Saponin fractions from fenugreek (*Trigonella foenum-graecum* L.) as dietary supplements for Nile tilapia (*Oreochromis niloticus* L.) and common carp (*Cyprinus carpio* L.)

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Dissertation

zur Erlangung des Grades eines Doktors

der Agrarwissenschaften

Fakultät Agrarwissenschaften

von

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aus Bremen

2012

Die vorliegende Arbeit wurde am 23.10.2012 von der Fakultät Agrarwissenschaften der Universität Hohenheim als "Dissertation zur Erlangung eines Doktors der Agrarwissenschaften" angenommen.

Tag der mündlichen Prüfung: 21.12.2012

1. Prodekan: Prof. Dr. M. Rodehutscord

Berichterstatter, 1. Prüfer: Prof. Dr. U. Focken

Mitberichterstatter, 2. Prüfer: Prof. Dr. M. Rodehutscord

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Table of content

Abbreviations

ALC	Apparant lipid conversion
ANOVA	Analysis of variance
AUE	Apparently unmetabolized energy
BM	body mass
BMG	body mass gain
DPF	days post fertilization
EE	energy expenditure / heat dissipation
ER	energy retention
FAO	Food and Agricultural Organization of the United Nations
FCR	feed conversion ratio
GH	Growth hormone
IGF-1	Insuline like growth factor-1
MT	17-α-methyletestosterone
MBM	metabolic body mass
MGR	metabolic growth rate
O_2C	oxygen consumption
PER	protein efficieny ratio
PPM	parts per million
PPV	protein productive value
QS	*Quillaja saponaria* saponins
SD	Standard deviation
SEM	Standard error of mean
SGR	specific growth rate
TS	*Trigonella foenum-graecum* saponins

Chapter 1

General introduction

The importance of Aquaculture for world food production

In 2010 the Food and Agricultural Organization of the United Nations (FAO) reported in their bi-annual report "State of World Fisheries and Aquaculture" (SOFIA 2010) that in 2008, 115 million of the 142 million tons of fish produced by capture fisheries and aquaculture were used for human consumption. Of these around 46 per cent (52.5 million tons) were produced by aquaculture alone. By far the larger share of that production was grown in freshwater (32.9 million tons) while marine aquaculture contributed 19.7 million tons. A closer look into the species composition production in 2008 reveals that cyprinids and, with a large gap in between, tilapias are quantitatively the dominant groups of fish (carps: 20.7 million tons, tilapias: 2.8 million tons, FishStat 2011) and are therefore crucial to the worldwide supply of animal protein. This is also reflected in the fact that fish accounted for 15.7% of the global population's animal protein intake in 2007 or around 17kg of fish per person.

Different regions and countries show different preferences for fish species and commodities. Especially in China and India but also other parts of Asia like Vietnam for example, people favor carp species over other whitefish like tilapia or pangasius catfish (*Pangasius* spp.). A high percentage of the Asian production is consumed locally or traded. China has turned by now into a net importer of fish and sea food although it is still the biggest producer in the aquaculture sector in absolute terms. In contrast, industrialized countries, mainly countries in Europe and the United States of America (USA) prefer marine fish, often already processed into fresh or frozen fillets.

Although carps account for by far the highest proportion of production, tilapias, especially Nile tilapia, *Oreochromis niloticus* (L.), have, in recent years shown a higher annual increase in production compared to most cyprinid species (Bostock *et al*. 2010). Overall, aquaculture is the fastest growing food producing sector with an average annual growth rate of 8.3 per cent between 1970 and 2008 (SOFIA 2010). However, this rate is appearing to slow down. Bostock *et al*. (2010) reported the annual average growth rate between 2004 and 2008 to be only 5.8 per cent. Nevertheless it is often proposed that aquaculture production is destined to make up for decreasing capture fisheries production (Deutsch *et al*. 2007).

Common carp, *Cyprinus carpio* (L.) and Nile tilapia, *Oreochromis niloticus* (L.), two important species for global freshwater aquaculture

As mentioned before, carps and tilapias are the two most important freshwater finfish taxa in aquaculture production. Of these, common carp and Nile tilapia are among the four quantitatively most produced fish species, with a total global production in 2009 of 3.216.203 and 2.542.960 tons, respectively (FishStat 2011). For 2011, Fitzsimmons *et al*. (2011) reported production rates for Nile tilapia above 3 million tons per year. It is thus widely accepted that freshwater aquaculture will increase in importance as will marine aquaculture.

Many species are under consideration as new candidates for aquaculture purposes but in all cases, no matter whether they are considered for production, for revenue, for national or international trade or merely for small scale urban aquaculture they all face the same problems. Many species are under consideration as new candidates for aquacultural purposes: for production, for revenue, for national or international trade or merely for small scale urban aquaculture. However, to be successful, any novel approach must be based on reliable

information on topics such as the reproduction cycles of the fish, artificial fertilization, larval nutrition, stocking densities, nutritional requirements, feeding levels, optimum water quality parameters and factors influencing meat yield and quality.
In the case of carp and tilapia which, historically, are probably the fish that have been cultured for the longest time (tilapia in ancient Egypt and carp in ancient China), this knowledge base has already been established and therefore it is likely that the production of these species will continue to increase in the future.
China dominates the worldwide production of common carp with a harvest of more than 2.4 million tons in 2009, followed by Indonesia (0.25 million tons) and Vietnam (0.11 million tons). Chinese production of Nile tilapia is smaller (1.26 million tons) but still outstrips that of other nations: Egypt (0.39 million tons), Indonesia (0.32 million tons), Thailand (0.21 million tons) and the Philippines (0.19 million tons) (FAO 2011).
Both carp and tilapia species are considered to be herbivorous or omnivorous (Bostock *et al.* 2010) with relatively little need for the inclusion of high levels of fishmeal in their diets. On average only 5% fishmeal is included in diets for carp and 6% for diets of tilapia (Deutsch *et al*. 2007). But in practice, the amount of fish meal that needs to be included in the diet depends on the production system. The more intensive the system, the greater the amount of fish meal included in the diet and the higher the quality of the feed. In highly intensive systems, it is of utmost importance to feed diets which fulfill all the nutritional requirements of the fish since amounts of nutrients that are normally available in natural food decrease with intensification level.
In Asia the common carp is mainly produced extensively or semi-intensively, often in polyculture with other cyprinids or in integrated agriculture-aquaculture systems (IAA) (Prein 2002). In these systems little compound feed is added if any.
Nile tilapias however, are found in all kinds of production systems from extensive polyculture in Asia or Africa to highly intensive recirculation systems in Israel or the USA. In highly intensive recirculation systems it is imperative to apply formulated diets fulfilling all the needs of the fish. As a result, feed frequently accounts for around 50% of the running cost of an aquaculture enterprise (NRC 2011).

There are several problems commonly associated with tilapia production. Probably the biggest drawback in extensive and semi-intensive tilapia production systems is the large number of offspring that are produced in mixed sex populations under pond conditions (Kaliba *et al*. 2006). This phenomenon originates in their precocious reproductive behavior. Both males and females can reach sexual maturity at only 10-15 g of body mass (own observations). Furthermore the mouth-breeding behavior of Nile tilapia females guarantees a high survival rate of the larvae and fry.
A high frequency of spawning, compared to other fish species, makes up for the relatively small batch sizes per spawn which mainly depend on size, age and nutritional status of the female and average around 600-1800 eggs per spawn per female. As a consequence only male monosex production of Nile tilapia is considered financially viable since in pond production systems, even a percentage as low as 2.5% of females can lower the average final body mass and therefore market value of the originally stocked males significantly even though the overall production may be slightly higher (Lovshin *et al*. 1990). In a study conducted in Tanzania it was reported that the mixed-sex production practiced there was not economically

viable without including predator control or switching to all-male culture (Kaliba *et al*. 2006). Another study came to a similar conclusion about Nile tilapia production in Kenya. It pointed out that mixed sex culture is economically unsustainable, male monosex culture is the system of choice with the highest financial returns, while predator controlled mixed sex culture is an intermediate solution (Kaliba *et al*. 2007).
The advantages of monosex fish cultures over mixed sex cultures were summarized by Beardmore *et al*. (2001) and are shown in Table 1.

Table 1: Potential benefits of monosex fish in aquaculture (after Beardmore *et al*. 2001), M = male, F = female

Potential benefit	Application to M/F monosex culture
Higher growth rate	M & F, depending on species
Preventing large energy diversion into:	
Gonad production	M
Courtship behavior	M
Production of uneconomic recruits	M & F
Reducing aggressive interactions	F
Greater uniformity of size at harvest	M & F
Avoiding undesirable impacts of sexual maturation on appearance and flesh quality	M & f
Reducing unwanted environmental impact through escapes	M

Several possibilities exist to control either the unwanted reproduction in mixed sex cultures or to directly produce male monosex populations of Nile tilapia.

Reproduction control and all male production

To control unwanted reproduction the above mentioned predator control system may be used. This is a polyculture in which the main crop of Nile tilapia is raised with a small number of piscivorous fish, e.g. African catfish, *Clarias gariepinus*, or largemouth bass, *Micropterus salmoides* (McGinty 1985).
Another system is one in which a mixed sex population is cultured in hapas or cages so that spawned eggs that the females are unable to pick up because they fall through the mesh of the hapa and die on the bottom of the pond.
Other systems involve attempts to control or alter the sex of the fish. The phenotypic sex in Nile tilapia is controlled by major and minor genetic factors and by temperature (Wessels & Hörstgen-Schwarck 2011). In *O. niloticus*, males have heterogametes (XY) and females are homogametic (XX) (Jalabert *et al*. 1971, cited in Wohlfarth & Wedekind 1992, Müller-Belecke & Hörstgen-Schwarck 1995) as opposed to i.e. *O. macrochir* where the females show heterogamety while males show homogamety (Jalabert *et al*. 1971, cited in Wohlfarth & Wedekind 1992).
At around 23-26 days after hatching the gonads are differentiated into ovaries or testes (Nakamura and Nagahama 1985, 1989), so 0-26 days after hatching is the time window for attempting to actively influence the phenotypical or functional sex of the fish.

Hand-sorting males and females is often mentioned as one potential method to establish male monosex populations. The sex can be distinguished by skilled workers at relatively early ages of the fish by close inspection of the genital papilla. However, the probability of error is relatively high (around 10%) depending on the skill of the worker and it is extremely time and labor intensive (Mair *et al*. 1997, Beardmore *et al*. 2001).

YY-supermale

The possibility of influencing the functional sex of Nile tilapias by the administration of androgens (masculinization) and estrogens (feminization) has lead to the development of the so called supermales or YY-male technology in which fish possess two Y-chromosomes as sex chromosomes. Their offspring are genetically all male (XY) and the sex ratios are usually more than 95% phenotypically male. The technique is described in detail by Mair *et al*. (1997).

Exceptions do exist where the offspring of YY-males are not 100% phenotypically male (on average > 95%, Devlin & Nagahama 2002) because of the presence of other genetic and temperature related factors (Tariq Ezaz *et al*. 2004, Wessels & Hörstgen-Schwarck 2011).

Temperature

It has been shown that *O. niloticu*s populations can produce higher percentages of males if 10 day post fertilization (dpf) fry are exposed to high temperatures (36°C). Tessema *et al*. (2006) temperature treated offspring of a Lake Manzala strain (Egypt) and found that in two thirds of the samples more than 80% of the fish were male. In contrast, the percentage of males in samples of temperature treated progeny from Lake Rudolph (Kenya) was on average around 61% with no male percentage above 80%. Although this type of research is imperative for an understanding of the functional mechanisms of sex determination in Nile tilapia it is, at the current state, hardly an applicable method for creating all male populations for aquaculture production.

Hybrid tilapia

Large numbers of all male progeny can reliably be produced by hybridization between two tilapine species. All-male offspring are for example produced by crossing *O. mossambicus* X *O. urolepis hornorum* or *O. niloticus* X *O. urolepis hornorum* while from 50 to 100% males are found after mating *O. niloticus* X *O. aureus* (Wohlfarth & Wedekind 1992). Despite the success in producing all male progeny by specific, interspecific or intergeneric hybridizations, several drawbacks were reported. Among them is the “constant vigilance” required to keep parental stocks pure, strain dependent low fecundity (and hence low fry production) and reduced acceptance by consumers because of unusual coloration and general appearance (Wohlfarth 1994).

Application of androgens

The most widely applied method for producing male monosex Nile tilapia populations is through dietary hormone administration (Popma & Green 1990). Although the most important androgen is a synthetic testosterone derivative, 17-α-methyltestosterone (MT), several other natural and synthetic androgens have been claimed to yield equal results (Pandian & Sheela 1995). As described above, the time of application is from first feeding until the 26th day after

hatching since by that time the gonads are fully developed. The effective concentration necessary to achieve a 100% masculinization is in the range of 40-60 mg MT kg^{-1} diet, depending on the mode of application (Pandian & Sheela 1995). However, evidence exists that MT is not only a potential threat to the environment but also to people coming into direct contact with it such as untrained personnel who mix or prepare the feed. Instead of being included in the feed, hormones may also be dissolved in a solvent and added to the water for treatment by immersion. However, it has been proved more practical if androgens are included in the diet.

Potential negative impacts of large scale 17-α-methyltestosterone utilization in aquaculture

The potentially detrimental impacts of the large scale utilization of MT in tilapia production systems have not been satisfactorily examined in the past. The major concerns which have been raised include effects on farm workers (carcinogenic, Velazquez & Alter 2007) and on consumer health and the impacts of MT enriched effluents on natural water bodies downstream from sex reversion facilities. Evidence for the androgenic potential of effluent water from MT fed fish ponds was provided by Hulak *et al*. (2008). They ran water from aquaria stocked with gynogenetic female common carp (*C. carpio*) fed with a diet containing 100 mg MT kg^{-1} through aquaria containing carp that had not been treated with MT and found that a high percentage of the fish in the second set of aquaria became masculinized. Earlier experiments in which MT was added to the water in aquaria containing common and ornamental common carp (koi) showed that between 46.7 and 96.6% of all female fish were sex inversed (Gomelsky *et al*. 1994). Further strong evidence was provided by Abucay and Mair (1997). They found that Nile tilapia controls kept in the same respective 900 L tanks as treatment groups for masculinization (40 mg kg^{-1} feed MT) and feminization (1000 mg kg^{-1} feed diethylstilboestrol) were also strongly impacted by the hormones.

Saponins

The name saponin is derived from the Latin word *sapo* which means soap because, like soap, saponins can form stable foams in combination with water. Saponins are glycosides consisting of a non-polar aglycone, called sapogenin, and one or more sugar side chains of various lengths. The aglycone is usually either steroidal or triterpenoidal in nature and both types are derived from a 30 carbon skeleton (Haralampidis *et al*. 2002, Vincken *et al*. 2007). Saponins are mainly produced by a great variety of plants from the two major plant classes Magnoliopsida and Liliopsida. The orders with the most numerous saponin-containing plant species are the Liliales (46 species), Fabales (42 species) and Apiales (26 species) (Vincken *et al*. 2007).

Compared to saponins from plants, relatively little is known about saponins produced by marine invertebrates like echinoderms (mainly starfish and sea cucumbers, Rio *et al*. 1965, Kitagawa 1988, D'Auria *et al*. 1992, Ebada *et al*. 2010), or sponges (Ebada *et al*. 2010).

Biological activities reported for various saponins isolated from marine organisms included antitumor-, antileukemic-, antifungal-, antibacterial-, cytotoxic-, piscicidal-, spermatostatic- and immunosuppressive activities, thrombin receptor antagonistic effects and inhibition of

platelet aggregation as reported by Ebada *et al*. (2010). A similar diversity of effects of plant saponins on animals is reported by Francis *et al*. (2002a) and it is likely that the range of potential biological activities is as diverse as the chemical structures of the compounds themselves.

Usually saponins are considered to act as a defense against microbial and predatory attacks on plants (Francis *et al*. 2002a). The high toxicity of saponins extracted from starfish indicates a similar function in echinoderms. However, Rio *et al*. (1965) found that the toxicity to fish was higher when saponins were present in the water than when they were injected intraperitoneally which implied that the toxins (saponins) were actively absorbed by gill membranes.

Sparg *et al*. (2004) consider saponins to be extremely toxic for poikilothermic animals while oral toxicity for mammals is thought to be low. Saponins in animal nutrition are generally considered to be anti-nutrients (Bureau *et al*. 1998, Francis *et al*. 2001a), reducing growth, lowering feed intake and damaging the intestinal mucosa. When dissolved in water, saponins can damage the gill epithelia of fish and are considered to be the active components in some traditionally used fish toxins (Francis *et al*. 2001a). Apart from the studies by Francis *et al*. (2001b, 2002b, c, d) only a few experiments have been published in which saponins have been added in low concentrations to fish diets

Previous studies using saponins as feed additives for fish

Up to now only a few studies have been conducted in which saponins, sapogenins or closely related plant derived substances have been used as feed additives to test for beneficial effects in freshwater finfish and to our knowledge none has been conducted in marine finfish or crustaceans. Around a decade ago Francis and colleagues conducted a series of experiments (Francis *et al*. 2001b, 2002b, c, d) using mainly commercially available saponin mixtures derived from *Quillaja saponaria* M. containing around 10% sapogenin (Sigma S 2149) as feed supplements for common carp and Nile tilapia. The inclusion levels ranged between 150 and 700 mg kg^{-1} diet and the diets were fed over one period or alternating periods of saponin supplementation and non saponin supplementation. In all experiments one or more beneficial effects in the saponin diet fed groups compared to control animals were observed.

Francis *et al*. (2001b) showed that 300 mg kg^{-1} *Quillaja saponaria* saponin in the diet of Nile tilapia increased the body mass, apparent lipid conversion, body lipid content, gross energy content, muscle cholesterol content and energy retention of the fish while the ash content and the unmetabolized energy were reduced compared to the control. One observation, which could not be proved, was that on average less females fed with saponins spawned than in hte control group.

Francis *et al*. (2002b) showed that Nile tilapia fed for six months with 150, 300, 500 and 700 mg kg^{-1} *Quillaja* saponin had higher body masses and increased specific growth rates (SGR) compared to control while a similar effect over two months was only observed in fish fed 150 and 300 mg kg^{-1} saponins. A completely different species, the common carp *Cyprinus carpio* L., belonging to the cyprinidae which do not have a true stomach (a common attribute for all carp-like fish) showed beneficial effects of a *Quillaja* saponin supplementation at lower concentrations than tilapia. An addition of 150 mg saponins kg^{-1} diet led to a higher final body mass compared to control fed fish and nutrient utilization parameters where improved

compared to the control group and the group fed 300 mg saponins kg^{-1} diet. Another striking result was that the metabolic rate and oxygen consumption per unit body mass gain of carp fed the low saponin concentration in the diet was lower than that of the control fish while the 300 mg kg^{-1} group had the highest metabolic rates and oxygen consumptions per unit body mass gain (Francis *et al*. 2002c). A different experimental design but with the same concentrations and fish species also resulted in the highest body mass gain and best nutrient utilization for carp fed continuously with 150 mg *Quillaja* saponins kg^{-1} diet compared to control. Carp fed week-wise alternately with supplemented and un-supplemented feed showed a response lying between the control and 150 ppm groups (Francis *et al*. 2002d). To this author's knowledge no studies, apart from those of Francis *et al*. (2001b, 2002b, c, d), have been published showing a beneficial influence of saponins on growth, metabolism and nutrient utilization.

Since the beginning of 2006 hormones and anti-biotic additives in sub-therapeutic levels have been prohibited in the EU (EC 1831/2003). Not only is the use of these compounds banned in the formulation of feeds within the E.U., but the importation into the EU of products from animals raised on anti-biotic or hormone supplemented feeds is also prohibited.

Although some studies have been made on the effects of saponins from *Q. saponaria* on fish, only a few saponin rich plants have been investigated for their potential beneficial effects in aquaculture. In one study, 9 g of extracts from *Tribulus terrestris*, a plant rich in steroidal saponins (Dinchev *et al*. 2008), were dissolved in 30 L of water and used to masculinize African catfish *Clarias gariepinus* by immersion (Turan & Cek 2007) resulting in 80% males compared to 55% males in the control group. Extracts from the same plant have also been successfully applied to masculinize convict cichlids *Cichlasoma nigrofasciatum* (Çek *et al*. 2007). Feeding freshly hatched Nile tilapia fry for 32 days with a high dose (2000 mg kg^{-1}) of *Quillaja saponaria* saponins inhibited reproduction in that group (Steinbronn *et al*. 2004).

Hypothesis and objectives of this thesis

Trigonella foenum-graecum, fenugreek, is a widespread plant cultured in many parts of Mediterranean Europe, Africa and Asia (Petropoulos 2002) and it is rich in steroidal saponins (Marker *et al*. 1947, Petropoulos 2002). During this study saponins derived from *T. foenum-graecum* and *Q. saponaria* and fractionated by HPLC were tested, along with other commercially available saponins and sapogenins, for their effects on sex ratio, gonad histology, growth performance, nutrient utilization and metabolic performance primarily of Nile tilapia but also of common carp.

It is postulated that the beneficial effects in the fish are, at least in one or more fractions, more pronounced because of the fractionation than the effects observed by Francis *et al*. (2001b, 2002b, c, d) and Steinbronn *et al*. (2004) with non-fractionated material.

This thesis consists of nine chapters, beginning with a general introduction (chapter 1), followed by chapters 2 to 5 which report experiments where saponins were tested for their potential use for sex inversion and inhibition of reproduction in Nile tilapia. Chapters 5 to 8 are dedicated to the evaluation of saponin fractions as growth promoters in Nile tilapia and common carp. Chapter 9 concludes the thesis with a general discussion and an outlook on necessary follow up studies for future research.

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Chapter 2

Treatment with saponins from *Trigonella foenum-graecum* and *Quillaja saponaria* influences sex ratio in Nile tilapia, *Oreochromis niloticus* L., larvae

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Proceedings of the 8th International Symposium on Tilapia in Aquaculture (I), pp 355-364, Cairo 2008, Egypt. Eds: Elghobashy H., Fitzsimmons K. and Diab A.

ISBN: 978-1-88807-18-9

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.

Introduction

Worldwide Nile tilapia (*Oreochromis niloticus*) production increased during the years 2001 to 2006 from 1.113.737 metrical tonnes (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-α-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.

Material and Methods

Saponin extraction

Prior to extraction plant material was ground and dried for 72 h. 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 sex *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 either 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 was 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 occurence 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-α-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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Chapter 3

Effects of saponin fractions 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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Journal of Applied Ichthyology, doi: 10.1111/jai. 12002

Journal of Applied Ichthyology

J. Appl. Ichthyol. (2012), 1–3
© 2012 Blackwell Verlag, Berlin
ISSN 0175–8659

Received: February 20, 2012
Accepted: June 10, 2012
doi: 10.1111/jai.12002

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.

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.

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.

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.

Acknowledgements

This work was partly funded by the DFG (German Research Foundation) under the project number BE 1133/19-2. The authors want to gratefully thank Dr. Heinz Streble for his guidance in the preparation and analyzation of thin sections for histology. Furthermore, we wish to thank Dr. Peter Lawrence and Mrs. Sabine Nugent for critically reading the manuscript.

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Chapter 4

Effects of fenugreek saponin fractions, a sapogenin, methyltestosterone and fadrozole on sex ratio and mortality of genetically female Nile tilapia, *Oreochromis niloticus* (L.) fry and occurrence of feed related jaw deformities

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Abstract

Different saponin fractions derived from fenugreek *Trigonella foenum-graecum* L., the commercially available sapogenin diosgenin, the non-steroidal aromatase inhibitor fadrozole and the synthetic androgen 17-α-methyltestosterone (MT) have been fed to first feeding genetically female Nile tilapia, *Oreochromis niloticus* (L.), fry. The experiment aimed to test the influence of the saponin fractions and the diosgenin on sex ratio and gene expression of male sex related genes during the experimental feeding period of four weeks and afterwards on the effect of the test substances on reproduction and fecundity of the females. However, high frequencies of occurrence (between 6 and 33%) of bone deformities, primarily in the jaw, prevented the experiment from being finished in the anticipated way. Here we describe the effects of the feed additives on sex ratio, mortality, reproduction and possible reasons for the jaw deformities.

Introduction

Large scale, commercially viable Nile tilapia, *Oreochromis niloticus* (L.), production depends on male monosex culture (Kaliba et al. 2006, 2007). Although the total fish yield per area is increased in the presence of small percentages of females, the average body mass and therefore the economic value is reduced due to precocious maturity and subsequent overcrowding and competition for food and feed (Lovshin et al. 1990). On a commercial scale, the most widely applied androgen, the synthetic 17-α-methlytestosterone (MT), is included in the diets of first feeding fry in concentrations of around 50 mg kg^{-1} (Pandian & Sheela 1995). In juvenile Nile tilapia, it takes around 23 to 26 days for the gonads to become differentiated into ovaries or testes (Nakamura & Nagahama 1985, 1989) and therefore the hormone is usually fed for four weeks after *O. niloticus* have hatched to produce all male populations.

A possible influence of saponins on the sex ratio of *O. niloticus* was reported by Francis et al. (2002a) where dietary inclusion of different concentrations (between 150 and 700 mg kg^{-1} diet) of *Quillaja saponaria* (Molina) saponins led to higher proportions of males. Another study reported no reproduction after supplementation with 2 g kg^{-1} diet of the same saponins to first feeding Nile tilapia fry (Steinbronn et al. 2004).

Saponins are secondary plant compounds occurring in many cultured and wild plants and consist of a steroidal or triterpenoid aglycone called sapogenin and one or more sugar side chains (Francis et al. 2002b). Besides plants, some lower marine invertebrates also produce saponins, probably as defense against predators (Rio et al. 1965; Ebada et al. 2010).

Up to 38 different saponins have been described to be present in *Q. saponaria* (Guo & Kenne 2000, Bankefors et al. 2008). Fractionation of these saponins led to the discovery of a fraction that caused inhibition of the enzyme aromatase *in vitro* that was stronger than that caused by the unfractionated material (Golan *et al*. 2008).

Aromatase is responsible for the aromatization of androgens (primarily androstenedione or testosterone) to estrogens (estrone and estradiol) (Banting & Ahmed 2009) and its inhibition *in vivo* was found to lead to high proportions of males or complete masculinization of Nile tilapia (Kwon et al. 2000; Afonso et al. 2001).

Another widely spread and cultivated plant rich in saponins is fenugreek *Trigonella foenum-graecum*. It is used as an herb and spice and also in traditional medicine in Mediterranean, central and East Asian regions. The saponins from fenugreek are predominantly steroidal saponins while *Q. saponaria* contains mainly triterpenoid saponins (Marker et al. 1947; Murakami et al. 2000; Bankefors *et al*. 2008). Fenugreek saponins showed high biological activity in aromatase inhibition assays *in vitro* (data not shown).
The intention of this study was to test two different saponin fractions with high biological activity *in vivo* and a commercially available sapogenin, diosgenin, for their effects on sex ratio, reproduction and gene expression on aromatase (CYP19A1) and an exclusively male related gene, tDMRT1 (*doublesex/mab-3* related transcription factor-1, Kobayashi *et al*. 2008). As positive controls, a validated androgen, 17-*a*-methyltestosterone (MT) and the non steroidal aromatase inhibitor fadrozole (FAD) were included in the study.

Material and methods

The saponins were extracted from *T. foenum-graecum* using the method of Marston and Oleszek (2000) with minor modifications. Ethanol extracts were fractionated using a reversed phase high-performance liquid chromatography (HPLC, CombiFlash RETRIEVE, Teledyne Isco, Lincoln, NE) and different consecutive methanol/water solutions (v/v, 40/60, 60/40, 90/10) resulting in three saponin eluates or fractions (40, 60 and 90%) of which the 40% eluate was discarded. The 60 and 90% eluates were chemically modified by autoclaving (140°C, 60 min.) to increase their biological activity.
The experimental diets were prepared at the Hebrew University of Jerusalem, Department of Animal Science at the Robert H. Smith Faculty of Agriculture in Rehovot.

Table 1: Proximate composition of the basal and grower diets used during this experiment (according to manufacturer). CP = crude protein, CL = crude lipids, CA = crude ash, CF = crude fiber, DE = digestible energy

	Israeli basal diet	Dutch basal diet	Dutch grower diet
CP (g kg^{-1})	480	450	440
CL (g kg^{-1})	55	100	110
CA (g kg^{-1})	84	86	95
CF (g kg^{-1})	22	13	15
DE (MJ kg^{-1})	16.5	17.7	17.7

As basal diet for the experiment a commercial Israeli tilapia larvae diet (Table 1) was used and the different additives (both saponin fractions, diosgenin, MT and FAD) dissolved in methanol and mixed with the diets which were afterwards freeze dried. The concentrations of the different additives are shown in Table 2.

Table 2: Concentration of the different additives in the Israeli experimental diets. (CMc3TS60 = chemically modified cluster 3 of the *Trigonella foenum-graecum* saponin 60% fraction, CMc3TS90 = chemically modified cluster 3 of the *T. foenum-graecum* saponin 90% fraction, MT = methyltestosterone, FAD = fadrozole)

Diet	Additive	Concentration (mg kg^{-1})
1	None	Control
2	CMc3TS60	2000
3	CMc3TS90	1600
4	Diosgenin	4000
5	MT	60
6	FAD	250

A total of 2000 genetically female *O. niloticus* larvae from the Hebrew University of Jerusalem, Department of Animal Science at the Robert H. Smith Faculty of Agriculture in Rehovot were divided into two groups, one experimental and one non-experimental. The experimental group (around 820 animals) was divided again into two groups, 720 fish which were stocked at a density of 40 fish per aquarium into 18 aquaria (2.5 L) of a flow-through system (FTS) and around 100 fish which were stocked into one extra aquarium of the same system. The fish of the non-experimental (around 1200) group were kept in an aquarium (45 L) of a recirculation aquaculture system (RAS). The water flow rate in the FTS was set for the first two weeks to 4 L h^{-1}, for weeks three and four to 5 L h^{-1} and afterwards to 6 L h^{-1}. The water flow rate in the RAS was between 170-200 L h^{-1}. In both systems the temperature was kept at 27 ± 1°C. The water quality was in the optimum range for Nile tilapia and the light regime was set to 12/12 light/dark. All fish were fed using automatic feeders (Rondomatic 400, Grässlin, St. Georgen, Germany) that provided feed six times a day. The capacity of the automatic feeders for the approximately 1200 fish in the RAS was insufficient to provide enough feed and therefore the fish were additionally fed by hand to apparent satiation once a day. The 18 x 40 fish in the FTS were fed the Israeli experimental and control diets in three randomized replicates per treatment. The 100 fish in the FTS and those in the RAS were fed with a different commercial basal diet from the Netherlands (Table 1). The experimental diets were fed for four weeks, followed by the Dutch basal diet. After six weeks, all fish from the FTS were moved into aquaria in the RAS. During the six weeks in the FTS, mortality was recorded for all experimental treatments; however it was not recorded for the 100 'extra' Fish in the FTS or for the 1200 fish in the RAS but their condition was checked daily. Four weeks after the stocking of all experimental groups into the RAS the feed for all fish, both those on the experimental diets and those on the Dutch commercial diet was changed to a Dutch grow out diet (Table 1).

Sixteen weeks after the experiment started, the number of fish was reduced to 10 in each aquarium. One male tilapia was put into each aquarium to test for reproduction. In each case, the male chosen was slightly bigger than any of the fish already present. Offspring-incubating females were taken, eggs or larvae removed, the females weighed and eggs or larvae counted and incubated. All fish sampled before the reproduction trial were killed by a sharp blow on the head and sexed. Fish sampled after the reproduction trial or which were found dead were also weighed to the nearest 0.1 g and sexed if possible (i.e. if gonads were still present). Sex

determination was conducted either microscopically according to Guerrero and Shelton (1974) or, if possible, macroscopically. All sampled fish were checked for visible body deformities. In addition, a spot-sample of 20 fish from the 1200 fish that had throughout been kept in the RAS was sexed around 10 weeks after the experiment started.
During the experiment, an increasing number of deformities were observed, especially in the jaw and gill region of the fish. It was therefore decided not to conduct the gene expression studies since we could not be sure that the gene expression levels of the intended target genes were not influenced by the deformities. All diets (both basal diets and all experimental diets) were analyzed for their content of phosphorous (P), calcium (CA), zinc (Zn), L-ascorbic acid (Vit C), vitamin A (Vit A), vitamin D_3 (Vit D) and α-tocopherol acetate equivalents (Vit E) by the Landesanstalt für Landwirtschaftliche Chemie (LA Chemie), University of Hohenheim, Stuttgart, Germany.
Statistical analysis was done using SPSS vers. 10.0 for mortality and differences in percentage of deformities between the different treatments. The one-way ANOVA procedure was used with mortality or percentage of deformities as a variable and treatment as the between-subjects factor. The significance level for all tests was set to 5%. To test for homogeneity of variance, a Levene test was applied and ANOVA only conducted if the Levene test was insignificant. If the one-way ANOVA showed a significant difference between the treatments, a Tukey's HSD test was applied post-hoc to test for the level of difference and p-values between the different groups.

Results

The observed mortalities for all treatments were high at around 30% while amongst the fadrozole fed fish two out of three died during the experimental feeding phase (Table 3).
Deformities as shown in Figure 1 were first observed at an average body mass of around 5 g in all treatments but in different rates of occurrence (Table 3). Feeding Nile tilapia with MT supplemented feed led to the lowest amount of jaw malformations (around 6%) while the treatment with FAD led to the highest rate of occurrence (33%) (Table 3). No statistical connection between severity of the deformities and the different treatments was observed. The deformities included mainly deformities of the jaw while in few but not quantified cases twisted pectoral fins, humpbacks and malformations of the gill operculum were also observed. The deformities were not assessed in detail and no x-radiographs were taken. The deformities did not prevent the fish from feeding although in some severe cases (Fig. 1) the mouth gape was too small for larger pellet sizes.
Total masculinization was achieved by the methyltestosterone treatment while the fadrozole treatment resulted in high masculinization rates but with high standard deviation. Two of the three replicates had medium rates of masculinization (53 and 69% males, respectively) while

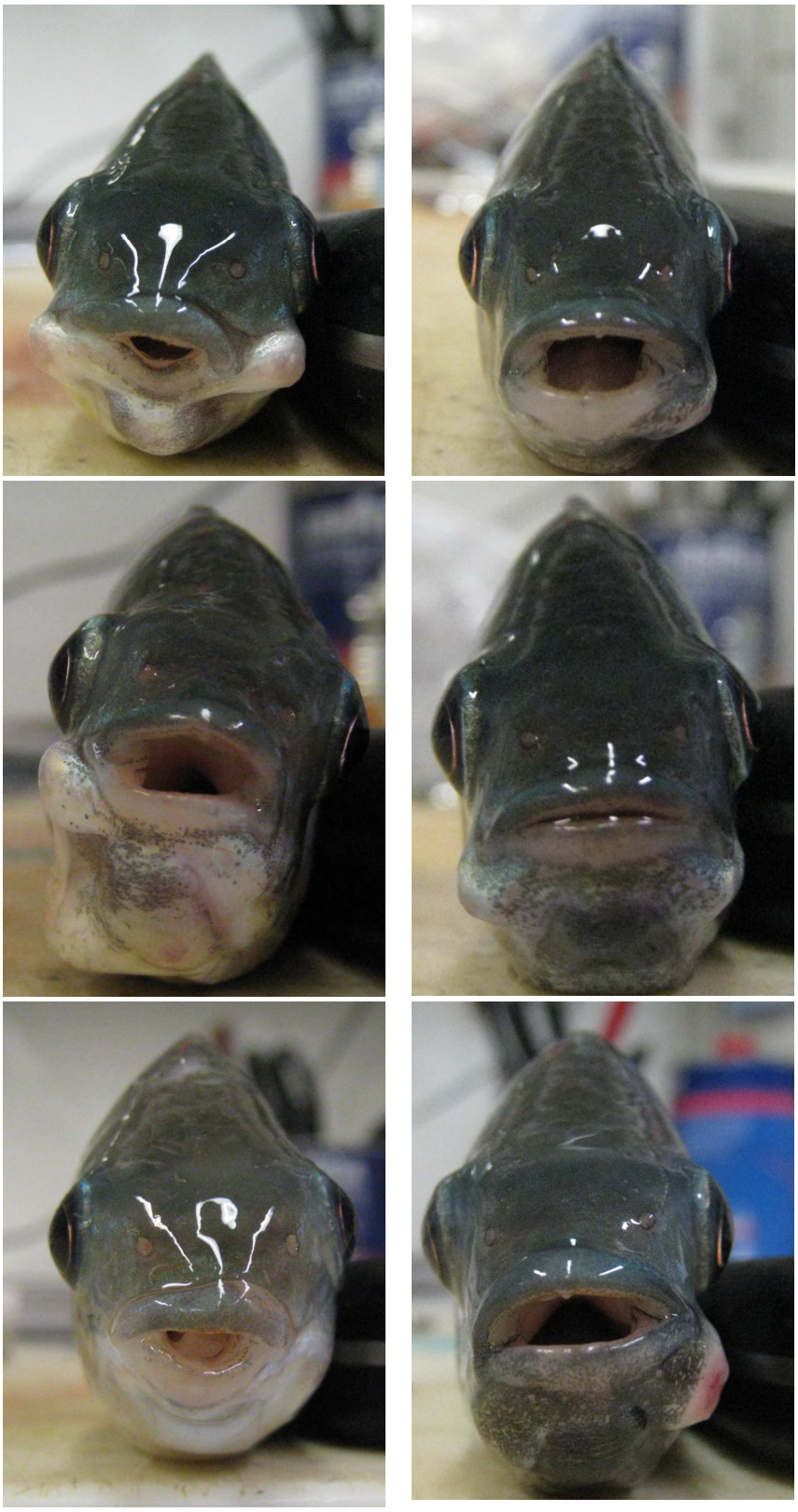

Figure 1: Examples of the observed jaw deformities with severe cases on the left side and less severe cases on the right side

in the third replicate a total masculinization (100%) was achieved. Percentages of males in both fenugreek saponin fraction groups and the diosgenin fed fish were below 5% while no male fish was found in the control group (Table 3).

Table 3: Observed mortality at the end of experimental feeding, occurrence of deformities and percentage of males for the different experimental groups. Values = mean ± SD, N = 3.
(CMc3TS60 = chemically modified cluster 3 of the *Trigonella foenum-graecum* saponin 60% fraction, CMc3TS90 = chemically modified cluster 3 of the *T. foenum-graecum* saponin 90% fraction, MT = methyltestosterone, FAD = fadrozole)

Treatment	Males (%)	Mortality (%)	N[†]	Deformities (%)	N[‡]
FTS DF[*]	0.00	Low[+]	~100	0.00	~100
RAS DF[#]	0.00	Low[+]	~1200	0.00	~1200
Control	0.00 ± 0.00	28.3 ± 5.20	106	19.9 ± 13.7	26
CMc3TS60	2.15 ± 3.72	36.7 ± 11.3	95	16.1 ± 7.2	25
CMc3TS90	3.57 ± 3.57	28.3 ± 3.82	101	9.37 ± 2.4	26
Diosgenin	1.19 ± 2.06	35.0 ± 13.9	104	11.9 ± 11.6	22
MT	100 ± 0.00	29.2 ± 3.82	108	6.13 ± 2.9	24
FAD	74.0 ± 23.8	64.2 ± 8.04	80	33.0 ± 11.5	24

[*] Flow-through system, fed entirely with Dutch feed, no SD given since no replicates available
[#] Recirculation aquaculture system, fed entirely with Dutch feed, no SD given since no replicates available
[+] Mortality not quantified but estimated to be not above 10%
[†] Sample size at the end of experimental feeding
[‡] Sample size after reduction to 10 fish per replicate

Neither the fish kept in the flow-through system and fed with non experimental feed nor the fish kept during the whole time in the recirculation aquaculture system showed any signs of increased mortality. They also did not develop any jaw or other body deformities comparable to those developed by the fish fed the experimental diets.

During the reproduction phase we observed increased aggression and aggression related mortality amongst fish fed initially with MT and FAD. We also checked daily in all other aquaria for signs of mating behavior between the stocked male and the fish of unknown sex. However, only three females spawned, two of them were incubating eggs and the third one was spotted when the eggs had already hatched. Two of those females were initially fed with CMc3TS60 and one with CMc3TS90. The average body mass (BM) for the fish fed with CMc3TS60 at spawning was 40.9 g. One female had jaw deformities while the other did not. The number of offspring from the un-deformed female was 289 eggs (7.2 eggs g^{-1} BM) and for the deformed female 71 larvae (1.7 g^{-1} BM). The body mass of the un-deformed female fed with CMc3TS90 was 55.1 g and 391 eggs were counted (7.1 g^{-1} BM) (Table 4).

Table 4: Observed reproduction data with body masses of the females, the counted eggs/larvae, the offspring per gram body mass, whether females showed deformity and the observed mortality in the offspring

	CMc3TS60	CMc3TS60	CMc3TS90
BM (g) of female	40.1	41.7	55.1
Eggs/larvae	289	71	391
Offspring / BM	7.2	1.7	7.1
Deformity	no	yes	no
Mortality offspring	low	low	total

The analysis for some macro- and micro minerals and vitamins associated with bone formation revealed only small differences between the two basal diets fed during the larval and fry phase on the one side and the diets and the published requirements on the other. Both diets were over-fortified in most minerals and vitamins compared to the published requirements. The only remarkable insufficiency in the Israeli diet was vitamin A compared to the published requirements. The Israeli diet was lower in zinc, vitamin C and vitamin E and higher in calcium and vitamin D compared to the Dutch diet (Table 5).

Table 5: Analysis of the two basal diets fed to the tilapia fry

	Israeli basal diet	Dutch basal diet	Requirement [a]
Phosphorous (%)	1.02	1.04	0.4
Calcium (%)	1.66	1.58	0.7
Zinc (mg kg^{-1})	154	235	20
Vit C (mg kg^{-1})	257	600	20
Vit A (mg kg^{-1})	1.2	8.1	1.8
Vit D (IU kg^{-1})	83	68	9
Vit E (mg kg^{-1})	180	200	60

[a] Requirements according to NRC 2011

Discussion

A replacement of MT by environmentally friendly “green” products to produce all male tilapia populations or to stop uncontrollable reproduction would be a desirable goal for the tilapia industry especially if such products could be administered in the form of a feed additive. Earlier publications by Francis *et al*. (2002a), Steinbronn *et al*. (2004) and Golan et al. (2008) pointed towards such effects in saponins and saponin fractions or eluates derived from *Quillaja saponaria*. Furthermore, unpublished *in vitro* results for *Trigonella foenum-graecum* saponin fractions pointed towards similar *in vitro* effects as observed by Golan *et al*. (2008). The results of this study do not support an application of the chemically modified fenugreek saponin eluates or diosgenin for masculinization in Nile tilapia. The commercially applied MT had the highest potential for sex inversion followed by fadrozole while in the plant derived saponin fractions and the sapogenin (diosgenin) only isolated examples of sex inversed fish were observed. The surprisingly low masculinization potential and high mortality rates during the experimental feeding phase for fadrozole can not be explained. Depending on treatment duration and fadrozole concentration, male sex ratios well above

80% have been reported in other studies (Kwon *et al*. 2000; Afonso *et al*. 2001; Higa et al. 2003)

The results obtained from the reproduction trial were too ambiguous to prove any effect of the saponin fractions or diosgenin on the reproductive ability of the experimental fish. No obvious reason was found why only three females spawned during the total eight months of the trial since the water quality, temperature and nutrient supply were in the optimum ranges for Nile tilapia. The limited space and the design of the aquaria (cubic with a standing pipe in the center and a negatively sloped bottom) might have had an impact.

Around 90% of the offspring of two of the spawning females survived while all eggs of the third female died. The reason for this could not be determined but it is likely that counting 391 eggs by hand took too long and the aeration was poor.

The three females who spawned were from groups whose diets included chemically modified saponins which implies that saponins do not completely inhibit reproduction. A replacement for methyltestosterone as a feed additive is desirable for large scale tilapia production due to its potentially negative impacts on human health (carcinogenic, Velazquez & Alter 2004) and the environment. To our knowledge no in-depth publication exists on the impacts of the effluents from farms or hatcheries using MT on a large scale on the aquatic environment downstream of the facilities. However, several publications report that MT can masculinize fish and invertebrates in aquaria adjacent to experimental systems or to ponds of outdoor systems (Gomelsky et al. 1994; Abucay & Mair 1997; Crane et al. 2006; Hulak et al. 2008).
Until it has been definitely proved that farm effluents do not influence downstream aquatic fauna it is best to assume that MT polluted waters do have the ability to alter sex ratios.

The observed deformities in fish fed during the experimental feeding phase with the Israeli basal diet are certainly caused by the Israeli diet. The severity of the deformities is obviously influenced by the treatments. No such deformities were observed in fish kept in either the flow-through system or the recirculation aquaculture system and fed with the Dutch basal feed. Certainly the treatments had a significant effect on the frequency of occurrence of the deformities as the fadrozole fed fish had the highest frequency followed by the control fed fish. No publications about these special types of malformations in the jaws have been found for *O. niloticus*. Several physiological, environmental, genetic, xenobiotic and nutritional factors have been mentioned as possible causes of skeletal deformities (Lall & Lewis-McCrea 2007) especially in larval and juvenile stages of fish. The observed jaw disorders during this experiment are clearly related to the diet and must therefore be due either to a deficiency or excess of one or more macro or micronutrients. The Israeli diet was comparatively low in lipids and therefore a deficiency in essential fatty acids (EFA) probably occurred during the crucial early developmental stages although in an older study purified diets with only 5% lipids or none at all did not result in such deformities (Chou & Shiau 1996). Nevertheless EFA have been reported to be important for bone formation in freshwater fish. However, these fish need only shorter chain poly unsaturated fatty acids (PUFA) like 18:3n-3 and 18:2n-6 because they are able to synthesize longer chain PUFA from the shorter ones (Lall & Lewis-McCrea 2007).

Genetic causes of the observed deformities can be excluded since these would have affected effect all the fish to a similar degree.

Jaw and vertebrae deformities in Atlantic salmon, *Salmo salar* L., produced in Chile have been reported to be caused by too high water temperatures and vitamin C deficiency (Roberts et al. 2001). Another influence of temperature on development in Atlantic halibut, *Hippoglossus hippoglossus* L., larvae was reported by Boll & Holmefjord (1988) who found that a significantly higher proportion of fish reared at 10°C had mouth deformities than those reared at 6°C and 2°C. In our experiments, environmental influences such as water pollutants and different water temperatures can also be excluded as causative factors since about one hundred fish were kept in the same system but fed with the Dutch basal diet and these fish were unaffected.

The disturbance of the retinoic acid receptor pathway regulating the osteoblasts in the jaw region was suggested to be the major factor in causing jaw deformities in Japanese flounder, *Paralichthys olivaceus* (Temminck & Schlegel), (Haga et al. 2003) but we had no way to check whether this was the case in the present study.

The most remarkable difference in the analysis of the two different basal diets fed during the initial weeks of the experiment was the vitamin A content which was considerably lower in the Israeli diet compared to the other basal diet and lower than recommended (NRC 2011).
Other than that none of the analyzed vitamins and minerals usually correlated (Lall & Lewis-McCrea 2007) with bone deformities were below the requirement levels as published in NRC (2011).

Although it is unlikely that the factor in the Israeli basal diet causing the jaw deformities also influences the sexual development, this possibility cannot be excluded. However, our results certainly show that the tested saponin fractions and the sapogenin diosgenin are not viable alternatives to MT if high degrees of masculinization are the required result. Regarding a potential reduction of reproduction potential caused by the saponin fractions, the lack of data allows for no clear conclusions and the experiment needs to be repeated under more favorable conditions and certainly with a different basal diet.

Acknowledgements
This work was partly financed by the German Research Foundation (DFG) under the grant BE 1133/13-2 for which we are grateful. We would like to thank Johannes Pucher, Florian Zuber and Christoph Schinkel for their support during the Christmas time in the fish laboratory.

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Chapter 5

Influence of short and long term feeding of *Quillaja saponaria* Molina saponins on sex ratio, growth performance and feed conversion of Nile tilapia, *Oreochromis niloticus* (L.)

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Abstract

Earlier experiments found positive influence of dietary supplementation with Quillaja saponaria Molina saponins on growth, nutrient utilization, sex ratio and metabolism of common carp Cyprino carpio (L.) and Nile tilapia Oreochromis niloticus (L.).

This experiment was conducted as a long term feeding experiment under field conditions in concrete ponds in Jericho, Palestinian Authority to evaluate commercial *Q. saponaria* saponins as potential alternative to 17-α-methyltestosterone treatment. The effects of saponins on growth, sex ratio, fish condition and reproduction were evaluated.

A total of 1000 genetically female *O. niloticus* were stocked before first feeding into the 25 tanks of a flow-through system at the Al-Quds University in Abu Dis, East Jerusalem. They were reared there for six weeks before being transferred to the field station in Jericho and being stocked into 25 hapas of approximately 640 L volume for the growth and reproduction trial. Five different treatments were applied, one control group (C) receiving a diet devoid of saponins, diets C10 and C25 which were fed for the first four experimental weeks with the control diet supplemented with *Q. saponaria* saponins containing 10 and 25% sapogenin, respectively and which were fed thereafter with the control diet. Diets QS10 and QS25 were supplemented throughout the experiment with the same saponins as C10 and C25. The overall growth performance of all groups was bad with body masses between 72 (C25) and 89 g (QS25) after 41 weeks. The QS25 group gained significantly more weight than all other groups although not showing a significantly reduced feed conversion. The condition factors of all groups were good and ranged between 1.61 (Control) and 1.69 (QS10) without statistical differences. No sex inversion occurred in any treatment and reproduction was found in all treatments but with different, non-significant intensity.

It is concluded that the tested saponins are no alternative to MT to produce male monosex populations or to inhibit reproduction of mixed sex tilapia. However, the effect on body mass gain needs to be more closely evaluated.

Introduction

Nile tilapia, *Oreochromis niloticus*, is one of the most important freshwater finfish species cultured globally. A production of over 3million tons has been predicted for *O. niloticus* in 2011 (Fitzsimmons et al. 2011).

Commercially viable production of Nile Tilapia in Tanzania and Kenya was only possible using male monosex culture (Kaliba et al. 2006, 2007). The fact that male monosex culture in commercial or industrial scale operations yields the highest financial returns is generally accepted.

The most widely applied technology to produce male monosex populations in tilapia is dietary application of 17-α-methyltestosterone (MT) or other androgens (Pandian & Sheela 1995). The phenotypic sex in tilapia can be influenced for the first four weeks after hatching because the gonads are not fully developed until 25-26 days after hatching (Nakamura & Nagahama 1985, 1989). However, besides being potentially carcinogenic (Velazquez & Alter 2004) and therefore posing a constant risk for workers preparing the feed on farm, MT must also be

considered to be environmentally harmful. Several experiments have shown that water in which fish were fed with MT containing diets had an influence on sex ratios of fish fed control diets (Gomelsky et al. 1994, Abucay & Mair 1997, Hulak et al. 2008). Methyltestosterone also affects invertebrates at low concentrations (Crane et al. 2006) which implies that effluent water downstream from facilities using MT for sex inversion poses a constant threat to natural aquatic vertebrate and invertebrate fauna.

Environmentally friendly and health safe substitutes for MT could be found amongst the class of chemical substance called saponins. They are glycosidic plant secondary compounds present in many cultivated plant orders or in marine invertebrates (Rio et al. 1965, D'Auria et al. 1992, Ebada et al. 2010). Saponins consist of an aglycone (sapogenin) and one or more sugar side chains, glycosidically linked to the sapogenin. The aglycone is usually either steroid or triterpenoid and both types are based on a 30 carbon skeleton molecule (Haralampidis et al. 2002, Vincken et al. 2007). They are considered to be toxic for fish and to be potent anti-nutritive factors in terrestrial protein sources for fish feeds like soy bean (Bureau et al. 1998, Francis et al. 2001a).
A previous study using *Quillaja saponaria* saponins (QS) reported that inclusion of 700 mg kg^{-1} in the diet influenced the sex ratio of Nile tilapia with higher male to female ratios in saponin fed groups compared to control groups (Francis et al. 2002a). Extracts from another saponin rich plant, *Tribulus terrestris* L., also showed a masculinizing effect on convict cichlid *Cichlasoma nigrofasciatum* Günther (Çek et al. 2008) and *Clarias gariepinus* Burchell (Turan & Cek 2007).
As well as its potential application as a source of environmentally friendly androgens Francis et al. (2001b, 2002b, c) reported that dietary QS led to an increase in growth and improved nutrient utilization in common carp *Cyprinus carpio* (L.) and Nile tilapia.
In this experiment we tested two saponin mixtures with different sapogenin content (purity) from *Q. saponaria*. One contained low sapogenin levels (~10%) and the other high levels (~25%). Their effects on sex ratio, growth performance, feed and nutrient utilization of all female Nile tilapia are reported. The saponins were either fed throughout the trial or during the first four weeks only.
Here we present only part of the results because the experiment is still ongoing.

Material & Methods

Three different diets were prepared, one diet was used as control and contained no saponins, and the other two diets consisted of the control diet plus 2000 mg kg^{-1} Sigma *Quillaja saponaria* saponin mixtures with 10 or 25% sapogenin content (S 2149 and S 4521, Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The composition of the basic diet fed during the first six weeks and the two diets fed alternately thereafter are given in Table 1. The saponins were added to the respective basic diet by dissolving them in a minimum volume of water and spraying the solution onto the feed while stirring. The control diet was sprayed with water not containing saponins. Excess water was removed by freeze drying and the feeds were then stored in a refrigerator at 6°C until required.

Table 1: Composition (according to manufacturer's description) of the different experimental diets used during the experiment.

	Starter feed	On growing feed	Alternate grower feed
CP (g kg^{-1})	480	400	350
CL (g kg^{-1})	55	90	50
CF (g kg^{-1})	22	50	51
Ash	84	65	59
DE (kJ g^{-1})	16.5	16.0	16.0

At the start of the experiment, five different treatment groups were randomly assigned to the 25 boxes of a flow-through system with five replicates per group. The control group (C) was fed throughout the experiment with the respective control diet, containing no saponins. Group C10 was fed for four weeks with feed containing 2000 mg kg^{-1} of the saponin mixture containing 10% sapogenin, group C25 was fed for the same time with the diet containing the 25% sapogenin saponin mix in the same concentration. Beginning in the fifth week groups C10 and C25 received the same diet as the control group for the rest of the experiment. Groups QS10 and QS25 were fed throughout the whole experimental time with the diets containing 10% sapogenin (QS10) and 25% sapogenin (QS25) at 2000 mg kg^{-1} diet, respectively.

A total of 1000 genetically female Nile tilapia *O. niloticus* were obtained from the Hebrew University of Jerusalem, Department of Animal Science at the Robert H. Smith Faculty of Agriculture in Rehovot. They were equally divided into the 25 2.5 L boxes (40 fish box^{-1}) of the flow-through system at the Department of Earth and Environmental Sciences, Faculty of Science and Technology, Al-Quds University, East Jerusalem, West Bank. The flow rates were on average set to 3.6 L h^{-1}, the temperature was kept at 28 ± 0.2°C under ambient light. One week after the start of the experiment the stocking density was reduced to 30 fish in each box.

To calculate the amount of feed for the first week, a body mass of 0.01 g was assumed for a single individual fish to avoid the exposure of the fry to the handling stress of the weighing procedure. Afterwards, a sub-sample of the fish was weighed for each box weekly and feed amounts calculated accordingly (Table 2). The boxes were cleaned once per week. Water quality was tested with commercial aquaria test kits (manufacturer according to availability, e.g. Merck and JBL) for ammonia, nitrite, and chlorine. Water levels of ammonia and nitrate

Table 2: Daily feeding rates during the experimental period before the reproduction trial started.

Week	Feeding level (% of BM)
1	30
2 – 4	20
4 – 6	15
6 – 19	10
19 – 23	7
23 – 25	5
25 – 27	4
27 – 30	2
30 – 35	3
35 – 36	4.5
36 – 39	4
39 – 40	3.5

were on average between 0.5 and 1.0 mg L^{-1} and never exceeded 2.0 mg L^{-1} for NH_4^+ during the indoor phase (first six weeks). For NO_2^- the water levels were on average between 0.05 and 0.1 mg L^{-1} and never exceeded 0.8 mg L^{-1} for a longer period during the indoor phase. During the pond phase these values never exceeded those of the indoor phase.
Initial high chlorine concentrations in the water (between 0.3 and 0.5 mg L^{-1}) during the first week caused the death of approximately 10-15% but after carbon filters were installed on day nine, mortality was minimal. Feed was provided five times per day by hand.

After six weeks the fish were transferred to an aquaculture research facility outside Jericho, West bank, for further grow-out. All surviving fish from each box were stocked into a corresponding hapa net supported by a frame with the size 80 cm * 80 cm * 120 cm (W * L * D) with a coarser meshed inner layer (10 mm) and a fine meshed outer layer (1 mm mesh size). The hapas were set up in different concrete ponds according to treatment with around 20 cm of the hapa net being above the water surface resulting in around 640 L volume. Groups QS10 and QS25 were each kept in separate ponds to prevent any influence of leached saponins in the water on the result. Groups C, C10 and C25 were divided at random between two more ponds. All ponds had the same water source, a nearby groundwater well. The water was changed periodically according to water quality parameters in the ponds and availability of water. During the wintertime the temperatures in the ponds dropped down to around 19°C and ponds were covered overnight with black polythene sheet to prevent excessive loss of heat. While the fish were in the ponds the basal diet was changed twice, according to availability, but the saponin levels were maintained (Table 1). The fish were group weighed every two weeks and feed allowance adjusted according to new body masses.
During wintertime water temperatures fell to suboptimal levels so the amount of feed was reduced. Feeding levels were increased when temperatures rose again in the spring. The lowest recorded temperatures during winter were around 21°C and the highest recorded temperatures were around 32°C during summertime.
After 40 weeks, a total of 212 fish were sampled from all treatments for proximate composition, blood sampling and macroscopic sex determination. A maximum of nine fish per hapa were taken if the number of fish in the hapa was 19 or more. Of these between two

and four fish per hapa were directly killed by a sharp blow on the head, weighed to the nearest 0.1 g, measured to the nearest 0.1 cm (total length, TL) and stored in ice slurry until all fish were sampled. Afterwards, they were transported to the fish-laboratory at the Al-Quds University and frozen at -8°C until analysis which took place about a week later. Between two and five fish per hapa were designated for blood sampling and were transported alive from Jericho to Rehovot (about 100 km). After anesthesia with 200 mg L^{-1} MS222, blood was sampled with heparinized syringes from the caudal vein. Results of blood analysis will be published elsewhere. The fish were then killed by a sharp blow on the head, weighed to the nearest gram, and their sex was determined by direct observation of the gonads.

To test for reproduction, the number of females per hapa was first reduced to eight females per hapa each and then two male *Oreochromis niloticus* X *O. aureus* of approximately 120 g BM, obtained from the aquaculture station at Dor, Israel, were stocked in each hapa resulting in a female to male ratio of 4:1. All males were marked by fin-clips. Every two weeks hapas were checked for breeding females and any offspring were removed, counted and weighed over a total period of six weeks. Offspring were grossly classified as eggs, developed eggs (i.e. when tail clearly distinguishable) and hatched eggs or larvae. For a better overview the results of the reproduction were pooled treatment-wise over the six weeks of the reproduction trial (Table 3).

Table 3: Pooled reproduction data for the different groups collected during six weeks

	Control	C10	C25	QS10	QS25
Eggs	178 ± 252	440 ± 700	130 ± 236	394 ± 716	620 ± 623
Developed eggs	0 ± 0	108 ± 182	109 ± 186	104 ± 169	198 ± 333
Larvae	66 ± 108	194 ± 225	143 ± 300	29 ± 64	447 ± 615
Average total offspring	244 ± 334	742 ± 749	382 ± 311	527 ± 708	1265 ± 1176
Average offspring / average female bodymass (g)	1.2 ± 1.9	1.4 ± 1.7	1.9 ± 1.4	1.9 ± 2.7	3.5 ± 3.7
Edematous ovaries (%)	80.0 ± 6.8	67.5 ± 20.9	67.5 ± 14.3	72.5 ± 20.5	60.0 ± 18.5

Values = mean ± SD (N = 5)

The trial for the effects of saponins on reproduction was carried out for 10 weeks with the first sampling for offspring after four weeks. During this time, the voluntary feed intake of the fish declined for no apparent reason so the daily feed allowance was reduced stepwise from 3.5% of BM to approximately 0.55% of BM to avoid wasting feed and polluting the water. Spot sampling for stomach content analysis was conducted after the reproduction trial ended. Stomachs and intestines were dissected and opened and visually analyzed for food categories (e.g. feed pellets, invertebrates and other). Fish not used in the proximate composition analysis, for blood sampling or for the reproduction trial were analyzed by the Israeli Central Fish Health Laboratory for ecto- and endoparasites and for general condition. Before the reproduction trial started, fish were treated with Ivermectin and ponds and hapas cleaned and scrubbed thoroughly.

Of the fish designated for the determination of proximal composition two per hapa were cut into small pieces with secateurs, autoclaved for 35 minutes at 121°C and homogenized with an Ultra-Turrax T25 (IKA-Labortechnik, Staufen, Germany). Afterwards they were freeze

dried and their water content determined by difference. Nitrogen content was determined with an element analyzer (C/N VarioMAX, Elementar Analysensysteme GmbH, Hanau, Germany) and the corresponding crude protein percentage calculated as (N (g) x 6.25 x 100)/weight of sample (g). Crude lipid was determined by Soxhlet and crude ash was determined by burning at 500°C for 6 hours in a muffle furnace (N11, Nabertherm, Lilienthal, Germany). Gross energy was determined with a bomb calorimeter (IKA C 700, IKA Labortechnik, Staufen, Germany).

To compare the effects of the different treatments the following parameters were calculated as follows:

Mortality (%)	100 - (surviving fish / stocked fish * 100)
Individual Body Mass Gain (IBMG (g))	Individual final body mass (g) – individual initial body mass (g)
Specific Growth Rate (SGR (% day^{-1}))	100 x [(ln final mass - ln initial mass) / days of experiment]
Feed Conversion Ratio (FCR)	Feed consumption (dry matter) / live body mass gain (g)
Condition factor (K)	100 * (BM (g) / TL $(cm)^3$)

For statistical analysis, the program SPSS 10.0 (IBM SPSS, Chicago, IL, USA) was used. The average final body mass at the end of the growth trial, the feed conversion ratios (FCR), the mortalities, the total length and the condition factor for the five different treatments were analyzed using the procedure “Compare means – one way ANOVA”. A test for homogeneity of variance (Levene’s test) was applied and the ANOVA only conducted when the Levene test was insignificant ($p > 0.05$). To test for normal distribution, a Kolmogorov-Smirnov test was applied. In cases where the ANOVA was significant, the Tukey’s HSD test was applied to test for differences between treatments.

Results

During the feeding experiment all fish accepted all feeds and ate them during the first two minutes after feeding. No adverse effects of saponin supplementation on behavior were observed at first, but behavior changed considerably shortly after the reproduction trial started. All fish, females and males, reduced their feed intake significantly and feed allowance was reduced stepwise to 0.55% of BM compared to 3.5% of BM before the reproduction trial started. Only a few ectoparasites were found by the fish health service but none were present on the fish’s skin in sufficient number to have caused any adverse effects. Among the parasites observed were: *Dactylogyrus* sp., *Trichodina* sp., *Sessilina* sp. and *Gyrodactylus* sp. in low to medium densities. One endoparasite (*Capillaria* sp.) was found but only infrequently.

Table 4: Initial and final body masses, feed conversion ratio (FCR) and mortality of the differently treated fish

	Control	C10	C25	QS10	QS25
Average initial BM (g)	0.01	0.01	0.01	0.01	0.01
Average final BM (g)	$80.0^{ab} \pm 5.7$	$79.8^{ab} \pm 6.7$	$71.9^{a} \pm 9.4$	$79.0^{ab} \pm 5.8$	$88.7^{b} \pm 1.6$
FCR	1.29 ± 0.06	1.22 ± 0.07	1.29 ± 0.06	1.31 ± 0.05	1.32 ± 0.06
Mortality (%)	31.3 ± 9.0	18.0 ± 20.8	24.0 ± 5.5	20.0 ± 10.3	17.3 ± 19.2
Condition Factor	1.61 ± 0.12	1.62 ± 0.13	1.64 ± 0.07	1.69 ± 0.11	1.65 ± 0.10

Values = mean ± SD (N = 5), different superscripts in the same row indicate significant differences (ANOVA, $p < 0.05$)

Around 20-30% of the fish died during the experiment. This level of mortality is relatively high (Table 4), but was not statistically different among treatments due to high variation in the replicates. The control fed fish had numerically the lowest survival rate (69%) as opposed to approximately 80% in the saponin fed fish (Table 4) which shows that neither short nor long term supplementation with saponins had any abnormally negative impact on the fish. The average body mass in the differently treated groups after four weeks of feeding (groups C10 and C25 were from now on fed with the control diet) was between 92 and 102 mg with no statistically significant differences. After 40 weeks, the fish fed continuously with QS25 throughout the whole experiment were significantly heavier than those in any of the other groups (Table 4). Their body mass was on average 10.9% higher than fish fed continuously with control feed. The growth advantage of QS25 over the other groups was observed from the 23rd week onwards. In contrast, fish fed for four weeks only with C25, grew less than those in the other groups but the difference was not statistically significant. By the end of the experiment, the fish in this group weighed 11.3% less than those in the control group. The condition factors ranged from 1.61 in the control group to 1.69 in the QS10 group with no statistical difference between the groups (Table 4).

No male fish were observed in any of the treated groups or the control. No sex inversion occurred.

No differences in proximate composition between the differently fed fish were found. Values for crude protein, crude lipids, crude ash and gross energy were similar among the treatments and are shown in Table 5.

Table 5: Proximate composition of the differently treated fish

	Control	C10	C25	QS10	QS25
CP (g kg^{-1} FM)	16.7 ± 0.30	16.6 ± 0.51	16.7 ± 0.26	16.5 ± 0.64	16.9 ± 0.43
CA (g kg^{-1} FM)	3.24 ± 0.21	3.25 ± 0.13	3.30 ± 0.18	3.26 ± 0.22	3.34 ± 0.10
CL (g kg^{-1} FM)	8.66 ± 0.89	8.26 ± 0.82	8.39 ± 1.03	8.41 ± 0.89	8.21 ± 0.78
GE (kJ g^{-1} FM)	7.47 ± 0.30	7.34 ± 0.38	7.34 ± 0.38	7.32 ± 0.45	7.36 ± 0.32

Values = mean ± SD (N = 5)

Statistically no differences in reproduction were found between the different groups but numerically the reproduction success was lowest for fish being fed the control diet throughout the experiment while fish fed continuously with saponins containing 25% sapogenin showed the highest reproduction success (Table 3). At the end of the reproduction trial all killed females were opened and high percentages (between 60% in the QS25 group and 80% in the

control group) of ovaries were strongly edematous (Table 3, Fig. 1). These ovary deformities were found in all treatments.

Discussion

This is the first time that commercial saponins have been used in a long-term experiment under field conditions. The results of this study are different from those of several studies in which commercially available saponins derived from *Quillaja saponaria* were fed to common carp *Cyprinus carpio* and *O. niloticus* under laboratory conditions. Among the differences between this experiment and the experiment reported by Francis et al. (2002a) was the duration of saponin supplementation and the sapogenin content of the *Q. saponaria* saponins. Francis and colleagues used a *Q. saponaria* extract containing around 10% sapogenin (Sigma S 2149), whereas during this study an extract with around 25% sapogenin content (Sigma S 4521) was additionally fed.

In comparison to the observations made by Francis et al. (2002a) (obtained with mixed sex Nile tilapia fry) no masculinization was observed during this study in which genetically female tilapia fry were stocked. An inhibitory effect of a saponin fraction derived from *Q. saponaria* on aromatase, the enzyme responsible for aromatization of androgens to estrogens (Banting & Ahmed 2009) was observed *in vitro* by Golan et al. (2008). Inhibition of aromatase frequently led to masculinization in Nile tilapia (Kwon et al. 2000, Afonso et al. 2001). Although Stadtlander et al. (2008) reported masculinization of mixed sex Nile tilapia fry with saponin fractions derived from *T. foenum-graecum* a larger scale repetition of that experiment resulted in no statistically significant differences (Stadtlander et al., unpublished). Extracts of another plant rich in steroidal saponins, *Tribulus terrestris*, have been reported to affect sex ratio in favor of males. Mixed sex convict cichlids *Cichlasoma nigrofasciatum* and mixed sex African catfish *Clarias gariepinus* had significantly higher proportions of males compared to controls after immersion treatments (Çek et al. 2007, Turan & Cek 2007). However, it must be said that comparisons between studies are difficult given the large variations in experimental conditions, saponin concentrations and mode of application (e.g. dietary application vs. immersion treatments).

In this study, a growth promoting effect was only observed in fish continuously fed with QS25 but not, as reported by Francis et al. (2001b), in fish fed long term with QS10. However, that difference might arise from the different saponin concentrations added to the feed (300 mg kg^{-1} in Francis et al. 2001b vs. 2000 mg kg^{-1}, this study). However, a significantly higher growth rate usually should be accompanied by a significantly reduced feed conversion ratio, which has not been observed during this experiment. Although no quantification of phytoplankton and invertebrate biomass has been undertaken, visual observations of algae and invertebrates, primarily midge larvae of the family Chironomidae, being attached to the hapas, were made. Spot sampling of stomach contents revealed algae like stomach contents although throughout the experiment compound feed was offered. It

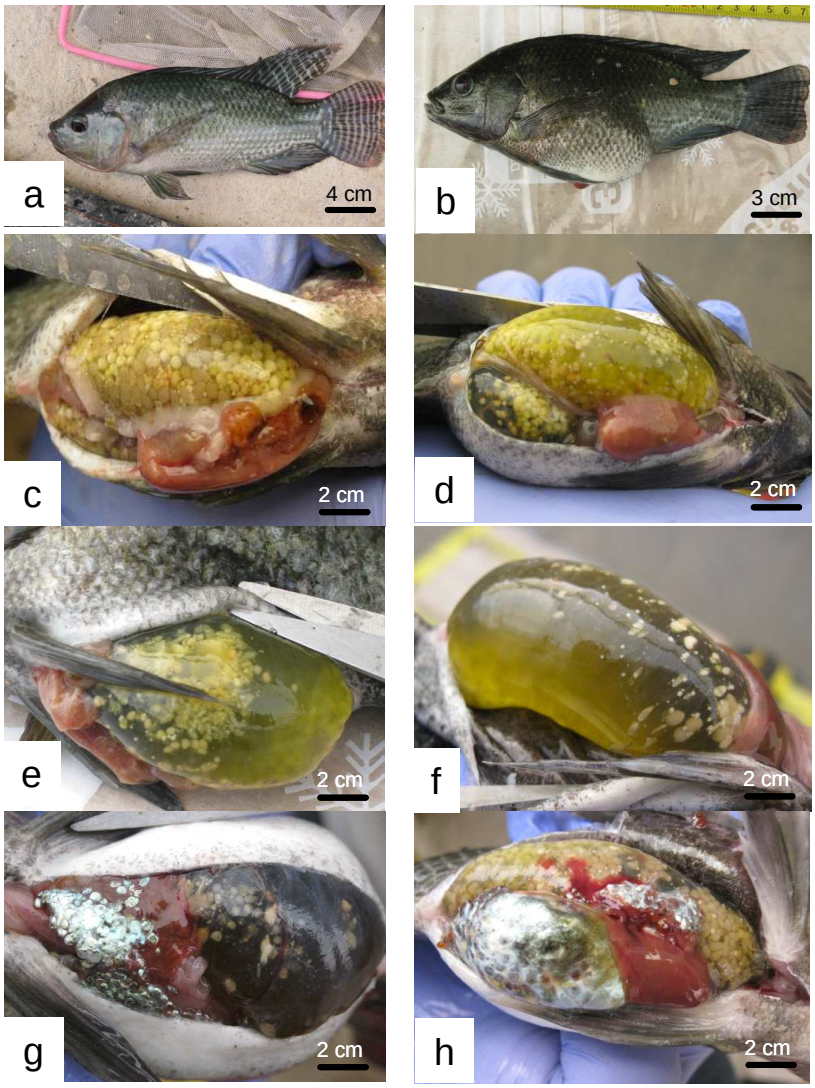

Figure 1: Pictures of Nile tilapia with normal appearance (a), swollen belly (b), little edematous and strongly edematous ovaries (c-f) and ova observed outside the ovary in the body cavity (g-h).

must be assumed that natural food and Aufwuchs from the hapa nets was consumed besides the offered compound feed and influenced growth and feed conversion. The five hapas of the QS25 group were stocked alone in one concrete pond of 16 m³ volume while the stocking density of the other ponds was comparably higher. Nevertheless no strong algae blooms were observed in any of the ponds and water temperature and ammonia and nitrite concentrations of all ponds were similar over the whole experimental period. Nile tilapia are generally considered herbivorous (Khallaf & Alne-na-ei 1987, Bwanika et al. 2004) while own observations suggest Nile tilapia to be highly opportunistic. The growth advantage observed by the QS25 group can therefore not be fully attributed to the saponin supplementation.

Despite low overall growth performance the condition factors in all groups were generally good with a numerical but statistically insignificant trend towards higher condition factors in fish fed continuously with saponins. *Quillaja saponaria* saponins are known and widely applied in vaccines because they are good adjuvants and they are forming immune stimulating complexes (Barr et al. 1998). A beneficial effect of long term supplementation with a potential pre-biotic substance might explain parts of the growth advantage accompanied by

numerically higher condition factors seen in the QS10 and QS25 groups. Short term saponin supplementation seemingly had rather detrimental effects on growth in the C25 group.
Although statistically not different from the control, fish fed short term (C25) with the same saponins in the same concentrations were actually smaller than fish from all other groups.
Reproduction occurred in all treatments but to an insignificantly different degree. Numerically the best reproduction performance was observed in fish fed continuously the *Q. saponaria* saponin containing 25% sapogenin. Previously Steinbronn et al. (2004) reported no reproduction after supplementation with *Q. saponaria* saponins containing around 10% sapogenin at the same concentration as used in this study (2000 mg kg^{-1}). During this experiment reproduction was observed in all treatments but with numerically different but statistically insignificant intensity. However, the observed reproduction success seems to be rather limited which could be attributed to the relatively high amounts of edematous ovaries found in females of all treatments.
Numerically the QS25 group showed the lowest incidence of edematous ovaries while the control fish showed the highest occurrence. Similar observations were made in another experiment conducted under controlled laboratory conditions with the same starter feed as used during this experiment. Fish fed with a completely different larval feed during that trial did not show the high occurrence of edematous ovaries. However, the true reason for the high deformities in the ovaries remains unknown although the saponins seem not to be the cause. The reduction in feed intake might be associated with the ovary deformities since as consequence of the swollen gonads all internal organs are compressed (Fig. 1) including the stomach.
The results of this experiment do not support an application of the tested commercially available *Q. saponaria* saponins as a substitute of methyltestosterone for masculinization. Furthermore, although the results are less conclusive, the reproduction seems not to be inhibited by the tested saponins. Although due to the edematous ovaries, no clear statements can be made and a repetition of this experiment should be conducted under more controlled conditions. The observed growth advantage of *O. niloticus* continuously fed *Q. saponaria* saponins containing 25% sapogenins can not clearly be attributed to the saponins and should be tested for long term under controlled conditions.

Acknowledgements
We wish to thank the German Research Foundation for partly funding this experiment under the project number BE 1133/13-2. Furthermore we are grateful for the assistance of the students Ali Hisham Aref Abu Sadoun, Samer Manwiar Jabarein, Farouq Eibasi Yousef Ebeisi and Guy Harlev for their help in the field and with the lyophilizer. We want to thank Mrs. Beatrix Fischer and Mr. Herrmann Baumgärtner for their help during the chemical analysis.

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Chapter 6

Effect of feed supplementation with saponins extracted from *Trigonella foenum-graecum* L. and *Quillaja saponaria* M. on growth performance, feed and nutrient utilization and metabolic efficiency in carp, *Cyprinus carpio* (L.)

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Abstract

Saponins are generally regarded as anti-nutritional factors in animal diets. However in some previous experiments it has been shown that low levels of *Quillaja saponaria* saponins in the diets of common carp, *Cyprinus carpio* (L.), and Nile tilapia, *Oreochromis niloticus* (L.), showed some potential as growth promoters.

We included low concentrations of eluated saponin fractions in the diets of common carp and measured the effects on growth, proximate composition, feed and nutrient utilization and oxygen consumption. Saponin fractions were derived from fenugreek, *Trigonella foenum-graecum* (L.), and the South American soap-bark tree, *Quillaja saponaria* (M.). Supplements were prepared using consecutive methanol fractionation of ethanol extracts resulting in 40%, 60% and 80% methanol saponin eluates. All three fenugreek eluates (40TS, 60TS, 80TS) and the 80% methanol eluate from *Q. saponaria* (80QS) were added at 150 mg kg^{-1} diet. Fish that were fed the diet supplemented with fenugreek saponins eluated with 60% methanol had numerically the best nutrient and feed utilization and growth performance. They consumed less oxygen and spent least energy per gram of protein accretion compared with the other groups.

Introduction

From 1970 to 2008 aquaculture showed an average annual growth rate of 8.3% (SOFIA 2011). Today almost every second fish consumed worldwide originates from aquaculture and this tendency is increasing (Cressy 2009, Naylor et al. 2009). With an annual production of 20.6 million metric tonnes (FAO 2010) Cyprinids are the most important group of cultivated freshwater fish. The common carp (*Cyprinus carpio* L.) is one of the worlds most commonly produced fish species and contributed just under 3 million metric tons in 2008 (FAO 2010).

Worldwide demand drives the continual increase in aquaculture production. Besides boosting production by simply building more and bigger aquaculture enterprises, increases in production can also be achieved by other means for instance by optimizing feed utilization, improving rearing techniques, increasing the sophistication of facilities, and augmenting disease resistance by the inclusion of immunostimulating additives in the feed.

One option is to use secondary compounds from plants. One such group of substances that may act as growth enhancers are the saponins. They are glycosidic compounds mainly produced by plants that are often activated after tissue damage and act for instance as antimicrobial defense substances (Gus-Mayer et al. 1994). Saponins can be found in a great variety of different plants (Vincken et al. 2009), including many cultured plants like the soy bean which is the most common plant protein source for aquaculture feeds. Saponins consist of a steroidal or triterpenoidal core structure called aglycone or sapogenin and one or more sugar side chains. Due to large variations in either the aglycone or the sugar moiety they produce very diverse biological effects in animals. A detailed review of the biological actions of saponins is given by Francis et al. (2002a).

Although in animal nutrition saponins generally are considered to be anti-nutritional factors (Francis et al. 2001) they have also been shown to have beneficial metabolic effects and to promote growth if they are included in the diet in small quantities. Francis, Makkar & Becker (2002b, c) included different concentrations of commercially available *Q. saponaria* saponins (Sigma S 2149) in the diet of *C. carpio* and found that 150 mg kg^{-1} increased the growth and reduced the oxygen consumption of the fish.

At least 38 different saponins have been identified in *Q. saponaria* (Bankefors et al. 2008, Guo & Kenne 2000) but up to date it is unclear which ones show what kind of effect and in what strength. In this study saponins have been extracted and fractionated from the bark of *Q. saponaria* and one fraction included in the diets of common carp as have fractions derived from the seeds of *T. foenum-graecum* L. (fenugreek), another saponin-rich middle eastern plant containing at least 19 different saponins (Murakami, Hishi, Matsuda & Yoshikawa 2000). Several beneficial effects of saponins in different animal species have been reported. Hassan et al. (2008) applied different concentrations of *Q. saponaria* saponins to zebrafish (*Danio rerio*) in a bio-assay and concluded that a concentration of 5 µg or less per milliliter of water functions as a growth promoter while 10 µg or more are lethal for the zebrafish embryos. Pham et al. (2006) showed that some of the saponins derived from *Q. saponaria* formed immuno-stimulatory-complexes (ISCOMs). Furthermore Quillaja saponins are applied as feed additives in terrestrial livestock for ammonia and odor control (Cheeke 2000).

Ethanol extract of *T. foenum-graecum* was found to be an excellent alternative to a well known anti-diabetic drug when tested in rats with artificially induced diabetes (Eidi et al.

2007). Diosgenin, a sapogenin present in fenugreek, stimulated ion transport in human cortical neuronal cells (Wang et al. 2006).

In this study we supplemented the feed of juvenile common carp (*C. carpio*).with different saponin eluates or fractions derived from fenugreek and the soap-bark tree and investigated the effects on growth, feed and nutrient utilization, and metabolic parameters.
The purpose of this study was to evaluate different saponin eluates as potential growth promoters in carp. The extracted saponins were fractionated to narrow the biological activity of saponins down to a certain fraction with the future goal in mind to find and isolate one or more active saponin(s) or sapogenin(s) which could be used as environmentally friendly growth promoters in aquaculture.

Materials and Methods

Saponin extraction

Plant materials (seeds from *T. foenum-graecum* and bark from *Q. saponaria*) were ground on a Wiley Mill (Arthur H. Thomas, Philadelphia, PA) with a mesh size of 2 mm and afterwards dried for three days at 55°C. Ten grams of the fine powder was defatted by the Soxhlet method with hexane (150 ml) as solvent for 3 hours. The residue was further treated with 70% ethanol in water (150 ml) for 9 hours. The solution was centrifuged (10 min., 40°C at 18 000 g) and the supernatant filtered through a Whatman paper No.1.
The filtrate was purified by flash chromatography (CombiFlash RETRIEVE, Teledyne Isco, Lincoln, NE) using consecutive methanol/water concentrations (v/v, 40/60, 60/40, 80/20) resulting in three different saponin eluates or fractions (40%, 60% and 80%). The active saponin mixtures have been subjected to a reversed phase preparative HPLC to isolate the active ingredients into pure individual compounds. They are currently under investigation to fully elucidate their structure by MS / 2-D NMR spectroscopy. The exact structures will be published separately in a different journal.

Based on earlier observations and an *in-vitro* study (Golan et al. 2008) all three fenugreek and the 80% *Quillaja* fractions were investigated.

Experimental Diets

Four saponin supplemented diets and one control diet were prepared. The ingredients and proximate composition of the basal diet are shown in Table 1 and Table 2. The saponin supplemented diets were prepared by adding 150 mg kg^{-1} of the saponin fraction to the control diet. The supplement (22.5 mg) was dissolved in 30 ml water and added to the feed (150 g) while stirring for 10 minutes. The control diet received the same amount of water without any

Table 1. Ingredients of the basal and experimental diets

	Diet				
Ingredient	Control	80TS	60TS	40TS	80QS
Trigonella 80% fraction (mg kg^{-1})		150			
Trigonella 60% fraction (mg kg^{-1})			150		
Trigonella 40% fraction (mg kg^{-1})				150	
Quillaja 80% fraction (mg kg^{-1})					150
Fish meal[a] (g kg^{-1})	500	500	500	500	500
Whole wheat meal (g kg^{-1})	420	420	420	420	420
Sunflower oil (g kg^{-1})	40	40	40	40	40
Vitamin premix[b] (g kg^{-1})	20	20	20	20	20
Mineral premix[b] (g kg^{-1})	20	20	20	20	20

[a]Norwegian fish meal obtained from Wuerttembergische Zentralgenossenschaft, Germany.
[b]Prepared after Gaye-Siessegger *et al.* (2004).

saponins. The resulting diets were termed C (control), 40TS, 60TS and 80TS (fenugreek saponin fraction eluated with 40, 60 or 80% methanol, respectively) and 80QS for the diet supplemented with saponins derived from *Q. saponaria* (Fig. 1). The feed mash was made into pellets using a domestic meat grinder (Bosch Comfort plus, Robert Bosch GmbH, Gerlingen, Germany) fitted with a 2 mm die. The pellets were dried at 40°C for 36 hours and afterwards kept at 6°C. The pellets sank in water and were accepted well by the fish.

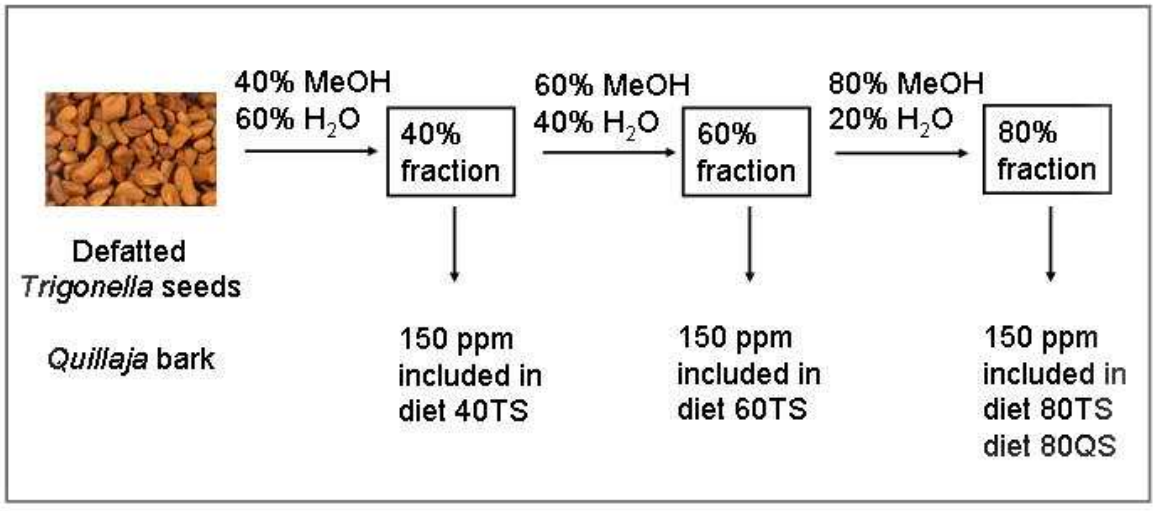

Fig. 1: Scheme of the consecutive methanol eluation/fractionation and incorporation of 150 ppm of the respective saponin fraction into the four different experimental diets, MeOH = methanol, TS = *Trigonella foenum-graecum* saponin, QS = *Quillaja saponaria* saponin

Table 2: Proximate composition and gross energy of the diets.

Diet	CP (g kg^{-1} DM)	CL	CA	GE (kJ g^{-1} DM)
Control	420	125	110	20.9
80TS	421	124	124	20.6
60TS	418	127	120	20.8
40TS	420	127	118	20.8
80QS	418	128	122	20.7

Crude Ash = CA, Crude Protein = CP, Crude Lipids = CL, Gross Energy = GE

Experimental set-up

Twenty-one common carp (*C. carpio*) from the Federal Research Center for Fisheries, Institute for Fisheries Ecology, Ahrensburg, Germany with an average body mass of 9.4 ± 1.2 g (mean ± SD) were divided into two groups, the initial group and the experimental group. The six fish of the initial group were killed by a sharp blow on the head and the fish carcasses stored at -20°C until further analysis. The 15 fish of the experimental group were placed individually in 15 chambers of a respirometric system (Focken et al. 1994) and the five different diets were randomly assigned to three fish each. Feed was offered at 5 times maintenance level (metabolic body mass ($kg^{0.8}$) * 3.6, Meyer-Burgdorff et al. 1989). The daily amount of feed was equally distributed between five feeding stations of an automatic feeder and offered at 9:00, 11:00, 13:00, 15:00 and 17:00. At least once per day the chambers were inspected for feed residues. The fish were weighed once a week and the amount of feed was adjusted according to the new body mass. For 24 hours after stocking and 24 hours before each weighing, feeding was stopped to allow time for defecation and minimize weighing errors**.** After weighing, each fish was kept in plastic buckets for approximately 5-10 minutes while its respirometric chamber was cleaned. The light regime was set to 12 hours light/dark and water temperature was maintained at 27 ± 1°C. Every day approximately 5-10% of the total water volume was changed. Total ammonia content was kept below 0.2 mg L^{-1}, nitrite below 0.1 mg L^{-1} and nitrate below 10 mg L^{-1}.

At the end of the five week experiment the fish were killed by a sharp blow on the head and frozen at -20°C until proximate chemical analysis was performed.

Chemical analysis

Prior to chemical analysis, the carcasses were autoclaved for 45 min at 121°C, homogenized with an Ultra-Turrax T25 (IKA-Labortechnik, Staufen, Germany), refrozen at -20°C and freeze-dried with a Lyovac GT 2 (SRK-Systemtechnik, Riedstadt, Germany).

The chemical analysis was conducted on each individual fish, including the six fish from the initial group, according to AOAC methods (1990). In brief, dry matter was determined by drying the material to constant mass over night at 105°C, crude protein (CP) was determined by macro-Kjeldahl with a conversion factor of CP = N*6.25, crude lipid was determined by the Sohxlet method, gross energy was measured by bomb calorimetry (IKA C 7000, Staufen, Germany) and ash content by ashing over night in a muffle oven at 500°C (Nabertherm N11, Lilienthal, Germany).

Calculations

The following parameters were calculated as shown:

Metabolic Body Mass (MBM ($kg^{0.8}$)	(Live body mass (g) / 1000)$^{0.8}$
Metabolic Growth Rate (MGR)	Live body mass gain (g) in one week / average metabolic live body mass ($kg^{0.8}$) during the same week (Dabrowski et al. 1986)
Specific Growth Rate (SGR (% day^{-1}))	100 x [(ln final mass - ln initial mass) / days of experiment]
Routine Metabolic Rate (RMR)	mean Oxygen consumption in 24 h (mg) / metabolic body mass ($kg^{0.8}$) x 24
Energy Expenditure (EE (kJ))	Oxygen uptake (g) x 14.86 (kJ g^{-1} O_2, Huisman 1976)
Energy Retention (ER (kJ))	Final gross energy (kJ) of fish – initial gross energy (kJ) of fish
Metabolizable Energy (ME), (kJ)	ER (kJ) + EE (kJ)
EE (% of GE fed)	EE (kJ) x 100 / Feed energy intake (kJ)
ER (% of GE fed)	ER (kJ) x 100 / Feed energy intake (kJ)
ME (% of GE fed)	ER (kJ) + EE (kJ) x 100 / Feed energy intake (kJ)
AUE (% of GE fed)	100 - EE (%) - ER(%)
O_2 consumption (g) / protein gain (g)	Total oxygen consumption (g) / total protein gain (g)
EE (kJ) / protein gain (g)	Total EE (kJ) / total protein gain (g)
Protein Efficiency Ratio (PER)	Live body mass gain (g) / feed protein intake (g)
Feed Conversion Ratio (FCR)	Feed consumption (dry matter) / live body mass gain (g)

Statistical analysis

All data were analyzed using the General Linear Models (GLM) or the Repeated Measures (for growth and metabolic growth rate) procedures of Statistical Analysis System (SAS, 1982) followed by a Scheffé post-hoc test to assess the significance of differences between different feeding groups. The values are expressed as mean ± SEM unless stated otherwise. All statements of significance were based on a probability of $p< 0.05$.

A power analysis was conducted using G*Power vers. 3.1.2 (Faul et al. 2007). A post-hoc power analysis was applied using the "F-test family – ANOVA: fixed effects, omnibus, one-way" procedure of the program. The determined effect size and achieved statistical power are reported in Table 6.

Results

General observations and behavior

No fish died during the experiment. The fish readily accepted all diets and consumed the feed during the first two minutes after feeding, which minimized leaching of saponins from the feed into the water.

Proximate chemical analysis

By the end of the experiment, all fish contained a higher percentage of lipids and a lower percentage of ash than the initial group. The percentage of protein increased slightly but the differences were not statistically significant (Table 3). Fish fed with 60TS had the lowest lipid content at the end of the experiment, gaining less than all other groups. The same group had also the lowest dry matter content at the end of the experiment (Table 3).

Table 3: Initial and final proximate chemical analysis of control and experimental fish.

Group	CP	CL	CA	DM	GE
		(g kg^{-1} FM)			(kJ g^{-1} FM)
Initial group	141 ± 2.19	24.7 ± 3.10	30.6 ± 0.72	194 ± 6.20	3.34 ± 0.21
Control	148 ± 2.31	49.9 ± 7.20	22.1 ± 0.26	214 ± 9.15	4.62 ± 0.33
80TS	146 ± 2.03	43.9 ± 0.36	22.6 ± 0.29	206 ± 0.86	4.37 ± 0.04
60TS	143 ± 2.08	36.1 ± 6.47	21.8 ± 1.09	196 ± 7.95	4.08 ± 0.19
40TS	149 ± 1.67	45.1 ± 1.96	21.7 ± 0.12	210 ± 3.99	4.41 ± 0.19
80QS	146 ± 1.79	46.2 ± 3.68	24.0 ± 1.36	212 ± 4.19	4.39 ± 0.11

Fresh Matter (FM), values are expressed as mean ± SEM, n = 3

Growth rates

At the end of the experiment, fish fed with the 60% fenugreek saponin eluate showed the highest relative body mass gain while those fed with the 40% eluate showed the lowest. The control group showed the second best percentage increase followed by the 80QS and the 80TS groups (Table 4). However, none of these results was statistically significant due to high variation within groups and small sample size. Specific growth rate as well as MGR (Fig. 2) showed similar results as the percentage body mass gain with the 60TS group showing the highest growth rates and the TS40 group the lowest. The sequence of the other groups was similar to the sequence in percentage growth (Table 4).

Metabolic rates

The metabolic rate of all groups of fish increased over the first three weeks and that of the 40TS group was highest. From week three till week five the metabolic rate decreased in all groups overall but fish fed 60TS and 80TS showed a slight increase from week four to week five. In week five, the fish fed with *Quillaja* saponins had a significantly lower metabolic rate than the other four groups, including the control (Fig. 3). Overall it appears that fish fed with 60TS and 80TS had the lowest oxygen consumption rates over the experimental period.

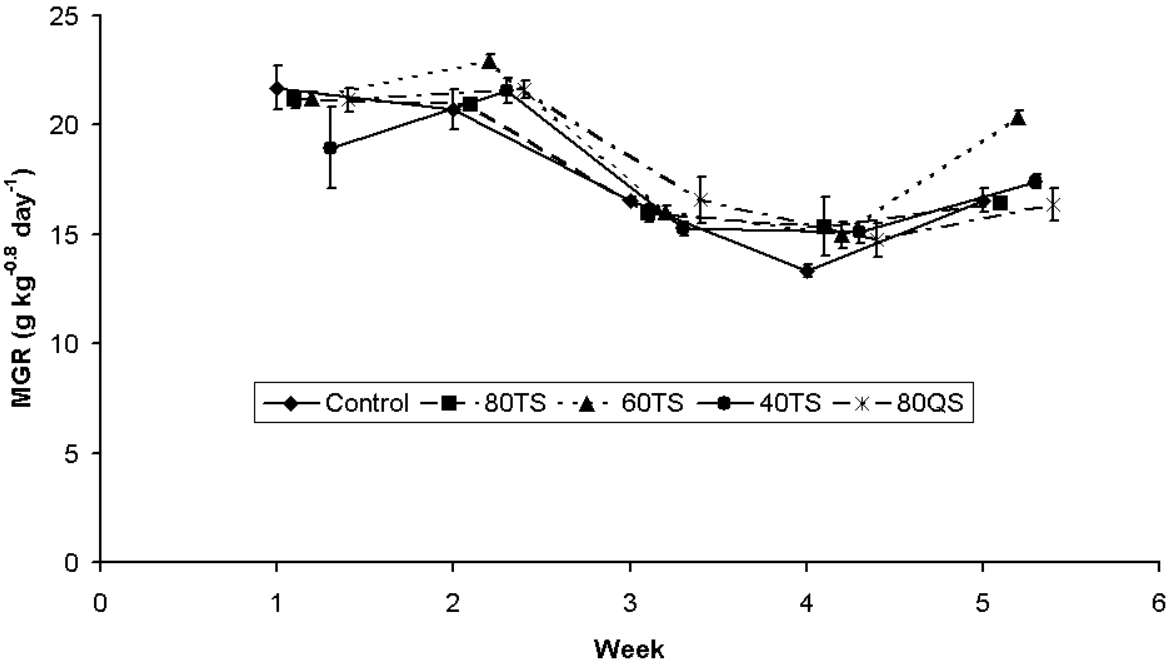

Fig. 2: Average metabolic growth rate of the control and experimental groups. Values are presented as mean ± SEM, n = 3

Feed conversion and protein efficiency

The feed conversion ratio (FCR) was generally good for all groups with values below 1. The best feed conversion was achieved in fish fed the 60% fenugreek methanol eluate of *T. foenum-graecum* (FCR = 0.88) while the highest feed conversion was found in the group fed the 80QS supplemented diet (FCR = 0.94).

Table 4: Growth performance and nutrient utilization.

	Control	80TS	60TS	40TS	80QS
Initial body mass (g)	9.1 ± 0.19	9.3 ± 0.64	9.5 ± 0.18	10.2 ± 0.74	8.9 ± 0.15
Final body mass (g)	38.1 ± 1.86	37.7 ± 3.09	40.9 ± 1.09	39.6 ± 2.31	36.8 ± 1.73
Body mass gain (g)	29.0 ± 1.74	28.5 ± 2.50	31.4 ± 0.98	29.4 ± 1.64	27.9 ± 1.88
Growth (%)	419 ± 13.0	407 ± 9.60	430 ± 7.20	391 ± 8.20	413 ± 21.3
SGR (% day^{-1})	4.09 ± 0.11	4.01 ± 0.08	4.16 ± 0.06	3.89 ± 0.07	4.04 ± 0.18
MGR (g $kg^{-0.8}$ day^{-1})	15.4 ± 0.44	15.1 ± 0.44	15.8 ± 0.23	15.0 ± 0.22	15.2 ± 0.65
FCR	0.91 ± 0.03	0.91 ± 0.03	0.88 ± 0.01	0.92 ± 0.01	0.94 ± 0.06
PER	2.47 ± 0.04	2.36 ± 0.11	2.54 ± 0.05	2.52 ± 0.06	2.42 ± 0.08
PPV (%)	37.1 ± 0.61	35.1 ± 1.68	36.7 ± 0.95	37.3 ± 0.87	35.1 ± 1.37
ALC (%)	43.0 ± 4.90	45.2 ± 1.51	57.6 ± 10.9	46.2 ± 0.71	47.8 ± 5.77
Feed intake (g DM)	26.2 ± 0.64	25.8 ± 1.20	27.4 ± 0.39	27.1 ± 0.97	26.0 ± 0.20

SGR = specific growth rate, MGR = metabolic growth rate, FCR = feed conversion ratio, PER = protein efficiency ratio, PPV = protein productive value, ALC = apparent lipid conversion. Values are expressed as mean ± SEM, n = 3

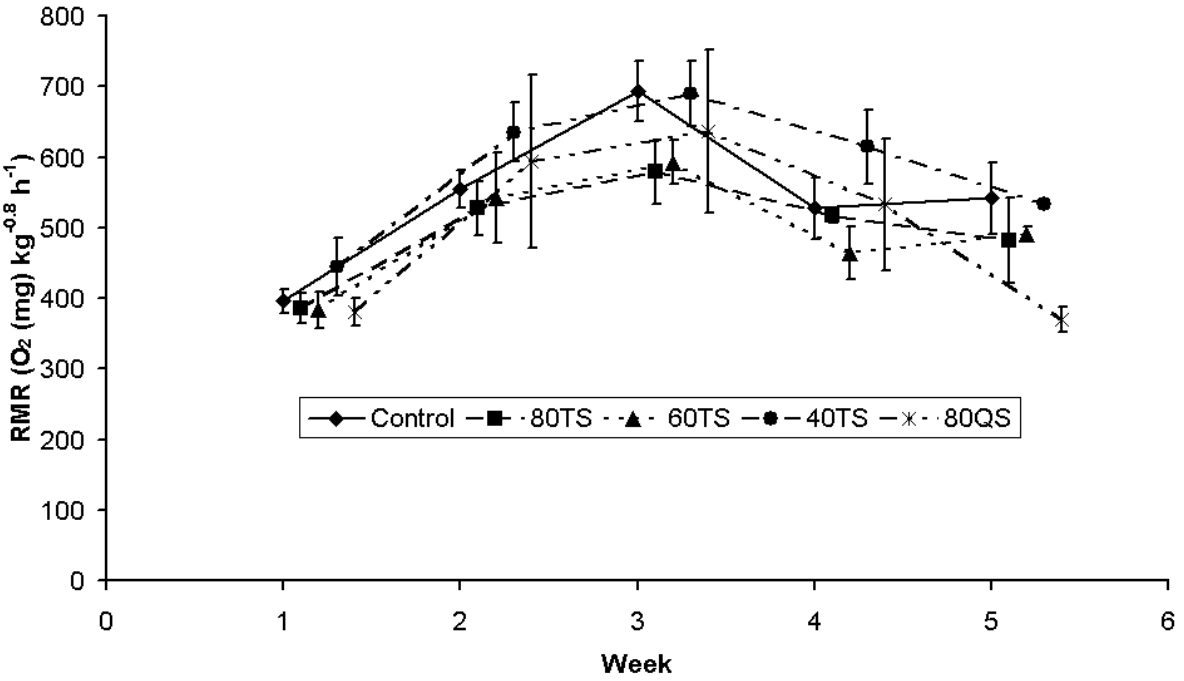

Fig. 3: Metabolic rates over the whole experimental period. Values are presented as mean ± SEM, n = 3, data points are spaced horizontally for better clarity

The protein efficiency ratio (PER) followed the same trend with the 60TS group showing the highest body mass gain per gram protein fed (2.54) followed by the 80TS group and the control. Again the fish fed the diet supplemented with saponins derived from *Q. saponaria* had the lowest value (PER = 2.42) (Table 4).

Energy utilization

The complete energy balance is shown in Table 5. Numerically all saponin supplemented feeding groups had lower energy retentions than the control group.

The 40TS group had the highest energy expenditure and the 60TS group the lowest.

The AUE (%) was highest for the 60TS group and lowest for the 40TS group (Table 5). The 60TS group consumed the lowest amount of oxygen per gram of retained protein while the 40TS group had the highest oxygen consumption per gram protein accretion. The dissipated heat is calculated from the oxygen consumption. Therefore the same trend within the various treatments as observed for oxygen consumption per gram protein accretion was observed for the heat dissipation per gram of protein accretion. However, due to low sample size these results showed no statistically significant differences.

Table 5: Energy balance for all five experimental groups.

	Control	80TS	60TS	40TS	80QS
Initial fish GE (kJ)	30.3 ± 0.62	30.8 ± 2.15	31.7 ± 0.60	33.8 ± 2.47	29.7 ± 0.50
Final fish GE (kJ)	175 ± 5.83	165 ± 15.0	166 ± 5.46	175 ± 12.9	162 ± 7.42
Ingested feed GE (kJ)	545 ± 16.4	535 ± 30.6	569 ± 9.87	563 ± 24.6	539 ± 5.18
ER (kJ)	145 ± 6.45	134 ± 13.0	135 ± 5.13	141 ± 10.6	132 ± 7.81
EE (kJ)	282 ± 13.1	260 ± 8.60	255 ± 4.80	318 ± 29.2	253 ± 31.6
ME (kJ)	427 ± 19.5	394 ± 17.1	390 ± 3.30	459 ± 37.4	385 ± 38.4
AUE (kJ)	118 ± 33.8	141 ± 22.3	179 ± 13.0	103 ± 13.1	154 ± 13.4
ER (% of GE fed)	26.5 ± 1.81	25.1 ± 1.09	23.7 ± 1.02	25.0 ± 1.22	24.5 ± 1.25
EE (% of GE fed)	52.0 ± 3.66	48.9 ± 3.39	44.9 ± 1.43	56.3 ± 2.88	46.9 ± 5.63
ME (% of GE fed)	78.7 ± 5.48	73.9 ± 3.20	68.6 ± 1.78	81.4 ± 3.25	71.3 ± 6.74
AUE (% of GE fed)	21.3 ± 5.48	26.1 ± 3.20	31.4 ± 1.78	18.6 ± 3.25	28.7 ± 6.74
g O_2 g^{-1} CP	4.39 ± 0.31	4.24 ± 0.38	4.02 ± 0.27	4.79 ± 0.24	4.15 ± 0.47
kJ EE g^{-1} CP	65.2 ± 4.66	63.0 ± 5.68	56.9 ± 2.97	71.1 ± 3.60	61.6 ± 7.05

Gross Energy (GE), Energy Retention (ER), Energy Expenditure (EE), Apparently Unutilized Energy (AUE), Metabolizable Energy (ME) and Oxygen Consumption and Energy Expenditure per gram Protein gain. Values are expressed as mean ± SEM, n = 3

Power analysis

The conducted power analysis showed that in all tested parameters the effect sizes obtained were generally high (above 0.4). However, the achieved powers were rather low ranging from 0.14 to 0.28 which leads to high type II error probabilities and therefore high probabilities of accepting a wrong null hypothesis (Table 6).

Table 6: Results of the power analysis (numerator df = 4, denominator df = 10, critical f = 3.478) showing the observed effect size and the statistical power

Parameter	Effect size f	Power
BMG (%)	0.5357	0.229
BMG (G)	0.5635	0.251
SGR (% day^{-1})	0.4777	0.188
MGR (g $kg^{-0.8}$ day^{-1})	0.4336	0.161
FCR	0.4125	0.149
PER	0.3988	0.142
PPV (%)	0.4429	0.166
ACL (%)	0.4955	0.200
OC (g)	0.5838	0.268
OC g^{-1} CP	0.4588	0.176
ME (%)	0.5974	0.279
ER (%)	0.5060	0.207

BMG = body mass gain, SGR = specific growth rate, MGR = metabolic growth rate, FCR = feed conversion ratio, PER = protein efficiency ratio, PPV = protein productive value, ACL = apparent lipid conversion, OC = oxygen consumption, OC g^{-1} CP = oxygen consumption per gram of crude protein accretion, ME = metabolizable energy, ER = energy retention

Discussion

In the past the metabolic efficiency of diets in animal production was frequently enhanced by adding growth promoting substances like synthetic steroid hormones or sub-therapeutic doses of anti-biotics. Most of the applied hormones were androgens but estrogens were also occasionally used (Lone & Matty 1980, Noppe et al. 2008). However, following public concerns about possible adverse health effects and environmental impacts of synthetic hormones and anti-biotics and their subsequent prohibition by the European Union (EC 1831|2003) efforts have been made to find "green" alternatives (Dibner & Richards 2005).

Possible alternatives might be found among the saponins, secondary plant compounds which can be found in at least 29 plant orders of which the Liliales and the Fabales have the most saponin containing plants (Vincken et al. 2007).

Saponins have either a steroidal or triterpenoidal core structure, called sapogenin or aglycone and one or more sugar side chains, glycosidically linked to the sapogenin.

In a previous study on common carp Francis et al. (2002b) demonstrated that saponins derived from *Quillaja saponaria* promoted growth. Carp that had been fed with 150 mg saponin kg^{-1} of feed grew by 372% and attained 18% higher average body mass than fish in the control group. In a second experiment carp fed the same diet and supplementation level with *Quillaja* saponins grew by 443% and attained a 20% higher average body mass and carp fed bi-weekly on a saponin supplemented or control diet did grow by 407% and had at the end of the experiment an average body mass of 10% above that of control fish (Francis et al. 2002c). In our study as compared to Francis et al. (2002b, c), carp which ate a similar basic diet supplemented at the same level but with saponins extracted from *Trigonella foenum-graecum* and eluted with 60% methanol increased their body mass on average by 430% or 27% above the average body mass of the control fish.

The 60TS group also had the lowest feed conversion and the highest protein efficiency ratio although the differences between all groups were small. In contrast to the studies of Francis et al. (2002b, c), the performance of fish whose diet was supplemented with 80% methanol *Quillaja* saponin eluate was poorer compared to control fed fish. Overall, fish fed 40TS showed the lowest performance in most of the parameters. However, none of the results obtained was statistically significant due to low sample numbers. The power analysis revealed that the observed effect sizes for all parameters were high with the smallest one being 0.3988. Due to the low N the observed powers were also low with the highest being 0.279 for the percentage of the metabolizable energy. A low statistical power means high probabilities for a type II error which, in case of ME (%), would be as high as 72.1%.

The difference of the performance of the *Quillaja* saponins in the studies by Francis et al. (2002b, c) and this study could be due to the different compositions in the saponins. It is likely that in the 80% methanol fraction of *Quillaja* other saponins are present and/or the ratios of the different saponins to each other vary compared with those in the commercially available *Quillaja* saponins used by Francis et al. (2002b, c) which contain total saponins extracted from *Quillaja saponaria*.

On the contrary certain evidence exists that the biological action of saponins is not due to the high activity of one single saponin but rather some kind of synergistic effect of saponin mixtures. In rat pituitary cells the highest release (about 22 times higher than the control) of

growth hormone (GH) was measured after stimulation with a crude methanol extract from fenugreek while purified saponins or sapogenins did not reach comparable levels (Shim et al. 2008). Single purified saponins extracted from the desert date (*Balanites aegyptiaca*) a common saponin rich plant from the Middle East did not show anti-diabetic effects while different mixtures of single saponins showed significant anti-diabetic activity (Kamel et al. 1991).

Saponins in animal nutrition are often regarded as growth inhibitors and anti-nutritional factors (Francis et al. 2001, Bureau et al. 1998) but our results and those of Francis *et al.* (2002b, c) show that it would be a generalization to declare all saponins as anti-nutrients.

Regarding soy-saponins Liener (1981) suggested removing saponins from the list of anti-nutrients. However, in his article he was only referring to adverse effects in chicks, rats and mice but not fish.

The reason for this apparent contradiction may be that saponins are a very diverse group of compounds whose biological actions are likely to be as diverse as their structures (Francis et al. 2002b). For instance, there exist at least 38 different saponins in *Q. saponaria* (Bankefors et al. 2008) and at least 19 in *T. foenum-graecum* (Murakami et al. 2000) and only by fractionation and later purification of single saponins can beneficial saponins be distinguished from detrimental ones. Given the possible synergistic effects of saponin mixtures or combinations of different purified saponins as described by Kamel et al. (1991) and Shim et al. (2008), the vast number of possible saponin combinations from plants like *T. foenum-graecum* or *Q. saponaria* calls for high-throughput *in vitro* assays instead of *in vivo* experiments.

Contrary to expectations, no statistically significant differences were found between the control and the saponin fractions treated groups on the one hand or within the treatment groups on the other. On the contrary it appears as if fish fed with the 40% methanol fraction from fenugreek tended to have inferior performance to control while only the 60% methanol eluate from fenugreek showed any potential as a plant derived growth promoter. We must conclude that the application of the tested saponin fractions in the applied concentrations, except for the 60% methanol eluated fenugreek saponin fraction, are not suited as plant derived growth promoters for common carp, *C. carpio*.

Acknowledgements

We thank Dr. Peter Lawrence for a critical reading of the manuscript, Herrmann Baumgärtner and Beatrix Fischer for their help and patience in the laboratory and Christoph Rieckmann for his support with the respirometric system. This work was partly financed by the German Research Foundation (DFG), project number BE 1133/13-1.

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Chapter 7

Evaluation of saponin fractions derived from *Trigonella foenum-graecum* and *Quillaja saponaria* for their effects on growth, nutrient utilization and body composition of Nile tilapia, *Oreochromis niloticus* (L.)

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Abstract

Saponins are generally regarded as anti-nutritional factors in aquaculture diets. However, previous experiments have shown that low dietary levels of saponins derived from *Quillaja saponaria* do have growth promoting effects on common carp and Nile tilapia. Based on these experiments, we conducted an experiment in which we fed eluated saponin fractions from *Q. saponaria* and *Trigonella foenum-graecum* (fenugreek) to Nile tilapia in a respirometric system allowing for continuous measurement of oxygen consumption. Saponins were eluated with consecutive methanol/water concentrations (v/v, 40/60, 60/40, 80/20) resulting in three different eluates for each plant. Fractions chosen were the 80% methanol eluate from *Q. saponaria* (80QS) and all three eluates from *T. foenum-graecum* (40TS, 60TS and 80TS). Three fish each were fed with low levels (150 mg kg^{-1} diet) of saponins in the diet and a control diet without saponins. Growth, feed and nutrient utilization, proximate composition, oxygen consumption and metabolic performance were evaluated.

The fish grew between 224 % (40TS) and 266 % (Control) over the eight week period. Feed conversion ratios were between 0.94 (80TS) and 1.15 (40TS) and protein efficiency ratios between 2.54 (80TS) and 2.16 (40TS). Due to low sample sizes, no statistical differences were found between control fish and saponin fed fish. However, numerically one of the tested saponin fractions (40TS) shows inferior performance.

It is concluded that the tested saponins in the tested concentrations are no potential growth promoter for Nile tilapia. On the contrary, one fraction appears to be a potent growth inhibitor.

Introduction

From 1970 till 2008 the worldwide aquaculture production for food fish has increased at average annual rate of 8.3% (SOFIA 2010). In 2009 approximately every second fish consumed by man was produced by aquaculture (Cressy 2009; Naylor et al. 2009). Although the largest proportion of farmed finfish are cyprinids with around 12 million metric tonnes (mmt) (FAO 2010), tilapia production, especially that of Nile tilapia *Oreochromis niloticus* L., is increasing rapidly. In 2002 1.1 mmt Nile tilapia were produced while in 2008, only six years later, the production had more than doubled to 2.3 mmt (FAO 2010).

Tilapias are considered herbi- and omnivorous but industrially manufactured tilapia feeds still often contain fishmeal as a protein source (New and Wijkström 2002; Furuya et al. 2004). As a general trend, inclusion levels of fishmeal in compound feeds are decreasing (Tacon and Metian 2008). Despite this, the dramatic rise in total fish and shrimp production worldwide means that there is an overall increase in the demand for fishmeal (Péron et al. 2010) to the extent that it is now a limiting resource.

Fish production may be increased by improving feed utilization which, in the past, was achieved by the addition of steroid hormones (androgens and estrogens) to diets (Lone & Matty 1980; Noppe et al. 2008). In the European Union however the use of steroids for animal production has been prohibited since 2006 (EC 1831/2003). Plant derived alternative growth enhancers could be a replacement for steroid hormones. Among the possible candidate substances are saponins, glycosidic compounds with either a steroid or triterpenoid core structure called aglycone or sapogenin. One or more sugar chains are glycosidically linked to the aglycone. Saponins are, because of the high diversity of sugars and aglycones, a very large group of compounds with a wide and variable range of biological activities as reviewed by Francis et al. (2002a). Saponins are mainly produced by various plants as defense against microbial or predatory attacks (Gus-Mayer et al. 1994), but also in some marine invertebrates like starfish (Rio et al. 1965).

In previous studies it has been shown that saponins derived from the South American soap bark tree (*Quillaja saponaria* M.), had a growth promoting effect in common carp, *Cyprinus carpio* (L.) and Nile tilapia, *Oreochromis niloticus* (L.) (Francis et al. 2001a; 2002b, c). Furthermore, a fractionation of saponins from *Q. saponaria* resulted in varying biological activities of *Q. saponaria* saponin fractions in an aromatase inhibition *in vitro* assay (Golan et al. 2008).

Fenugreek (*Trigonella foenum-graecum* L.), commonly cultivated in the Middle East and Asia, is another saponin rich plant (Marker 1947; Skaltsa 2002). It is traditionally used as a medicinal plant and a herb for various dishes and is reported to exhibit antidiabetic and hypocholesterolaemic effects (Al-Habori and Raman 1998). A fenugreek methanol extract and a newly isolated saponin were recently reported to increase secretion of growth hormone in rat pituitary cells in vitro (Shim et al. 2008).

We conducted an eight week experiment in a computer-controlled respirometric system (Focken et al. 1994) to evaluate the effects of low supplementation levels of saponin fractions derived from *T. foenum-graecum* and *Q. saponaria* in the diets on proximate composition, growth, oxygen consumption and metabolic performance of Nile tilapia, *Oreochromis niloticus* (L.).

Material and Methods

Saponin extraction

Saponins were extracted from fenugreek seeds (*T. foenum-graecum* L.) seeds and *Quillaja saponaria* bark generally according to Marston and Oleszek (2000). Ethanol extracts were fractionated by flash chromatography (CombiFlash RETRIEVE, Teledyne Isco, Lincoln, NE) and consecutive methanol/water solutions (v/v, 40/60, 60/40, 80/20) resulting in three saponin eluates or fractions (40, 60 and 80%). All three fractions of fenugreek and the 80% methanol fraction from *Q. saponaria* were used for this experiment.

Table 1: Ingredients of the basal and experimental diets and chemical composition of the standard diet

	Diet				
Ingredient	Control	40TS	60TS	80TS	80QS
Trigonella 40% Fraction (mg kg^{-1})		150			
Trigonella 60% Fraction (mg kg^{-1})			150		
Trigonella 80% Fraction (mg kg^{-1})				150	
Quillaja 80% Fraction (mg kg^{-1})					150
Fish meal[a] (g kg^{-1})	500	500	500	500	500
Whole wheat meal (g kg^{-1})	420	420	420	420	420
Sunflower oil (g kg^{-1})	40	40	40	40	40
Vitamin premix[b] (g kg^{-1})	20	20	20	20	20
Mineral premix[b] (g kg^{-1})	20	20	20	20	20
Standard diet	DM (%)	CA (g kg^{-1} DM)	CP (g kg^{-1} DM)	CL (g kg^{-1} DM)	GE (kJ g^{-1} DM)
	93.2	119	419	126	20.8

DM = dry matter, CA = crude ash, CP = crude protein, CL = crude lipids, GE = gross energy
[a]Brown fish meal „Seelöwe“ obtained from Vereinigte Fischmehlwerke Cuxhaven VFC, Germany.
[b]Prepared after Gaye-Siessegger et al. (2004).

Experimental Diets

Based on a standard diet, which also served as a control diet, four experimental feeds containing saponins were prepared. The ingredients, proximate composition and ingredients of the diets are shown in Table 1. The 80% methanol fraction derived from *Quillaja saponaria* and all three fractions from *Trigonella foenum-graecum* were added at a concentration of 150 ppm (150 mg kg^{-1} feed). The saponins where dissolved in water and if necessary the dissolution was reinforced with short ultra-sound treatments. The saponin solutions were added under continuous stirring to the feed mash. The resulting diets were named according to their supplementation, C (control), 40TS, 60TS and 80TS for the three diets supplemented with fenugreek saponin fractions and 80QS for the *Q. saponaria* saponin fraction. The feed mash was made into pellets (2 mm diameter) using a domestic food processor with meat mincing attachment (Bosch Comfort plus). The pellets were dried in a compartment dryer at 40 °C for 36 hours and stored at 6 °C until needed.

Experimental set-up, sampling and chemical analysis

A total of 20 mixed sex *Oreochromis niloticus* (Manzala strain, obtained from the Georg-August-University Göttingen, Department of Aquaculture and Ecology) were divided into 6 groups, 5 experimental groups each containing three animals and one initial group, containing five animals. The body mass at the beginning of the experiment was 37.8 ± 8.7 g (average ± SD). Due to the high variance in the initial fish size, the fish groups were chosen in a way that the variance inside the groups was high but between the groups there were no significant differences (ANOVA, $p < 0.05$).

The initial group was anesthetized by immersion in water containing 250 mg L^{-1} MS 222 (Tricaine Methane-Sulfonate) and the fish were killed through decapitation while still unconscious.

The 15 experimental fish were stocked individually into the 15 boxes of a respirometric system with a volume of 15 liters each (Focken et al. 1994). The water temperature was kept at 27 ± 1 °C and the light regime was set to 12 h light and 12 h dark. The five different diets (Control, 40TS, 60TS, 80TS and 80QS) were randomly assigned in three replicates to the chambers. The feed was applied at three times energy maintenance requirement (10.5 g $kg^{-0.8}$ d^{-1}, Meyer-Burgdorff et al. 1989) per day, divided into three feeding stations. Feed was applied via automatic feeders (Rondomatic 400, Grässlin GmbH, St. Georgen, Germany) at 9:00, 13:00 and 17:00. Once every week, all fish were weighed to the nearest 0.01 g, respirometric-chambers cleaned and amount of feed adjusted according to the new body mass. While the chambers were cleaned, the respective fish were kept in 15 L buckets with well aerated water. After stocking at the start of the experiment and before each weighing fish were starved for 24 hours.

Every day approximately 5-10% of the total water volume was exchanged, total ammonia-N was kept below 0.2 mg L^{-1}, total nitrite-N was kept below 0.15 mg L^{-1} and total nitrate-N was kept below 50 mg L^{-1}. The flow rates in the respirometric boxes were adjusted to 0.5 L min^{-1}.

At the end of the 8 week feeding experiment all fish from the respirometric system were killed and fish carcasses were stored in a -20 °C freezer until further analysis.

For the determination of the proximate composition the fish were cut into pieces and autoclaved for 45 minutes at 121 °C before homogenization with an Ultra-turrax T25 (IKA Labortechnik, Staufen, Germany). All fish were analyzed individually. Homogenate was refrozen at -20 °C before freeze drying (SRK Systemtechnik, Riedstadt, Germany). Proximate chemical analysis was conducted according to AOAC methods (1990).

Briefly, dry matter was determined by drying the material to constant weight for at least 8 hours at 105 °C. For crude protein (CP) determination the macro Kjeldahl (Büchi B 324 + 719 S Titrino, Büchi Labortechnik GmbH, Essen, Deutschland) method with a conversion factor of CP = N*6.25 was used, crude lipids were determined by the Soxhlet method using petrol ether as solvent, gross energy was determined by bomb calorimetry (IKA C 7000, IKA Labortechnik, Staufen, Germany) and ash content by ashing over night in a muffle oven at 500 °C (Nabertherm N11, Nabertherm, Lilienthal, Germany).

Calculations

The following parameters were calculated as shown:

Metabolic Body Mass (MBM ($kg^{0.8}$)	(Live body mass (g) / 1000)$^{0.8}$
Metabolic Growth Rate (MGR (g $kg^{-0.8}$ day^{-1})	Live body mass gain (g) / average metabolic live body mass ($kg^{0.8}$) / experimental period (Dabrowski et al. 1986)
Specific Growth Rate (SGR (% day^{-1}))	100 x [(ln final mass - ln initial mass) / days of experiment]
Routine Metabolic Rate (RMR)	mean oxygen consumption in 24 h (mg) / metabolic body mass ($kg^{0.8}$) x 24
Energy Expenditure (EE (kJ))	Oxygen uptake (g) x 14.86 (kJ g^{-1} O_2, Huisman 1976)
Energy Retention (ER (kJ))	Final gross energy (kJ) of fish – initial gross energy (kJ) of fish
Metabolizable Energy (ME), (kJ)	ER (kJ) + EE (kJ)
EE (% of GE fed)	EE (kJ) x 100 / Feed energy intake (kJ)
ER (% of GE fed)	ER (kJ) x 100 / Feed energy intake (kJ)
ME (% of GE fed)	ER (kJ) + EE (kJ) x 100 / Feed energy intake (kJ)
AUE (% of GE fed)	100 - EE (%) - ER(%)
O_2 consumption (g) / protein gain (g)	Total oxygen consumption (g) / total protein gain (g)
EE (kJ) / protein gain (g)	Total EE (kJ) / total protein gain (g)
Protein Efficiency Ratio (PER)	Live body mass gain (g) / feed protein intake (g)
Protein Productive Value (PPV (%))	Total protein gain (g) x 100 / total protein fed (g)
Apparent lipid conversion (%)	Total lipid gain (g) x 100 / total lipid fed (g)
Feed Conversion Ratio (FCR)	Feed consumption (dry matter) / live body mass gain (g)

Statistical analysis

All data are shown as mean ± standard deviation (SD) if not stated otherwise. All statistical analyses were performed using SPSS 10.0 (IBM SPSS, Chicago, IL, USA). A Levene's test was used to test for homogeneity of variance and a one-way ANOVA was used to test for differences between groups at a significance level of $p = 0.05$.

Due to the low sample sizes a statistical power analysis was conducted using G*Power vers. 3.1.2 (Faul et al. 2007). Post-hoc power analyses were conducted using the ANOVA: fixed effects, omnibus, one-way procedure of the F-test family to determine the achieved statistical power and the observed effect size. The results of the power analysis are shown in table 5.

Results

All the diets were accepted by the fish equally well and eaten during the first two minutes after provision of the pellets. No differences in behavior between the fish of the different treatments were observed.

Although, over the time of the experiment, relative amount of protein and lipids increased and ash decreased in all groups, no statistical differences were found between either the different groups for proximate composition at the end of the experiment or between the initial group and the experimental groups (Table 2).

Table 2: Initial and final proximate chemical analysis of control and experimental fish on fresh matter basis.

	Initial group	Control	40TS	60TS	80TS	80QS
Crude protein (g kg^{-1})	159 ± 4.35	163 ± 6.88	165 ± 3.62	160 ± 6.76	163 ± 3.47	164 ± 5.27
Crude lipids (g kg^{-1})	62.2 ± 8.63	67.4 ± 2.20	67.2 ± 10.1	61.9 ± 9.61	70.2 ± 5.55	67.4 ± 11.4
Crude ash (g kg^{-1})	53.9 ± 2.78	46.5 ± 2.06	47.6 ± 0.97	45.9 ± 5.13	47.6 ± 2.56	45.7 ± 0.11
Dry matter (g kg^{-1})	283 ± 14.2	283 ± 7.14	286 ± 10.2	273 ± 16.7	287 ± 8.35	283 ± 14.9
Gross energy (kJ g^{-1})	6.40 ± 0.62	6.54 ± 0.06	6.51 ± 0.43	6.23 ± 0.45	6.63 ± 0.29	6.54 ± 0.45

values are expressed as mean ± SD, n = 3

No statistical differences were found between the different treatments for growth, neither relative growth nor absolute growth. However, there were numerical differences which might have been more pronounced given a higher sample size. The best growth was observed in the control group closely followed by 80TS. The lowest growth was observed for fish fed 40TS (Table 3). Basically the same result (numerically but not statistically different) was found for the evaluated feed utilization parameters where 40TS shows the lowest performance and control and 80TS fed fish showed the highest performance (Table 3).

Table 3: Growth performance, feed conversion and nutrient utilization of the tilapia.

	Control	40TS	60TS	80TS	80QS
Initial body mass (g)	31.3 ± 8.25	39.0 ± 3.86	33.4 ± 8.21	35.8 ± 8.37	35.1 ± 2.52
Final body mass (g)	81.9 ± 14.5	86.3 ± 9.88	82.0 ± 5.13	92.5 ± 18.2	89.6 ± 14.0
Body mass gain (g)	50.7 ± 6.29	47.3 ± 11.9	48.6 ± 4.11	56.8 ± 10.2	54.5 ± 12.3
Growth (%)	266 ± 21.6	224 ± 41.4	253 ± 43.4	260 ± 15.0	255 ± 30.6
SGR (% day^{-1})	1.74 ± 0.15	1.42 ± 0.33	1.64 ± 0.32	1.71 ± 0.10	1.66 ± 0.22
MGR (g $kg^{-0.8}$ day^{-1})	7.01 ± 0.34	5.82 ± 1.44	6.60 ± 1.21	7.05 ± 0.36	6.85 ± 1.06
Feed conversion ratio	0.96 ± 0.04	1.15 ± 0.30	1.03 ± 0.20	0.94 ± 0.05	0.98 ± 0.15
Protein efficiency ratio	2.49 ± 0.11	2.16 ± 0.55	2.36 ± 0.42	2.54 ± 0.13	2.47 ± 0.37
Protein productive value (%)	41.4 ± 4.40	36.8 ± 9.40	38.0 ± 4.14	42.1 ± 0.61	41.3 ± 3.92
Apparent lipid convers. (%)	62.1 ± 4.56	56.2 ± 25.0	52.2 ± 17.2	67.2 ± 7.44	61.6 ± 14.6
Feed intake (g DM)	48.7 ± 8.23	52.2 ± 2.71	49.7 ± 5.80	53.3 ± 9.02	52.3 ± 5.05

Values are expressed as mean ± SD, n = 3

Although no significant differences were found, generally the control showed the lowest metabolic rates, followed by fish fed with 40TS while fish fed with 60TS and 80TS had the highest metabolic rates (Figure 1). A similar result was obtained for the oxygen consumption per gram of protein gained. Control fed fish consumed numerically least oxygen per gram of

protein accretion while fish fed 60TS had the highest oxygen consumption per gram protein gained.

Energy retention was highest for fish fed 80TS which in combination with a relatively low energy expenditure resulted, numerically, in the highest metabolizable energy. Energy retention and energy expenditure were numerically lowest in the 40TS group resulting in the lowest metabolizable energy (Table 4).

Table 4: Energy balance for all five groups.

	Control	40TS	60TS	80TS	80QS
Initial fish GE (kJ)	200 ± 52.7	249 ± 24.6	213 ± 52.5	228 ± 53.5	224 ± 16.1
Final fish GE (kJ)	535 ± 89.5	564 ± 94.1	512 ± 63.5	616 ± 143	587 ± 107
Ingested feed GE (kJ)	1010 ± 171	1084 ± 56.2	1033 ± 120	1106 ± 187	1085 ± 105
ER (kJ)	336 ± 36.8	314 ± 111	299 ± 45.6	388 ± 90.8	363 ± 92.5
EE (kJ)	390 ± 102	404 ± 30.8	455 ± 176	466 ± 107	455 ± 90.0
ME (kJ)	725 ± 138	718 ± 127	754 ± 173	853 ± 163	818 ± 182
AUE (kJ)	285 ± 47.2	366 ± 145	279 ± 52.6	253 ± 97.5	267 ± 78.7
ER (% of GE fed)	33.5 ± 1.89	29.0 ± 10.5	29.2 ± 5.22	34.8 ± 2.71	33.1 ± 5.52
EE (% of GE fed)	38.2 ± 4.68	37.4 ± 4.50	43.2 ± 11.7	42.5 ± 8.84	41.6 ± 4.44
ME (% of GE fed)	71.7 ± 3.57	66.4 ± 12.9	72.4 ± 8.27	77.2 ± 7.20	74.8 ± 9.95
AUE (% of GE fed)	28.3 ± 3.57	33.6 ± 12.9	27.6 ± 8.27	22.8 ± 7.20	25.2 ± 9.95
Cons. O_2 (g) / protein gain (g)	3.12 ± 0.66	3.55 ± 1.10	3.90 ± 1.53	3.37 ± 0.74	3.36 ± 0.18

Gross Energy (GE), Energy Retention (ER), Energy Expenditure (EE), Apparently Unutilized Energy (AUE), Metabolizable Energy (ME) and Oxygen Consumption per gram Protein gain. Values are expressed as mean ± SD, n = 3

The power analysis revealed medium to high effect sizes (generally an effect size of 0.1 is considered as small, 0.25 as medium and >0.4 as large) ranging from 0.31 to 0.48 however the statistical powers were very low, ranging from 0.1 to 0.19 (Table 5).

Discussion

In the past saponins were often described as anti nutritional factors present in many plant derived protein sources (Francis et al. 2001b). Soy bean is the main plant protein source used in commercial aquaculture diets and is rich in saponins unless they are specially treated. Bureau et al. (1998) showed that alcohol extracts derived from soybean have severe detrimental effects on feed intake and subsequent growth of chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*O. mykiss*).

Only a few studies have been published in which small inclusion levels of saponins were used as a growth promoter in fish. It has been shown that inclusion levels of 150 mg *Quillaja saponaria* saponin kg^{-1} diet had several beneficial effects, including higher body mass gain, improved feed conversion, protein utilization and energy retention in common carp, *Cyprinus carpio* (Francis et al. 2002b, c). In Nile tilapia similar effects were observed at inclusion levels of 150 and 300 mg *Q. saponaria* saponin kg^{-1} diet (Francis et al. 2001b). Several differences between the experimental designs might influence the various results. Saponins

Table 5: Results of the power analysis (numerator df = 4, denominator df = 10, critical f = 3.478) showing the observed effect size and the statistical power

	Effect size	Power
BMG (%)	0.4799	0.189
BMG (g)	0.4122	0.149
SGR (% day^{-1})	0.4696	0.183
MGR (g $kg^{-0.8}$ day^{-1})	0.4704	0.1830
FCR	0.4424	0.1657
PER	0.4144	0.150
PPV (%)	0.4195	0.153
ALC	0.3687	0.127
O_2	0.3182	0.105
EE/CP	0.3062	0.101
ER (%)	0.4216	0.154
ME (%)	0.4650	0.180

Body mass gain (BMG), specific growth rate (SGR), metabolic growth rate (MGR), feed conversion ratio (FCR), protein efficiency ratio (PER), protein productive value (PPV), apparent lipid conversion (ALC), oxygen consumption (O^2), energy expenditure per gram retained crude protein (EE/CP), energy retention (ER), metabolizable energy (ME)

derived from *Q. saponaria* are generally triterpenoid while saponins from *T. foenum-graecum* are mainly steroidal (Marker et al. 1947; Cheeke 2000). Furthermore in all three experiments by Francis et al. (2001, 2002b, c) a crude saponin mixture was used and up to now 38 different saponins have been described in *Q. saponaria* (Guo and Kenne 2000; Bankefors et al. 2008).

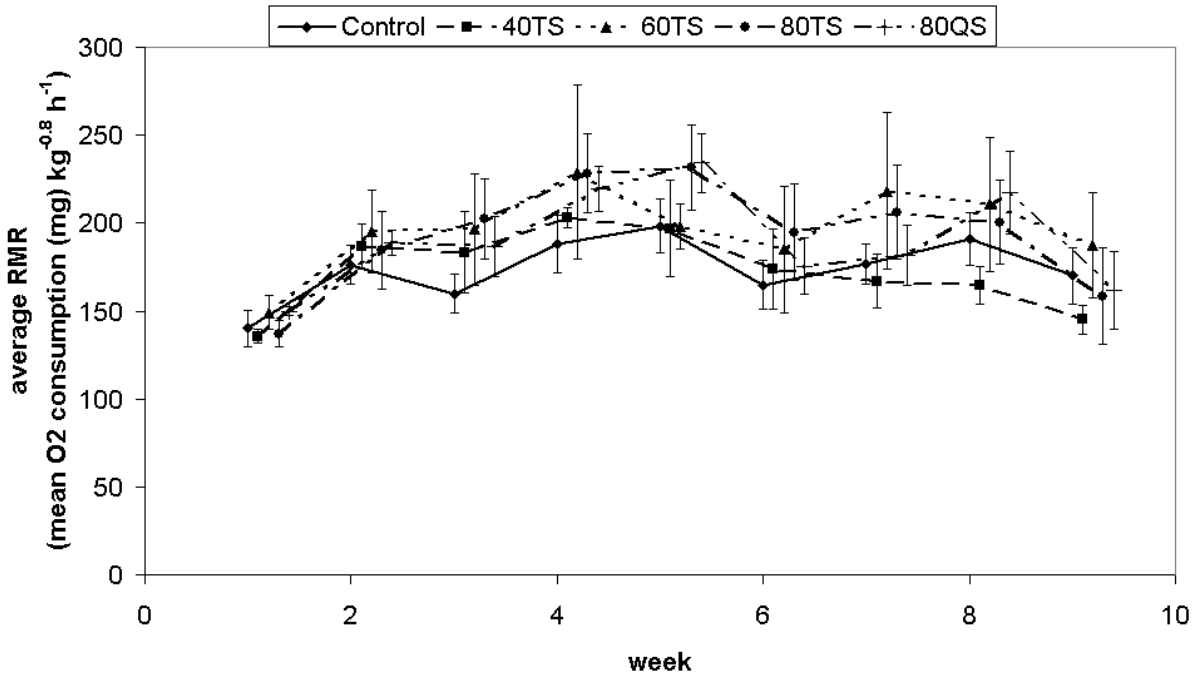

Figure 1: Average routine metabolic rates of the different treatments. Values are expressed as mean ± SEM, n = 3

In our experiment the saponins were consecutively fractionated by HPLC eluation with increasing methanol concentrations and different eluates were fed to the fish.
In other studies fractionation of crude saponins has resulted in different results. In one study saponins derived from the desert date, *Balanites aegyptiaca*, were evaluated for their antidiabetic activity in streptozotocin induced diabetic mice. Some saponins did not yield a result when applied individually but showed significant antidiabetic activity when applied in combination with one or more other saponins (Kamel et al. 1991). In an *in vitro* experiment it has been shown that a crude methanol extract of *T. foenum-graecum* and one individual saponin derived from that extract had the highest effect on GH secretion of rat pituitary cells while other saponin fractions and individual saponins showed lower activity compared to control (Shim et al. 2008). Furthermore saponins derived from *Quillaja saponaria* and fractionated in the same way as in our experiment were tested in an *in vitro* bio assay for their effect on aromatase and the 80% methanol eluate showed the highest enzymatic inhibition while the 40 and 60% eluates showed lower inhibitions (Golan et al. 2008). A fractionation seems sometimes to improve the biological activity while in other cases the opposite is the case.
The results of this experiment do not support a potential application of the tested saponin fractions in the applied concentrations from *T. foenum-graecum* and *Q. saponaria* as growth promoters for Nile tilapia. We did not observe increased growth rates or body mass gains for the saponin fed groups, nor did the saponin fractions improve the feed conversion, nutrient utilization or oxygen consumption compared to the control group. Numerically the 80TS fed fish were similar or slightly superior to the control fed fish in terms of growth, feed conversion, nutrient utilization and oxygen consumption while the other saponin fed fish were numerically inferior which points to the different saponin fractions having different effects. While the 80TS fraction had no effect or even slightly beneficial effects on tilapia performance, the 40TS eluate seems to have acted act as an anti-nutrient and the 60TS and 80QS eluates appear to be neither beneficial nor detrimental for Nile tilapia. Because of the restrictions in sample size inherent in our experimental system, we performed a statistical power analysis. The statistical power is defined as $1-\beta$ where β is the probability of a type II or false negative error i.e. the erroneous rejection of the null hypothesis. Given the low powers observed in our experiment, ranging from 0.1 to 0.19 and the relatively high effect sizes, it is therefore likely that the tested saponins may well have effects on the evaluated parameters which have not been recognized due to the low sample sizes. Based on our present results however, we must conclude that the tested saponin eluates in the tested concentrations are not useful as growth promoters for Nile tilapia. The data even suggest that saponin fraction 40TS might reveal itself as a potent anti-nutrient if tested in an experiment with higher sample sizes. The apparently conflicting underlying physiological actions of these saponin fractions is obscure, so further research should include determination of their effects on blood and endocrine parameters as well as their effects on nutrient digestion.

Acknowledgments

This work was partly financed by the German Research Foundation DFG under the project BE 1133/13-2. The authors' whish to thank Ms. Beatrix Fischer and Mr. Herrmann Baumgärtner for their efforts and help in the analytical work and Dr. Peter Lawrence and Ms. Sabine Nugent for critically reading the manuscript.

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Chapter 8

Effects of saponin fractions from *Trigonella foenum-graecum* and *Balanites aegyptiaca* on gene expression of GH, IGF-1 and their respective receptors, growth, nutrient utilization, body composition, oxygen consumption and plasma IGF-1 in Nile tilapia, *Oreochromis niloticus* (L.)

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Proceedings of the 9th International Symposium on Tilapia in Aquaculture, pp 245-259, Shanghai 2011, China. Eds: Liping L. and Fitzsimmons K.

ISBN: 978-1-888807-19-6

Abstract

Saponins in aquaculture feedstuffs are generally considered anti-nutritional. However, in several experiments it was shown that low level (150 ppm) supplementation with saponins from *Quillaja saponaria*, the South American soap bark tree, yielded several beneficial effects. Among them were improved growth rates, feed conversion efficiency, protein utilization and reduced oxygen consumption per unit body mass gain in common carp (*Cyprinus carpio*). In Nile tilapia, *Oreochromis niloticus*, supplementation levels of 300 ppm showed similar beneficial effects as the 150 ppm inclusion in carp diets.

Based on the above mentioned results an experiment was conducted in which saponin fractions (eluated with 60% or 80% methanol) from fenugreek (*Trigonella foenum-graecum* L.) and a methanol extract from the Egyptian desert date (*Balanites aegyptiaca* L.) were fed at different concentrations to 15 individually stocked Nile tilapia (19.1 ± 0.6 g, mean ± SD) in a respirometer system. Five treatments, namely a control diet (no saponin), three fenugreek saponin diets and one desert date saponin diet were fed to three replicates each.

Every week the fish were weighed and feed allowance was calculated accordingly. At the end of the eight week experiment the fish were anaesthetized and killed. IGF-1 levels in plasma were determined using a radio-immuno-assay, expression of genes encoding for IGF-1, GH and their receptors were determined using semi-quantitative reverse transcriptase real time PCR and proximate composition determined.

Fish fed with 60% *T. foenum-graecum* saponins at a concentration of 300 mg kg^{-1} showed the highest performance. Their expression levels of GH and IGF-1 genes were highest followed by control. The other groups had a significantly lower expression of GH and IGF-1. These results were also reflected in the numerically best growth and feed utilization parameters and the lowest oxygen consumption.

On the contrary, all other saponin supplementations resulted in reduced performance with considerably higher oxygen consumptions for fish fed 600 mg kg^{-1} 60% fenugreek saponins.

Results of gene expression levels strongly correlated with other performance parameters.

The obtained results suggest that the 60% MeOH eluated *Trigonella foenum-graecum* saponin fraction has a potential as natural growth promoter depending on applied concentration.

Introduction

With the rapidly growing aquaculture industry the demand for high quality fish feeds is increasing. At current and expected aquaculture growth rates the demand will eventually outgrow the availability of fish meal as highly digestible protein source (Hardy 2010). As a consequence the inclusion levels of plant derived proteins are increasing for formulated fish and crustacean feeds. However these plant derived ingredients have, in comparison to fish meal, a serious drawback since they always contain one or more anti-nutrients like protease inhibitors, lectins, gossypol, phytic acid, tannins or saponins (Francis *et al*. 2001a).

While saponins are generally considered as anti-nutrient, it has been shown in several experiments that low concentrations of *Quillaja saponaria* (South American soap-bark tree) saponins in the diet are improving the oxygen consumption, nutrient utilization and growth performance of carp, *Cyprinus carpio* and Nile tilapia, *Oreochromis niloticus*, respectively. When fed to common carp (*Cyprinus carpio*) at 150 mg kg^{-1} in the diet, *Quillaja saponaria*

saponin supplementation resulted in significantly increased final body mass, reduced the oxygen uptake per unit body mass gain and improved the protein and energy utilizations compared to the control (Francis *et al*. 2002a, b). Nile tilapia fed with 300 ppm *Q. saponaria* saponins in their diet showed a significantly higher body mass gain and increased energy retention when compared to the control fish (Francis *et al*. 2001b).

Saponins are glycosidic compounds mainly produced by plants that are often activated after tissue damage and act for instance as antimicrobial defense substances (Gus-Mayer *et al.* 1994). Saponins can be found in a great variety of different plants, including many cultured plants like soybean which is the most common plant protein source for aquaculture. Some marine invertebrates like starfish also produce saponins most likely as a chemical defense against predators (Rio *et al.* 1965).

Saponins consist of a steroidal or triterpenoidal core structure called aglycone or sapogenin and one or more sugar side chains. Due to the large variations in either the aglycone or the sugar moiety they produce very diverse biological effects in animals. A detailed review of the biological actions of saponins is given by Francis *et al.* (2002c).

Saponin fractions were derived from two different plants, one being Fenugreek, *Trigonella foenum-graecum*, the other one being the Egyptian desert date, *Balanites aegyptiaca*. Both are frequently occurring and cultivated in the Middle East and are rich in different saponins (Marker *et al*. 1947, Dawidar and Fayez 1969, Hosny *et al*. 1992, Kamel 1998, Murakami *et al.* 2000). Both plants are commonly used in traditional folk medicine in the Middle East. Ethanol extract of *T. foenum-graecum* was considered an excellent alternative to a well known anti-diabetic drug as tested in artificially induced diabetic rats (Eidi *et al*. 2007). Diosgenin, a sapogenin present in fenugreek, did stimulate ion transport in human cortical neuronal cells (Wang *et al.* 2006).

The desert date is traditionally used as an anti-diabetic drug in folk medicine in Egypt and other parts of northern Africa and the Middle East. Kamel *et al.* (1991) were able to show that aqueous extracts of the desert date mesocarps and its fractions reduced the blood glucose levels significantly. A totally different application of the desert date was demonstrated by Chapagain *et al*. (2008) who used saponins extracted from a root derived callus as a larvicidal agent against the mosquito *Aedes aegypti*, the major vector for dengue fever and dengue hemorrhagic fever.

The saponins were added in low concentrations to the diets of Nile tilapia. To test whether saponin fractions derived from Fenugreek and the desert date yield similar results as obtained for carp and tilapia by Francis et al. (2001b, 2002a, b), an eight week feeding experiment with Nile tilapia was conducted. Based on previous trials two eluates from fenugreek (one in two concentrations) and a methanol extract from the desert date were chosen and tested for their effects on gene expression of GH, IGF-1 and their receptors, IGF-1 plasma levels, growth performance, oxygen consumption, nutrient utilization and chemical composition.

Material and methods

Experimental set-up

A total of 20 male Nile tilapia, *O. niloticus*, with a body mass of 19.0 ± 0.5 g (mean ± SD) were divided in two groups. At the start of the experiment five fish were killed by a sharp

blow on the head and immediately frozen at -20°C for subsequent analysis of chemical composition. The fish were obtained from the University of Göttingen, Department of Aquaculture and Water Ecology.

The other fifteen fish were individually stocked in 12-L chambers of a fully computer controlled respirometric system (Focken *et al.* 1994).

The flow rates were adjusted to 0.3 L min^{-1} and the temperature was kept at 27°C. The light cycle was set to 12/12 light/dark and water quality was analyzed once per week. Once weekly the fish were weighed to the nearest 0.1 g and the feed ration adjusted accordingly. After the individual weighing the fish were kept for 5 to 10 minutes in a bucket with well aerated water while the respective respirometer chamber was cleaned. The feed rations were calculated as four times (14 g $kg^{-0.8}$ day^{-1}) the daily energy maintenance requirement (3.5 g $kg^{-0.8}$ day^{-1}) on metabolic body mass basis.

A standard diet was prepared according to Table 1 which also served as control diet. The different saponin fractions were added to the standard diet in different concentrations (Table 1) resulting in four saponin supplemented diets termed according to the included fraction and its concentration, for example 60TS600 refers to the 60% methanol extracted *Trigonella* saponin eluate or fraction included at 600 ppm while BA stands for *Balanites* saponin. The five diets were randomly assigned to the 15 chambers in triplicates.

Table 1: Ingredients of the basal and experimental diets and chemical composition of the standard diet

	Diet				
Ingredient	Control	60TS300	60TS600	80TS300	80BA300
Trigonella 60% Fraction (mg kg^{-1})		300	600		
Trigonella 80% Fraction (mg kg^{-1})				300	
Balanites 80% Fraction (mg kg^{-1})					300
Fish meal[a] (g kg^{-1})	500	500	500	500	500
Whole wheat meal (g kg^{-1})	420	420	420	420	420
Sunflower oil (g kg^{-1})	40	40	40	40	40
Vitamin premix[b] (g kg^{-1})	20	20	20	20	20
Mineral premix[b] (g kg^{-1})	20	20	20	20	20
Standard diet	DM (%)	CA (g kg^{-1} DM)	CP (g kg^{-1} DM)	CL (g kg^{-1} DM)	GE (kJ g^{-1} DM)
	93.2	119	419	126	20.8

DM = dry matter, CA = crude ash, CP = crude protein, CL = crude lipids, GE = gross energy

[a]Norwegian fish meal obtained from Wuerttembergische Zentralgenossenschaft, Germany.

[b]Prepared after Gaye-Siessegger *et al.* (2004).

At the end of the eight week feeding period all experimental fish were anaesthetized with 200 ppm MS 222, weighed, blood drawn from the caudal vein and killed with a sharp blow to the head. Afterwards, brain, liver and muscle samples were taken and stored on liquid nitrogen for later gene expression analysis while the carcasses were kept at -20°C for later proximate composition analysis. For the chemical analysis the fish were chopped while still frozen, autoclaved for 30 minutes at 120°C, homogenized with an Ultra-Turrax T25 (IKA-Labortechnik, Staufen, Germany), refrozen and freeze dried. Water content was calculated by difference from body mass at slaughter and dry matter mass after freeze drying. Basically the

chemical analysis was conducted according to AOAC (1990) on each individual fish. In brief, dry matter was determined by drying over night to constant mass at 105°C, ash was determined by ashing over night at 500°C, crude lipid (CL) was determined by a modified Smedes method (Smedes 1999, Schlechtriem *et al*. 2003). Crude protein (CP) was determined using a C/N-analyzer (C/N VarioMAX, Elementar Analysensysteme GmbH, Germany) and N x 6.25 = CP. Gross energy was determined using a bomb calorimeter (IKA C 700, IKA Labortechnik, Staufen, Germany) and benzoic acid as standard.
Saponins were extracted from fenugreek seeds (*T. foenum-graecum* L.) seeds generally according to Marston and Oleszek (2000). Ethanol extracts were fractionated using a reversed phase HPLC and different consecutive methanol/water solutions (v/v, 40/60, 60/40, 80/20) resulting in three saponin eluates or fractions (40, 60 and 80%) of which the 40% eluate was discarded. An 80% methanol extract of *Balanites aegyptiaca* was produced by grinding 5 g of seeds to a fine powder and mixing with 80% methanol over night. Afterwards the extract was centrifuged (5 minutes at 2400 g) and the supernatant collected and evaporated in a rotary evaporator at 40°C. After another MeOH washing step with 80% MeOH and subsequent centrifugation the extract was washed with 10 ml butanol, centrifuged, butanol phase incubated over night at 6°C and next morning evaporated at 45°C. The material was dissolved in aqua dest. and freeze dried before use.

Calculations
The following parameters were calculated as shown:

Metabolic Body Mass (MBM ($kg^{0.8}$)	(Live body mass (g) / 1000)$^{0.8}$
Metabolic Growth Rate (MGR (g $kg^{-0.8}$ day^{-1})	Live body mass gain (g) / average metabolic live body mass ($kg^{0.8}$) / experimental period (Dabrowski *et al*. 1986)
Specific Growth Rate (SGR (% day^{-1}))	100 x [(ln final mass - ln initial mass) / days of experiment]
Routine Metabolic Rate (RMR)	mean oxygen consumption in 24 h (mg) / metabolic body mass ($kg^{0.8}$) x 24
Energy Expenditure (EE (kJ))	Oxygen uptake (g) x 14.86 (kJ g^{-1} O_2, Huisman 1976)
Energy Retention (ER (kJ))	Final gross energy (kJ) of fish – initial gross energy (kJ) of fish
Metabolizable Energy (ME), (kJ)	ER (kJ) + EE (kJ)
EE (% of GE fed)	EE (kJ) x 100 / Feed energy intake (kJ)
ER (% of GE fed)	ER (kJ) x 100 / Feed energy intake (kJ)
ME (% of GE fed)	ER (kJ) + EE (kJ) x 100 / Feed energy intake (kJ)
AUE (% of GE fed)	100 - EE (%) - ER(%)
O_2 consumption (g) / protein gain (g)	Total oxygen consumption (g) / total protein gain (g)
EE (kJ) / protein gain (g)	Total EE (kJ) / total protein gain (g)

Protein Efficiency Ratio (PER)	Live body mass gain (g) / feed protein intake (g)
Protein Productive Value (PPV (%))	Total protein gain (g) x 100 / total protein fed (g)
Apparent lipid conversion (%)	Total lipid gain (g) x 100 / total lipid fed (g)
Feed Conversion Ratio (FCR)	Feed consumption (dry matter) / live body mass gain (g)

Radio-Immuno-Assay

Blood was drawn from each fish with a heparinized 1 ml syringe from the caudal vein after anaesthetizing the fish with 200 ppm MS 222. The blood was centrifuged at 4°C and 2,500 g for 5 minutes and the plasma was frozen at -20°C. For the determination of the plasma levels of IGF-1 a fish IGF-1 RIA kit from GroPep [including Anti Barramundi IGF-1 Polyclonal Antiserum (Rabbit) and Recombinant Barramundi IGF-1 (*Lates calcarifer*)] (catalogue nos. PAF1 and YU100 respectively) was used following the basic methodology of Claus and Weiler (1996) with some minor changes.

Isolation of total RNA

Extraction of total RNA from brain (including pituitary), liver and muscle tissues of Nile tilapia was carried out using TRIzol® Reagent (cat#15596-026, Invitrogen, Germany) according to the manufacturer's instructions with minor modifications. Tissue samples were homogenized in 1 ml of TRIzol® Reagent per 50 mg of the tissue. RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water.

Total RNA was treated with 1 unit of RQ1 RNAse-free DNAse I (Invitrogen, Germany) to digest DNA residues, re-suspended in DEPC-treated water and quantified photospectrometrically at 260 nm. Purity of total RNA was assessed by the 260/280 nm ratio which was between 1.8 and 2.1. Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis. Aliquots were used immediately for reverse transcription (RT) otherwise they were stored at -80°C.

Reverse transcription (RT) reaction

The complete Poly(A)$^+$ RNA isolated from Nile tilapia tissues was reverse transcribed into cDNA with a total volume of 20 µl using RevertAid^{TM} First Strand cDNA Synthesis Kit (MBI Fermentas, Germany) according to manufacturers instructions. The RT reaction was carried out at 25°C for 10 min, followed by 1 h at 42°C, and finished with a denaturation step at 99°C for 5 min. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until subjected to DNA amplification using the quantitative Real Time polymerase chain reaction (RT-qPCR).

Quantitative Real Time-Polymerase Chain Reaction (RT-qPCR)

An iQ5-BIO-RAD Cycler (Hercules, CA, USA) was used to determine the Nile tilapia cDNA copy number. PCR reactions were set up in 25 µL reaction mixtures containing 12.5 µL 1× SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 µL 0.2 µM sense and

antisense primers, 6.5 µL distilled water, and 5 µL of cDNA template. The reaction program was allocated to 3 steps. First step was at 95.0°C for 3 min. Second step consisted of 50 cycles in which each cycle divided to 3 sub-steps: (a) at 95.0°C for 15 sec; (b) at 60°C for 30 sec; and (c) at 72.0°C for 30 sec. The third step consisted of 71 cycles which started at 60.0°C and then increased about 0.5°C every 10 sec up to 95.0°C. At the end of each RT-qPCR a melting curve analysis was performed at 95.0°C to check the quality of the used primers. Each analysis included a non template control.
The sequences of specific primers of the genes used and sequence references are listed in Table 2. The quantitative values of RT-qPCR of GH, IGF-1 and their receptor genes were normalized to the bases of β-actin gene expression.

Table 2. Primer sequences used for RT-qPCR

Gene	Primer sequence (5'–3')[a]	Sequence references
GH	F: GAA CTG ATG CCA GCC ATG A R: AGC TAC AGA GTG CAG TTT G	Ber and Daniel (1992)
GHR-1	F: CCA TCA GAT GAG CAA CTT CTG AAA AGT R: ACT TCC TGG TGA ATC AGC CTT A	Jiao *et al.* (2006)
GHR-2	F: CAC AGA CTT CTA CGC TCA GGT CA R: TGA GTT GCT GTC CAG GAG ACA	Kajimura *et al.* (2004)
IGF-1	F: GTC TGT GGA GAG CGA GGC TTT R: AAC CTT GGG TGC TCT TGG CAT G	Schmid *et al.* (2003)
IGF-1 R_a	F: CTAAGGGCGTGGTTAAGCAC R: TTGTTGGCGTTGAGGTATGC	Greene and Chen (1999)
IGF-1 R_b	F: AGG GAC GAG CCA GAG ACG R:TTC AGA GGA GGG AGG TTG	Greene and Chen (1999)
β-actin	F: GTG ATG TGA CGC TGG ACC AAT C R: CCA TGT CAT CCC AGT TGG TCA CAA T	Hwang *et al.* (2003)

[a] F: forward primer; R: reverse primer.

Statistical Analysis

All data was analyzed using SPSS version 10.0 (IBM SPSS, Chicago, IL, USA). All data is presented as mean ± SEM if not stated otherwise. To test for homogeneity of variance a Levene test was applied while the test for normal distribution was conducted with a Kolmogorov-Smirnov test. To test for significant differences between the groups all data was subjected to an ANOVA with a subsequent Scheffé post-hoc test. Pearson's correlation coefficient was used to check for correlations among parameters. Statistical significance level was $p < 0.05$.

Results

Observations, growth performance, oxygen consumption and nutrient utilization

All fish accepted the respective diets and ate the provided feed during the first two minutes. No abnormal behavior or signs of stress were observed.

Table 3: Initial and final proximate chemical analysis of control and experimental fish on fresh matter basis.

	Initial group	Control	60TS300	60TS600	80TS300	80BA300
Crude protein (%)	11.8 ± 0.17	15.2 ± 0.50	15.1 ± 0.15	15.3 ± 0.21	14.8 ± 0.23	15.3 ± 0.10
Crude lipids (%)	3.3 ± 0.10	6.9 ± 0.28	6.8 ± 0.18	6.1 ± 0.36	6.3 ± 0.28	6.2 ± 0.62
Crude ash (%)	4.0 ± 0.12	4.5 ± 0.14	4.4 ± 0.10	4.4 ± 0.07	4.4 ± 0.11	4.4 ± 0.11
Dry matter (%)	22.9 ± 0.19	27.4 ± 1.06	27.1 ± 0.12	26.1 ± 0.23	25.9 ± 0.37	26.1 ± 0.80
Gross energy (kJ g^{-1})	4.51 ± 0.12	6.08 ± 0.35	5.94 ± 0.12	5.62 ± 0.14	5.63 ± 0.20	5.67 ± 0.24

values are expressed as mean ± SEM, n = 3

Over the experimental period all groups gained similarly in protein, lipids and subsequently in energy (Table 3). Fish fed with either control or 60TS300 feed showed a numerically higher apparent lipid conversion compared to the other saponin fed groups (Table 4).
Fish fed with 60TS300 grew numerically best in terms of body mass gain and final body mass compared to all other groups. The other saponin fed groups showed the lowest growth response while the control fed fish grew close to the 60TS300 group (Table 4).

Table 4: Growth performance, nutrient utilization and IGF-1 plasma levels of tilapia.

	Control	60TS300	60TS600	80TS300	80BA300
Initial body mass (g)	19.2 ± 0.34	18.9 ± 0.32	18.7 ± 0.26	18.9 ± 0.41	19.3 ± 0.06
Final body mass (g)	52.0 ± 6.25	54.0 ± 6.21	44.3 ± 2.17	45.8 ± 4.93	47.0 ± 3.04
Body mass gain (g)	32.8 ± 6.03	35.1 ± 6.38	25.6 ± 2.41	27.0 ± 5.09	27.2 ± 2.98
Growth (%)	270 ± 29.3	286 ± 35.0	238 ± 14.8	244 ± 27.9	243 ± 15.0
SGR (% day^{-1})	1.75 ± 0.19	1.85 ± 0.23	1.54 ± 0.11	1.56 ± 0.22	1.58 ± 0.11
MGR (g $kg^{-0.8}$ day^{-1})	6.47 ± 0.81	6.86 ± 0.98	5.52 ± 0.44	5.66 ± 0.88	5.74 ± 0.46
Feed conversion ratio	1.13 ± 0.13	1.12 ± 0.17	1.33 ± 0.09	1.35 ± 0.22	1.31 ± 0.10
Protein efficiency ratio	2.17 ± 0.29	2.21 ± 0.29	1.81 ± 0.13	1.85 ± 0.27	1.85 ± 0.14
Protein productive value (%)	34.4 ± 3.33	34.7 ± 4.29	29.5 ± 1.31	28.6 ± 3.16	30.0 ± 1.98
Apparent lipid convers. (%)	62.5 ± 7.04	62.1 ± 4.66	46.3 ± 5.43	48.5 ± 2.13	47.8 ± 5.18
Feed intake (g DM)	35.6 ± 1.76	37.3 ± 2.20	33.6 ± 0.92	34.3 ± 1.95	35.5 ± 1.18
IGF-1 plasma level (ng ml^{-1})	23.8 ± 2.68	23.1 ± 3.63	22.5 ± 3.46	25.3 ± 2.29	23.5 ± 5.65

values are expressed as mean ± SEM, n = 3

The same results can be observed in all measured and calculated growth performance and nutrient utilization parameters. Fish fed with 60TS300 always showed better or equal numerical values compared to the control group while the 60TS600 group exhibited the lowest performance. Strong positive correlations were found between feed utilization (FCR, PER and PPV) and growth performance (MGR, SGR, FBM and BMG) ($p < 0.01$).
Somewhat different to the growth and nutrient utilization performances was the oxygen consumption. It was highest for fish fed with 600 mg kg^{-1} of the 60% *Trigonella* fraction fed fish while it was lowest in fish fed with only 300 mg kg^{-1} of the same fraction. It can be seen from Fig. 1 that the oxygen consumption in the 60TS600 treatment increases from week 4

onwards together with a dramatically increasing standard error of mean. This is caused by a dramatically increasing oxygen consumption of one replicate in the respective treatment. The control and the other two saponin fed groups had an oxygen consumption between those two groups (Fig. 1). The oxygen consumption per gram of protein gain was lowest for fish fed with 60TS300 followed by control fed fish, while it was highest for fish fed with 60TS600 (Table 5). Logically, a similar result is gained for the heat dissipation (energy expenditure) since it is calculated from the oxygen consumption (Huisman 1976). Negative correlations were found for the energy expenditure per unit of protein gain and growth performance (MGR, SGR, FBM and BMG) showing that lower oxygen consumptions resulted in higher growth performance ($p < 0.05$).

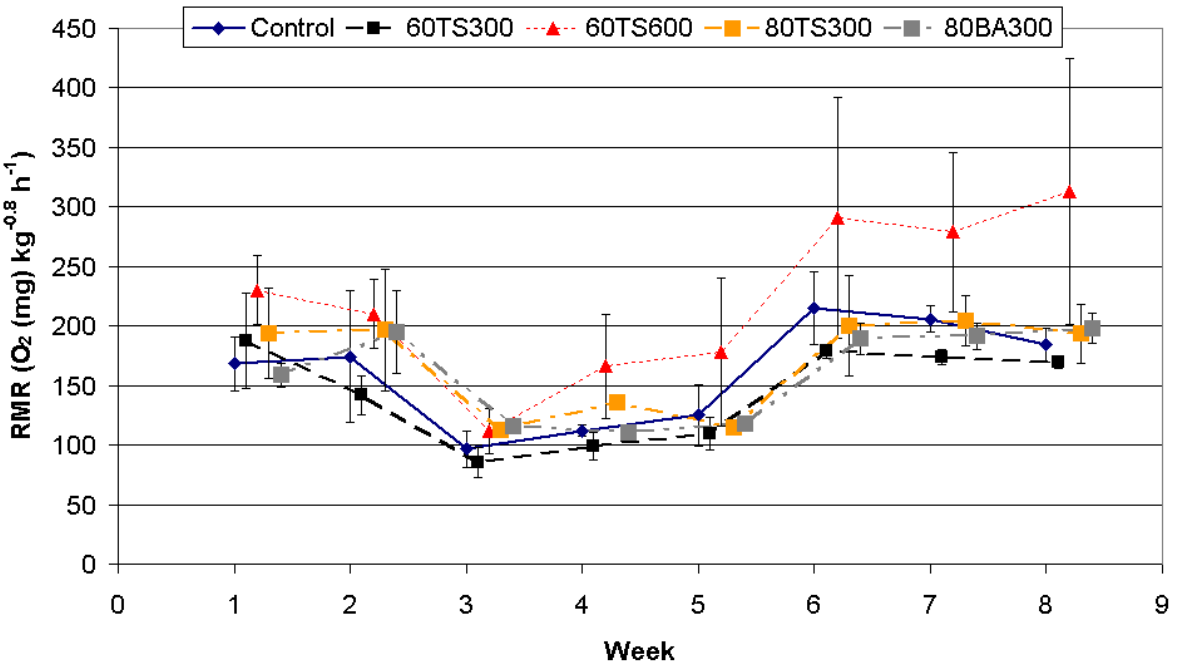

Figure 1: Average weekly routine metabolic rate over the experimental period. Values are presented as mean ± SEM, n = 3

Gene expression and IGF-1 plasma level

Expression of GH in brain and pituitary was highest for fish fed 60TS300 followed by control while the other saponin fed groups showed a significantly reduced expression of GH. A similar result was obtained for the expression of GHR-2 expression in brain and pituitary but not in liver and muscle tissue. IGF-1 expression was significantly lower for all saponin treated groups compared to control except 60TS300 which was numerically even higher than control. No differences were found between groups and tissues in expression of GHR-1, IGF-1 R_a and IGF-1 R_b. and between plasma levels of IGF-1 (Fig. 2 and 3). Expression levels of GH did strongly correlate to growth related parameters like BMG ($r = 0.99$, $p < 0.01$), MGR ($r = 0.99$, $p < 0.01$), SGR ($r = 0.99$, $p < 0.001$), nutrient utilization parameters like FCR ($r = -0.98$, $p < 0.005$), PER ($r = 0.98$, $p < 0.005$), PPV ($r = 0.97$, $p < 0.01$) and ER ($r = 0.96$, $p < 0.01$).

Similar correlations but not as strongly pronounced were found between expression of IGF-1 and performance related parameters (see Table 6).

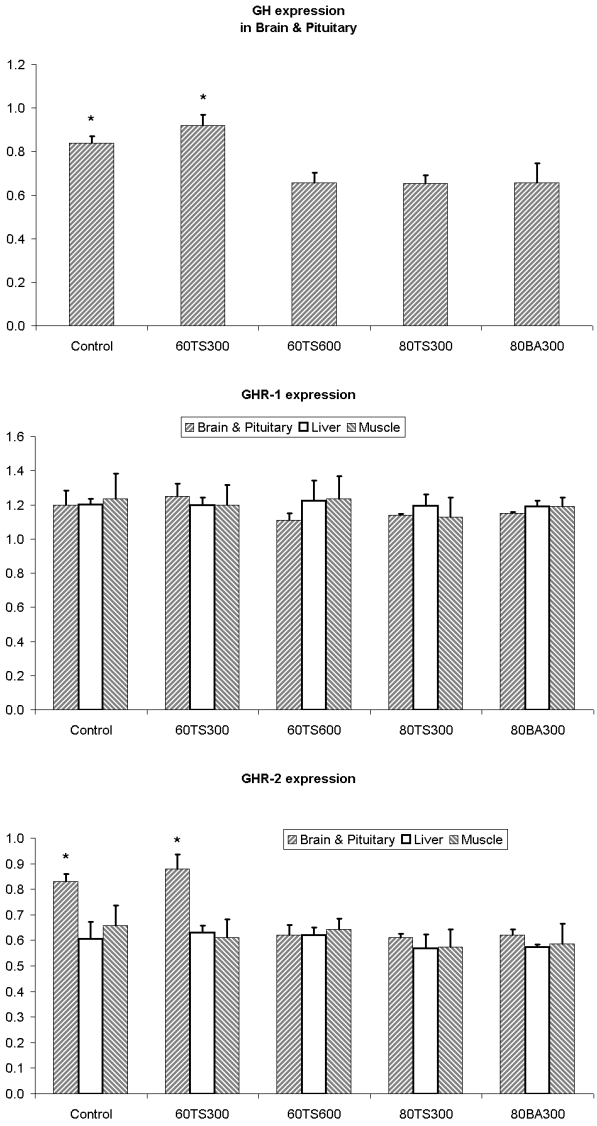

Figure 2: Expression of GH in brain and pituitary and GH receptors 1 and 2 in brain & pituitary, liver and muscle, respectively. Values are presented as mean ± SD, n = 3, * = $p < 0.01$

Discussion

To our knowledge, this is the first experiment where a traditional growth trial has been combined with proximate composition analysis, respirometry and gene expression to evaluate the effects of supplementation with saponin fractions in Nile tilapia. Furthermore this is the

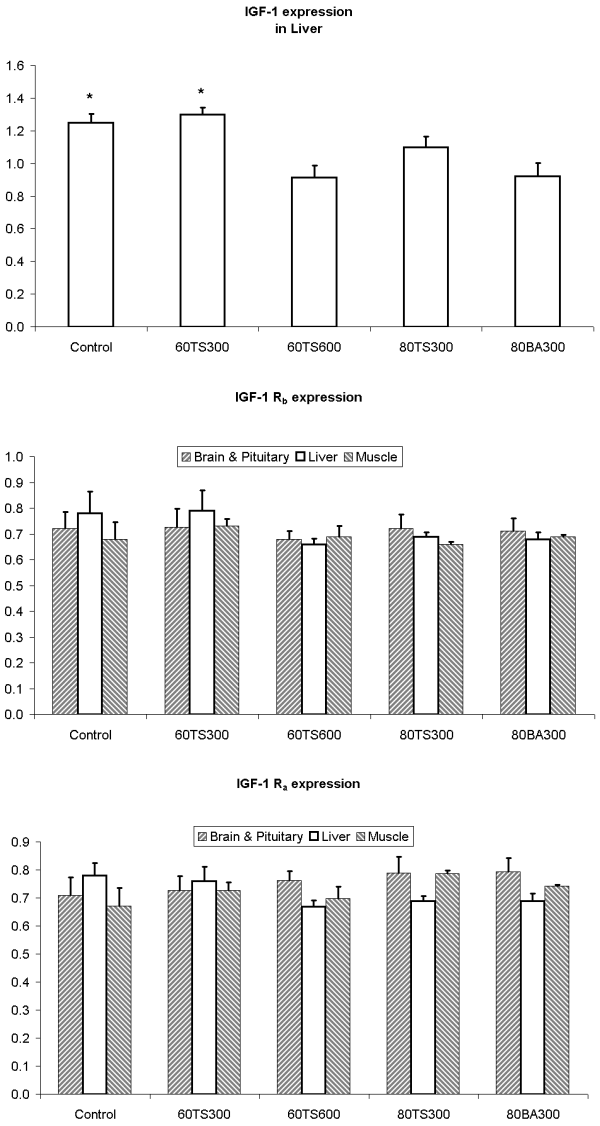

Figure 3: Gene expression of IGF-1 in liver and IGF-1 receptors a and b in brain & pituitary, liver and muscle, respectively. Values are presented as mean ± SD, n = 3, * = $p < 0.01$

first time that saponin extracts have been fractionated to be tested as possible natural growth promoters.

While the observed differences between growth, oxygen consumption and proximate composition between control and treatments were not statistically significant due to low number of replicates (which is limited by the number of boxes of the respirometer system), the expression of GH, GHR-2 and IGF-1 showed significant differences between treatments. The expression of GH in the pituitary and brain and the expression of IGF-1 in the liver were significantly reduced for all saponin treatments except 60TS300 which had numerically even higher expressions of these genes than the control. In our experiment the expressions of GH and IGF-1 genes directly reflected the overall performance including the growth rates and the various evaluated feed utilization and metabolic parameters of the respective treatments. All results point towards a performance depression in all saponin supplemented treatments except the 300 ppm supplementation with the 60% methanol fractionated saponin eluated from *T. foenum-graecum* seeds. High expression levels of GH and IGF-1 genes resulted in

numerically highest growth rates and best nutrient utilization while significantly reduced gene expressions of GH and IGF-1 resulted in numerically lowest growth and inferior nutrient utilization.

Table 5: Energy balance for all five groups.

	Control	60TS300	60TS600	80TS300	80BA300
Initial fish GE (kJ)	86.8 ± 1.55	85.4 ± 1.43	84.3 ± 1.15	85.1 ± 1.85	87.0 ± 0.29
Final fish GE (kJ)	314 ± 32.4	321 ± 38.3	249 ± 16.1	256 ± 18.9	265 ± 11.5
Ingested feed GE (kJ)	739 ± 36.6	775 ± 45.6	698 ± 19.1	711 ± 40.5	738 ± 24.4
ER (kJ)	227 ± 32.0	235 ± 38.8	165 ± 17.3	171 ± 19.7	178 ± 11.4
EE (kJ)	201 ± 32.7	186 ± 20.7	258 ± 59.2	198 ± 12.7	197 ± 4.35
ME (kJ)	429 ± 64.7	422 ± 54.7	423 ± 45.9	369 ± 15.5	376 ± 14.6
AUE (kJ)	310 ± 30.7	353 ± 9.25	275 ± 64.9	342 ± 35.7	362 ± 15.7
ER (% of GE fed)	30.5 ± 2.85	30.0 ± 3.45	23.6 ± 2.04	23.9 ± 1.66	24.2 ± 1.23
EE (% of GE fed)	27.0 ± 3.14	23.9 ± 1.63	37.5 ± 9.68	28.2 ± 2.14	26.8 ± 0.29
ME (% of GE fed)	57.5 ± 5.99	53.9 ± 4.11	61.1 ± 8.46	52.1 ± 2.57	50.9 ± 1.24
AUE (% of GE fed)	42.5 ± 5.99	46.1 ± 4.11	38.9 ± 8.46	47.9 ± 2.57	49.1 ± 1.24
Cons. O_2 (g) / protein gain (g)	2.61 ± 0.13	2.36 ± 0.26	4.34 ± 1.30	3.45 ± 0.74	3.00 ± 0.23
EE (kJ) / protein gain (g)	38.7 ± 1.93	35.0 ± 3.87	64.5 ± 19.4	51.2 ± 11.0	44.6 ± 3.48

Gross Energy (GE), Energy Retention (ER), Energy Expenditure (EE), Apparently Unutilized Energy (AUE), Metabolizable Energy (ME) and Oxygen Consumption and Energy Expenditure per gram Protein gain. Values are expressed as mean ± SEM, n = 3

Generally an over-expression of GH in transgenic fish results in significantly higher growth rates as reported for coho salmon (Devlin *et al*. 2004), Atlantic salmon (Du *et al*. 1992), Arctic charr (Pitkänen *et al*. 1999), rohu (Venugopal *et al*. 2004), common carp (Hinits and Moav 1999), channel catfish (Dunham *et al*. 1999) and Nile tilapia (Rahman and Maclean 1999).

In an *in vitro* study it has been shown that saponins extracted from Fenugreek significantly stimulated GH release in rat pituitary cells. The most potent substance proved to be a crude methanol extract of *T. foenum-graecum* (~ 22-fold higher GH release compared to control) while dioscin (~18-fold) and fenugreek saponin I (~13-fold) where also highly potent (Shim *et al*. 2008). Although no direct growth promotion was observed in GH transgenic tilapia, a significantly improved feed utilization was reported by Martínez *et al*. (2000). The differences between the different treatments compared to control, although we did not measure GH release, were far less pronounced as the above mentioned results. Feeding common carp with 150 mg per kg diet for eight weeks with crude *Quillaja saponaria* saponins resulted in significantly increased final body mass and improved oxygen consumption while a similar effect was yielded in tilapia when the saponin inclusion level was raised to 300 mg kg^{-1} diet (Francis *et al*. 2001b, 2002 a, b). The two main differences between those experiments and our experiment where the nature of the saponins on one side and the degree of saponin fractionation on the other side. *Q. saponaria* saponins are of triterpenoid nature (Guo & Kenne 2000) while *T. foenum-graecum* (Marker *et al*. 1947, Murakami *et al*. 2000) and *B. aegyptiaca* (Marker *et al*. 1947, Dawidar and Fayez 1969) saponins are of steroidal nature. The goal of this experiment was to test if saponins derived from two common and widespread Middle Eastern plants can be used as environmentally friendly growth promoters. To do so a

Table 6: Pearson's correlation coefficients for selected growth and nutrient utilization parameters and for gene expression. * = $p < 0.05$, ** = $p < 0.01$

	BMG (%)	ER (%)	EE (%)	PPV (%)	PER	MGR	SGR	O2 g^{-1} CP gain	FCR	GH expr. (brain&pituitary)
BMG (%)	-									
ER (%)	0.945*	-								
EE (%)	-0.685	-0.601	-							
PPV (%)	0.945*	0.983**	-0.562	-						
PER	0.979**	0.991**	-0.647	0.978**	-					
MGR	0.997**	0.955*	-0.702	0.960**	0.984**	-				
SGR	0.997**	0.951*	-0.677	0.961**	0.981**	0.999**	-			
O2 g^{-1} CP gain	-0.835	-0.808	0.946*	-0.789	-0.831	-0.859	-0.840	-		
FCR	-0.955*	-0.991*	0.583	-0.998**	-0.988**	-0.968**	-0.967**	0.801	-	
GH expr. (brain&pituitary)	0.990**	0.959**	-0.592	0.972**	0.983**	0.990**	0.993**	-0.780	-0.976**	-
IGF-1 expr (liver)	0.920*	0.897*	-0.679	0.826*	0.924	0.904*	0.895*	-0.774	-0.855	0.888*

fractionation was conducted with the future goal in mind to identify and purify a single compound responsible for a growth promoting effect as experienced in the experiments of Francis and his colleagues. However, evidence exists that the biological activity of saponins is not the consequence of one single biologically active saponin or sapogenin which can be extracted and purified but rather that saponin mixtures exhibit the highest biological activity. As mentioned above, the highest release of GH in rat pituitary cells was measured after stimulation with crude methanol extract derived from fenugreek (Shim *et al*. 2008) and Kamel *et al*. (1991) showed that single saponins derived from *B. aegyptiaca* showed no anti-diabetic activity while different mixtures of single extracted saponins showed significant anti-diabetic activity. Also Francis *et al*. (2001b, 2002a, b) used crude *Quillaja* saponin mixtures yielding far more pronounced effects than we observed in our experiment. Nevertheless we found a tendency that certain saponin fractions were less detrimental for growth and nutrient utilization (60% methanol fraction) while another fraction of the same plant seemed to exhibit stronger anti-nutritional activity (80% methanol fraction) when applied in the same concentration of the diet. Furthermore the concentration of the saponin matters since 600 ppm of the 60% MeOH extract from Fenugreek in the diet yielded similar bad results as 300 ppm of the 80% MeOH extract from Fenugreek. The variance in the oxygen consumption experienced in the 600 ppm treatment of the 60% Fenugreek methanol fraction is starting to increase from the 4^{th} week onwards. The cause is a strongly increasing oxygen consumption of a single fish while the other two fish's metabolic rates only increase slightly which could be explained by increasing body mass. That might point towards a rising inability of the fish's metabolism to cope with higher amounts of this specific saponin fraction.

Despite a potential application of saponins as growth promoter in aquaculture they might also be used to influence the sex ratio *in vivo*. This is especially interesting for tilapia since the commercial tilapia production depends on male monosex cultures which are at the moment mainly produced by application of a synthetic androgen, 17-α-methyletestosterone.

Commercial extracts of *Tribulus terrestris*, known as Gokshura, had a significant effect on sex ratio and in the highest dose also on growth of the African catfish, *Clarias gariepinus* (Turan and Çek 2007). Furthermore, the same extract was able to increase the sex ratio of male convict cichlids (*Cichlasoma nigrofasciatum*) (Çek *et al*. 2007).

Gokshura is reported to be rich in steroidal saponins but with varying compositions depending on origin (Dinchev *et al*. 2008).

Up to now only very little attention has been paid to effects and potential of saponins as feed additives in aquaculture feeds. While saponins are in use in terrestrial livestock feeds for example to control ammonia and odor (Cheeke 1999) they are generally still considered anti-nutrients for aquatic animals.

We conclude that the saponin fractions derived from Fenugreek and the desert date in the applied concentrations are no possible alternative for prohibited antibiotics as growth promoters in Nile tilapia production. However, a certain potential as growth promoter might be found in the 60% MeOH saponin fraction from Fenugreek or in crude saponin extracts and that the effects of the saponins are likely to be concentration depending.

More studies are needed to test a possible optimum dietary concentration of that saponin fraction (60TS). Furthermore, the decreased performance of other saponin treated fish, including the higher concentration of 60TS, points toward anti-nutritional effects. The combined results are in good coherence since high expression levels of GH and IGF-1 genes

positively correlated with parameters related to growth, nutrient digestion and metabolic efficiency.

Acknowledgements
This work was partly financed by the German Research Foundation DFG under the project number 1133-13/2. Furthermore we wish to thank Beatrix Fischer, Herrmann Baumgärtner and Dominique Lorenz for their help and support in the analytical work and Dr. Peter Lawrence for the critical reading of the manuscript.

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Chapter 9

General Discussion

General discussion and conclusion

In the work presented here, tilapia diets were supplemented with commercial and small-scale extracts of saponins, saponin fractions or eluates, and sapogenins. The goals of the work were twofold. Firstly to test whether these supplements can be used as a replacement for 17-α-methyltestosterone (MT) to inhibit reproduction or invert the sex of *Oreochromis niloticus* and secondly whether they can act as growth promoters.

Due to the potential of MT to masculinize or have other negative impacts on aquatic organisms (fish, Gomelsky et al. 1994, Abucay & Mair 1997, Hulak et al. 2008; invertebrates, Oehlmann & Schulte-Oehlmann 2003, Crane et al. 2008) and to its carcinogenicity (Velazquez & Alter 2004) it would be highly desirable if the tilapia industry could find a plant-derived "green" alternative for MT. The trade in fish and other animals fed during their production phase with hormones (among certain other additives) is prohibited in the European Union (EU) since 2006 (EC 1831|2003). The potential danger for consumers of MT sex-inversed Nile tilapia seems to be rather small. Goudie et al. (1986a, b) fed tilapia with a diet containing radiolabelled MT. When they switched the fish to a non-treated diet, the radioactivity decreased exponentially until, after 21 days, only 5 ng MT g^{-1} tissue remained (< 1% radioactivity of the initial radioactivity level).

However, in the EU, the consumer's acceptance of any type of hormone, anti-biotic treated animal product or genetically modified organism is generally very low. The production of hormone free tilapia fillets could therefore result in a considerably increased acceptance.

An influence of *Quillaja saponaria* saponins on sex ratio, growth, nutrient utilization and metabolic performance was reported by Francis et al. (2001a, 2002a, b, c). The main differences between the work performed by Francis and colleagues and that conducted in this study are the plant species from which the saponins were derived and the purity of the saponins themselves. During this study saponins were extracted from either fenugreek *Trigonella foenum-graecum* or *Quillaja saponaria* and afterwards fractionated by HPLC while Francis et al. (2001a, 2002a, b, c) used commercially available saponin mixtures (S 2149, Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Saponins from *Q. saponaria* are predominantly of the triterpenoid type (Bankefors et al. 2008) while the ones from *T. foenum-graecum* are steroidal (Marker et al. 1947, Murakami et al. 2000).

Effect of saponins on sex ratio, gonad histology and reproduction

Four experiments (Chapters 2-5) were conducted in which the effects of saponin fractions and a commercially available sapogenin on sex ratio, reproduction and gonad histology of Nile tilapia were tested. The saponin fractions were derived from *Quillaja saponaria* and *Trigonella foenum-graecum*. Diosgenin, a pure commercial steroidal sapogenin which was also applied, is the aglycone of dioscin, a saponin present in, among others, *T. foenum-graecum* (Marker et al. 1947). Except for the experiment described in chapter 2 no influence of saponins or the sapogenin on the sex ratio were observed. In the experiment described in chapter 2, two fractions were found to have a significant influence on the sex ratio of mixed sex Nile tilapia. These were the 60% and 80% methanol eluated fenugreek saponins, each included at a level of 150 mg kg^{-1} diet.

In chapter 3 a similar fraction, the 90% methanol eluated fenugreek fraction and diosgenin were applied in the same and higher concentrations (150 mg kg^{-1} and 300 mg kg^{-1}) but this time with higher stocking densities and considerably increased total sample sizes. No statistically significant influences on sex ratios were found but large variations in sex ratios among the replicates of the same treatment were observed with marked masculinization in one replicate and feminization in another. Histological analysis was done for sub-samples of these remarkable replicates but no differences between specimens from treatments and control were found. In chapters 4 and 5 genetically female tilapia, provided by the Hebrew University of Jerusalem, Department of Animal Science at the Robert H. Smith Faculty of Agriculture in Rehovot, were used for the experiment. No statistically significant difference was observed between saponins extracted from *T. foenum-graecum*, diosgenin and commercial *Q. saponaria* saponins and the negative controls. It must therefore be assumed that the effects observed in chapters 2 (significant differences between two treatments and control) and 3 (high variation in the same treatment but different replicates) were random effects caused by inhomogeneous initial stocking of the mixed sex tilapia fry at the beginning of the experiment.

For a statistical evaluation of the influence of saponins on reproduction too little data is currently available. Of the three females observed spawning in the experiment described in chapter 4, all three were fed with saponins eluted from *T. foenum-graecum*. However, no statistical evaluation would be possible with that limited data. Results presented in chapter 5 suggest that commercial *Q. saponaria* saponins had no influence on reproduction.

Very few publications exist in which saponins, saponin extracts or sapogenins have been used to sex inverse or otherwise influence reproduction in fish or tilapia. The herb *Tribulus terrestris* is rich in steroidal saponins (Kostova & Dinchev 2005) the nature of which depends on their origin (Ganzera et al. 2001). Extracts of *T. terrestris* have been used to masculinize convict cichlids *Cichlasoma nigrofasciatum* and African catfish *Clarias gariepinus*. Turan & Çek (2007) boiled commercially available extracts of *T. terrestris* in water and added the resulting solution to water in aquaria to give concentrations of 0, 0.1, 0.2 and 0.3 g L^{-1} of the original extract. *C. nigrofasciatum* mixed sex fry were raised in each aquarium (35 per aquarium, three replicates) from three days after hatching. All tested concentrations resulted in a significantly higher percentage of males compared to the control. The highest number of males occurred in the highest concentration (Çek et al. 2007). A similar experimental setup with higher concentrations (0, 3, 6 and 9 g L^{-1}) was used to test the influence of *T. terrestris* extract on sex ratios of one day old mixed sex hatchlings of *C. gariepinus*. All treatments resulted in a significantly greater number of males compared to the control with the highest numbers of males in the highest concentrations (Turan & Cek 2007).

Effects of saponins on growth, nutrient utilization and metabolic performance

Improving growth and nutrient utilization is not necessarily simply a matter of boosting the growth of the fish. Improved nutrient utilization, metabolic performance and improved health compared to control diets are also desirable effects of a feed additive for aquaculture. An increased production with the same input or the same production with reduced input both improve the productivity and commercial viability of an aquaculture operation.

In comparison to Francis et al. (2001a, 2002b, c) no significant differences for growth, nutrient utilization and metabolic performance between control and any fish fed with saponin fractions, either from *T. foenum-graecum* or from *Q. saponaria*, were found in carp or tilapia. However, several remarkable results were found consistently in the different experiments described in chapters 5-8. In all fish fed with the 40/60% (v/v) methanol/water eluated fenugreek saponin fraction, the evaluated parameters were numerically inferior to those of the control or other saponin fed fish. In carp, a supplementation level of 150 mg kg^{-1} diet of the 60% fenugreek saponin fraction led to numerically superior growth and performance (chapter 6) while the same concentration of the same eluate led in tilapia to similar growth and performance compared to control fed fish (chapter 7). Raising the concentration of the 60% fenugreek saponin fraction to 300 mg kg^{-1} led to numerically improved growth and performance of tilapia. Further increase of the same fraction to 600 mg kg^{-1} diet resulted again in inferior performance compared to the control (chapter 8). The 80/90% methanol fraction derived from fenugreek (during the course of the project the eluent concentration of the methanol/water mixture was changed from 80/20 methanol/water to 90/10 methanol/water) showed numerically slightly improved values of feed conversion, protein and lipid utilization in only one experiment but in the other two experiments its performance was equal or inferior to control fed fish. The 80% saponin fraction derived from *Q. saponaria*, which showed the highest aromatase inhibition *in vitro* (Golan et al. 2008), led only to results similar to those in control fed fish. The reported advantages of dietary supplementation with crude *Q. saponaria* saponin mixtures (Francis et al. 2001a, 2002a, b, c) in carp and tilapia could not be observed. This finding indicates that different fractions of the *Q. saponaria* eluate are responsible for inhibition of aromatase and for the effects on growth and metabolism. These 'fractions' may be either different single saponins or combination of saponins.
Combinations of single, previously extracted saponins from the target plants resulted in synergistic effects as described in Shim et al. (2008) and Kamel et al. (1991) for *T. foenum-graecum* and *B. aegyptiaca*, respectively.

Results from the experiment at the Jericho field station (chapter 5) show a significantly higher growth of all female fish continuously fed with a crude saponin mixture from *Q. saponaria* containing around 25% sapogenin. In comparison, fish fed continuously with *Q. saponaria* saponins containing around 10% sapogenin did not show a similar response nor did fish fed short term (for the first 28 days after hatching) with either saponin mixture.

A significantly reduced oxygen consumption per gram of body mass gain has been found by Francis et al. (2002c) in *C. carpio* fed for eight weeks with a commercially available *Q. saponaria* saponin mixture. Common carp fed with a diet containing 150 mg kg^{-1} of fenugreek saponin eluted by 60 % methanol showed a numerically but statistically insignificant reduction in oxygen consumption per gram of protein accretion compared to the control (chapter 6) while tilapia fed with the same fenugreek fraction in the same concentration had a numerically but also insignificant increase in oxygen consumption per gram of protein accretion (chapter 7). In contrast, a diet supplemented with 300 mg kg^{-1} of the same eluate resulted in a numerically reduced O_2 consumption per gram protein gain in Nile tilapia while a concentration of 600 mg kg^{-1} of diet resulted again in a numerically increased

O_2 consumption per gram protein gain compared to control (chapter 8). These results clearly show a concentration dependent reaction of the fish to saponin supplementation and point towards a beneficial effect of saponins on protein turnover.

Mode of action of saponins

Probably the most interesting but also the most difficult question often asked at conferences is, "How do saponins work?". That question is also one which can not be answered easily or probably answered in this context at all. One of the problems is the high diversity of saponins. In *Quillaja saponaria* alone at least 38 different triterpenoid saponins have been described (Guo & Kenne 2000, Bankefors et al. 2008) and in *Trigonella foenum-graecum* at least 19 different steroidal saponins have been isolated and characterized (Murakami et al. 2000). Another major difference between the two plants used during the course of this thesis is the structure of their aglycones. In *Q. saponaria* the aglycone of the different saponins has a triterpenoid structure while *T. foenum-graecum* has a steroidal one.

The inclusion of a single methyl-group at the 17th C-atom through α-alkylation instead a hydroxyl-group makes the difference between orally active (17-α-methyltestosterone) and orally inactive (testosterone) androgens (Shahidi 2001). Single saponins might have a beneficial or desired effect on fish growth, nutrient utilization, nutrient retention or reproduction, while another pure saponin from the same plant, present in the same extract might have a detrimental effect. Therefore the individual saponins present in a saponin extract and their relative quantity might cause beneficial or detrimental effects, or the effects may even cancel each other out. The idea during this study was to isolate fractions specifically responsible for beneficial (on growth or reproduction) or detrimental effects using HPLC fractionation of crude methanol extracts of mainly *T. foenum-graecum* but also of *Q. saponaria*. Results in chapters 6 and 7 show that saponin fractions eluated from *T. foenum-graecum* with 40/60 (v/v) methanol/water yielded numerically inferior effects on growth, nutrient utilization and metabolism. Those eluted with 60/40 (v/v) methanol/water (chapters 6 and 8) gave results that are numerically superior to the control. This implies that groups of saponins with similar chemical characteristics produce similar biological effects, either beneficial or detrimental.

Saponin fractions from *Q. saponaria* (Golan et al. 2008) and *T. foenum-graecum* (unpublished results) had inhibiting effects on the enzyme aromatase *in vitro* which usually leads to masculinization (Kwon et al. 2000, Afonso et al. 2001). The degree of masculinization after administration of non steroidal aromatase inhibitors is generally concentration and time of application dependent (Afonso et al. 2001, Gao et al. 2010).

One big difference between the *in vitro* (conducted by the Israeli partners) and *in vivo* experiments described in chapters 2-8 was the mode of application. In the *in vitro* experiments a direct contact of the test substance with the target tissue took place, while in the *in vivo* experiments the test substances were applied with the feed. In this case, therefore, the saponins had to pass the stomach and had probably been chemically altered due to the low pH of around 1.4 to 1.5 in Nile tilapia stomachs (Getachew 1989) before reaching the intestine. Furthermore they had to pass the intestinal membrane before being absorbed into the blood.

Unpublished results seem to show that saponins administered to Nile tilapia via the feed have afterwards been found in the blood of the fish, which shows that saponins can in fact pass through membranes.
Some published studies point towards more complex or even systemic modes of action. A large increase in growth hormone (GH) secretion of rat pituitary cells *in vitro* was reported after stimulation with a crude methanol extract of fenugreek (22-fold increase compared to control) and with dioscin, the saponin that has diosgenin as aglycone (18-fold increase compared to control) (Shim et al. 2008). Other tested single saponins or sapogenins did not have similarly marked effects on GH secretion, except for one newly identified saponin, fenugreek saponin I (Shim et al. 2008). These results indicate systemic effects. Crude methanol extract affects GH secretion the most and although dioscin seems to cause a great part of that response, unknown factors present in the methanol extract appear to act synergistically so that the effect of dioscin is increased even more. Similar results have earlier been reported for dioscin extracted from another saponin rich plant, *Dioscorea batatas*. The 70% methanol extract led to the greatest increase in GH secretion *in vitro* (rat pituitary cells) and *in vivo* (after intravenous injection). The *in vitro* increase was 10-fold compared to control while the *in vivo* GH-release was doubled compared to control (Lee et al. 2007). Saponins extracted from *Balanites aegyptiaca*, a saponin rich plant commonly called desert date, were tested for their anti-diabetic efficiency. Compounds previously extracted and isolated from *B. aegyptiaca* and orally administered to streptozotocin induced diabetic mice in varying combinations produced the greatest reduction in blood glucose when all of the isolated compounds where used. No anti-diabetic effect was found when single compounds were administered (Kamel et al. 1991).

Effects of the same compound, *Q. saponaria* saponins, reported by Francis et al. (2001a) on growth and nutrient utilization on the one hand and by Francis et al. (2002a) on the sex ratio of Nile tilapias on the other indicate the presence of typical anabolic-androgenic effects commonly produced by androgens (Shahidi 2001). They generally exhibit two effects simultaneously, by being anabolic (growth promoting) and androgenic (promoting male sexual characteristics or changing sex completely). These effects can not be completely separated from one another since both are mediated via the same receptor, the androgen receptor (AR). However, at supraphysiologic levels, androgens might exert their anabolic effects by interaction with glucocorticoid receptors thereby decreasing protein catabolism (Shahidi 2001). Furthermore, differences also exist in the intensity of these effects relative to each other with some hormones acting in a more androgenic than anabolic manner while others show the opposite behaviour. The effect of steroid hormones on genital tissues, secondary sex organs and extragenital tissues varies greatly between species and is also dependent on concentration, treatment duration and mode of application (Kochakian 1961, 1975, Shahidi 2001). Testosterone for example is hepatically inactivated after oral administration through oxidation of the 17-β-hydroxy group, but if C17 undergoes α-alkylation the product, 17-α-Methyltestosterone, becomes orally active and hepatic degradation is inhibited (Shahidi 2001).
In the long term experiment described in chapter 5 only a growth promoting effect was found and that solely in the diet containing *Q. saponaria* saponin with 25% sapogenin content. No

males and therefore no sex inversion were observed during the one year period of culturing, implying that the observed effects are likely not mediated through the androgen receptor and are therefore not androgenic-anabolic effects.
A beneficial effect of saponins not so far mentioned is a potential immunostimulant effect. Some saponins, especially those extracted from *Q. saponaria*, are known to form Immuno Stimulatory Complexes (ISCOMs) and are widely used in vaccinations for humans and animals (Barr et al. 1998, Pham et al. 2006) although it seems that the effect on fish is significantly less pronounced than that on humans (Francis et al. 2002d).

CONCLUSION

In general saponins have been regarded as antinutrients in fish nutrition (Bureau et al. 1998, Francis et al. 2001b). During this study is was possible to back up previous results published by Francis et al. (2001, 2002a, b, c) showing that low dietary concentrations of saponins can not generally be regarded as antinutrients. Through fractionation of the *T. foenum-graecum* saponins it was possible to provide information showing that certain fractions act in a more detrimental manner while others can be considered as potential growth promoters. The saponin fractions used in the present series of experiments were administered as food additives in various concentrations but under these conditions, none proved to be suitable as a plant derived substitute for 17-α-methyltestosterone either for the production of male monosex cultures of Nile tilapia or as an inhibitor of reproduction in mixed sex tilapia populations. On the other hand, the observed effects on growth, nutrient utilization and metabolism proved more promising (though a trifle ambiguous) and should be investigated in greater detail to evaluate a potential application for these fractions as feed additive for aquaculturally valuable species. Ongoing research should also include investigations into the effects of saponins on gut flora and the immune system. Although saponins are naturally occurring secondary compounds in plants, their purification, concentration and application in fish feeds might have negative impacts on the environment or on the health of farm workers. If, in the future, potent saponin extracts are put into use, a prerequisite would be to conduct studies to ensure that they are non toxic and environmentally harmless.

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Acknowledgements

First I want to mention that the sequence of persons named here is not relevant to the “level of my gratitude” for that person. Without each person mentioned here by name my doctoral thesis would have never been finished.

I want to thank the DFG for partly financing me and large parts of this study in the course of the project BE 1133/13-2.

I want to deeply thank the following persons:

Prof. Dr. Klaus Becker for giving me the chance to work as a Ph.D. student at his institute. He proofed to be always a rationale scientist with excellent organizational skills from whom much can be learned.

Prof. Dr. Ulfert Focken for accepting me as his Ph.D. student and for the good guidance in scientific and daily life questions, no matter about which topic.

Dr. Mark Prein for his constant cheerful mood and all his advices during the time he worked as project coordinator at the Department of Aquaculture Systems.

Dr. Peter Lawrence (of course also his lovely wife Kate Lawrence) for the many excellent advices and all the numerous readings of half finished and finished manuscripts and chapters of this thesis and of course for the many nice evenings with a Weizen or Kellerpils in the “Franziska” or “Garbe”, respectively.

Herrmann Baumgärtner and Beatrix Fischer for keeping lots of work and stress away from me and for being the persons they are.

Sabine Nugent for helping in uncountable administrative issues with the university and telling me always the newest stories about her daughter.

Johannes Pucher for being exactly the colleague one could ask for although he decided to stay ¾ of the year in Vietnam because of the culinary advantage over our “Mensa”.

Dominique Lorenz, Florian Zuber and Christoph Schinkel for being elite HiWis (each one of them in her/his own way).

And last and least all the other persons of the institute and in the university who actually helped me during the hardship of a typical Ph.D. study.

Summary

The future role and importance of aquaculture for the world food supply is more and more recognized by the public. High quality feed for semi-intensive and intensive aquaculture operations are necessary to guarantee a future continuous growth of fish production. Nutrient utilization efficiency has in the past been augmented by addition of feed additives, mainly anti-biotics and hormones. That practice is prohibited in the European Union since 1st of January 2006 and the consumer acceptance of products produced in that way is low. Around a decade ago saponins, secondary plant metabolites, have been found to be potential alternatives for aquafeeds. Saponinmixtures derived from the South American soap bark tree *Quillaja saponaria*, have been found to have beneficial effects on growth, feed and nutrient utilization and on metabolism of common carp *Cyprinus carpio* and Nile tilapia *Oreochromis niloticus*. Furthermore, in one of the early experiments a sex ratio in favor of males was observed in saponin fed tilapia compared to control fish not supplemented with saponins. An influence on sex ratio of Nile tilapia would be highly desirable since industrially Nile tilapia production is only financially viable if all male populations are produced since mixed sex tilapia populations tend to reproduce uncontrollable. The production of all male tilapia populations is mainly conducted by feeding the potentially environmentally hazardous and carcinogenic synthetic hormone 17-α-methyltestosterone to sexually undeveloped tilapia fry. The above mentioned problems led to a joint research project with partners in Israel and Palestine in which this dissertation has been conducted. The aim of the project was the fractionation of crude saponin extract derived from fenugreek *Trigonella foenum-graecum* and its testing *in vitro* and *in vivo*. It was assumed that a higher biological activity could be achieved if saponin fractions instead of crude mixtures would be applied. After in vitro testing, different saponin fractions or eluates, have been tested on their effects on growth, feed and nutrient utilization, metabolism, proximate composition, gene expression of GH and IGF-1, sex ratio, reproduction and gonad histology. Most of the feeding experiments were conducted with Nile tilapia but one experiment was also conducted with carp. Three of the feeding experiments have been conducted in a system capable of measuring continuous respiration of the fish. Another experiment has been conducted at a field station at Jericho, Palestine.

The experiments conducted to evaluate the influence of saponin fractions on sex ratio of undifferentiated tilapia fry have been conducted primarily in a flow-through system. The tested saponin fractions and a tested sapogenin are not suitable to produce male monosex tilapia populations. The sex ratios after supplementation of diets of mixed sexed tilapia fry did only in one case show a significantly higher proportion of males than the control. In a larger scale repetition of that experiment the previous observations could statistically not be proofed. It must be assumed to be random effects or be the result of initially biased sex ratios after stocking undifferentiated tilapia fry. An experiment in which genetically female tilapias were fed with two fenugreek saponin fractions and positive and negative controls supported that finding. A long term feeding experiment conducted at Jericho revealed no influence of the long and short term supplemented *Q. saponaria* saponin mixtures on sex ratio and reproduction of genetically female tilapia. A similar laboratory experiment with fenugreek saponin fractions could not be evaluated in that regard.

The experiments evaluating the effects of the saponin fractions on growth, feed and nutrient utilization, gene expression of GH and IGF-1 and proximate composition revealed no significant differences. But in all experiments one fraction eluated with 60%/40% (v/v) methanol/water showed numerically improved values compared to control and other fractions. Furthermore one fraction eluated with 40%/60%/ (v/v) methanol/water gave constantly numerically inferior results of tested parameters compared to control. That supports the conclusion that the 40% methanol fraction contains saponins generally referred to as anti-nutrients. In only one experiment, conducted at Jericho, significantly higher growth was observed after long term supplementation with a *Q. saponaria* saponin mixture containing elevated sapogenin content.

The presented data in this thesis is not supporting an application of the tested saponin fractions as environmentally friendly alternative to methyltestosterone to produce all male populations of tilapia or to inhibit uncontrollable reproduction. Additional experiments are needed to evaluate different modes of application like immersion treatments or injections since during the experimental work of this thesis all tested saponins were added to the feed.

An application of the tested fenugreek saponin fractions as growth promoters yields a higher potential although the experimental results are based upon low sample sizes due to capacity restrictions of the respirometric system. Therefore a repetition under near commercial or commercial conditions must be considered.

Zusammenfassung

Die zukünftige Bedeutung der Aquakultur für die Welternährung wird mehr und mehr auch von der breiten Öffentlichkeit erkannt. Wichtige Vorraussetzung für das weitere, stetige Wachstum der Aquakulturproduktion ist die ausreichende Produktion von hochwertigem Futter für semi-intensive und intensive Produktion. Die Verwertungseffizienz ist in der Vergangenheit im europäischen Raum oft durch die Zugabe von Wachstumsbeschleunigern, z.B. Antibiotika und Hormone, gesteigert wurden. Der Einsatz solcher Futteradditive ist in der EU seit 1. Januar 2006 nicht mehr zulässig und genießt zudem einen schlechten Ruf bei den Konsumenten. Vor ungefähr einer Dekade wurden Saponine, sekundäre Pflanzenmetabolite, als mögliche Alternativen zu den Futteradditiven entdeckt. Saponinmischungen von *Quillaja saponaria*, dem südamerikanischem Seifenrindenbaum, zeigten bei Karpfen *Cyprinus carpio* und Niltilapien *Oreochromis niloticu*s positive Wirkungen auf Wachstum, Futter- und Nährstoffverwertung sowie auf den Metabolismus. Weiterhin wurde in einem Experiment ein höherer Anteil an männlichen Tilapien in einer Saponin-gefütterten Gruppe im Vergleich zur Kontrolle gefunden. Dies wäre ein weiterer attraktiver Effekt von Saponinen bei Tilapien, da kommerzielle Aquakultur von Niltilapien nur finanziell rentabel ist, wenn rein-männliche Populationen zur Produktion verwendet werden. Gemischtgeschlechtliche Populationen unkontrollierten Nachwuchs erzeugen und die Weibchen Maulbrüter sind. Die Erzeugung rein männlicher Tilapienpopulationen wird derzeit größtenteils durch das potentiell Umwelt-gefährdende und krebserregende synthetische Hormon 17-*a*-Methyltestosteron gewährleistet.
Aus den oben genannten Problemen hat sich ein Forschungsvorhaben mit Partnern in Israel und Palästina entwickelt, in dessen Rahmen diese Dissertation entstanden ist. Ziel des Vorhabens war das Fraktionieren durch Hochleistungs-Flüssigkeits-Chromatographie und Testen von Saponinen des Bockshornklees *Trigonella foenum-graecum*. Es wurde davon ausgegangen, daß eine erhöhte biologische Aktivität gefunden wird, wenn statt grober Saponinmischungen Fraktionen als Futteradditive genutzt werden. Verschiedene, *in vitro* getestete Saponinfraktionen bzw. Saponineluate wurden in verschiedenen Fütterungsexperimenten auf ihre Wirkung auf Wachstum, Futter- und Nährstoffverwertung, Metabolismus, grobchemische Zusammensetzung, Genexpression von GH und IGF-1, Geschlechterverhältnis, Reproduktion und Gonadenhistologie getestet. Primär wurden dafür Niltilapien genommen aber auch ein Fütterungsexperiment an Karpfen durchgeführt.
Drei der Fütterungsexperimente wurden zum Zweck der kontinuierlichen Sauerstoffverbrauchsmessung in einer computergesteuerten Respirationsmessanlage durchgeführt, ein weiteres in Außenanlagen in Jericho, Palästina.
Die Experimente zur Feststellung des Einflusses von Bockshornklee Saponinfraktionen auf das Geschlechterverhältnis von undifferenzierten Tilapienlarven wurden primär in einer Durchflußanlage durchgeführt.
Für die Erzeugung von rein männlichen Tilapienpopulationen eignen sich die in dieser These getesteten Saponinfraktionen von *T. foenum-graecum* und *Q. saponaria* und ein ebenfalls getestetes Sapogenin nicht. Die Geschlechterverhältnisse nach Fütterung von gemischtgeschlechtlichen Tilapienlarven wiesen nur in einem Experiment signifikant mehr Männchen auf, als bei den Fischen, die mit Kontrollfutter gefüttert wurden. In einem größer angelegten Wiederholungsexperiment konnten die vorhergehenden Beobachtungen statisitisch

nicht bestätigt werden und müssen zufälligen Effekten oder einer von vornherein beim Besatz der Becken entstandenen Ungleichverteilung der Geschlechter zugeordnet werden. Dies bestätigte sich durch ein Experiment in dem genetisch weibliche Tilapien mit zwei Saponinfraktionen vom Bockshornklee sowie Positiv- und Negativkontrollen gefüttert wurden. Ebenfalls wurde bei einem Langzeitexperiment in Jericho kein Einfluß auf das Geschlecht und die Vermehrung nach kurz- und langfristiger Fütterung von rein weiblichen Tilapien mit Saponinmischungen von *Q. saponaria* im Vergleich zu Fischen die ohne Zusatz von Saponinen gefüttert wurden, gefunden. Ein Laborexperiment, in welchem Bockshornklee Saponinfraktionen getestet werden sollten, konnte diesbezüglich nicht ausgewertet werden.
Bei den Experimenten zur Evaluierung der Effekte von den getesteten Saponinen auf das Wachstum, die Futter- und Nährstoffverwertung, die Genexpression von GH und IGF-1 sowie die grobchemische Zusammensetzung und den Metabolismus gab es keine statistisch signifikanten Ergebnisse. Es gibt aber in allen Experimenten Hinweise darauf, daß eine bestimmte Bockshornklee Saponinfraktion, eluiert mit 60%/40% (v/v) Methanol/Wasser, bessere Ergebnisse liefert als die Kontrollen und die anderen Saponinfraktionen. Die 40%/60% (v/v) Methanol/Wasser Saponinfraktion liefert über alle durchgeführten Exeperimente die numerisch schlechtesten Ergebnisse. Dies läßt darauf schließen, daß diese Fraktion die oftmals in der Literatur beschriebenen „Anti-Nutrients“ enthält. Nur ein signifikantes Wachstumsergebnis konnte mit kontinuierlicher Fütterung von einer *Q. saponaria* Saponinmischung mit hohem Sapogeningehalt in Jericho erzielt werden.
Die in dieser These gefundenen Ergebnisse lassen nicht hoffen, die getesteten Saponinfraktionen als umweltfreundlichen Ersatz für Methyltestosteron zur Erzeugung von rein männlichen Tilapienpopulationen oder zur Unterbindung von unkontrollierter Vermehrung nutzen zu können. Jedoch sollten noch Experimente zur Verabreichungsmethode durchgeführt werden, da sämtliche in dieser These durchgeführten Experimente Fütterungsexperimente waren. Es müßten, um die besprochenen Saponinfraktionen als potentielle Ersatzstoffe für Methyltestosterone ausschließen zu können, noch Tauchbehandlungen und Injektionen getestet werden.
Größeres Potential bieten die Bockshornklee Saponinfraktionen bei einem möglichen Einsatz als Wachstumsförderer, wobei die erzielten Ergebnisse auf Experimenten mit kleinen Stichprobenmengen, anlagenbedingt, beruhen. Daher müßte in diesem Forschungsfeld ein „upscaling“ stattfinden, so daß die Saponinfraktionen unter nahezu kommerziellen Bedingungen getestet werden könnten.

Erklärung

Hiermit versichere ich, dass ich diese Arbeit selbstständig und auschliesslich mit den angegebenen Mitteln angefertigt habe. Sämtliche Zitate sind im Text als solche kenntlich gemacht. Diese Arbeit wurde bislang noch nicht in dieser oder anderer Form einer Prüfungsbehörde vorgelegt.

Bad Säckingen, 13.04.2012

Timo Stadtlander

Curriculum Vitae

Personal data

Timo Alexander Stadtlander

Place of birth:	Bremen, Germany
Family status:	single
Nationality:	German
Date of birth:	11 June 1979

Scientific Work Experience

01 '12 -	Projectleader aquaculture at the Research Institute for Organic Agriculture (FiBL), Switzerland
04 '07 – 12 '11	Junior scientist in a trilateral German Research Foundation funded project entitled: "Increasing productivity and efficiency of Nile tilapia production using plant saponins and introduction of its culture in areas under the jurisdiction of the Palestinian Authority (PA)", BE 1133/13-2 PhD topic: "Effects of saponin fractions on Nile tilapia, *Oreochromis niloticus* (L.), and carp, *Cyprinus carpio* (L.), performance and reproduction" Responsible for the departmental aquaculture research laboratories since October 2009 Teaching of fish anatomy, high value coldwater species and fish feed production in two different M.Sc. modules ("Intensive Aquaculture Systems" and "Integrated Aquaculture Systems") at the University of Hohenheim
10 '05 – 04 '06	Department of Ichthyology and Fisheries Research, Rhodes University, Grahamstown, South Africa. Fisheries biological methods like catch data collection (length, weight, sex, gonad stage, otolith collection, ageing and stomach content analysis) and data processing
07 '04 – 07 '05	Department of Marine Biology, University of Bremen, study project, fatty acid-biomarker analysis and stomach content analysis of two arctic fish species
10 '99 – 06 '05	Student assistant at the Department of Production Engineering of the University of Bremen
2002 – 2004	Participant on various expeditions aboard the German research ships "FS Meteor" and "FS Polarstern", a French ("N/O L'Atalante") and an US ("RV Oregon II") research vessel. -Operation and maintenance of CTD und ADCP units -Deployment and recovery of deep-sea moorings -Operation of long-lines

-Operation of various plankton nets like multinets, bongonets and a rectangular midwater trawl (RMT)

Education

04 ‘07 – 12 ‘11	Ph.D. student at Hohenheim University, Stuttgart, Germany “Department of Aquaculture Systems and Animal Nutrition in the Tropics and Subtropics”
09 ‘06	University of Bremen, Diploma (M.Sc.) in biology
06 ‘06	Diploma (M.Sc.) thesis: “Comparative biology of the smallmouth yellowfish *Labeobarbus aeneus* in Glen Melville Reservoir and the Great Fish River, Eastern Cape, South Africa“
07 ‘05	Study project with title “Vergleich der Ernährungsgewohnheiten von *Boreogadus saida* und *Triglops murrayi* zweier arktischer Fischarten”
10 ‘02	University of Bremen, main subject marine biology, secondary subject zoology, tertiary subject ecology
04 ‘02	University of Bremen, undergraduate examination
10 ‘99	University of Bremen, start of studies; general Biology
09 ‘98 – 06 ‘99	Military service in the German army
07 ‘98	High school graduation at “Gymnasium Vorkampsweg”, Bremen

Research Interests

- Fish nutrition, reproduction and culture
- Fisheries biology and fish ecology of marine and freshwater species
- Marine biology and topics regarding marine ecosystems
- Food web analysis of aquatic ecosystems
- Field work in remote and pristine areas

Other Work Experience

12 ‘06 – 01 ‘07	Volunteer for an Animal Home in Bremen
11 ‘06	Technical support of the Conference “10th EEA Science Conference“ in Hamburg
09 ‘06	Assistance in organisation and technical support of the conference “ISRS European Meeting“ in Bremen
07 ‘06 – 12 ‘06	DAL-Shipping Agent Bremen, part time job in the documentation
06 ‘04 – 09 ‘04	Brewery Beck & Co Bremen, student trainee in the human resources

08 ‘01 – 09 ‘01 Daimler-Chrysler Bremen, student job at the assembly line

Special and Language Skills

- Course on “Laboratory Animal Science category B” (GV-SOLAS)

- European drivers license for car and motorcycle

- Divers certificates PADI Open Water Diver and PADI Advanced Open Water Diver

- Fluent in written and spoken English

- Basics in Spanish

- Very good computer skills for soft- and hardware

Other interests

- Since 2003 member of the D.E.G. (German Elasmobranch Association for the protection of sharks and rays)

- Late summer 2003: Course about the behaviour of White Sharks, Gaansbai, South Africa

- Various sports like running, kayaking, diving, snorkelling and weight lifting

-Enjoying a good, thrilling novel or have a chilled beer with friends

References

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Publications:

T. Stadtlander, Khalil W.K.B., Focken U. and Becker K; 2013. Effects of low and medium levels of red alga Nori (*Porphyra yezoensis* Ueda) in the diets on growth, feed utilization and metabolism in intensively fed Nile tilapia, *Oreochromis niloticus* (L.). Aquaculture Nutrition 19(1), 64-73, doi: 10.1111/j.1365-2095.2012.00940.x.

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Bad Säckingen, 27/03/2013

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Einfluss von Saponinfraktionen des Bockshornklees, Trigonella foenum-graecum (L.), auf das Geschlechterverhältnis und die Reproduktion von Niltilapien, Oreochromis niloticus (L.) und auf Wachstum, Nährstoffverwertung und Metabolismus von Niltilapien und Karpfen, Cyprinus carpio (L.)