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Short communication

Effects of a saponin fraction extracted from *Trigonella foenum-graecum* L. and two commercially available saponins on sex ratio and gonad histology of Nile tilapa fry, *Oreochromis niloticus* (L.)

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Introduction

Over three million tonnes (t) of tilapia, mostly Nile tilapia (*Oreochromis niloticus*, L.), are produced annually making it the second most abundantly produced freshwater fish (FAO, 2010). Tilapia are mouthbreeders that often produce stunted populations under pond conditions; one means of prevention is to produce all-male fish with the additional advantage that males usually grow faster than females. All-male populations can be achieved by supplementing feed with androgens such as 17-α-Methyltestosterone (MT) during days 10–25 post-hatch (Pandian and Sheela, 1995). However, MT is considered to be carcinogenic (Velazquez and Alter, 2004), and Hulak et al. (2008) also showed that effluents of systems in which carp were fed diets containing MT caused masculinization of female fish. Furthermore, in aquaculture the application of hormones to fish destined for human consumption is prohibited in the European Union under directive 96/22/EC, article 5, which also prohibits import of animal products produced with hormones.

Kwon et al. (2000) showed that Fadrozole, a non-steroidal compound, caused masculinization in tilapia by inhibiting aromatase, which is the enzyme responsible for the conversion of endogenous androgens to estrogens. Steinbronn et al. (2004) were able to show that a dose of 2000 ppm *Quillaja* saponins (Sigma S-2149) inhibited reproduction of tilapia after dietary application for 32 days to first-feeding fry, suggesting saponins as a possible alternative to MT. These secondary plant compounds consist of either a steroid or triterpenoid basic structure (aglycone or sapogenin) plus one or more sugar side chains (Francis et al., 2002a).

In a previous experiment a saponin fraction from the soapbark tree (*Quillaja saponaria* M.) inhibited aromatase *in vitro* (Golan et al., 2008). The fenugreek plant (*Trigonella foenum-graecum* L), widely cultivated in the Middle East and Asia, also has a high saponin content. The experiment was therefore conducted to test whether saponin fractions from *Q. saponaria* and from *T. foenum-graecum* were able to influence the sex ratio and gonad histology of Nile tilapia.

Materials and methods

Saponins were extracted from fenugreek (*T. foenum-graecum* L.) according to Marston and Oleszek (2000). Ethanol extracts were fractionated using a reversed phase HPLC and different methanol/water solutions (v/v, 40/60, 60/40, 90/10) resulting in three saponin eluates or fractions (40, 60 and 90%). The 90% methanol fraction and two commercial saponins (*Quillaja* saponin, Sigma S4521 and Diosgenin, Sigma D1634) were added to the diets in concentrations shown in Table 1. The saponins were dissolved in ethanol (99.8%) and sprayed on a commercial tilapia diet (TilapiCo Crumble Excellent, 200–300 μm; Coppens Int., the Netherlands). The same amount of ethanol without saponins was sprayed on the control diet. Feed was dried in a drying oven and refrigerated at 6°C.

A total of 1080 Nile tilapia larvae each of 10 mg body mass (BM) were evenly distributed into 27 aquaria (40 fish per aquarium) each with 2.5 L capacity and connected to a flow-through system. The flow rate was adjusted to 4 L h^{-1} (weeks 1 and 2), increased to 5 L h^{-1} (week 3) and then to 6 L h^{-1} (week 4) resembling 160, 200 and 240% water exchange per hour in the respective weeks. The nine treatments (Table 1) were randomly assigned to three aquaria each.

Fish were fed *ad libitum* by an automatic feeder five times per day. Once per day, feed residues and feces were removed by siphoning. Temperature was kept constant at 26 ± 0.1°C and the light regime set to 12 h L/12 h D under fluorescent light with a surface intensity of 300 Lx. Experimental feed was provided for 4 weeks beginning on the 13th day post-fertilization (5 days post-hatch). At the end of the 4-week period the fish were transferred to 45 L aquaria in a recirculating aquaculture system kept at 26 ± 1°C. Water flow rate in each aquarium was set to 160 L h^{-1}, water was filtered by a mechanical filter, with approximately 30% bypassed through a trickling biofilter. Water quality was measured weekly and ammonia-N kept below 0.2 mg L^{-1}, nitrite-N below 0.05 mg L^{-1} and nitrate-N below 15 mg L^{-1}. Circa 8

Table 1
Experimental diets and nominal concentrations of saponins

Diet	Saponin type	Conc. ppm
Control	None	0
150TS90	90% *Trigonella* fraction	150
300TS90	90% *Trigonella* fraction	300
150QS	Commercial *Quillaja*	150
300QS	Commercial *Quillaja*	300
1000QS	Commercial *Quillaja*	1000
150DS	Commercial Diosgenin	150
300DS	Commercial Diosgenin	300
1000DS	Commercial Diosgenin	1000

–10% of the water was exchanged weekly. A commercial tilapia diet containing no saponins (TilapiCo Start Premium, Coppens Int., the Netherlands) was provided by automatic feeders five times a day *ad libitum*. Feed residues and feces were removed once per day. All deaths were recorded. In week 12, the fish were killed with a sharp blow to the head. The gonads were removed and either stored in 4% buffered formalin for histology or in 0.9% saline solution for immediate determination of functional sex. The sex was determined microscopically in all gonads of the surviving tilapia (n = 944) using the gonad squash method (Guerrero and Shelton, 1974). A total of 108 gonads, 12 per treatment, were randomly chosen for histological investigation. After initial sex determination, 18 gonad samples from fish in treatments where the variability between replicates and gonads of control fish was high were preferentially analyzed histologically according to Streble and Bäuerle (2007); the results showed no significant trends, thus no additional gonads were investigated histologically. Values for the percentage of males in each group were tested for significant differences by one-way ANOVA using SPSS 10.0. A total of 15 fish per treatment was randomly chosen; body mass (to the nearest 0.01 g) and total length (to the nearest mm) were then measured and tested by one-way ANOVA for differences.

Results

All diets were well accepted by all fish; although the fenugreek saponin-fed fish showed the highest mortalities of 16.7% (150TS90) and 17.1% (300TS90), there was no statistical difference among the treatments, including control. Overall mortality averaged 12.6 ± 6.2% (mean ± SD) and could not be attributed to any obvious cause. Statistically, no differences either in percentage of males, mortality or growth, were found among the treatments (Table 2). However, some treatments showed a high variability, with one replicate having elevated numbers of males and, in the same treatment, another replicate showing a high number of females, resulting in high standard deviations (150TS90, 300TS90, 300DS and 1000QS). The histological analysis did not reveal any distinctive anatomical changes or intersex states.

Discussion

The percentages of males obtained provided no evidence for masculinization effects in any of the applied treatments. In single replicates of different treatments, 69, 68 and 65% males were achieved (300TS90, 1000QS and 300QS, respectively). However, since there were also treatments in which single replicates yielded high percentages of females (72 and 63% in 150TS90 and 300DS, respectively), it seems unlikely that the elevated ratios of males and females in these replicates were caused by the applied saponins. Since in the respective treatments all conditions were equal, the high variability points toward randomly skewed sex ratios in the stocked larvae. The single replicates seem to show some effect of the applied saponins, but the pooled data per treatment shows that neither the types of saponins nor their concentrations influence sexual differentiation in Nile tilapia fry. These results are supported by the histological investigation, which did not show any abnormal development of the gonads.

Table 2
Percentage of males, number of sampled fish, mortality, body mass (BM) and total length (**TL**) in various treatments at end of experiment

Treatment	% males	N	Mortality %	BM (g)	TL (cm)
Control	53 ± 2.9	108	10.0 ± 5.0	8.86 ± 1.01	13.4 ± 4.2
150TS90	47 ± 17.1	100	16.7 ± 2.9	8.97 ± 0.93	13.3 ± 4.0
300TS90	56 ± 12.6	98	18.3 ± 12.3	9.37 ± 0.85	15.3 ± 3.9
150QS	52 ± 6.1	104	13.3 ± 2.9	9.43 ± 1.10	16.3 ± 5.3
300QS	57 ± 8.2	103	14.2 ± 3.8	9.12 ± 1.21	14.9 ± 6.5
1000QS	53 ± 12.8	105	12.5 ± 4.3	8.58 ± 1.03	12.6 ± 4.9
150DS	52 ± 8.0	106	11.7 ± 8.8	8.80 ± 0.97	13.5 ± 4.4
300DS	46 ± 13.8	110	8.3 ± 3.8	8.55 ± 0.90	12.0 ± 3.6
1000DS	47 ± 3.4	110	8.3 ± 6.3	8.93 ± 0.87	13.9 ± 3.7
Difference	n.s.	n.s.	n.s.	n.s.	n.s.

n.s., not significant.
Values = mean ± SD from three replicates per treatment.

In a previous experiment, Francis et al. (2002b) showed that feeding Nile tilapia fry with *Quillaja* saponin supplemented feed significantly changed the sex ratio in favor of males. Furthermore, it was reported by Francis et al. (2001) that *Quillaja* saponins administered orally with the diet also had a growth promoting effect in Nile tilapia; in their experiment, fish fed a concentration of 300 ppm had a significantly higher total weight gain compared to other treatments and control. Generally substances that exhibit an androgenic action also act anabolically, since both actions are mediated via the androgen receptor and cannot be separated from each other, although some steroids are more anabolic than androgenic and *vice versa* (Shahidi, 2001). If *Quillaja* saponins are acting as anabolic growth promoters they must also have a certain androgenic activity. However, masculinization may not necessarily be due to androgen application but can also be achieved by inhibition of the enzyme aromatase. In an *in vitro* experiment Golan et al. (2008) showed that a *Q. saponaria* saponin extract inhibited aromatase. In studies reporting successful masculinization after aromatase inhibition no improved growth accompanying the sex reversal was mentioned (e.g. Kwon et al., 2000; Afonso et al., 2001). It seems as if two different mechanisms were responsible for the effects described by Francis et al. (2002a,b) and Golan et al. (2008). The first possibly an androgenic-anabolic action, while the latter described the aromatase inhibition by saponins. Methyltestosterone is usually considered to be a potent androgen as well as an anabolic growth promoter (Lone and Matty, 1980; Shahidi, 2001). However, there is some evidence that MT might not act on sex differentiation through androgenic/anabolic action but through aromatase inhibition (Mor et al., 2001). Since in this study no influence of saponins on

the sex ratio or on length and body mass was observed, none of the two possible actions were found in the tested saponins in the applied concentrations. The saponins extracted from *T. foenum-graecum* and the commercial *Quillaja* saponins and Diosgenin are not potential replacements for MT to achieve sex inversion when applied with the feed in the tested concentrations; they also did not show a growth promoting effect. In order to elucidate the difference between the results *in vitro* by Golan et al. (2008) and this *in vivo* experiment, absorption of saponins in the fish intestine should be further investigated. However, as reported by Steinbronn et al. (2004), *Quillaja* application did prevent reproduction of Nile tilapia when applied at a high concentration (2000 ppm) but not at low concentrations (150 and 500 ppm). This does not necessarily require masculinization of females. Inhibiting the reproduction of mixed sex Nile tilapia would also largely increase the profitability of such a production system, Therefore another experiment will be conducted to investigate the ability of saponins derived from *T. foenum-graecum* to inhibit reproduction.

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References

Afonso, L. O. B.; Wassermann, G. J.; de Oliveira, R. T., 2001: Sex reversal in Nile tilapia (*Oreochromis niloticus*) using a nonsteroidal aromatase inhibitor. J. Exp. Zool. **290**, 177–181.

FAO, 2010: Fisheries Department, Fishery Information, Data and Statistics Unit. FISHSTAT Plus Version 2.3. 2000, Database Aquaculture Production 1950–2008. FAO, Rome.

Francis, G.; Makkar, H. P. S.; Becker, K., 2001: Effects of *Quillaja* saponins on growth, metabolism, egg production and muscle cholesterol of individually reared Nile tilapia (*Orecohromis niloticus*). Comp. Biochem. Phys. C **129**, 105–114.

Francis, G.; Kerem, Z.; Makkar, H. P. S.; Becker, K., 2002a: The biological action of saponins in animal systems: a review. Brit. J. Nutr. **88**, 587–605.

Francis, G.; Levavi-Sivan, B.; Avitan, A.; Becker, K., 2002b: Effects of long term feeding of *Quillaja* saponins on sex ratio, muscle and serum cholesterol and serum LH level in Nile tilapia (*Oreochromis niloticus* (L.)). Comp. Biochem. Phys. C **133**, 593–603.

Golan, M.; Avitan, A.; Qutob, M.; Dweik, H.; Abu-Lafi, S.; Focken, U.; Francis, G.; Becker, K.; Kerem, Z.; Levavi-Sivan, B., 2008: *Quillaja* saponins inhibit tilapia aromatase activity in vitro. Cybium **32**(Suppl.), 80–82.

Guerrero, R. D.; Shelton, W. L., 1974: An aceto-carmine squash technique for sexing juvenile fishes. Prog. Fish Cult. **36**, 56.

Hulak, M.; Paroulek, M.; Simek, P.; Kocour, M.; Gela, D.; Rodina, M.; Linhart, O., 2008: Water polluted by 17α-methyltestosterone provides successful male sex inversion of common carp (*Cyprinus carpio* L.) from gynogenetic offspring. J. Appl. Ichthyol. **24**, 707–710.

Kwon, J. Y.; Haghpanah, V.; Kogson-Hurtado, L. M.; McAndrew, B. J.; Penman, D. J., 2000: Masculinization of genetic female Nile tilapia (*Oreochromis niloticus*) by dietary administration of an aromatase inhibitor during sexual differentiation. J. Exp. Zool. **287**, 46–53.

Lone, K. P.; Matty, A. J., 1980: The effect of methyltestosterone on growth and body composition of common carp (*Cyprinus carpio* L.). Gen. Comp. Endocrinol. **40**, 409–424.

Marston, A.; Oleszek, W. (Eds), 2000: Saponins in food, feedstuffs and medicinal plants. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Mor, G.; Eliza, M.; Song, J.; Wiita, B.; Chen, S.; Naftolin, F., 2001: 17α-Methyl testosterone is a competitive inhibitor of aromatase activity in Jar choriocarcinoma cells and macrophage-like THP-1 cells in culture. J. Steroid Biochem. **79**, 239–246.

Pandian, T. J.; Sheela, S. G., 1995: Hormonal induction of sex reversal in fish. Aquaculture **138**, 1–22.

Shahidi, N. T., 2001: A review of the chemistry, biological action, and clinical applications of Anabolic-Androgenic steroids. Clin. Ther. **23**, 1355–1390.

Steinbronn, S.; Hussain, M. A.; Francis, G.; Focken, U.; Becker, K., 2004: Impact of dietary *Quillaja* saponins on growth, sex ratio and reproduction of Nile tilapia *Oreochromis niloticus* under field conditions in zrBangladesh, VII Asian Fisheries Forum, Penang, Malaysia (Book of Abstracts, Vol. I. p. 57).

Streble, H.; Bäuerle, A., 2007: Histologie der Tiere: ein Farbatlas. Spektrum, Heidelberg (in German).

Velazquez, I.; Alter, B. P., 2004: Androgens and liver tumors: Fanconi's anemia and non-Fanconi's conditions. Am. J. Hematol. **77**, 257–267.

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