but this property changes when quercetin is present at high concentrations (Liu et al., 2004). Even though, quercetin interaction with Cu$^{2+}$ is quite remarkable, specially as a chelating agent, it can also be altered by pH (Mira et al., 2002; Torregani et
and presence of either free or conjugated quercetin (Turner et al., 2004; Vestergaard et al., 2005). Several authors agree that quercetin can have an important role in control of copper-overload toxicity conditions (Kuo et al., 2001), the other biochemical events, such as Cu²⁺-mediated ascorbic acid induced lipid peroxidation (Ramanathan and Das, 1993) by inactivation of Cu²⁺ ions in the organisms, possibly by increasing its bioaccumulation by precipitation in biologically inactive forms after chelation, suggest other possible interactions that were not explored in fish thus far.

Tilapia was found to be highly susceptible to UV-irradiation damage. Behavioral modification expressed as inactivity and external lesions (whitening) on dorsal portion of the body observed in our experiment were similar to the signs described by Charron et al. (2000) in newly hatched zebrafish after exposure to direct ambient UV light, equivalent of 1.95 W/m². Also, observed lesions on head skin based on histological analyses following exposure to 0.99 J/cm² for 1 minute, corresponded to similar tegument alterations reported in other fish species after UV exposure. Histological analyses of cutthroat trout (O. clarki) provided evidence of sunburns 48 hours after exposure to artificial sunlight manifested by separation of mucus cells, necrosis and inflammation of both epidermis and endodermis (Blazer et al., 1997). Sole (S. solea) displayed reduction in size of mucus cells, and increased epidermal thickness after 2.5 KJ bio eff/m² exposure (McFadzen et al., 2000). An increased cellular hyperplasia was observed in Japanese medaka (Armstrong et al., 2002), as well as increased secretion of mucus after UV exposure (Fabacher et al., 1995). Tilapia
skin damage described in Figure 5.6, matches the previously reported skin UV induced changes in other species. Therefore, we can suggest that tilapia can be placed in the category of UV sensitive fish species.

The observed concentrations of total ascorbic acid in tilapia skin tissue are related to the level of inclusion on the experimental diet; it was evident that diets with 1000 mg/kg of ascorbate (C+Q- and C+Q+) resulted in higher skin ascorbic acid concentrations (Figure 5.3). This fact corresponds to observations made in other fish species. Wang et al. (2003) for instance reported a correlation, independently of the dietary ascorbic acid source (either L-ascorbyl-2-monophosphate-Ca or L-ascorbyl-2-monophosphate-Na/Ca) between dietary and liver and muscle AA concentrations in Korean rockfish (Sebastes schlegeli). Wahli et al., (2003) indicated that variations on AA inclusion level in diet (20, 150 and 1000 mg/kg) had a direct effect on the pool of vitamin C in several tissues including skin, muscle and liver. More importantly in skin AA concentrations were particularly relevant given the increased requirement during a wound healing process after inflicted skin damage in rainbow trout.

The observed variations of ascorbic acid expressed as formation of dehydroascorbic acid in skin tissue (Table 5.4) can be considered noteworthy, as a potential evidence of the exerted free radical scavenging effect in skin tissue after UV exposure. Many authors remark the direct impact of ascorbic acid as an endogenous defense against UV radiation as an antioxidant (Boyle et al., 2000; Rijnkels et al., 2003). Also the observed levels of residual available AA on its reduced form in both C+Q- and C+Q+ after UV-irradiation exposure is noticeable
as a new finding, given the fact that in exposed fish the reduced form of ascorbic acid 24 h after acute UV exposure is significantly lower in groups fed low ascorbic acid inclusion diets. Higher consumption of ascorbic acid will be reflected in more protection after an acute stress conditions, such as direct ultraviolet light exposure.

Quercetin it is known to interact with ascorbic acid at different levels. Quercetin is capable to reduce ascorbic acid, therefore acting as a protective agent by allowing re-utilization of the molecule (Bors et al., 1995). Our results do not indicate a significant sparing effect of quercetin on UV exposed fish by either a higher concentrations of reduced ascorbic acid or attenuation of the formation of dehydroascorbic acid in skin, and the observed variations could be exclusively related to higher supplementation rates of AA in the diet.

Quercetin detection on skin was virtually negligible in most of the analyzed fish skin samples. Several studies have addressed the uptake of quercetin and other flavonoids in skin cells, but in most cases the experiments have been conducted in vitro. Saija et al. (2003) indicates that the efficacy of antioxidant protection in vitro can be improved using specific quercetin semi-synthetic derivatives. Kim (2001) showed that the presence of quercetin in fibroblast cell culture media, protected effectively against UV irradiation reducing lactic dehydrogenase release (cell leaching) by 30 to 40%. Skaper et al. (1997) exposed human skin fibroblasts and keratinocytes to buthionine sulfoximine (BSO), an irreversible inhibitor of glutathione (GSH) in the presence of quercetin and ascorbic acid. Their results indicated that quercetin effectively protects
against GSH depletion and that the presence of ascorbic acid lowered the EC_{50} required to yield a protective effect in this skin cells. Alternatively, effective absorption by permeation of quercetin and other flavonoids through the *stratum corneum* has been reported after topical applications on skin fragments. In the presence of quercetin it resulted in a reduction of UV induced peroxidation of phosphatidylcholine vesicles that were used as a membrane model (Bonina *et al.*, 1996).

Quercetin presence in liver of experimental fish can be considered, to some extent, evidence of absorption and metabolization of this phytochemical in tilapia. The significant reduction of quercetin levels as non-metabolized compound in liver tissue that was observed (Figure 5.5), could be an indication of a considerable degradation of the compound after reduction (H^+ donation) due to an increased systemic free radical production that usually follows exposure to UV irradiation (Bonina *et al.*, 1996; Kim, 2001; Rijnkels *et al.*, 2003).

However, to explain in detail whether quercetin is being absorbed in tilapia requires more studies. There is no consensus of which molecular form will easier pass through the intestinal barrier, withstand liver metabolism and get to the circulatory system to assure delivery to other tissues, in this case either free or conjugated forms (Walle *et al.*, 2003). Although it has been discussed that free forms are absorbed to a higher degree (Hollman and Katan, 1997), different studies did not agree on this assertion. Monoglucoside forms derived from free quercetin were better absorbed in rat small intestine (Gee *et al.*, 2000) and consumption of quercetin as glucosides sources (onions) in humans, increased
the levels in plasma of glycosidic forms of the flavonoid (Boyle et al., 2000). At this moment there are no references that have directly addressed the absorption of quercetin and other flavonoids in fish, thus research needs to address this.

Determination of the variations and availability of ascorbic acid and quercetin in tilapia skin tissue as an indirect measure of interaction between both dietary antioxidants did not prove to be conclusive to elucidate the extent of the interaction. Measurement of alternative biochemical responses could provide this information. Changes in formation or depletion of specific endogenous antioxidants are reported in several studies. Consequently, quantification of endogenous antioxidants of non dietary origin such as GSH might provide a better understanding of the prevalence and efficacy of quercetin as an active in vivo antioxidant after consumption in tilapia when exposed to acute UV irradiation.

In conclusion, we suggest that variations in observed ascorbic acid concentrations were related to dietary inclusion level and less to a sparing effect of quercetin. Quercetin was absorbed to a certain degree; and conceivably quercetin antioxidant activity could be more evident at low ascorbic acid inclusion in the diet.
Figure 5.1 Molecular structures of L-ascorbic acid (AA) and quercetin (QUER).
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>C-Q-</th>
<th>C-Q+</th>
<th>C+Q-</th>
<th>C+Q+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (vitamin free)</td>
<td>36.0</td>
<td>36.0</td>
<td>36.0</td>
<td>36.0</td>
</tr>
<tr>
<td>Gelatin</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Dextrin</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>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Cod liver oil</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>
</tr>
<tr>
<td>Vitamin mix&lt;sup&gt;2&lt;/sup&gt;</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Mineral mix&lt;sup&gt;3&lt;/sup&gt;</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Carboxymethylcellulose</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Phospitan C&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.02</td>
<td>0.02</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.0</td>
<td>1.0</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>1.18</td>
<td>0.18</td>
<td>1.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

<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>Bernahrt 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), 50% AA equivalent.

Table 5.1 Composition of experimental diets for first feeding tilapia expressed as percentage of dry matter per 100 g. 100 mg/kg AA (C-Q-), 100 mg/kg AA + quercetin 10 g/kg (C-Q+), 1000 mg/kg AA (C+Q-), 1000 mg/kg AA + quercetin 10 g/kg (C+Q+).
<table>
<thead>
<tr>
<th>Treatment (Diet)</th>
<th>Final individual weight (g)</th>
<th>Survival(%)</th>
<th>HSI</th>
<th>SGR (%/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-Q-</td>
<td>8.2±0.7</td>
<td>51.1±7.2</td>
<td>1.23±0.42</td>
<td>5.4±0.1</td>
</tr>
<tr>
<td>C-Q+</td>
<td>7.9±2.6</td>
<td>53.4±13.6</td>
<td>1.25±0.55</td>
<td>5.2±0.1</td>
</tr>
<tr>
<td>C+Q-</td>
<td>7.9±1.8</td>
<td>56.7±5.8</td>
<td>1.09±0.43</td>
<td>5.2±0.1</td>
</tr>
<tr>
<td>C+Q+</td>
<td>6.8±0.8</td>
<td>52.8±10.9</td>
<td>1.18±0.63</td>
<td>5.3±0.1</td>
</tr>
</tbody>
</table>

Table 5.2 Observed performance in Nile tilapia (*Orechromis niloticus*) after 19 weeks feeding with experimental diets. Final individual weight, survival, hepatosomatic index (HIS) and specific growth rates (SGR %/day) values n= 3 (mean±SD). Diets: 100 mg/kg AA (C-Q-), 100 mg/kg AA + quercetin 10 g/kg (C-Q+), 1000 mg/kg AA (C+Q-), 1000 mg/kg AA + quercetin 10 g/kg (C+Q+).
<table>
<thead>
<tr>
<th>Component</th>
<th>C-Q-</th>
<th>C-Q+</th>
<th>C+Q-</th>
<th>C+Q+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture %</td>
<td>75.9±0.53</td>
<td>77.8±1.31</td>
<td>77.5±1.73</td>
<td>77.1±1.00</td>
</tr>
<tr>
<td>Protein</td>
<td>62.4±0.55</td>
<td>64.1±1.77</td>
<td>64.6±2.61</td>
<td>65.0±1.41</td>
</tr>
<tr>
<td>Ash %</td>
<td>13.8±0.44</td>
<td>14.5±0.65</td>
<td>14.5±0.31</td>
<td>14.2±0.23</td>
</tr>
<tr>
<td>Lipid analysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total lipid %</td>
<td>22.3±0.41</td>
<td>19.4±1.43</td>
<td>20.6±2.62</td>
<td>19.7±0.96</td>
</tr>
<tr>
<td>Neutral Lipids %</td>
<td>76.9±5.5</td>
<td>77.9±2.6</td>
<td>77.9±3.5</td>
<td>76.2±4.7</td>
</tr>
<tr>
<td>Macrominerals (mg/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphorus (P)</td>
<td>21.1±0.68</td>
<td>22.0±0.62</td>
<td>21.3±0.66</td>
<td>21.6±0.23</td>
</tr>
<tr>
<td>Potassium (K)</td>
<td>11.6±0.21</td>
<td>12.0±0.20</td>
<td>11.7±0.30</td>
<td>12.2±0.09</td>
</tr>
<tr>
<td>Calcium (Ca)</td>
<td>29.1±0.86</td>
<td>30.9±1.59</td>
<td>29.6±0.85</td>
<td>29.5±0.53</td>
</tr>
<tr>
<td>Magnesium (Mg)</td>
<td>1.1±0.04</td>
<td>1.1±0.03</td>
<td>1.1±0.03</td>
<td>1.1±0.01</td>
</tr>
<tr>
<td>Sodium (Na)</td>
<td>4.7±0.05</td>
<td>5.0±0.08</td>
<td>4.8±0.25</td>
<td>5.0±0.02</td>
</tr>
<tr>
<td>Trace elements (µg/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aluminum (Al)</td>
<td>12.2±0.72</td>
<td>16.8±6.37</td>
<td>17.2±1.92</td>
<td>16.5±4.18</td>
</tr>
<tr>
<td>Boron (B)</td>
<td>0.38±0.18</td>
<td>&lt;0.250</td>
<td>0.33±0.14</td>
<td>0.59±0.41</td>
</tr>
<tr>
<td>Copper (Cu)</td>
<td>71.9±35.9</td>
<td>88.7±78.1</td>
<td>78.6±55.1</td>
<td>321.5±305.5</td>
</tr>
<tr>
<td>Iron (Fe)</td>
<td>75.5±2.48</td>
<td>63.7±6.30</td>
<td>84.6±9.75</td>
<td>69.0±6.37</td>
</tr>
<tr>
<td>Manganese (Mn)</td>
<td>11.9±1.00</td>
<td>18.9±1.08</td>
<td>13.1±2.12</td>
<td>21.1±3.32</td>
</tr>
<tr>
<td>Molybdenum (Mo)</td>
<td>&lt;0.250</td>
<td>&lt;0.250</td>
<td>&lt;0.250</td>
<td>&lt;0.250</td>
</tr>
<tr>
<td>Zinc (Zn)</td>
<td>174.8±3.96</td>
<td>198.4±13.6</td>
<td>174.7±17.0</td>
<td>208.8±8.43</td>
</tr>
</tbody>
</table>

Table 5.3 Proximate and mineral composition of tilapia juveniles body after 19 weeks feeding with experimental diets. Values are indicated as mean±SD (n=3). Diets: 100 mg/kg AA (C-Q-), 100 mg/kg AA + quercetin 10 g/kg (C-Q+), 1000 mg/kg AA (C+Q-), 1000 mg/kg AA + quercetin 10 g/kg (C+Q+).
Figure 5.2 Progress of individual mean body weight of Nile tilapia fed with experimental diets for 19 weeks. Values are mean ± SEM of triplicate groups (n=3). Diets: 100 mg/kg AA (C-Q-), 100 mg/kg AA + quercetin 10 g/kg (C-Q+), 1000 mg/kg AA (C+Q-), 1000 mg/kg AA + quercetin 10 g/kg (C+Q+).
Table 5.4 Observed concentrations of dehydroascorbic acid (DHA) in skin tissue prior and 24h and 7 days after the second UV acute exposure. Values are indicated as mean±SD (n=6). Diets: 100 mg/kg AA (C-Q-), 100 mg/kg AA + quercetin 10 g/kg (C-Q+), 1000 mg/kg AA (C+Q-), 1000 mg/kg AA + quercetin 10 g/kg (C+Q+).
### Reduced ascorbic acid (µg/g)

<table>
<thead>
<tr>
<th>Diet</th>
<th>Prior UV</th>
<th>Control (24 h)</th>
<th>UV (24 h)</th>
<th>Control (7 d)</th>
<th>UV (7 d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-Q-</td>
<td>3.60±2.12&lt;sup&gt;a1&lt;/sup&gt;</td>
<td>0.07±0.17&lt;sup&gt;b1&lt;/sup&gt;</td>
<td>0±0&lt;sup&gt;b1&lt;/sup&gt;</td>
<td>0.32±0.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.60±0.97&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C-Q+</td>
<td>4.42±1.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.86±1.27&lt;sup&gt;b1&lt;/sup&gt;</td>
<td>0.06±0.14&lt;sup&gt;b1&lt;/sup&gt;</td>
<td>0.18±0.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.10±0.25&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C+Q-</td>
<td>1.44±0.78&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.62±1.38</td>
<td>1.62±1.36</td>
<td>0±0</td>
<td>1.15±1.23</td>
</tr>
<tr>
<td>C+Q+</td>
<td>4.22±2.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.21±2.29&lt;sup&gt;a2&lt;/sup&gt;</td>
<td>3.09±6.36&lt;sup&gt;a2&lt;/sup&gt;</td>
<td>0.23±0.55&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.32±0.79&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different letters indicate significant differences (P<0.05) per row, different numbers indicate significant differences (P<0.05) per column.

Table 5.5 Observed reduced ascorbic acid concentrations in skin tissue prior to and after 24h and 7 days of second UV acute exposure. Values are indicated as mean±SD (n=6). Diets: 100 mg/kg AA (C-Q-), 100 mg/kg AA + quercetin 10 g/kg (C-Q+), 1000 mg/kg AA (C+Q-), 1000 mg/kg AA + quercetin 10 g/kg (C+Q+).
Figure 5.3 Total ascorbic acid concentrations in skin tissue (µg g⁻¹ wet tissue) observed prior (A), 24 h control (B) and UV (C) and 7 days control (D) and UV (E) after 2nd UV irradiation acute exposure (0.9 J/cm²). Data are means ± SD (n=6) for total ascorbic acid concentrations (µg/g). Different letters indicate significant differences (P> 0.05) per sampling. Diets: 100 mg/kg AA (C-Q-), 100 mg/kg AA + quercetin 10 g/kg (C-Q+), 1000 mg/kg AA (C+Q-), 1000 mg/kg AA + quercetin 10 g/kg (C+Q+).
Figure 5.3
Figure 5.4 Chromatograms for quercetin (A) in skin tissue samples and (B) liver tissue samples determined with electrochemical detection. Top shows quercetin standard 200 ng/ml, bottom shows tissue chromatograms.
Figure 5.5 Quercetin concentrations in liver tissue observed 24h after 2\textsuperscript{nd} UV irradiation acute exposure (0.9 J/cm\textsuperscript{2}). Data are means ± SD (n=6). Means with the same letter are not significantly different (P > 0.05). Diets: 100 mg/kg AA + quercetin 10 g/kg (C-Q+), 1000 mg/kg AA + quercetin 10 g/kg (C+Q+).
Figure 5.6 Head skin in control (unexposed) fish (a) and lesions and in UV-exposed fish (b, c and d) observed by histological analysis display cell alteration that include hyperplasia, intracellular edema and necrosis (arrows). Hematoxilin-eosin stained 400–1000x. a) Control treatment. b) UV exposed –C-Q. c) UV exposed –C+Q. d) UV exposed +C-Q.
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