TREATMENT WITH SAPONINS FROM TRIGONELLA FOENUM-GRAECUM AND QUILLAJA SAPONARIA INFLUENCES SEX RATIO IN NILE TILAPIA (OREOCHROMIS NILOTICUS) LARVAE

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Abstract

Nile tilapia production is one of the most rapidly increasing aquaculture industrial sectors worldwide. Mixed sex culture is not sustainable on a long term basis because of the high rate of reproduction, increasing feeding competition in ponds and low harvest of large individuals. The most common method to prevent unwanted reproduction and subsequent overcrowding is an all male culture which is mainly achieved by synthetic hormone treatment with 17-a-methyl-testosterone (MT). Since the application of synthetic hormones in animal feed is prohibited in many countries, including the whole of the EU, due to environmental and health concerns, endocrine active plant derived substances with a similar effect could serve as a substitute for MT.

In previous experiments saponin supplementation in fish diets has shown a potential to influence sex ratio and reproduction success.

During this experiment we tested the effects of steroidal saponins derived from fenugreek (Trigonella foenum-graecum, TS) and soap bark tree (Quillaja saponaria, QS) as feed supplement in mixed sex tilapia populations for their influence on sex ratio.

Saponins were extracted by the conventional Soxhlet method with hexane/ethanol and fractionated and isolated by consecutive methanol concentrations of 40, 60 and 80% (TS) and 80% (QS). Extracted saponins and commercially available Quillaja saponin (Sigma) were added to the diets of Nile tilapia larvae in two different concentrations, 150 and 1000 ppm, respectively, with Sigma-saponin in 1000 ppm only. Fish were fed with experimental diets and standard diet as control for four weeks and afterwards raised on standard diets only.

Sex ratio was determined micro- and macroscopically after approximately 4 months.

Percentage of non-females ranged from 52% (40% TS, 150 ppm) to 73% (80% QS, 150 ppm) with two treatments (80% TS 150 ppm and 60% TS 150 ppm) being significantly different ($p < 0.05$) from the expected 50:50 ratio.

Although further work is needed to identify the most effective fraction, single saponin or mode of action of Trigonella saponins. However, it is obvious that plant derived saponins have a significant masculinization effect in tilapia larvae.

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INTRODUCTION

Worldwide Nile tilapia (*Oreochromis niloticus*) production increased during the years 2001 to 2006 from 1.113.737 metrical tones (mt) to 1.988.726 mt resembling a growth of 79% (FAO 2008), and thus making it one of the fastest growing fresh- and brackishwater fish aquaculture productions, respectively. One of the major drawbacks in commercial tilapia production is the precocious maturity and following uncontrolled reproduction, resulting in increasing competition for feed followed by stunted growth and low commercial value (Baroiller and Toguyeni 1996, Wassermann and Afonso 2003). Monosex production systems show several advantages over mixed sex production systems like the choice for the faster growing sex of the species to be produced, lower environmental impact through escapes, preventing energy diversions into gonad production, courtship behavior and unwanted reproduction, reducing aggressive interactions during courtship behavior and larger uniformity of size at harvest (Beardmore *et al.* 2001).

In small scale Nile tilapia farming systems in Africa, only monosex production systems were found to be financially sustainable on a long term basis with mixed sex culture being unprofitable and culture systems including predator control, being an intermediate solution (Kaliba *et al.* 2006, 2007).

To achieve single sex cultures, several methods have been developed. Hand sorting is among these methods and needs a high degree of experience; it is very labor intensive and even with a 90% rate of success there will still be considerable reproduction under pond conditions. Production with genetically male tilapia offers a good alternative but for small scale farmers in remote locations neither the broodstock nor fingerlings for stocking may be available to guarantee a continuous supply of fry throughout the year. Hormonal treatment is frequently used to sex reverse tilapia and achieve male monosex populations (Beardmore *et al.* 2001). Several hormones and hormone analogues are used to achieve this goal. Among the most frequently used synthetic hormones is 17-□-methytestosterone (MT) which works well in at least 23 species belonging to six families, namely Salmonidae (8 species), Cichlidae (5 species), Cyprinidae (5 species), Anabantidae (1 species), Poecilidae (3 species) and Cyprinodontidae (1 species) (Pandian and Sheela 1995). The critical period for hormone application, either through the diet or by immersion, is within 23-26 days after hatching of larvae since that resembles the time span when functional sex of Nile tilapia can be influenced (Nakamura and Nagahama 1985, 1989).

A serious drawback for tilapia farmers who intend to sell their products internationally is that administering hormones to food fish or import of hormone treated fish is, however, forbidden in several countries (among them the European

Union) for concerns regarding consumer health and safety of the aquatic environment (EU Directive 1996/22, modified by Directive 2003/74). Endocrine active plant derived substances, showing a similar effect on tilapia or other food fish as MT does, would be an alternative to synthetic hormones and would have a greater acceptance among consumers and will most likely possess a lower environmental risk. For that reason the interest in plant derived alternatives to natural or synthetic hormones is internationally increasing.

One candidate group of plant secondary compounds are saponins which are glycosides produced by many plant families (Fenwick et al. 1991) and in some marine invertebrates (Riguera 1997). They consist of an either triterpenoidal or steroidal aglycone (sapogenin) and a higly variable sugar moiety resulting in a great variety of saponins. In general, triterpenoidal saponins are predominant in cultivated crops while steroidal saponins occur mostly in wild plants used as herbs or for medicine (Fenwick *et al.* 1991).

In previous experiments a growth promoting effect of steroidal saponins derived from the South American soap bark tree Quillaja saponaria (Sigma, S2104) and administered through the diet to common carp (Cyprinus carpio) and Nile tilapia could be shown (Francis et al. 2001, 2002a). Furthermore a reduced egg production in tilapia was observed by Francis *et al.* (2001). A change in the anticipated sex ratio of 50:50 males: females with a significantly higher percentage of males was observed by Francis et al. (2002b) when Nile tilapia where fed diets supplemented with 700 ppm Quillaja saponins.

Immersion experiments conducted by Çek *et al.* in 2007 resulted in 87.2% male convict cichlids (*Cichlasoma nigrofasciatum*) after treatment with Tribulus terrestris extract once per week for two months. The bioactive compounds in T. terrestris are most likely steroidal saponins with Protodioscin being the most dominant one (Çek *et al.* 2007).

Despite saponins there are other bioactive plant compounds which show some potential for an application as masculinization agent in aquaculture. Supplementation of a fish meal diet with dried and ground roots from Red Kwao Kreua (Butea superba) resulted in a significantly increased percentage of males in the Ghana strain of O. niloticus. The active compounds in B. superba were found to be Daidzein and Genistein, belonging to the Isoflavones which are believed to act as phytoestrogens (Mengumphan *et al.* 2006).

In the work presented here we used saponins extracted from Quillaja saponaria and Trigonella foenum graecum (fenugreek) and commercially available Quillaja saponins (Sigma, S2104). The different saponin fractions and concentrations were fed to 7 days post hatch mixed sex Nile tilapia larvae to test for their effect on the sex ratio.

MATERIALS AND METHODS

Saponin extraction

Prior to extraction plant material was ground and dried for 72 h. Saponins were extracted by Soxhlet apparatus using hexane as solvent. Defatted material was dried at room temperature and further treated with 70% ethanol. Residue was centrifuged and filtered. Filtrate was purified by flash chromatography using consecutive methanol/water concentrations (v/v, 40/60, 60/40 and 80/20) resulting in three fractions (40, 60 and 80% eluted saponin) termed TS40, TS60 and TS80. Only the 80% fraction from Quillaja saponaria was used in the experiment, termed QS80.

Experimental set-up

At an age of 6 days after hatching 20 mixed sexes *O. niloticus* larvae were stocked in triplicates into containers with a volume of 2.5 L and connected to a flow through system. Water temperature was kept at 26 ± 1°C and flow rate was adjusted to 4 l h^{-1}. From week 7 the flow rate was raised to 5 l h^{-1} and from week 9 onwards the flow rate was increased to 6 l h^{-1}. At week 10 the larvae were transferred to 40 l aquaria in a recirculation system until they were sacrificed.

Treatments included nine different experimental and one standard diet serving as control (C). The test diets were produced from standard diet supplemented with 40, 60 or 80% eluted fenugreek (TS) and 80% eluted Quillaja (QS) saponins and were administered in two different concentrations, 150 ppm and 1000 ppm, respectively (see also Table 1). Saponins were solubilised in water and added to the diets under continuous stirring. Commercially available Quillaja saponins (Sigma, S2149) were only added to the feed in 1000 ppm. Feeding started one day after stocking and feed allowance was five times per day ad libitum. Pellet size was adjusted to the size of the fish. After four weeks all diets were changed to standard diet.

After 10 weeks, fish were killed and sex determined, either microscopically by the gonad squash method (Guerrero and Shelton 1974) or, if sex was clearly distinguishable after gonads were excised, macroscopically. Females were counted as females and males and those with undifferentiated sex were counted as non-females. Although intersex fish were observed, they were counted to the sex which made up the largest portion of the gonad.

Statistical Analysis

Data was analysed using STATISTICA Version 6. Data was tested by Students t-test against the expected 50% occurrence of females and for a difference in frequency of occurrence of non-females between experimental diets and control diet.

Table 1. Feed composition and saponin supplementation levels and sources

Treatment	Supplementation	Concentration
Control (C) 91.8% Dry Matter (DM) As percent of DM: 43.5% Crude Protein (CP) 10.9% Crude Lipid (CL) 13.0% Crude Ash (CA) 18.7 kJ g^{-1} Gross Energy	None	None
80TS150	80% eluted Trigonella foenum-graecum saponins (TS)	150 ppm
60TS150	60% TS	
40TS150	40% TS	
80QS150	80% eluted Quillaja saponaria saponins (QS)	
80TS1000	80% TS	1000 ppm
60TS1000	60% TS	
40TS1000	40% TS	
80QS1000	80% QS	
SQS1000	Sigma Quillaja saponin	

Results

All diets were accepted well by the fish and no diet related mortality was observed during the period of experimental feeding or thereafter. Due to water flow failure in one of the triplicates of 40TS150 all fish died.

Mortality ranged from 26.7 ± 11.8% (SD; n=3) in treatment SQS1000 to 55 ± 14.7% (SD; n=3) in treatment 80QS1000 but was not found to be correlated to treatments. In the control the observed mortality was 46.7 ± 20.1% (SD; n=3). All mortality was accounted to agonistic and cannibalistic behaviour.

In all treatments, including the control, a higher percentage of non-females than females were observed. The mean percentage of pooled treatments ranged from 51.9 ± 2.7% (SD; n=2) non-females in 40TS150 to 73.2 ± 22.2% (SD; n=3) non-females in 80QS150 with two treatments showing a significantly higher ratio of non-females to females than the expected 50:50 ratio. In the diet supplemented with 80TS150 70.0 ± 3.0% (SD; n=3) non-females (t-test against 50% expectancy, $p < 0.01$) and in treatment 60TS150 65.0 ± 3.0% (SD; n=3) males and undifferentiated fish (t-test against 50% expectancy, $p < 0.05$) occurred.

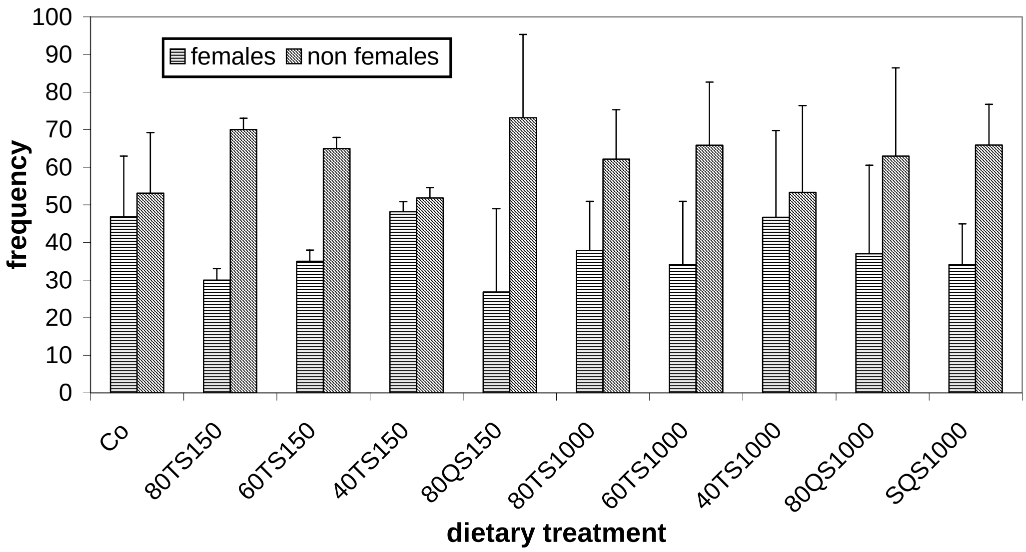

Figure 1. Frequency of occurrence of females and non-females after feeding with different experimental feeds. Co = control, 80TS150 = 80% eluate of Trigonella saponins with a concentration of 150 ppm

In single triplicates the highest ratios of males and undifferentiated fish to females was obtained in treatment 80QS150 with 88.2% followed by 80QS1000 (83.3%).

DISCUSSION

As was shown earlier in several experiments by Francis et al. (2001, 2002a, 2002b, 2002c), saponins derived from the soap bark tree Quillaja saponaria showed potential as growth enhancers in common carp and Nile tilapia. Furthermore they influenced the sex ratio resulting in a higher percentage of males in tilapia. Saponins extracted from T. terrestris, with Protodioscin as the most dominant saponin, also resulted in a higher percentage of male convict cichlids (*Cichlasoma nigrofasciatum*) (Çek et al. 2007) after immersion treatments.

In our experiment we showed that saponins extracted and eluted from T. foenum-graecum with 60 and 80% methanol and fed at 150 ppm through a fish meal based diet resulted in a significantly increased non-female to female ratio. The saponin fractions extracted from Q. saponaria with 80% methanol and administered at 150 ppm resulted not only in an elevated mean percentage of non-females but showed in one triplicate with 88.2% the highest percentage of males and undifferentiated fish of the whole experiment. However, due to the high standard deviation these results were statistically not significant.

One possible mode of action of steroidal saponins is through inhibition of the aromatase enzyme. The steroidogenic enzyme aromatase is the key enzyme in conversion of androgens (androstenedione and testosterone) to estrogens (estrone and estradiol) (Ryan 1959, Pasmanik and Callard 1988, Kwon et al. 2002). In an in vitro assay using Nile tilapia ovarian microsomes the 80% eluted saponin fraction derived from the soap bark tree (Q. saponaria) showed the highest potency in aromatase inhibition (Golan et al. in press).

Although the most active saponins, mode of action, concentrations for the different fish species, duration of treatments and method of application (dietary or immersion treatment) still have to be determined, saponins as a substance group show high potential as substitute for synthetic or natural hormones in sex inversion treatments.

As long as hormone treatments are prohibited in several countries, tilapia producers who intend to export to or produce in these countries will have to look for alternatives to natural or synthetic sex hormones. Although several studies are dealing with the effects of xenobiotics and other endocrine active substances on the aquatic environment and the ichthyofauna (e.g.: Sumpter 1995, Sumpter and Jobling 1995, Leaños-Castañeda *et al.* 2002, Jobling *et al.* 2002), up to now no studies were conducted to evaluate the impact of large scale application of synthetic hormones, especially 17-□-Methyltestosterone, in aquaculture production. A study to evaluate the impact of the synthetic sex hormone Ethynylestradiol (EE_2) on reproductive success and mechanisms of disruption showed that a short-term exposure of 40 days to EE_2 resulted in no effect on the treated mature fish. A life-long exposure to EE_2, however, resulted in a lowered fecundity and complete failure of reproduction with no fertilization in the F1 generation of the tested Zebrafish (*Danio rerio*) (Nash et al. 2004).

Detailed evaluations of the long-term impact of commercial scale treatments of already in use synthetic sex hormones and of possible alternatives like saponins on consumer’s health and the aquatic environment are needed. But even if it can be proven that there is no negative effect of synthetic hormones on humans and flora and fauna, the consumers, at least in the EU, will most likely prefer tilapia produced with the help of natural substances.

CONCLUSION

We could show that saponin fractions, eluted with 60 and 80% Methanol from Trigonella foenum-graecum and Quillaja saponaria and administered through the feed at 150 ppm, show the potential to serve as masculinizing agents. However more work is needed to further purify these saponin fractions or even elute single saponins which show the highest bioactivity. Furthermore the most effective concentration and mode of application for each fish species intended to sex inverse must be found. Before saponins can be used for large scale commercial sex inversion treatments they have to be proven harmless for man and environment. This work will continue and will include the evaluation of the fate of administered saponins in the fish flesh and in the water and will also include the effects of saponins on energy metabolism, protein turnover and on gene expression of hormones related to growth and reproduction in Nile tilapia.

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