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Effects of low dietary levels of saponins on two common culture fish – common carp (*Cyprinus carpio* L.) and Nile tilapia (*Oreochromis niloticus* (L.))

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in den Tropen und Subtropen der Universität Hohenheim
Fachgebiet Aquakultur-Systeme und Tierernährung
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Dissertation
zur Erlangung des Grades eines Doktors
der Agrarwissenschaften

Der Fakultät IV – Agrarwissenschaften II
(Agrarökonomie, Agrartechnik und Tierproduktion)

von
George Francis
aus Kerala, Indien

2001

Die vorliegende Arbeit wurde am 27-11-2001 von der Fakultät IV – Agrarwissenschaften II – der Universität Hohenheim als „Dissertation zur Erlangung des grades eines Doktors der Agrarwissenschaften“ angenommen.

Tag der mündlichen Prüfung:	14-12-2001
Berichterstatter bzw. 1. Prüfer:	Prof. Dr. K. Becker
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2 Prüfer:	Prof. Dr. V. Hoffmann
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Dekan:	Prof. Dr. S. Dabbert

Acknowledgements

First of all I thank Prof. Dr. Klaus Becker for allowing me do Ph. D. at his Institute in Hohenheim. It was his unreserved support and guidance, and stimulating enthusiasm that steered me through to the end of the work in stipulated time. I am grateful to Dr. H. P. S. Makkar for his valuable advice and suggestions, which helped me to stay on course throughout the research work. I am also thankful to Dr. Ulfert Focken for devoting a lot of time for me particularly during the initial period of my stay in Germany and helping me both in academic and non-academic matters.

I am thankful to each and every one of my colleagues at the Institute and friends for numerous favours and great company during these years. Dr. Peter Lawrence and Hartmut Richter require special mention for critically reading parts of this thesis.

I also express my gratitude to KAAD (Catholic Academic Foreigner Service) for financial support during the study period.

Finally, I lovingly acknowledge the sufferings of my wife and kids, who sacrificed a lot to keep me in high spirits during this period.

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List of Abbreviations

Abbreviations used in text

ALC	apparent lipid conversion
AMR	average metabolic rate
AS-1	*Asparagus officinalis* saponin 1
AUE	apparently unmetabolised energy
B-cell	Bursa of Fabricius lymphocyte
BSA	bovine serum albumin
C	control diet
CNS	cental nervous system
CO	cytochrtome *c*-oxidase
DDMP	2, 3-dihydro-2, 5-dihydroxy-6-methyl-4H-pyran-4-one
DHS-1	dehydrosoysaponin 1
DNA	deoxyribonucleic acid
dpf	days post fertilisation
DS-1	de-acylated *Quillaja* saponin 1
DS-2	de-acylated *Quillaja* saponin 2
E_2	estradiol
EE	energy expenditure
ER	energy retention
FAO	Food and Agricultural Organisation
FCE	feed conversion efficiency
FCR	feed conversion ratio
GS	*Gypsophila* saponins
GSI	The gonado-somatic index
GSR	Raw *Gypsophila* saponin
HDL	high density lipid
HL-60	human leukemia cell line
HMF	high molecular fraction
i. p.	intraperitoneally
IgE	immunoglobulin E
IgG	immunoglobulin G
ISCOM	immune stimulating complex
ISI	intestine somatic index
$kg^{-0.8}$	metabolic body weight
LDH	lactate dehydrogenase
LDL	low density lipid
LH	luteinising hormone
LSI	liver somatic index
MGR	metabolic growth rate
MR	metabolic rate
OPMG	oxygen consumed per unit body mass gain
OSW-1	3β, 16 β, 17α-tri-trihydroxycholest-5-en-22-one 16-O-(2-O-4-methoxybenzoyl- β-D-xylopyranosyl)-(1→3)-(2-O-acetyl-α-L-arabinopyranoside)
PBS	phosphate buffer solution
PER	protein efficiency ratio
pH	hydrogen ion concentration
PPV	protein productive value
QH-B	a *Quillaja* fraction
QS	*Quillaja* saponins
QS-21	*Quillaja* saponin 21
Quil-A	partially purified *Quillaja* saponin
Rf	retention factor- ratio of the distance covered by the spot of interest to the distance travelled by the solvent front or a chosen standard.
RMR	routine metabolc rate
SMR	standard metabolic rate
taGtH	tilapia gonado-tropic hormone
T-cell	thymus lymphocyte
TEER	transepithelial electrical resistance
TLC	thin layer chromatography
TPD	transmural potential difference
UK	United Kingdom
YBE	*Yucca* butanol extract
YS	*Yucca schidigera*
β LG	β-lactoglobulin

Units

°C	degrees centigrade
µg kg^{-1}	microgram per kilogram
µl	microlitre
A°	angstrom units
g kg^{-1}	gram per kilogram
g	unit for measuring centrifugation force
h	hour
kJ	kilojoule
l	litre
m.t.	metric tonnes
mg kg^{-1}	milligram per kilogram
mg l^{-1}	milligram per litre
ng	nanogram
ppm	parts per million

vitamin A	retinol
vitamin E	tocopherols

Common abbreviations

e. g.	for example
et al.	and others

Statistical terms

ANOVA	analysis of cariance
P	probability
SD	standard deviation

Chemical elements and compounds

Ca	calcium
Fe	iron
N	nitrogen
Na	sodium
Zn	zinc
$CHCl_3$	chloroform
H_2O	water
H_2SO_4	sulphuric acid
HOAc	acetic acid
KH_2PO_4	potassium di-hydrogen phosphate
MeOH	methanol
NaOH	sodium hydroxide
NH_3	ammonia

General Introduction

1. Background

As estimated by FAO, the human demand for food fish will climb from the current level of about 90 million metric tonnes (mmt) to about 110 mmt by the year 2010. By the year 2030, aquaculture will dominate fish supplies, and more than half of the fish consumed is likely to originate from this sector (FAO 2000). In fact, aquaculture has become the fastest growing food production sector of the world, with an average annual increase of about 10% since 1984 compared to 3 % increase for livestock meat and 1.6 % increase for capture fisheries (FAO, 1997). To sustain such high rates of increase in aquaculture production, a matching increase in the levels of production of fish feeds is required. Aqua-feed production is currently one of the fastest expanding agricultural industries of the world, with annual growth rates in excess of 30% per year. The projected total production of aqua-feeds in the year 2010 range from 25 mmt (Tacon 2001) to 32.6 mmt (IFOMA 2000) against an approximate production estimate of about 13 mmt in the year 2000. Almost one third of the 122 million tonnes of fish harvested in the year 1997 was converted into fishmeal or fish oil to be used in producing animal feed, including aquaculture feed. FAO (1999) estimates that about 40 % of the total aqua-feed production was for carnivorous finfish species, 35 % for non-carnivorous species, and 25 % for shrimp. Thus, even though the bulk of fishmeal is used in salmon, trout and marine fish farming in western countries, freshwater fish farming, largely that of carp, also consumes a substantial proportion. Given the current very rapid increase in the intensification of fresh-water farming in Asia, particularly in China, intense future competition for limited global supplies of fishmeal and fish oil are likely (Sargent and Tacon, 1999; Naylor et al., 2000). Fishmeal production is also rather localised in some regions of the world, as a result of which it is more expensive and difficult to obtain in many countries practising aquaculture. The need for alternative protein sources to replace fishmeal in aqua-feeds is therefore obvious and was strongly recommended by the Second International Symposium on Sustainable Aquaculture (1998) in Oslo, Norway.

The main limiting factor to their use as food and feed is the presence of a wide variety of anti-nutritional substances (Francis et al., 2001). These antinutrients exert a negative impact on the nutritional quality of the proteins in the plant material that contain them. A number of these compounds have however been reported to have positive effects in domestic animals and humans, when present in low levels in the diet. The aim of this research work has been to study the effects of one such antinutrient, saponin, when present at low levels in the diets of

two important species of culture fish, namely common carp (*Cyprinus carpio*) and Nile tilapia (*Oreochromis niloticus*).

2. About saponins

Saponins are steroid or triterpenoid glycosides found in many of the potential, alternate plant-derived feed ingredients for fish such as legumes (ranging between 18 and 41 mg per kg in various legume seeds; 67 mg kg^{-1} in defatted roasted soybean flour; Fenwick et al., 1991). When added to water, they are highly toxic to fish because of the damage caused to the respiratory epithelium of the gills by their detergent action. Newinger (1994) identified saponins as the main active compounds present in the most effective fish poisoning plants in Africa. They are also considered to be the active components of many traditionally-used fish poisons, such as mahua oil cake. Saponins in lupin seed meal (1.1%) and alfalfa (*Medicago sativa*, < 0.30 % in low saponin varieties to > 1.5 % in high saponin varieties) could have been important contributing factors for the lower growth performance of rainbow trout (de la Higuera et al., 1988), and tilapia (Olvera-Novoa et al., 1990; Yousif et al., 1994) fed diets containing high levels of these ingredients. Krogdahl et al. (1995), however, did not find any negative effects of saponins included in the diet of Atlantic salmon at levels similar to that expected to be found in a soybean meal (30-40 %) based diet. Bureau et al. (1998) observed extensive damage to the intestinal mucosa in rainbow trout and Chinook salmon fed diets containing over 1.5 g per kg *Quillaja* bark saponin. The condition of the intestine of these fish was similar to that of fish fed the raw soybean meal diet indicating the role of the saponins in causing the damage. The negative effects of saponins could be caused by the well-known effects of these surface active components on biological membranes. Saponins at low levels were, however found to increase transport of nutrients across rat intestinal membranes (Onning et al., 1996) and increase activity of some gut and liver enzymes in carp (Serrano et al., 1998).

3. The hypotheses

The primary hypothesis was that saponins, at low dietary levels, would not harm the intestinal mucosa of fish, but might make utilisation of feed more efficient, due to their effects on the mucosal membrane structure and functioning and thereby increase growth.

During the course of experiments using tilapia it was observed that egg production was inhibited by dietary saponins. Consequently, we developed a second hypothesis that dietary

saponins might have a negative effect on reproductive activity of Nile tilapia. The results reported here, in addition to being relevant to applied aquaculture, could also contribute to the knowledge about effects of low dietary levels of saponins in animal systems, based on the fish model.

4. The experimental approach

The saponins used were either obtained from commercial sources or extracted and concentrated from plant material.

The feeding experiments to test the effects of supplementation of saponins in diets of carp and tilapia were conducted in a computer controlled respirometer system (Figure 1; technical details described in Focken et al., 1994) which allowed feeding and continuous measurement of oxygen consumption of individual fish. Data on oxygen consumption from feeding experiments together with initial and final carcass chemical composition values were used to set up complete energy budgets for fish, showing proportions for indigestible, metabolized and retained energy. These provide comprehensive information on growth physiology of the experimental fish and how it is affected by feed supplements. Some supplementary feeding trials including the one to assess the effect of dietary saponins on the reproductive development in tilapia fry were conducted in aquaria forming part of re-circulating systems. Parameters such as serum cholesterol and hormone levels and muscle cholesterol levels were also measured to get insights into the various aspects of dietary saponin activity.

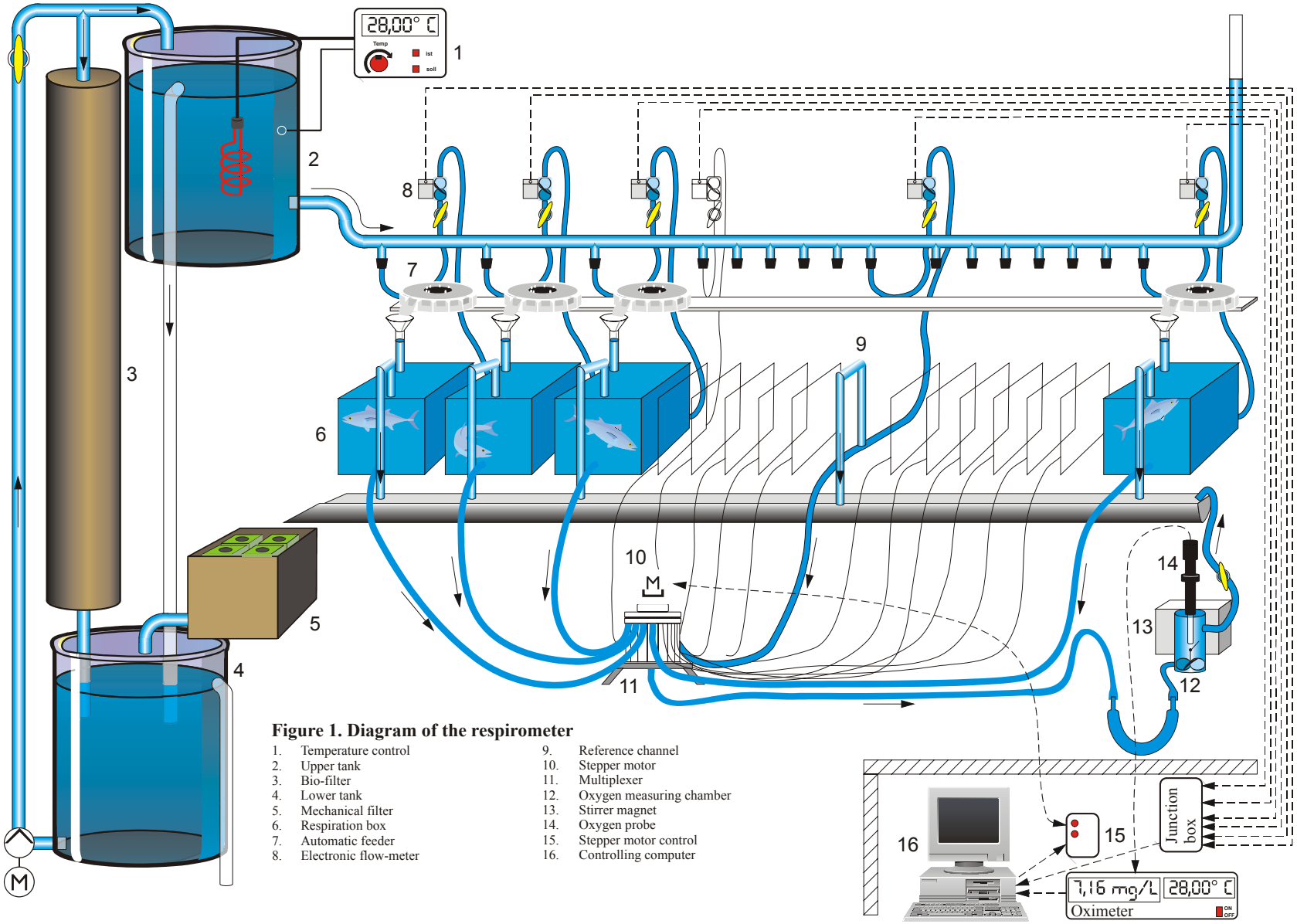

Figure 1. Diagram of the respirometer

1. Temperature control
2. Upper tank
3. Bio-filter
4. Lower tank
5. Mechanical filter
6. Respiration box
7. Automatic feeder
8. Electronic flow-meter
9. Reference channel
10. Stepper motor
11. Multiplexer
12. Oxygen measuring chamber
13. Stirrer magnet
14. Oxygen probe
15. Stepper motor control
16. Controlling computer

Chapter 1

The biological action of saponins in animal systems – a review

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Revised version accepted for publication in the British Journal of Nutrition

Abstract

Saponins are steroid or triterpenoid glycosides, common in a large number of plants and plant products that are currently or potentially important in human and animal nutrition. Several biological effects have been ascribed to saponins. Extensive research has been carried out into the membrane permeabilizing, immunostimulant, and hypocholesteremic properties of saponins and they have also been found to significantly affect growth, feed intake and reproduction in animals. These structurally diverse compounds have also been observed to inhibit the growth of malignant cells, to kill molluscs, to be antioxidants, to impair the digestion of protein and the uptake of vitamins and minerals in the gut, to cause hypoglycaemia, to scavenge free radicals and to act as anti-fungal and antiviral agents. These compounds can thus affect the animal body in a host of different ways both positive and negative.

Key words: Saponins, steroid, triterpenoid, biological activity

1. Introduction

The saponins are naturally occurring surface-active glycosides. They are mainly produced by plants, but also by lower marine animals and some bacteria (Yoshiki et al., 1998; Riguera, 1997). They derive their name from their ability to form stable, soap-like foams in aqueous solutions. This easily observable character has attracted the human interest from ancient times. Saponins consist of a sugar moiety usually containing glucose, galactose, glucuronic acid, xylose, rhamnose or methylpentose, glycosidically linked to a hydrophobic aglycone (sapogenin) which may be triterpenoid (Fig 1a) or steroid (Fig 1b) in nature. The aglycone

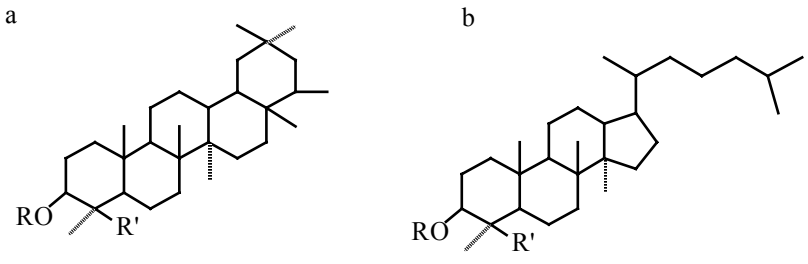

Figure 1. Basic structures of sapogenins: a triterpenoid (a) and a steroid (b)

may contain one or more unsaturated C-C bonds. The oligosaccharide chain is normally attached at the C-3 position (monodesmosidic), but many saponins have an additional sugar

moiety at C-26 or C-28 position (bidesmosidic). The great complexity of saponin structure arises from the variability of the aglycone structure, the nature of the side chains and the position of attachment of these moieties on the aglycone. Experiments demonstrating the physiological, immunological and pharmacological properties of saponins have provoked considerable clinical interest in these substances.

There have been quite a few reviews in recent years of published reports about various properties of saponins (Kensil, 1996, Sen et al., 1998, Barr et al., 1998, Yoshiki et al., 1998). The purpose of this review is to provide an overview of their extremely diverse biological activities and relate these to their structure as far as the available literature permits. It is hoped that the information collated here will provide the reader with a comprehensive background on the effects of saponins in animal systems.

2. Occurrence

Saponins occur constitutively in great many plant species, wild plants and cultivated crops. In cultivated crops the triterpenoid saponins are generally predominant, while steroid saponins are common in plants used as herbs or for their health-promoting properties (Fenwick et al., 1991). Triterpenoid saponins have been detected in many legumes such as soybean, beans, peas, alfalfa, etc. and also in alliums, tea, spinach, sugar beet, quinoa, liquorice, sunflower, horse chestnut, and ginseng. Steroid saponins are found in oats, capsicum peppers, aubergene, tomato seed, alliums, asparagus, yam, fenugreek and ginseng. One example of an extensively studied group of saponins is produced from *Quillaja saponaria*, a tree native to the Andeans, whose barks were pealed and extracted by the natives as a shampooing agent, and by the Shamans as an overall curing agent. Generally immature plants of a species have been found to have higher saponin contents than more mature plants of the same species (Fenwick et al., 1991).

A number of factors such as physiological age, environmental and agronomic factors, have been shown to affect the saponin content of plants (see review by Yoshiki et al., 1998). Reports reviewed here indicate that saponins increase on sprouting in soybeans, alfalfa, mung beans, and peas but decrease in moth bean, and that light has a profound effect on the saponin content during germination. Saponin content increases during light-irradiated germination, but decreases when light is excluded.

3. Role in plants

The physiological role of saponins in plants are not yet fully understood. While there are a number of publications describing their identification in plants, and their multiple effects in animal cells and on fungi and bacteria, only a few have addressed their function in plant cells. Many saponins are known to be antimicrobial, to inhibit mould, and to protect plants from insect attack. The observation that saponin levels are higher in immature plants than mature plants (Fenwick et al., 1991) supports the idea that saponins play a role in plant-protection. Immature plants are more prone to environmental attack and therefore, need more effective protection mechanisms. Saponins may thus be considered a part of plants' defense systems, and as such have been included in a large group of protective molecules found in plants' named "phytoanticipins" or "phytoprotectants" (Morrissey and Osbourn, 1999). The first term describes those saponins, such as A and B avenacosides from oat, that are activated by the plants' enzyme in response to tissue damage or pathogen attack (Gus-Mayer et.al 1994). The second describes those saponins that have a general anti-microbial or anti-insect activity. A glycosylated triterpenoid saponin from *Pisum sativum* was purified and characterized as a specific inhibitor of diguanylate cyclase, a key regulatory enzyme in the synthesis of cellulose (Ohana et al., 1998). It has also been suggested that saponins could be a source of monosaccharides for the plant (see review by Barr et al., 1998).

4. Isolation and characterization of saponins

The unique chemical nature of saponins demand tedious and sophisticated techniques for their isolation, structure elucidation and analysis. The task of isolating saponins from plant material is complicated also by the occurrence of many closely related substances in plant tissues, and by the fact most of the saponins lack a chromophore. Thus, for many years, the complete characterization of saponins from even well known saponin-containing plants was not achieved. However, recently, renewed interest in medicinal plants and foods alongside the dramatic evolution of analytical tools have resulted in a burst of publications presenting numerous novel saponins. The modern methods available for the separation and analysis of saponins have been well reviewed by Marston et al. (2000), Muir et al. (2000) and Schopke (2000). These methods will be only briefly outlined in this review.

There are several strategies available for the isolation of saponins. As a general rule, they begin with the extraction of the plant material with aqueous methanol or ethanol. Further processing of the extract is carried out after evaporation under reduced pressure, dissolution in a small amount of water and phase separation into n-butanol. It is currently recognized that this step is sometimes undesirable, since only those saponins with short oligosaccharide side chains will eventually be extracted into the butanolic phase. A further purification is then carried out, which involves liquid chromatography over a silica gel column, or a gradient elution from a polymeric support or liquid-liquid partition chromatography, or, as most commonly practiced, HPLC separation. In most cases, certain of the above steps have to be repeated with a change of support or eluent to achieve high purity.

Once the saponin has been purified, it may be subjected to the analytical methods including mass – spectrometry (MS), proton and carbon nuclear magnetic resonance (NMR), and infra red spectroscopy. Other classical methods are used to ascertain the presence of saponins in a crude plant extract, and to elucidate their composition throughout purification steps. TLC and staining with dehydrating reagents containing aromatic aldehydes (such as anisyl aldehyde in sulphuric acid) are commonly used. The pure saponin may also be hydrolyzed to verify the nature of its glycosidic moieties.

5. Biological effects in animals

5.1. Effects on cell membranes

a. Permeabilization, and effects on other membrane properties

A large number of the biological effects of saponins have been ascribed to their action on membranes. In fact, their specific ability to form pores in membranes has contributed to their common use in physiological research (Menin et al, 2001, Plock et al, 2001, Choi et al, 2001, Authi et al, 1998 and El Izzi et al, 1992). Saponins have long been known to have a lytic action on red cell membranes and this property has been used for their detection. The haemolytic action of saponins is believed to be the result of the affinity of the aglycone moiety for membrane sterols, particularly cholesterol (Glauert et al., 1962), with which they form insoluble complexes (Bangham and Horne, 1962). The amount of glycosides required for permeabilization was much lower for cholesterol-rich lipid layers than cholesterol-free membranes (Gögelein and Hüby, 1984). Isolated cell membranes from human erythrocytes

when treated with saponin developed holes of 40 to 50 A° diameter as against the 80A° holes produced in artificial membranes (Seeman et al., 1973). Compared to the reversible perforations caused by substances such as vitamin A, the membrane holes or defects produced by saponins were long lasting and such membranes were then permanently permeable to large molecules like ferritin (Seeman, 1974). The lesions that are caused by saponins are thought to be a micelle-like aggregation of saponins and cholesterol in the plane of the membrane, possibly with saponin molecules arranged in a ring with their hydrophobic moieties combined with cholesterol around the outer perimeter (Seeman, 1974; Bangham and Horne, 1962). Other reports indicate that the interactions between saponins and biological membranes are much more complex and that other factors may also be involved. Brain et al. (1990), showed that insertion of the aglycone into the lipid bilayer is independent of the presence of cholesterol. Saponins could induce a permeability change on liposomal membrane without cholesterol when they are glycosylated at both C-3 and C-28 (bidesmosidic) of the oleanolic aglycone (Hu et al., 1996). Abe et al. (1978b) observed no close and direct relationship between the haemolytic activity of saikosaponin (from *Bupleurum falcatum*) and membrane-permeabilizing activity, nor was it correlated with either their surface or interfacial tension lowering properties (Pillion et al., 1996; Steurer et al., 1999). Efficacy as absorption enhancing agents across nasal mucosa in rats was greatest in those *Quillaja* saponins with the lowest haemolytic titres and critical micellar concentration (CMC) values (Pillion et al., 1996).

Cholesterol enrichment was shown to have an inhibitory effect on many membrane ATPases, as it may directly interact with the boundary lipids of ATPase and alter the intermolecular hydrogen bonds of the protein. Ginsenosides (from *Panax quinquefolius* and *Panax japonicus*) share the steroid backbone and amphipathic nature with cholesterol. The desacyl-jego-saponin (from *Styrax japonica*) and gensenoside-Rd, which had little or no effect on membrane permeability, were capable of stimulating Na^{+}-Ca^{+} exchange activity in canine cardiac sarcolemmal vesicles (Choi et al., 2001; Yamasaki et al., 1987). However, not all ginsenosides alter the Na^{+}-K^{+}-ATPase and Ca^{2+}-Mg^{2+}-ATPase activity in neurones in the same manner (Choi et al., 2001). It is possible that some ginsenosides interact with membrane cholesterol and displace it from the immediate environment of ATPases. Since removal of cholesterol will lead to an increase in membrane fluidity, conformational changes that ATPases undergo during their transport cycle may be facilitated. The inotropic action of saponins, supposed to be caused by their effect on membrane Ca^{2+} channels, also did not have any simple correlation with membrane permeability effect (Enomoto et al., 1986) indicating

involvement of other mechanisms. Saponins such as ophiopogonins and ginsenocides hemagglutinated human, rabbit, and sheep erythrocytes but were not haemolytic (Takechi and Tanaka, 1995a). They were thus able to bind to the membrane lipids of erythrocytes and form bridges between the cells. Other studies (Gögelein and Hüby, 1984) explained the increase in electrical conductance caused by saponins in planar lipid bi-layers, to be due to fluctuating membrane channels. Soysaponin I, III and dehydrosoysaponin, DHS-I (isolated from *Desmodium adscendens*) have been shown to be able to open large calcium dependent potassium (maxi-K) conductance channels causing membrane hyperpolarization, suppression of electrical activity and relaxation of smooth muscle (McManus et al., 1993).On the other hand there are also reports of the ability of saponins to block membrane ion channels on neurons (Kai et al., 1998) and human neutrophils (Bei et al., 1998).

The side chains present on the aglycone such as sugar chains (Segal et al., 1974; Santos et al., 1997), acyl recidues (Matsuda et al., 1997) or the epoxy-framework system (Abe et al., 1978a) are also supposed to contribute to the effects on membranes. These effects however were not uniform e. g. some saponins with acyl residue (lablaboside D) did not show hemolytic activity (Oda et al., 2000), while in another work, only acylated triterpenoids saponins in low concentrations were able to alter membrane activity (Melzig et al., 2001). In the later case, it was suggested that only acetylated saponins may integrate transiently into membranes, thus inducing pore-like structures. It has been suggested that the hemolytic activity of saponins increase with increasing numbers of polar groups in the aglycone moiety (Namba et al., 1973). Carbohydrate chain length was shown to influence the manifestation of physiological activity of oligosides (Kuznetzova et al., 1982). Steroid and triterpenoid saponins with a single sugar chain (monodesmosides) were found to have strong haemolytic activity, whereas those with two sugar chains (bidesmosides) showed less activity (Woldemichael and Wink, 2001; Fukuda et al., 1985). Even though monodesmoside saponins are generally considered to be more active than bidesmosides there are exceptions. e.g., among 15 synthetic methyl ursolate glycosides, di and triglycosides showed much higher haemolytic activity than monoglycosides (Takechi and Tanaka, 1995b). It was also observed that an increase in sugar moieties enhanced the effects of saponins on sarcolemmal membrane Ca^{2+} permeability (Yamasaki et al., 1987). The permeabilizing activity of the native avenacin A-1 was completely abolished after one, two, or all three sugar residues were hydrolysed to yield monodeglucosyl, bis-deglucosyl, and aglycone derivatives respectively (Armah et al., 1999). De-acylated *Quillaja* saponins DS-1 and DS-2 which differ only in the absence of one glucose residue, differed significantly in their ability to stimulate absorption of insuln despite

having similar CMC and haemolytic strength (Pillion et al., 1996). The stereochemistry of the terminal sugar on the saccharide chain appears to be an important feature in conferring activity on the saponin molecule because of its ability to affect the overall shape of the molecule (Gee et al., 1998). Increased saccharide branching and increased permeabilizing activity were observed to have no direct relationship with haemolytic properties in saponins (Price et al., 1994).

The interaction between saponins and membrane lipids, thus seems to be complicated, and to be affected by other components of the membranes. The composition of the target membrane, the type of side chain, and the nature of the aglycone to which these are attached are all features which appear to be necessary to produce a permeabilizing effect (Attele et al., 1999; Gee et al., 1998). Membrane fluidity controls the enzyme activity of biological membranes and has an important role in ion transport (Ma and Xiao, 1998) and the ability of saponins to affect this parameter may explain their effects on cellular function. Cellular membranes may exist under conditions of curvature stress, being close to the hexagonal phase transition. Consequently, the physicochemical properties of these membranes are sensitive to changes in membrane components and lipophilic agents, which may modulate curvature stress. Membrane proteins are thought to be localised selectively in cholesterol-rich domains (ACh receptor) or in cholesterol-poor domains (the sarcoplasmic Ca^{2+}-ATPase). Therefore, the biophysical properties of the different domains, rather than the bulk lipid, may selectively influence transmembrane protein function and mimic specificity at the effector level. Saponins may interact with the polar heads of membrane phospholipids and the -OH group of cholesterol through OH groups at C-3 or C-28, which will result in their later ability to form micelle like aggregates. Moreover, their hydrophobic aglycone backbone could intercalate into the hydrophobic interior of the bilayer. Both of these effects may contribute to altering the lipid environment around membrane proteins. It has become increasingly evident that the lipid environment of membrane proteins, including ion channels, transporters, and receptors, plays an important role in their function. Abe et al. (1978a) proposed that the effects of saikosaponins on the secondary biochemical responses could have been mediated by alterations in the metabolism of proteins or glycoproteins in the plasma membranes.

The precise details of the interactions between saponins and membranes need more elucidation so that a clearer correlation is possible between structure and activity. It seems likely that different mechanisms such as formation of the saponin-cholesterol complex, alterations in the organisation of sarcolemmal membrane phospholipids, and the formation of phospholipid breakdown products such as phosphatidic acid are all involved in the actions of

saponins on membranes (Yamasaki et al., 1987). The three-dimentional orientation of the saponins may also play an important role in their bio-activity (Fenwick et al., 1991). It is however safe to conclude that since cell membranes are important sites mediating inter and intracellular events, many saponins within a specific concentration range act as stimulators or inhibitors of normal cellular functions through changes in the cell membrane structure (Rao and Sung, 1995).

b. Effects on nutrient uptake through the intestinal membrane

Johnson et al. (1986) found that some saponins increase the permeability of intestinal mucosal cells *in vitro*, inhibit active mucosal transport and facilitate uptake of substances that are normally not absorbed. Saponins (from *Gypsophila*, *Quillaja*, clover, guar and alfalfa) also lowered transmural potential difference (TPD-the electrochemical gradient that acts as a driving force for active nutrient transport across the brush border membrane of the intestine) across the small intestine of the rat (Gee et al., 1989). Here, the stereo-structure of the saccharide chain appeared to be an important feature in conferring this ability. Soysaponins with a non-acidic triterpenoid moiety coupled to a straight chain tri-saccharide caused only a small drop in TPD in Wistar rat intestines *in vitro* whereas saponins from *Gypsophila*, alfalfa and guar, which contain acidic triterpenes coupled to branched tetrasaccharide moieties caused significant changes in TPD. Among alfalfa saponins (glycosides of medicagenic acid), bidesmosides containing 4 sugar moieties reduced rat-intestine TPD *in vitro* in a similar fashion to the monodesmoside and to medicagenic acid itself (one exception was 3Glc, 28 Glc-medicagenic acid which had no effect on TPD) (Oleszek et al., 1994). The tridesmoside of zahnic acid, which is only weakly haemolytic and which neither inhibits fungal growth nor forms insoluble complexes with cholesterol, was the most active compound, giving further evidence of the complexity of the interactions between saponins and membranes.

An increase in the apparent permeability of the brush border observed at sublethal levels of saponins may have important implications for the uptake of macromolecules, such as allergens, whose passage through the epithelium is normally somewhat restricted (Gee et al., 1996). Rats fed the unwashed bitter quinoa flour or cereal diets (containing saponins that caused rapid depolarisation of the small intestinal mucosa, even at concentrations of less than 50 $\mu g\ ml^{-1}$) based on this material demonstrated growth impairment and decreased food conversion efficiency (Gee et al., 1993). The presence of *Gypsophila* saponins enhanced the uptake of β LG, a milk allergen in the jejunal loops' tips of brown Norway rats (Gee et al., 1997). Histological investigation of the mucosal epithelium exposed to saponin revealed

damage, especially of the villi. Avenacosides which were haemolytic in the crude extract significantly increased the passage of ovalbumin in rat intestine *in vitro* at a concentration of 1 mg ml^{-1} but did not affect the active transport of glucose (Onning et al., 1996). However, when given *in vivo* in the same experiment the saponins did not affect the transport of bovine serum albumin (BSA) across the intestine wall. *Quillaja* saponins were able to increase uptake of HGG (human gamma globulin) into the body, when fed to tilapia or administered by anal incubation (Anderson, 1992; Jenkins et al., 1991). Rat intestines have been shown to offset the damage to small intestinal mucosal cells caused by dietary saponins (up to 1.5 %) by continuous proliferation (Gee and Johnson, 1988).

Some reports describe obstruction of the absorption of micronutrients by dietary saponins. *Gypsophila* saponins in the diet depressed mean liver iron concentrations and total liver iron by impairing iron absorption (Southon et al., 1988). Probably the saponin formed complexes with dietary iron rendering it unavailable for absorption. It has been reported that lucerne saponins increased excretion of Fe and Mg when present in rat diets, and reduced plasma Ca and Zn in pigs. These saponins could complex with Fe and Zn *in vitro* and this complex formation might have hindered their absorption. Triterpenoid saponins from *Gypsophila* and *Quillaja* when administered at 0.9% of the diet appeared to interfere with the absorption of vitamin A and E in chicks (Jenkins and Atwal, 1994). Feeding a steroid saponin (sarsasaponin) up to levels of 0.9 % had no effect on any of these parameters.

The mechanism of action of saponins on the intestinal membranes *in vivo* is not yet clearly understood. Ingested saponins are exposed to many potential ligands in the intestine like bile salts, dietary cholesterol and membrane sterols of the mucosal cells, and nutrients or antinutrients in food, all of which may reduce or enhance their effectiveness. More research is called for on these aspects.

5.2. Effects on animal growth and feed intake

Animal nutritionists have generally considered saponins to be deleterious compounds. In ruminants and other domestic animals the dietary saponins have significant effects on all phases of metabolism, from the ingestion of feed to the excretion of wastes (Cheeke, 1996). Alfalfa and soy are the only examples of saponin-rich plants that serve extensively in human, ruminant and poultry diets. Lately, a number of studies have reported both beneficial and adverse effects of these compounds in a variety of animals (see review by Sen et al., 1998).

a. Effects in Ruminants

Yucca schidigera plant extract (the plant is native to south and central America where it is used as animal feed, and like many other saponin rich plants, as a herbal medicine) has been found to improve growth, feed efficiency and health in ruminants (Mader and Brumm, 1987). *Quillaja* saponins increased the efficiency of *in vitro* rumen-microbial protein synthesis and decreased degradability of feed protein (Makkar and Becker, 1996). Partially hydrolysed alfalfa saponins administered intra-ruminally resulted in a significant reduction in the total protozoa count in the rumen of sheep (Lu and Jorgensen, 1987) which may be the reason for the decrease in feed protein degradability. *Yucca* extract can also bind ammonia when ruminal ammonia concentrations are high, and release it again when ruminal ammonia is low, thus providing a continuous and adequate supply of ammonia for microbial protein synthesis (Hussain and Cheeke, 1995). A positive effect of *Yucca* saponins in ruminant nutrition was attributed to the enhancement of the entrapment of ammonia nitrogen from urea supplemented straw (Makkar et al., 1999). This increases the availability of nutrients to rumen bacteria and reduces environmental damage by decreasing losses of ammonia to the air. Supplementation of feed with leaves of *Sesbania sesban,* known for its high saponin content, has been found to have the potential to improve protein flow from the rumen by suppressing protozoal action there (Newbold et al., 1997) but rumen bacteria were observed to be capable of metabolising the antiprotozoal factor. Saponins may also be degraded by the saliva of sheep fed saponin rich foods for a long time (Odenyo et al., 1997; Teferedegne, 2000). GC-MS analysis of tissue samples (bile, urine, rumen, duodenum, jejunum, colon and rectum, and faeces) from sheep fed *Narthecium ossifragum* (containing mainly sarsasapogenin and smilagenin) for 6 days, followed by 20 mg d^{-1} of [20,23,23-H-2(3)]sarsasapogenin on the seventh day revealed only negligible levels of deuterium-labelled sarsasapogenins in these organs (Flaoyen et al., 2001). Ingested saponins were quickly hydrolysed in the rumen to free sapogenins and, in part, epimerized at C-3 into episapogenins. The absorption of free sapogenins appeared to occur in the jejunum. The concentration of sapogenins in faeces reached a plateau 108 h after dosing started.

The positive effects of saponins were more pronounced when they were directly administered into the rumen rather than added to the feed (Odenyo et al., 1997). Killeen et al. (1998) proposed that a surfactant/ flocculant action of saponins on the feed constituents that alters the rate of digestion, would account for the substrate-dependent nature of the effect of *Y. schidigera* on rumen dry matter and nitrogen digestibility. This substrate-dependency might also be due to negative effects of saponins on specific bacterial populations (Hussain and

Cheeke, 1995). Wang et al. (2000a and b) observed that supplementation with *Yucca* extracts might be beneficial to ruminants fed a high grain diet. *Yucca* saponins were found to have a direct negative effect on cellulolytic bacteria while being harmless to amylolytic bacteria, suggesting the possibility to use of saponins for 'designing' the rumen population. Other reports point to the fact that some of the effects of dietary saponins in lambs are sex-dependent. Bosler et al., (1997) found that both male and female lambs fed up to 40 mg kg^{-1} *Quillaja* saponin mixed with a basal diet had significantly higher average daily weight gains than controls but that the difference in weight gain was lower in the females. The dietary saponins reduced the fat deposits around the kidney in females while increasing it in males.
Dietary saponins were often suspected of having a role in causing ruminant bloat (Sen et al., 1998; Cheeke, 1996), but clear experimental proof for this is lacking in the literature. The lack of any positive effect in experiments where *Yucca* saponins were fed to ruminants (e. g. Wu et al., 1994) might have been because adapted animals were used.

b. Effects in fish

Saponins have been reported to be highly toxic to fish because of their damaging effect on the respiratory epithelia (Roy et al., 1990). They are also considered to be the active components of many traditionally used fish poisons, like mahua oil cake (Francis et al, 2001a). Fish have also been shown to exhibit stress reactions to the presence of saponins in water. Roy and Munshi (1989) reported that the oxygen uptake of perch, *Anabas testudineus,* increased with a concomitant increase in the RBC, hemoglobin and hematocrit levels, after the fish had been in water containing 5 mg l^{-1} *Quillaja* saponin for 24 hours. *Penaeus japonicus* that had been previously exposed to concentrations of 20 mg l^{-1} of saponin for 24 h increased both respiration rate and metabolism (measured as increase in oxygen uptake and ammonia excretion) during a 6 hour detoxification process (Chen and Chen, 1997). Bureau et al. (1998) observed that *Quillaja* saponins damaged the intestinal mucosa in rainbow trout and Chinook salmon at dietary levels above 1.5 g per kg. The condition of the intestines of these fish was similar to that of fish fed a raw soybean meal diet indicating the role of saponins in causing the damage. Krogdahl et al. (1995), however, did not find any negative effects when saponins were included in the diet of Atlantic salmon at levels similar to those likely to be found in a soybean meal (30-40 %) based diet. In the same study, an alcohol extract of soybean meal caused growth retardation, altered intestinal morphology, and depressed mucosal enzyme activity in the lower intestine. Dietary levels of triterpenoid *Quillaja* saponins of 150 mg kg^{-1} showed potential for promoting growth and nutrient utilisation in common carp and tilapia

(Francis et al., 2001b, c). Growth in common carp was significantly higher than control only when there was a continuous dietary supply of the saponins (Francis et al., 2001d). The growth promoting effect was not pronounced in common carp fed steroid *Yucca schidigera* saponins (G. Francis, unpublished observations). The effects of dietary saponins in fish such as higher growth, reduced oxygen consumption and metabolic rate, and inhibition of egg production in fish that we observed (Francis et al., 2001b, c, d) point to a possible systemic effect. The mechanisms whereby *Quillaja* saponins increased the growth and food conversion efficiency in fish remain to be ascertained.

c. Effects in other monogastrics

Legume grains form the staple food in a large part of the world, and serve also as the largest source for plant protein. Surprisingly there are only scarce reports on the effects of soysaponins (common among other legumes as well) on mammals, birds and cold blood organisms but for a few reports during the late 60's to the early 70's of the 20th century. Soybean saponins did not impair growth of chicks when added at five times the concentration in a normal soybean supplemented diet (Ishaaya et al., 1969). In the same work they observed no effect on the growth response of rats and mice or on the amount of ingested food. *Y. schidigera* extract has also been found to improve growth, feed efficiency and health in poultry and pigs by mechanisms that are not as yet understood (Johnston et al., 1981, 1982; Mader and Brumm, 1987; Anthony et al., 1994). Male Wistar rats, given 10 and 100 mg per 300 g body weight of fenugreek extract mixed with their food had significantly higher feed intake and appetite (Petit et al., 1993). The circadian rhythm of feeding behaviour was modified so that the rats fed the fenugreek extract ate continuously during 24h rather than just at night (Petit et al., 1995).

There are also numerous reports of negative effects of dietary saponins. Dietary saponins depressed growth, feed consumption in gerbils and egg production in poultry (Sim et al., 1984; Terapunduwat and Tasaki, 1986; Potter et al., 1993; Jenkins and Atwal, 1994). These negative effects have been ascribed to several properties of saponins such as reduced feed intake caused by the astringent and irritating taste of saponins (Oleszek et al., 1994), reduction in intestinal motility (Klita et al., 1996), reduction in protein digestibility (Shimoyamada et al., 1998) and damage to the intestinal membrane and inhibition of nutrient transport described earlier in this review.

When ginsenoside R-b1 and R-b2 were anaerobically incubated with human intestinal microflora, these ginsenosides were metabolized to 20-O-beta -D-glucopyranosyl-20(S)-

protopanaxadiol (compound K) and 20(S)-protopanaxadiol. Several kinds of intestinal bacteria hydrolyzed these ginsenosides (Bae et al., 2000). Breakdown products of saponins formed in the intestine may also have profound effects (Wakabayashi et al., 1998) which sometimes exceed those of the parent saponins. More investigations are required into the fate of ingested saponins in the digestive tract of ruminants and monogastrics.

5. 3. Effects on protein digestion

Saponins reduce protein digestibility probably by the formation of sparingly digestible saponin–protein complexes (Potter et al., 1993). Endogenous saponin affected the chymotrypsic hydrolysis of soybean protein particularly glycinin (Shimoyamada et al., 1998). The heat stability of BSA (bovine serum albumin) was increased by the addition of soysaponin due to electrostatic and hydrophobic interactions. The digestibility of the BSA-soysaponin complex was much lower than that of free BSA indicating that complexing with saponin had an obstructing effect. Soysaponin seemed to slightly activate α-chymotrypsin, but the effect was not great (Ikedo et al., 1996). It has also been shown that casein and *Quillaja* saponin form complexes of high molecular weight at elevated temperatures (78°C). Starfish saponin accelerated the thermal aggregation of actomyosin from walleye pollack muscle (Ishisaki et al., 1997). This enhancing effect increased concomitantly with increase in the ratio of starfish saponin to actomyosin. *Quillaja* saponin was also reported to accelerate the thermal aggregation of actomyosin (Ishisaki et al., 1997). The same research group showed that tea saponins had the ability to suppress the heat denaturation of salt soluble proteins from fish, while *Quillaja* saponin accelerated the reaction indicating a lack of uniformity in the way saponins act with regard to these effects.

A large number of foods and feed materials contain both substance families. The nature of the interactions between specific dietary saponins and proteins would influence the nutritive value of a diet, and hence these interactions need to be studied to elucidate possible structure-activity relationships.

5. 4. Hypoglycemic activity

Saponins isolated from plants like fenugreek have been shown to have hypoglycaemic effects. Male Wistar rats given 10 and 100 mg per 300 g body weight of fenugreek extract mixed with food had chronically higher plasma insulin levels, probably caused by stimulation of the β-cells (Petit et al, 1993). Dietary supplementation with fenugreek extract also stabilised feed

consumption in diabetic rats (Petit et al., 1995). The active components of the butanol extract of a 1:1 mixture of *Phellodendron cortex* and *Aralia cortex* were found to be able to regulate blood glucose levels (Kim et al., 1998a). Tectorigenin and kaikasaponin III from *Pueraria thunbergiana* has strong hypoglycemic and hypolipidemic effects on rats with streptozotocin-induced diabetes (Lee et al., 2000b). Studies of the effects of oleanolic acid glycosides on rats orally loaded with glucose led Matsuda et al. (1999a) to conclude that these compounds given orally did not have insulin-like or insulin-releasing activity. Their hypoglyceamic action was due to suppression of the transfer of glucose from the stomach to the small intestine and the inhibition of glucose transport across the brush border of the small intestine.

5. 5. Effects on cholesterol metabolism

A number of studies have investigated the role of saponins in lowering serum cholesterol levels in a variety of animals including humans. Large mixed micelles formed by the interaction of saponins with bile acids account for their increased excretion when consuming saponin rich foods such as soybean, lucerne and chickpea (Oakenfull and Sidhu, 1990; Oakenfull, 1986). *Gypsophila* saponins produced hypocholesteremia in rats fed low iron diets (Southon et al., 1988). Fenugreek seed saponins had a similar effect in both humans and animals (see review by Al-Habori and Raman, 1998). The synthetic saponin, tiqueside, decreased intestinal cholesterol absorption without interfering with the entero-hepatic bile acid re-circulation in chow-fed hamsters (Harwood et al., 1993). The same substance dose-dependently inhibited cholesterol absorption in humans, resulting in a reduction in serum LDL cholesterol levels (Harris et al., 1997), and a casein diet supplemented with *Quillaja* saponins resulted in reduction in LDL cholesterol and LDL/ HDL ratios in gerbils (Potter et al., 1993). The ethanol extract of de-fatted fenugreek seeds inhibited taurocholate and deoxycholate absorption *in vitro,* in a dose dependent manner in everted intestinal sacs (Stark and Madar, 1993). Morehouse et al. (1999) found that the synthetic saponins tiqueside and pamaqueside were much more potent than naturally occurring saponins such as those from alfalfa in preventing hypercholesterolemia in rabbits. The *in vivo* potency of pamaquecide was 10 fold that of tiqueside even though it differs from tiqueside only by an additional keto group. The mechanism of action was luminal but did not involve stoichiometric complexation with cholesterol. Other suggested mechanisms of action of saponins include delaying the intestinal absorption of dietary fat by inhibiting pancreatic lipase activity (Han et al., 2000).
Not all reports, however, agree on the anticholesteraemic activity of saponins. Calvert and Blight (1981) noticed no hypocholesteremic effect of soybean saponins in

hypercholesterolaemic men. Removal of saponins from alfalfa plant material did not reduce its capacity for affecting bile acid adsorption (Story et al., 1984). On the other hand, saponin-free alfalfa was found to reduce cholesterol accumulation in the liver of cholesterol fed rats, indicating involvement of other factors. Even though alfalfa saponins, bound significant quantities of cholesterol *in vitro,* dietary alfalfa sprouts did not prevent accumulation of cholesterol. Sugano et al. (1990) reported that the undigested high molecular fraction (HMF) of soybean protein significantly reduced serum cholesterol levels, but soybean saponins at the dietary levels equivalent to those contained in the high molecular fraction did not. Results from our laboratory showed that the level of muscle cholesterol in tilapia fed small amounts (up to 300 mg kg^{-1}) of *Quillaja* saponins was higher than that of controls (Francis et al., 2001c).

There is also evidence of increased cholesterol synthesis to compensate for saponin-induced excretion. Chick fed triterpenoid saponins from *Gypsophila* and *Quillaja* at 0.9% of diet had marked increases in cholesterol excretion, but no changes in excretion of bile acids (Jenkins and Atwal, 1994). Blood levels of total cholesterol and high-density lipoprotein cholesterol were unaffected. Dietary sarsasaponin failed to lower the cholesterol content of egg yolk or the serum of white-leghorn laying hens even though it increased the excretion of cholesterol and decreased the transfer of dietary cholesterol to the eggs (Sim et al., 1984).

Since the hypocholesterolemic action of saponin takes place in the intestinal lumen, factors such as quantity of saponins and cholesterol, and the presence of other ligands of both these compounds may play a role. Knowledge of the nature of the interaction between the particular saponin and cholestrol, and the nature of the cholestrol moieties and other ligands in the diet are essential to arrive at an effective dietary dose of that particular saponin that could have a significant anticholesteremic effect. It has also been noted that a saponin-induced reduction of serum cholesterol occurred only when a hypercholesteraemic diet had been fed (Jenkins and Atwal, 1994).

5. 6. Effects on animal reproduction

The negative effects of saponins on animal reproduction have long been known. Saponins from broom weed (*Gutierrezia* sp) and lechuguilla (*Agave lecheguilla*) or commercial pharmaceutical grade saponins caused abortion or death or both in rabbits, goats and cows when administered intravenously at concentrations above 2.3 mg kg^{-1} body weight (Dollahite et al., 1962). Saponins isolated from the crude extract of *Gleditischia horrida*, *Costus speciosus* Sm and *Phytolacca dodecandra* caused sterility in mice (Chou et al., 1971; Tewary

et al., 1973; Stolzenberg and Parkhurst, 1976). Quin and Xu (1998) found that the butanol extract of *Mussaenda pubescens* was capable of terminating pregnancy in rats. Extracts of this plant are used as a contraceptive in the Fujian province of China.

Saponins were found to be extremely strong stimulators of luteinising hormone (LH) release from cultured hypophysial cells (El Izzi et al., 1989; Benie et al. 1989; El Izzi et al., 1992) but their action was neutralized in the presence of serum. Saponin-rich extracts from *Petersianthus macrocarpus* injected into female rats stimulated uterine growth, lowered LH release, and blocked the oestrous cycle (Benie et al. 1989). The steroidal saponin OSW-1 isolated from *Ornithogalum saundersiae* injected into rats at a level of 9 μg kg^{-1} on the morning of proestrous inhibited the onset of the next proestrous (Tamura et al., 1997). Treatment on the day of dioestrous did not affect the oestrous cycle. OSW-1 inhibited ovarian estrogen secretion. This may have been the cause of the effects on the oestrus cycle, due to the gene translation inhibition exerted by OSW-1 and, thereby, on the proliferation of the granulosa cells of the ovary. The substance had no obvious effects on the hypothalamus-pituitary system (Tamura et al., 1997). Dietary *Quillaja* saponins inhibited egg production in tilapia and caused an imbalance in the sex ratio in favour of males in tilapia fry (G. Francis, unpublished observations).

Saponins also reportedly affect functioning of the male reproductive system. The gonado-somatic index (GSI) of 6 month old male tilapia fed a diet containing *Quillaja* saponins during the initial part of the life cycle were significantly higher than that of the control, whereas male tilapia receiving a continous supply of dietary saponins tended to have lower GSI (G. Francis, unpublished). On the other hand, saponin-rich extracts of *Pfaffia paniculata* improved the copulatory performance of sexually sluggish/impotent rats while being ineffective in sexually potent rats (Arletti et al., 1999). The authors felt that the plant extracts acted by increasing central noradrenergic and dopaminergic tone, and possibly oxytocinergic transmission.

Saponins have been shown to have both positive and negative effects on the viability of human sperm cells *in vitro*. A saponin fraction prepared from the roots of *Panax notoginseng* (Burt.) F. H. Chen (Ginseng root) increased motility as well as progression of sperm at the 60^{th} minute or the 120^{th} minute (Chen et al., 1998). Purified *Sesbania sesban* saponins were however found to be spermicidal at 1-1.3 mg ml^{-1} (Dorsaz et al., 1988).

The antifertility activity of saponins may have been due to their abortifacient; antizygotic and anti-implantation properties (Tewary et al., 1973). They may also affect the structure of the uterine endometrium, which prevents implantation (Stolzenberg and Parkhurst, 1976). The

stimulation of LH release by pituitary cells *in vitro* may be caused by their permeabilizing effect on cells (El Izzi et al., 1992). LH release from pituitary cells was inhibited in animals given saponins *in vivo* (Benie et al. 1989) or when cells were placed in serum.

5. 7. Effects on the immune system

A trend towards the use of highly purified recombinant proteins in modern vaccine development, and the need to find vaccines capable of eliminating virus-infected or malignant cells, have stimulated research on saponin adjuvants (Barr et al., 1998). Saponins from different sources have been reported to have widespread immunostimulant effects. Addition of crude *Quillaja* saponin, Quilayanin, Quil-A (a partially purified *Quillaja* saponin mixture used in veterinary vaccines) and glycyrrhizic acid preparations to mouse spleen cell cultures resulted in significant T and B cell proliferation (Chavali et al., 1987). B-cells were induced to proliferate in the presence of the crude saponin and T-cells in the presence of Quil-A. Quillayanin and glycyrrhizic acid stimulated both T and B-lymphocytes equally. Triterpenoid saponins from different sources were found to have mitogenic effects on mouse spleen and thymus cells and on human mononuclear cells and macrophages *in vitro* (Plohmann et al., 1997). *Silene fortunei* saponins showed significant enhancement of granulocyte phagocytosis *in vitro* at 10-100 μg/ml but did not show any activating effect on T lymphocytes (Lacaille-Dubois et al., 1999). Purified *Quillaja* saponins boosted antibody production without producing any reaginic antibodies (So et al., 1997) in contrast to partially purified Quil A, *Gypsophila* saponins, or saponins from other unnamed sources, all of which have produced increased levels of IgE when added to vaccines (Kensil et al., 1991, 1998; Sasaki et al., 1998; also see Sjolander et al., 1998).

Saponin based adjuvants have the unique ability to stimulate the cell mediated immune system, as well as to enhance antibody production and have the advantage that only a low dose is needed for adjuvant activity (Oda et al., 2000). Purified *Quillaja* saponins such as QS-21 are capable of amplifying the immune response (Barr et al., 1998). The immune stimulating action of saponins may be attributed to different mechanisms, such as protection of the antigen from digestive degradation, complex formation with virus to present it in an immunogenetically more active form, or activation of the interaction between antigens and lymphocytes by increasing the permeability of intestinal membranes (Maharaj et al., 1986). Saponins reportedly induced production of cytokines such as interleukins and interferons that might mediate their immunostimulant effects (Jie et al., 1984; Kensil, 1996) such as antigen localisation (Kensil, 1996) and spleen cell proliferation (Jie et al., 1984).

ISCOM vaccines (immune stimulating complexes-formed by the combination of cholesterol, saponin, phospholipid and amphiphatic proteins) formulated with Quil-A or other *Quillaja* saponin preparations induced specific cytotoxic T-lymphocyte responses (Coulter et al., 1998). ISCOMs have been reported to induce antibody responses and/or protective immunity in guinea pig, turkey, cat, rabbit, dog, seal, sheep, pig, cow, horse and monkeys (Sjolander et al., 1998). They also have been shown to induce cytotoxic T lymphocyte responses in mice (Barr et al., 1998). Although the mechanisms by which saponins or ISCOMs promote and modulate immune responses are not clearly understood, it is likely that they interact with antigen presenting cells to induce many of these responses. They may become incorporated into cell or endosomal membranes, thereby exposing the incorporated antigen to cytosolic proteases. Co-incorporation of antigen and saponin in the ISCOM might improve targeting of the antigen and adjuvant to the same antigen- presenting cell.

There is also evidence that saponins may increase the immune response by increasing the uptake of antigens from the gut and other membranes. Oral administration of *Panax ginseng* C. A. Meyer saponins (Jie et al., 1984), *Quillaja* saponins (Maharaj et al., 1986), and the butanol extract of *Lonicera japonica* (Lee et al., 1998), and DS-1 administered on the nasal mucosa (Recchia et al., 1995), all stimulated the immune responses *in vivo*.

The adjuvant action of saponins is not so pronounced in some of the non-mammalian species tested. e.g. saponin adjuvant when injected intraperitoneally had little effect on the humoral immune response in rainbow trout (Cossarni-Dunier, 1985). Neither did the use of Quil-A produce any improvement in the immune response to *Yersinia ruckeri*, the causative agent of enteric redmouth disease in rainbow trout despite some enhancement of the *in vivo* bacterial clearance (Grayson et al., 1987).

The efficiency of saponins as immune stimulants also depends on their chemical nature. Optimum primary antibody responses were most marked with *Quillaja* saponins, whereas secondary responses were nearly the same for saponins from *Quillaja* and *Gypsophila*, lower but detectable with saponins from *Saponaria officinalis*, and absent altogether with other saponin extracts from soybean, alfalfa, *Chenopodium quinoa* and *Glycyrrhiza radix* (Kensil, 1996).

The aspects of saponin structure that cause their adjuvant activity is probably more related to the totality of their molecular structure rather than to any individual component. The adjuvant activity of saponins was thought to be related to branched sugar chains or aldehyde groups (Bromford et al., 1992) or to an acyl residue bearing the aglycone (Kensil et al., 1996). Later soysaponins and lablabosides were found to show strong adjuvant activity despite lacking

acyl residues and possessing only unbranched sugar chains (Oda et al., 2000). Also most of the escins that have acyl residues and branched sugar chains did not show adjuvant activity. Adjuvant activity and toxicity, but not the cholesterol binding capacity of QH-B, a *Quillaja* fraction, declined on peroxidate oxidation due to alterations in the structure of the sugars galactose and xylose. Modification of the apiose moiety may influence adjuvant activity but not toxicity *in vivo* (Ronnberg et al., 1997). Oda et al.(2000) concluded that the overall juxtaposition of hydrophilic and hydrophobic functional groups, rather than the structures of individual groups is the essential element that confers adjuvanticity. Soysaponins, lablabosides and QS-21 which possess adjuvant action have only two to four oxygen atoms equally distributed around the aglycone, and may retain the typical amphipathic features. On the other hand, escins without adjuvant activity have seven oxygen atoms, with five localised around one side of the aglycone, thus reducing its hydrophobic and adjuvant nature.

5. 8. Cytostatic effects on malignant cells

Saponins isolated from plants and animals have been shown to inhibit the growth of cancer cells *in vitro* (Kuznetzova et al., 1982; Rao and Sung, 1995; Marino et al., 1998; Konoshima et al., 1998; Podolak et al., 1998). Saponins from different sources (soybean and *Gypsophila*) induced significant morphological changes in human colon carcinoma cells and appeared to act by different mechanisms. *Gypsophila* saponins acted on the plasma membrane and soybean saponins acted initially on the cytoplasmic material and then cell membranes (Sung et al., 1995). Ruscogenin glycoside with three acetyl groups attached to the inner galactosyl moiety, and its corresponding 26-glucosyloxyfurostanol saponin isolated from the under ground parts of *Ruscus aculeatus*, displayed cytostatic ability against HL-60 cells (Mimaki et al., 1998a). The ginseoside metabolite M1 produced in the intestine by the action of microbes on ingested ginseng saponins caused apoptotic cell death through transcriptional regulation of several proteins associated with cell growth (Wakabayashi et al., 1998). Saponins might also exert an anti-cancer effect at the intestinal level. Bile acids are metabolised by intestinal microbes to form secondary bile acids which are implicated as causative agents of colon cancer. The binding of saponins to bile acids in the intestine could reduce the availability of bile acids to the microbial population, thus reducing the formation of carcinogenic substances in the colon (Cheeke, 1996).

The selective action against malignant cells might be due to a destructive interaction between the altered cell membrane structure of such cells and saponins. The cytostatic activity against cancer cells cannot, however be directly related to haemolytic activity. Mimaki et al. (1998b)

found that the furostanol saponins that had higher cytostatic activity against HL-60 cells had no haemolytic activity.

5. 9. Molluscicidal effect

The molluscicidal properties of saponins was first observed by Lemma (1965) who noticed the toxic effects of extracts of unripe berries of *Phytolacca dodecandra* on river snails in Ethiopia. Efforts were then mounted to utilise this property of saponins to control diseases like schistosomiasis which are transmitted by molluscs. Saponins extracted from many other sources were also seen to have similar molluscicidal property. Purified *Sesbania sesban* saponins at 3 to 25 mg kg^{-1} (Dorsaz et al., 1988) and purified saponin mixtures from *Maesa lanceolata* at above 5 ppm (Sindambiwe et al., 1998) have been found to be active against *Biomphalaria glabrata*. Monodesmodic triglycosides from the pericarp of the fruits of *Sapindus rarak* at 6.25 to 12.5 mg kg^{-1} (Hamburger et al., 1992) and a spirostanol glycoside from the inflorescence of *Yucca aloifolia* (Kishor and Sati, 1990) were also molluscicides. The molluscicidal activity of the saponins may be due to their damaging effect on the soft body wall of the molluscs.

5. 10. Antifungal activity

A variety of saponins have been shown to be fungicidal and at least one deactivates viruses (purified saponin mixture from *Maesa lanceolata*; Sindambiwe et al., 1998). The major mechanism suggested for the antifungal activity of saponins is their interaction with membrane sterols as described earlier in this review. Numerous recent papers describe the antimicrobial activity of various saponins, and an identification method based on their activity against *Trichoderma viride* has been developed some decades ago. Kalopanaxsaponins A and I isolated from *Kalopanax pinctus* exhibited strong and specific antifungal activity against *Candida albicans* and *Cryptococcus neoformans* (minimum inhibitory concentration of 25µg ml^{-1}; Kim et al., 1998b). A saponin, AS-1, from the lower leaves of *Asparagus officinalis* has antifungal properties in concentrations of 0.5 to 8 µg ml^{-1} depending on the type of fungus (Shimoyamada et al., 1990). The monodesmosidic spirostanol saponins from *Yucca schidigera* destroys certain food-deteriorating yeasts, film-forming yeasts, and dermatophytic yeasts and fungi (Miyakoshi et al., 2000). It was observed that those saponins having a branched-chain trisaccharide moiety without any oxygen containing groups at C-2 and C-12 exhibited the anti-yeast activity, while saponins with 2β-hydroxyl or 12 keto groups showed

very weak or no activity. A saponin with a disaccharide moiety exhibited relatively low activity and the aglycones or bidesmodic furostanol saponins showed no activity. Kalopanaxsaponins A and I showed antifungal activity only against fungi causing dermatomycosis (Kim et al., 1998a). Synthetic steroid saponins prepared by Takechi et al. (1999) were both antifungal and haemolytic but in many cases haemolytic triterpenoid saponins show little antifungal activity.

The antifungal activity of food originated substances has attracted an applicational research. Some reports describe the anti-yeast activity of saponins as having an anti-food deteriorating effect (Miyakoshi et al., 2000)

5. 11. Antioxidant effects

Soybean DDMP (2, 3-dihydro-2, 5-dihydroxy-6-methyl-4H-pyran-4-one) saponins are able to scavenge superoxides by forming hydroperoxide intermediates, thus preventing bio-molecular damage by free radicals (Yoshiki and Okubo, 1995). The scavenging activity was found to reside in the DDMP moiety (Yoshiki et al., 1995) and this saponin is widely distributed among legumes, such as kidney beans, peanuts, chickpeas, clover, and Japanese bushclover (Yoshiki et al., 1998). The soysaponin βg is pro-oxidative by itself because of its tendency to attract hydrogen , but when present along with an electron donor suc as gallic acid this saponin has a strong reducing effect (Yoshiki et al., 1998). Hederagenin glycosides from *Kalopanax pinctus* could effectively prevent the metabolic activation of aflatoxin B-1 or scavenge the electrophilic intermediate capable of inducing mutation (Lee et al., 2000a). Hederagenin was found to be the essential moiety for antimutagenicity. Soysaponins were also shown to have antimutagenic activity in mammalian cells (Berhow et al., 2000) probably because of their ability to decrease microsomal enzyme activity (Sindambiwe et al., 1998).

5. 12. Other miscellaneous effects

Ginseng extract (40 or 80 mg kg^{-1}) significantly improved learning ability and cognitive functions in brain damaged rats in a dose dependent manner, and enhanced the strategic performance of normal rats. These results are thought to be due to membrane stabilising effects such as the inhibition of Na^{+} and Ca^{2+} channels (Zhao and McDaniel, 1998). Ginseng total saponins injected intracerebroventricularly at doses of 0.1 to 1 μg inhibited stress-induced hypothalamo-pituitary-adrenal responses by inducing NO production in the brain (Kim et al., 1998c). This may be beneficial in preventing the harmful effects of excessive

increases in plasma corticosterone on the target organs in stressful circumstances. Ginsengosides were shown to inhibit catecholamine secretion from bovine adrenal chromaffin cells (Kudo et al., 1998).

The addition of 1 to 10 µg of soysaponin to the diet of the oriental clouded larva, *Coliaserate poliographus* increased their food intake (Matsuda et al., 1998).

Panax notoginseng saponins (PNS) at 200 mg kg^{-1}, when administered intraperitoneally significantly inhibited abnormal increases in platelet aggregation and platelet adhesiveness in rats subjected to permanent occlusion of the middle cerebral artery. Similar effects were observerved in *in vitro* preperations. The anti-cerebral ischemic effect of PNS is probably due to changes in the rank and structure of functional membrane proteins induced by fluidity of membranes which lead to changes in protein activities. Platelet membrane glycoproteins play a key role in the adhesion of platelets to the walls of blood vessels, in the accretion of platelets and in the formation of thromboses (Ma and Xiao, 1998).

Glycyrrhetinic acid, the active ingredient of licorice is a highly efficient inhibitor of 11β-dehydrogenase and this was claimed to be the reason for its mineralocorticoid-like activity (Monder et al., 1989).

Ivy, *Hedera colchica* contains several saponins including β-Hederin, Hederacolchiside A1 and α-Hederin. The latter has a wide spectrum biological activity. As well as being a haemolytic and antispasmodic, it is also effective against fungi and amoebae. All three compounds are effective in the treatment of leischmanaisis caused by *Leishmania infantum* in both the extracellular and intracellular stages, and they also strongly inhibit the proliferation of human monocytes (Delmas et al., 2000). The addition of a glucose unit in the carbohydrate chain of oleanolic acid (Hederacolchiside A1) greatly enhances its interaction with both parasite and human cells. The replacement of oleanolic acid by hederagenin does not significantly modify the pharmacological properties of the compounds but the mechanism of inhibition changes. In both parasite and human cells inhibition occurs through reactions on the external cell membranes but in the latter there is also a large decrease in DNA synthesis in the nucleus. Yucca saponins also inhibit the growth of the promastigote of *Leishmania Spp.* This effect of pure saponins was quantified using flow cytometry and microscopic methods (Plock et al., 2001).

The triterpenoid saponins from *Glinus oppositifolius* inhibit the reproduction of *Plasmodium falciparum* (Traore et al., 2000).

The saponin Momordin Ic significantly and dose-dependently inhibits gastric emptying at levels of 12.5 to 50 mg kg^{-1} (Matsuda et al., 1999b). The inhibitory activity is dependent on

the level of serum glucose and mediated at least in part by the capsaicin-sensitive sensory nerves and the CNS.

Theasaponin E-1 from seeds of the tea plant *Camellia sinensis* L. var. Assamica PIERRE exhibit considerable gastroprotective activity and the whole saponin fraction from teaseed protects gastric mucosal lesions induced by ethanol in rats (Murakami et al., 1999).

The oral administration of 200 mg $kg^{-1}day^{-1}$ of saponins from *Hernaria glabra* for 30 days, results in a significant decrease in blood pressure in hypersensitive rats and affects salt and water transport in the renal tubules (Rhiouani et al., 1999)

6. Conclusions

A survey of experimental results indicates that the saponin mixtures present in plants and plant products possess diverse biological effects when present in the animal body. Most of the experiments to study the biological effects of saponins have been done using crude saponin extracts from plant sources rather than purified compounds. Because of the presence of a large number of structurally different saponins in plant extracts, it is difficult to pinpoint the individual saponins responsible for the observed effects. Some properties such as the membrane permeabilising activity seem to be possessed by a large number of saponins, whether triterpenoid or steroid. A number of studies have been conducted on immunostimulation, using mainly *Quillaja* saponins. In this case the adjuvant effect of specific saponins on the immune system function has been conclusively proved using purified individual saponins in animal models. Whether these observed effects are reproducable in humans will be known only after the results of ongoing human clinical trials are made public. Other properties ascribed to saponins such as their ability to lower the cholesterol content of the serum of animals when present in the diet or the beneficial effects that some of them are reported to have on the efficiency of feed utilisation in ruminants and monogastrics need further experimental proof to be conclusively established. Further tests are also required to determine whether the observations on the growth promoting effects of low levels of crude *Quillaja* saponin mixtures in carp and tilapia are repeatable in other cold and warm water fish and animal species. The search for natural, biologically active, and renewable plant products that could be used to replace hazardous synthetic growth stimulants have great current and future relevance. Another important field which calls for further investigation is that regarding the effects that saponins might have on animal and human reproductive cycles and hormonal

balance but various other observed effects would have to be taken into consideration before they are recommended for use in human medicine. More *in vivo* experiments using purified individual compounds are also needed to identify the individual compounds responsible for the observed effects. In view of their widespread presence in animal and human diets it would be particularly interesting to investigate the fate of saponins in the intestine of animals, their mechanisms of action at the intestinal level and their systemic effects if they are actually absorbed into the blood.

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Chapter 2

Dietary supplementation with a *Quillaja* saponin mixture improves growth performance and metabolic efficiency in common carp (*Cyprinus carpio* L.)

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Abstract

The effects of supplementation of a saponin mixture in diets of common carp were studied using a respirometer system which allowed feeding and continuous measurement of oxygen consumption of individual fish. Five fish each, weighing approximately19 g, were fed either a normal diet (C group), a diet supplemented with 150 mg kg^{-1} (S150 group) or 300 mg kg^{-1} (S300 group) *Quillaja* saponins. At the end of the experimental period (8 weeks), the weight gain of the S150 group (372 %) was significantly ($P< 0.05$) higher than that of the control group (327 %) and that of the S300 group (325 %), which was similar to the control group. The average metabolic rate (mg $kg^{-0.8}$ h^{-1}) and oxygen consumption per unit body weight did not differ among the groups. Up to four weeks the average feed conversion ratio and metabolic growth rate values of the S150 group were significantly ($P<0.05$) better than those of the C group. Later the differences narrowed and were no longer significant at the end of the experiment. The average protein utilisation values at the end of the experiment were highest in the S150 group. The S150 group also had the highest average energy retention value. The average apparently unutilised energy was lowest for the S300 group. It is concluded that *Quillaja* saponin mixture could act as a growth stimulant for common carp when present at a level of 150 mg kg^{-1}in the diet.

Key words: *Quillaja* saponin, *Cyprinus carpio,* respirometry, growth, protein, energy, carcass composition.

1. Introduction

The increase of food production on land and in aquaculture, especially by improving the metabolic efficiency in food utilisation is at the frontier of agricultural development. The use of synthetic substances that were found to increase the efficiency of feed utilisation by animals such as antibiotics and steroid hormones as animal feed additives are currently prohibited in several countries. In this context, search for natural, biologically active, and renewable plant products that could be used to replace hazardous synthetic growth stimulants are relevant. One such group of substances is saponins present in many wild plants and cultivated crops. They are steroid or triterpenoid glycosides synthesised in plants as ‘defence compounds’ in their ‘evolutionary arms race’ against herbivores. They are known to inhibit mould and protect plants from insect attack (Hostettmann and Marston, 1995). When present in the diet in moderate to high quantities, they are believed to have several negative effects on

animals (Cheeke, 1996). Dietary saponins derived from different plants have been implicated as the causative factor for depression of feed intake and reduction in weight gain in salmonid fish (Bureau et al., 1998), accentuation of ruminant bloat and causation of photosensitization (Cheeke, 1996), inhibition in gut of active uptake of nutrients (Johnson et al., 1986), including vitamins (Jenkins and Atwal, 1994) and minerals (Southon et al., 1988), anti-fertility action in animals (Tewary et al., 1973; Quin and Xu, 1998) and reduction in protein digestibility (Shimoyamada et al., 1998). Contrarily, there have also been reports of beneficial effects of saponins. They are able to bind to ammonia and are hence considered to reduce release of this gas into the atmosphere of stables (Cheeke, 1996; Makkar et al., 1999), reduce the rumen protozoa number and thus increase the efficiency of rumen microbial protein production (Newbold et al.; 1997, Makkar et al., 1998), lower serum cholesterol level which might reduce the risk of cardiovascular diseases (Sugano et al. 1990; Harwood et al., 1993), and stimulate the immune system when given along with vaccines (Kensil, 1996).

One property considered to be universal for saponins is their high toxicity to fish when present in water. Newinger (1994) identified saponins as the main active compounds present in the most effective fish poisoning plants in Africa. They are also considered to be the active components of many traditionally used fish poisons, like mahua oil cake (Francis et al., 2001). Consequently, fish bioassay used to be a prominent method for their detection in crude extracts.

Saponins are present in relatively large concentrations in many alternate plant protein sources for culture fish (Francis et al., 2001). Given the importance of such alternate nutritional sources in future aquaculture feeds it is imperative that the effects of purified saponin concentrate when present in the diets of common culture fish species are studied in more detail. The present study explores the effects of low dietary quantities of a commercially available *Quillaja* saponin mixture (QS) on growth, feed utilisation efficiency, and energy metabolism in common carp.

2. Materials and methods

Feeds

Three experimental diets, a basal or control diet (C), and two with added saponin (S150 and S300) were prepared. The ingredients and chemical composition of the basal diet are shown in Table 1. The basal or control diet, which was obtained in pellet form, was ground and then repelleted (about 2 mm diammeter). The saponin (QS; Sigma no. 2149; Sigma, St. Louis, USA), was first dissolved in demineralised water and then mixed thoroughly with the basal

feed at levels of 150 mg kg^{-1} (S150) and 300 mg kg^{-1} (S300). The moist pellets were frozen, freeze-dried and stored in a freezer at -18°C, until use.

Experimental fish

Carp (*Cyprinus carpio* L.) of about 1.0 g weight were obtained from the Federal Fishery Research Agency, Institute for Coastal and Inland Fishery, Ahrensberg, Germany. They were brought to Hohenheim and grown in 200 l aquaria maintained at 23 ± 1°C. They were fed a diet containing approximately 420 g kg^{-1} protein, 100 g kg^{-1} lipid, 100 g kg^{-1} ash and an energy content of 20 kJ g^{-1} on dry matter basis, until their body weight reached approximately 20 g. They were then fed the same diet at a level that ensured maintenance of weight (3.2 g $kg^{-0.8}$ day^{-1}; Meyer-Burgdorff et al., 1989). Five days prior to the start of the experiment, 25 carp of comparable body mass (~ 19 g) were selected from a large population, weighed and 15 were placed individually in 15 respiration chambers of a device previously described by Focken et al. (1994). The remaining ten fish were killed by a sharp blow to the forehead and preserved for determination of the initial chemical composition. During acclimatisation in the chambers (5 days), the fish continued to receive maintenance levels of the feed. The fish were starved for one day prior to the start of feeding the experimental feeds. At the start of the experiment the three experimental diets namely C, S150 and S300, were assigned each to 5 fish in a random manner.

Table 1. Ingredients and chemical composition of the basal diet

Ingredients (g kg^{-1})	
Fish Meal[a]	500
Whole wheat meal	420
Sunflower oil	40
Vitamin mixture[b]	20
Mineral mixture[c]	20

Chemical compostion (g kg^{-1} in DM)	
Crude protein	417
Lipid	84
Ash	113
Gross energy (MJ kg^{-1} DM^{-1})	20.1

[a] Norwegian fish meal obtained from Württembergische Zentralgenossenschaft, Germany.
[b]/[c] prepared after Meyer-Burgdorff et al. (1989)

The experimental set-up

During the experiment the fish were kept in respiration chambers (5 litres capacity) of the fully automated, computer controlled fish respirometer system (Focken et al., 1994). Thirty two measurements of oxygen consumption per individual fish were made every 24 h and recorded on the hard disk of the computer, which controls the system. The system was lit with fluorescent tubes to give a day length of 12 h. The water flow rate through the respirometer chambers, which was controlled by electronic flow meters connected to each chamber, was adjusted (0.4 to 0.65 l min^{-1}) to keep the oxygen saturation in water above 75 % in the chambers. Every day about a tenth of the water in the system was replaced. The water

temperature was maintained at 27 ± 0.1°C. Once a week, when the fish were weighed, the chambers were cleaned and the oxygen probe calibrated. During cleaning of chambers the fish were kept in plastic buckets containing water taken out from the system. The entire process of weighing and cleaning the system took approximately 3 h. During the experimental period all water quality parameters were maintained at acceptable levels (pH – 7-7.9; total NH_3 – 0.1-0.2 mg l^{-1}; nitrite – 0.07-0.1 mg l^{-1}, and nitrate – 1-3 mg l^{-1}).

Feed regime

The fish were fed individually at a level of 16 g $kg^{-0.8}$ day^{-1} in 6 equal instalments using automatic feeders attached to the respiration boxes. The fish were weighed once a week and the ration for each fish was adjusted according to its body weight. The fish were then fed at the same rate until the next weighing. There was no feeding on the weighing day.

At the end of eight weeks the experiment was terminated and the fish were weighed, killed by a sharp blow to the forehead and immediately frozen. Prior to analysis, the carcasses were autoclaved for 30 min at 120°C, homogenised, refrozen and freeze-dried.

Chemical analyses

The chemical composition of the experimental diets and the freeze-dried fish was analysed according to official methods (Neumann and Basler, 1983) i.e. dry matter by drying to a constant weight at 105°C, crude protein by macro-Kjeldahl (N x 6.25), lipids by extraction with petroleum ether, and gross energy by bomb calorimetry (IKA C 7000).

Calculations and statistical analysis

All calculations were performed for each fish individually. Feed conversion ratio (FCR) was calculated as feed consumption (dry matter)/ live weight gain, and Metabolic Growth Rate (MGR) as live weight gain (g)/ average metabolic live weight ($kg^{0.8}$)/ day. The average Metabolic Rate was calculated as mg oxygen consumed $kg^{-0.8}$ h^{-1}. The Standard Metabolic Rate (SMR) was taken as the lowest metabolic rate sustained for 3 h by the undisturbed animal that had not been fed for the preceding 24 h. The relevant oxygen consumption values for calculating SMR were obtained during the 24 h period when the fish were not fed after a 5 day acclimatisation period in the respirometer chambers on a maintenance ration. Oxygen uptake (g) x 14.85 kJ g^{-1} gave the energy expenditure during the whole experiment (Huisman, 1976), and the energy apparently not metabolised (AUE) was calculated by subtracting energy expenditure and energy retention (ER; gross energy gain of the carcass) from the gross energy

of the feed consumed. Protein Productive Value (PPV) was calculated as protein gain x 100/ feed protein.

The data were subjected to ANOVA and statistical comparisons between the feeding groups were made using the Duncan's Multiple Range test (Statistica for Windows, release 5.1 H, '97 edition). The significance of observed differences was tested at $P < 0.05$. The values are reported as Means (sd).

3. Results

Feed intake and behaviour of carp

All fish ate the feed provided completely. Since the fish ate the feed pellets as they dropped into the respirometer box there was little chance for the saponins to be leached into the water. There was no mortality or abnormal behaviour of fish in any of the treatment groups during the experimental period.

Growth rates

At the end of the experimental period (8 weeks) the percent weight gain of the S150 group was 372 %, which was significantly higher than the control group (327 %), while that of the

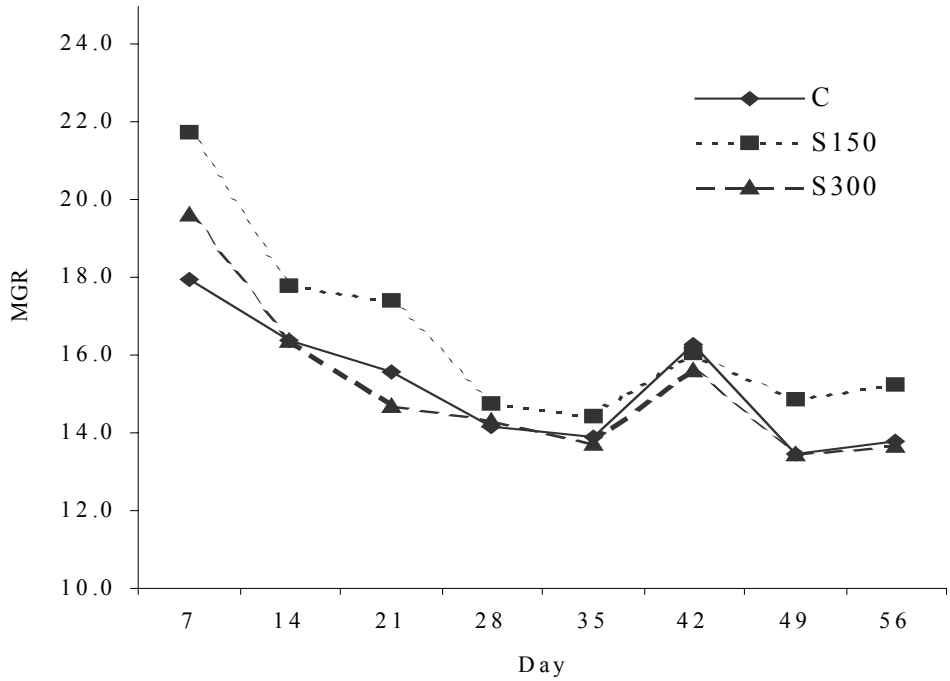

Figure 1. Average weekly Metabolic Growth Rate (MGR) of carp fed the experimental diets

S300 group was similar to the control group (325 %). An 18 % difference between the final average body weight of the C and S150 groups was observed. Also the MGR of the S150 group was significantly higher than the C group after the first week of feeding. However the difference between the growth rates tended to decrease as the experiment progressed. Growth rate in the S300 group was also higher than the C group after the first week but subsequently decreased, as the experiment progressed, to levels lower than the C group (Fig 1).

Table 2. Initial and final body composition of experimental fish

	Initial		Final					
			C		S150		S300	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Dry matter (DM), % of fresh matter	19.8	0.8	21.1	0.4	21.2	0.8	21.7	1.4
Crude protein(N x 6.25), % of DM	57.4	2.3	63.2	1.8	62.3	1.4	62.5	2.2
Crude fat, %	17.8	2.9	20.4	3.0	20.7	2.0	22.5	2.5
Ash, %	12.7	0.6	10.5	0.4	10.5	10.5	10.9	0.6
Gross energy, kJ g^{-1}	22.7	0.4	23.2	0.7	24.0	0.8	23.4	0.9

C-fish fed the control feed
S150-fish fed diet containing 150 mg kg^{-1} *Quillaja* saponin
S300-fish fed diet containing 300 mg kg^{-1} *Quillaja* saponin

Whole body carcass composition

The chemical composition of the experimental fish in the three groups at the end of the experiment was similar (Table 2).

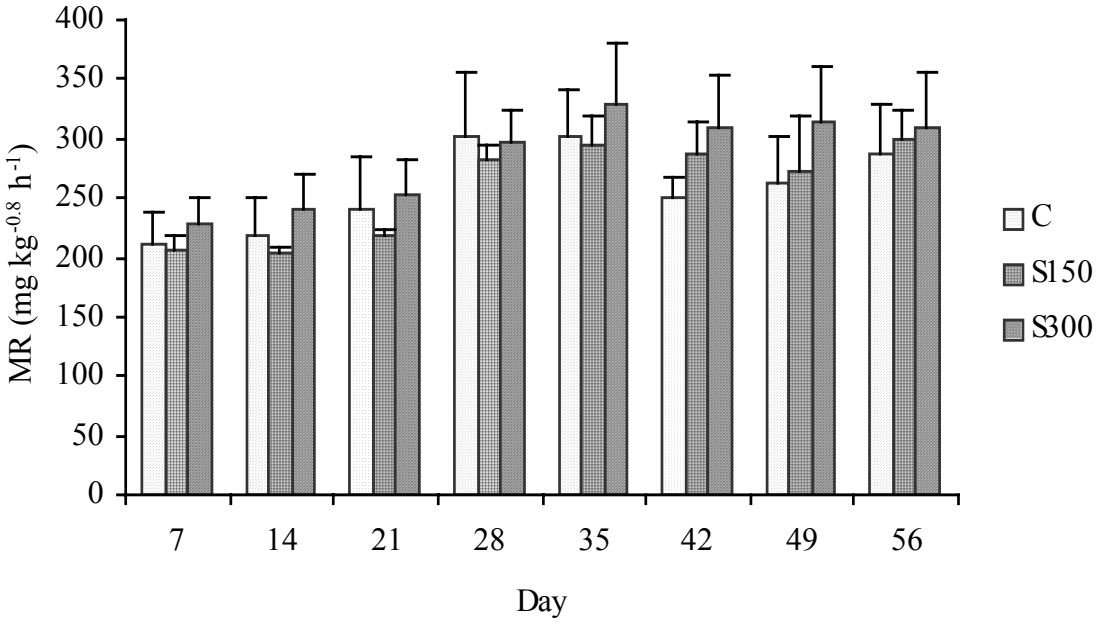

Figure 2. Weekly average Metabolic Rate (MR) of carp fed the experimental diets

Oxygen consumption

The average SMR values measured as mg $kg^{-0.8}$ h^{-1} of the experimental groups before the start of the feeding trial were 72 (11) for the C group, 73 (10) for the S150 group and 76 (13) for the S300 group. The weekly average metabolic rates (mg $kg^{-0.8}$ h^{-1}) for the whole experimental period are shown in Fig 2. The average metabolic rates, during the first 5 weeks of the experiment were lower (not statistically significant) in the S150 group as compared to the control. The values of the C and S150 groups were, however, similar at the end of the experiment. The S300 group had consistently the highest average metabolic rates from the beginning till the end of the experiment. The average oxygen consumption per unit body weight gained at the end of the experiment, was the highest (12.7 % higher than control) in the S300 group and lowest in the S150 group (6.8 % lower than the C group).

Feed conversion and efficiencies

The calculated average values of feed consumed, feed conversion ratio and protein and lipid efficiency values are presented in Table 3. The FCR values in the S150 group was significantly lower than those of the control initially but later it followed a pattern similar to the MGR. The S150 group had the highest values for PPV and PER. The differences in the average values of PPV and PER though considerable, were not statistically significant.

Table 3. Feed consumption and assimilation in the experimental groups (n=5)

	C		S150		S300	
	Mean	SD	Mean	SD	Mean	SD
Initial live mass (g)	18.5	1.6	19.7	1.1	18.9	1.4
Final live mass (g)	79.0[b]	4.9	93.1[a]	10.4	80.4[b]	8.2
Feed consumption (g dry matter)	53.6	3.6	59.5	3.9	54.7	2.7
Food conversion ratio, FCR (g g^{-1})	0.89	0.04	0.82	0.07	0.90	0.10
Productive protein value ,PPV (%)	37.8	1.5	40.4	3.0	38.2	2.3

C-fish fed the control feed
S150-fish fed diet containing 150 mg kg^{-1} *Quillaja* saponin
S300-fish fed diet containing 300 mg kg^{-1} *Quillaja* saponin
FCR = feed consumed/ live weight gain
PPV = total protein gain/ total protein fed x 100

Energy utilisation

The complete energy budget of the fish in the three groups is given in Table 4. There were considerable differences in the energy retained and excreted between the groups. The S150 group had the highest energy retention value. The AUE value (a measure of energy excreted

through faeces) was lowest in the S300 group. The energy expenditure per unit feed energy was similar in the C and S150 groups, but higher in the S300 group.

Table 4. Energy budget of carp fed the experimental diets (n=5)

	C		S150		S300	
	Mean	SD	Mean	SD	Mean	SD
Initial GE content of carcass (kJ)	83.0	7.0	88.4	4.8	84.7	6.2
Final GE content of carcass (kJ)	385	27	475	72	407	47
Feed GE uptake (kJ)	1076	72	1194	78	1098	53
Energy expenditure (EE[a]; % of GE fed)	39.6	5.1	40.0	2.0	43.7	5.8
Energy retention (ER[b]; % of GE fed)	28.2	2.0	32.2	4.4	29.4	3.8
Apparently unmetabolised energy (AUE[c]; % of GE fed)	32.2	5.6	27.8	3.9	26.9	8.0

C-fish fed the control feed
S150-fish fed diet containing 150 mg kg^{-1} *Quillaja* saponin
S300-fish fed diet containing 300 mg kg^{-1} *Quillaja* saponin
[a] EE = oxygen uptake (g) x 14.85 kJ g^{-1}
[b] ER = energy retention
[c] AUE = energy fed - energy expenditure as heat - energy retention.

4. Discussion

The results obtained indicated that supplementation with QS at a level of 150 mg kg^{-1} caused an 18 % increase in the average weight of carp over control. The values of other parameters such as FCR, ER, AUE and PPV, though not statistically different**,** were also numerically better in the S150 group. It is possible that the lack of any statistical difference was because of the low number of fish and the high variability within treatment groups.

The physiological effects of saponins on fish have been controversial; some authors reporting positive and others negative influences. They depress blood parameters such as hematocrit and hemoglobin levels and red cell count in several species of fish (Roy et al., 1990; Homechaudhuri and Banerjee, 1991; Homechaudhuri et al., 1991). Contrary to this, Roy and Munshi (1989) reported increased oxygen uptake of the climbing perch (*Anabas testudineus*) with a concomitant increase in red blood corpuscles, hemoglobin and hematocrit levels, after the fish were exposed to QS at 5 mg l^{-1} for 24 h. Bureau et al. (1998) observed reduced feed intake and growth in chinook salmon and rainbow trout fed diets containing soybean saponin extracts or *Quillaja* bark saponins, both at a level of 0.3 % in the diet.

Serrano. et al. (1998) have shown that the same QS mixture as used in this experiment, when present in the diet of carp, stimulated some gut and liver enzymes. The activity of the gut

enzymes trypsin and amylase were significantly stimulated at 300 mg kg^{-1} and 450 mg kg^{-1} levels and that of the liver enzymes, cytochrome *c*-oxidase (CO) and lactate dehydrogenase (LDH) were significantly higher at 150 mg kg^{-1} QS. These results indicate actions of the QS both at the intestinal and general metabolic levels. The higher FCR and the PPV values of the S150 group could be attributed to the increased digestion and absorption of food nutrients. Onning et al. (1996) have previously reported that saponins significantly increased passage of macromolecules (ovalbumin) across rat intestine *in vitro*. The tendency for lower average values for AUE observed in the saponin fed fish in the current experiment indicate higher absorption and lower excretion of nutrients.

The carp of the S300 group had the highest oxygen consumption (and metabolic rate) throughout the experiment indicating continuous higher metabolic activity. The reasons for this is not clear, but could possibly be a result of physiological stress induced by the higher dietary saponin content.

Further studies are required to obtain more information regarding the mechanisms by which dietary QS brought about the observed effects in common carp. Finally, the QS used in the current experiment could also have biologically active compounds other than saponins. Efforts are being made to procure purified compounds in sufficient quantities for future trials.

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Chapter 3

Effects of cyclic and regular feeding of a *Quillaja* saponin supplemented diet on growth and metabolism of common carp (*Cyprinus carpio* L.)

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Abstract

Fifteen common carp weighing 19.2 ± 2.9 g were reared individually in chambers of a respirometer system which allowed feeding, and continuous measurement of oxygen consumption. The fish were divided into three groups of 5 each, and were given control feed (C group), control feed supplemented with 150 mg kg^{-1} of *Quillaja* saponins (QS) (S150 group), and control and the saponin supplemented diet during alternate weeks (S150b group), for 56 days. Supplementation with QS at a level of 150 mg kg^{-1} caused a significant ($p<0.05$) increase in metabolic growth rate, food conversion efficiency, protein productive value (PPV) and apparent lipid conversion (ALC) of carp, even as the metabolic rate was lower than the C group. The efficiency of energy utilisation indicated by parameters such as energy expenditure (EE), energy retention (ER) and amount of oxygen consumed per unit body mass gain (OPM) were also significantly ($p<0.05$) better in the S150 group. The performance of the S150b group was intermediate between the C and S150 group in this experiment. Possible reasons for the effects of dietary QS are discussed. It was concluded that there has to be continuous dietary supply of QS at the level of 150 mg kg^{-1}, for maximum positive effects of these substances on growth of common carp.

1. Introduction

Saponins are present in many plant products that are potential aquaculture feed ingredients (Francis et al. 2001). The negative effects of several alternative fish feed ingredients such as legume seed meals on the growth performance of culture fish, has been attributed, at least partially, to the occurrence of saponins in the diet (Bureau et al. 1998). However, the antinutritional status of saponins has been questioned and some of them have been postulated to have positive effects in animals (Liener, 1994). In a previous publication we described the positive effect on growth of common carp at a dietary level of 150 mg kg^{-1} *Quillaja* saponins (QS) when (Francis et al. 2001a). Inclusion of QS mixture at 300 mg kg^{-1} in the diet of the same fish species did not have any apparent negative effect after 56 days of feeding. It was however observed that the difference in growth rate between the fish consuming the saponin supplemented diet and the control diet was highest during the initial week of the feeding trial. The difference in growth rate declined thereafter and then picked up again towards the end of the experiment. We hypothesised that the fish adapted to the dietary saponin mixture, at a level of 150 mg kg^{-1}, leading to a decrease in its growth promoting effects after an

initial stage. The current experiment was therefore intended to test whether the growth promoting effects of QS could be prolonged during the entire experimental period, by feeding diets containing QS intermittently. Common carp having similar initial weight as in the previous experiment were divided into three groups and were given control feed, control feed supplemented with 150 mg kg^{-1} of QS, and control and the supplemented diet during alternate weeks.

2. Materials and methods

Experimental fish

Carp (*Cyprinus carpio* L.) were obtained from the Federal Fishery Research Agency, Institute for Coastal and Inland Fishery, Ahrensberg, Germany. They were brought to Hohenheim when they weighed about 1 g and grown in 200 litre aquaria at 23 ± 1°C. They were fed a diet containing approximately 42 % protein, 10 % lipid, 10 % ash and an energy content of 20 kJ g^{-1} on a dry matter basis, until they reached an average weight of about 20 g. Once most of the fish attained this weight, they were fed the same diet at a level that maintained carp weight constant (3.2 g $kg^{-0.8}$ day^{-1} at 23°C; Meyer-Burgdorff 1989). Five days prior to the start of the experiment, 15 carp weighing 19.2 ± 2.9 g were selected from a large population and placed individually in respiration chambers (details explained elsewhere). Ten fish of comparable body weight, as the experimental fish, were killed and preserved for determining the initial chemical composition. During acclimatisation (5 days) in the experimental set up, the fish continued to receive maintenance levels of feed. The fish were starved for one day immediately previous to the start of the experiment. They were divided into 3 groups of 5 fish each randomly and were named C, S150, and S150b based on the types of feed and feeding regime.

Table 1. Ingredients and chemical composition of the basal diet

Ingredients (%)	
Fish Meal[a]	50
Whole wheat meal	42
Sunflower oil	4
Vitamin mixture[b]	2
Mineral mixture[c]	2
Chemical compostion (% in DM)	
Crude protein	40.1
Lipid	8.8
Ash	10.8
Gross energy (MJ kg^{-1} DM^{-1})	19.5

[a] Fish meal obtained from Württembergische Zentralgenossenschaft, Germany.
[b] and [c] prepared after Meyer-Burgdorff et al.(1989)

Feeds and feeding regime

The experimental diets, namely C and S150, were prepared from the same basal diet to ensure uniformity of composition. The ingredients and chemical composition of the basal diet used for the experiments are shown in Table 1. The basal diet was ground and then pelleted (pellets of about 2 mm diammeter) without any additions in the case of the control feed (C). The saponin mixture (*Quillaja* saponin; Sigma no. 2149; Sigma, St. Louis, USA) at the rate of 150 mg kg^{-1} was dissolved in demineralised water, mixed thoroughly with the powdered feed using a mixer, and pelleted to form the S150 feed. The moist pellets were frozen, freeze-dried and stored at -18°C until use. The C group was fed the control feed and the S150 group the S150 feed. The S150b group fish were fed the C feed and S150 feed during alternate weeks, with S150 feed being fed during the first week.

After the acclimatisation period in the respirometer, the fish were fed individually at a level of 16 g $kg^{-0.8}day^{-1}$ in 6 equal instalments using automatic feeders attached to the respiration boxes. The fish were weighed individually once a week and the feed ration for each fish was adjusted according to its body mass. There was no feeding on the weighing day.

At the end of eight weeks the experiment was terminated. The fish were weighed, killed, and carefully dissected to determine the weight of the liver and intestine. After weighing, these organs were put back without any loss of material and the complete material was stored at –18°C until analysis. Prior to analysis, the carcasses were autoclaved for 30 minutes at 120°C, homogenised, refrozen and freeze dried.

Experimental set-up

The fish were kept in respiration chambers (5 l capacity) of the computer controlled fish respirometer system during the experimental period. The mechanical and technical details of the system were as described previously (Focken et al. 1994). Thirty-two measurements of oxygen consumption per individual fish were made every 24 h. All the data were recorded on the hard disk of the computer. The system was lit with fluorescent tubes to give a day length of 12 h. The water temperature was maintained at 27 ± 0.1°C, and the flow rate of water through the respiration chambers was adjusted (0.4 to 0.65 l min^{-1}) to keep the oxygen saturation 75 %. Water in the system was continuously passed through an attached bio-filter to facilitate microbial degradation of the metabolites that may be present in it. In addition, every day, about a tenth of the

water in the system was replaced. Once a week, when the fish were weighed, the system was cleaned and the oxygen probe calibrated. This took approximately 3 h, during which time the fish were kept in containers with water taken from the system.

The water quality parameters were maintained within a critical range during the experimental period (pH: 7-7.9; total NH_3: 0.1-0.2 mg l^{-1}; nitrite: 0.07-0.1 mg l^{-1}, and nitrate: 1-3 mg l^{-1}).

Chemical analyses

The chemical composition of the experimental diets and the freeze dried fish were analysed according to official methods (Neumann and Basler 1983) i.e. dry matter was measured by drying to constant weight at 105°C, crude protein as macro-Kjeldahl (N x 6.25), lipids by extraction with petroleum ether and gross energy by bomb calorimetry (IKA C 7000) with benzoic acid standard.

Calculations and statistical analysis

All calculations were performed for each fish individually. Feed Conversion Efficiency (FCE) was calculated as live weight gain/ feed consumption (dry matter), and Metabolic Growth Rate (MGR) as live weight gain (g)/ average metabolic live weight ($kg^{0.8}$)/ day. The average Metabolic Rate was calculated as mg oxygen consumed $kg^{-0.8}$ h^{-1} on a weekly basis. The Standard Metabolic Rate (SMR) was taken as the lowest metabolic rate sustained for 3 hours by the undisturbed animal that had not been fed for the preceding 24 hours (Ultsch et al., 1980). This calculation was done using the oxygen consumption values recorded on the day during which the fish were starved, before experimental feeding started. Oxygen uptake (g) x 14.85 (kJ g^{-1}) gave the energy expenditure (EE) during the whole experiment (Huisman 1976) and the energy apparently not metabolised (AUE) was calculated by subtracting energy expenditure and energy retention (ER; gross energy gain of the carcass) from the gross energy of the feed consumed. The Protein Productive Value (PPV, %) and Apparent Lipid Conversion (ALC, %) were calculated as protein gain x 100/ feed protein and lipid gain x 100/ feed lipid and Protein Efficiency Ratio (PER) was calculated as live weight gain (g)/ protein fed (g). The Liver Somatic Index (LSI) and the Intestine Somatic Index (ISI) were calculated as wet liver weight/ wet body weight and wet intestine weight/ wet body weight respectively.

The data were subjected to ANOVA and statistical comparisons between the feeding groups were made using the Duncan's Multiple Range test (Statistica for

Windows, release 5.1 H, '97 edition.). The significance of observed differences was tested at $p < 0.05$. The values presented in the text are Mean ± Standard Deviation.

3. Results

Feed intake and behaviour of carp.

All the experimental fish consumed the feed provided completely and there was no mortality of fish during the experiment. Since the fish in all treatment groups ate the feed pellets as quickly as they dropped into the respirometer box, there was little chance that the saponins might have leached into the water. Abnormal behaviour of any kind was not noticed during the experiment in any of the treatment groups.

Growth rates

At the end of the experiment the average MGR of the S150 group was significantly ($P< 0.05$) higher than the C group whereas the average MGR value of the S150b group was between those of C and S150 groups. The weekly changes in the MGR during the experimental period are presented in Fig. 1.

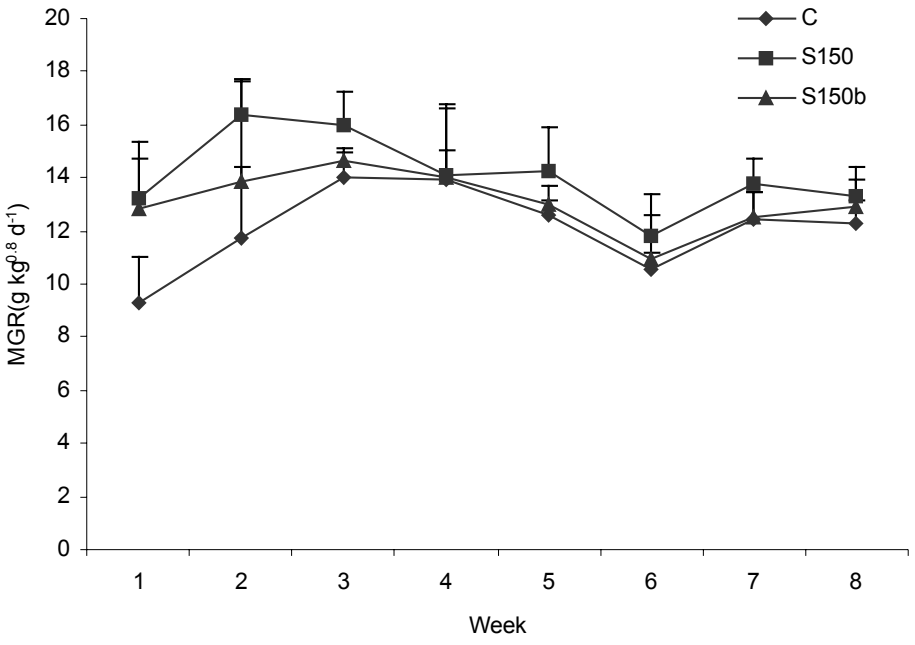

Figure 1. Average weekly Metabolic Growth Rate (MGR) of the experimental carp

Whole body carcass composition

There were differences in the carcass chemical composition of the experimental fish (Table 2). The S150 group had significantly higher average dry matter, lipid and gross energy content as compared to the C group. The average protein and ash contents of the carcass were higher in the case of the C group, but the differences from the corresponding values of S150 group were not significant. The average values of all these carcass composition parameters of the S150b group were intermediate between the C and S150 groups.

Table 2. Initial and final body composition of experimental fish.

	Initial		C[1]		S150[2]		S150b[3]	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Number of fish			5		5		5	
Initial live mass, g	19.5	2.4	19.3	1.6	19.9	0.8	19.9	1.7
Final live mass, g			73.6^{b}	6.4	88.2^{a}	10.3	81.1^{ab}	6.9
Dry matter (DM), % of fresh matter	20.7	0.8	19.7^{b}	0.5	20.5^{a}	0.3	20.4^{ab}	0.7
Crude protein(N x 6.25), % of DM	60.1	2.3	65.5	3.1	63.3	1.5	64.5	1.9
Petroleum ether extract, %	15.8	2.9	16.1^{b}	2.9	21.2^{a}	1.5	18.5^{ab}	4.3
Ash, %	12.3	0.6	11.4	0.8	10.4	0.5	10.5	1.0
Gross energy, kJ g^{-1}	20.0	0.4	22.0^{b}	0.5	23.0^{a}	0.6	22.7^{ab}	0.8

Mean values which do not share the same superscripts differ significantly at $p < 0.05$
[1]C = fish fed control feed
[2]S150 = fish fed control feed containing 150mg/ kg Quillaja bark saponin
[3]S150b = fish fed control feed and control feed containing 150mg/ kg Quillaja bark saponin during alternate weeks

Metabolic rates and oxygen consumption

The SMR were 98.5 ± 15.0, 79.1 ± 17.1 and 111.0 ± 26.3 respectively for the C, S150 and S150b groups. The average metabolic rate (mg $kg^{-0.8}$ h^{-1}) at the end of the experiment was highest in the C group (319 ± 24) and lowest in the S150 group (269 ± 29). Carp in the S150b group, that had the highest SMR before the experimental feeding started, however, decreased to levels below that of the control group (300 ± 27.2). The weekly average metabolic rates are presented in Fig. 2. The average oxygen consumption per unit body mass gained (OPM, g g^{-1}) was significantly higher in the control group (0.63 ± 0.05) as compared to the S150 group (0.49 ± 0.07). The average OPM value of the S150b group was between those of the C and S150 groups (0.56 ± 0.05).

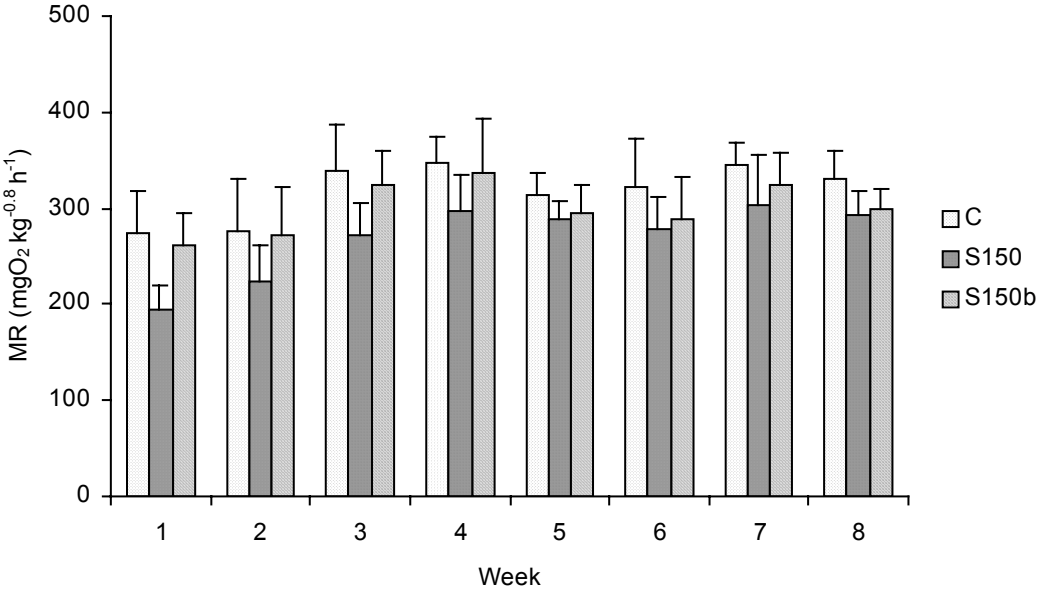

Figure. 2. Weekly average Metabolic Rate (MR) of the experimental carp

Feed conversion and protein and lipid efficiencies

The average FCE values in the S150 group were significantly higher than those of control. The average FCE value of the S150b group was in between the C and S150 groups. The S150 group had significantly higher average values for PPV and ALC. The values of feed consumption and utilisation parameters are presented in Table 3.

Table 3. Body mass, feed consumption and food conversion parameters of carp fed the experimental diets

Experimental group	C[1]		S150[2]		S150b[3]	
	Mean	SD	Mean	SD	Mean	SD
Initial live mass (g)	19.3	1.6	19.9	0.8	19.9	1.7
Final live mass (g)	73.6[b]	6.4	88.2[a]	10.3	81.1[ab]	6.9
Feed consumption (g dry matter)	53.8	3.3	59.7	4.3	57.4	3.1
Food conversion efficiency, FCE[4] (g g^{-1})	1.02[b]	0.04	1.16[a]	0.09	1.08[ab]	0.05
Protein productive value ,PPV[5] (%)	32.3[b]	2.1	36.7[a]	3.3	34.8[ab]	1.8
Protein efficiency ratio, PER[6]	2.5[b]	0.1	2.8[a]	0.2	2.6[ab]	0.1
Apparent lipid conversion, ALC[7] (%)	36.6[b]	9.6	61.5[a]	3.7	49.2[ab]	16.4

Mean values which do not share the same superscripts differ significantly at $p < 0.05$
[1]C = fish fed control feed
[2]S150 = fish fed control feed containing 150mg/ kg Quillaja bark saponin
[3]S150b = fish fed control feed and control feed containing 150mg/ kg Quillaja bark saponin during alternate weeks
[4]FCE = live weight gain/ feed consumed (dry matter)
[5]PER = wet weight gain/ protein fed
[6]PPV = total protein gain/ total protein fed x 100
[7]ALC = total lipid gain/ total lipid fed x 100

Energy utilisation

A complete energy budget of the fish in the different experimental groups was set up (Table 4). There were differences in the energy utilisation parameters between the treatment groups. The S150 group had significantly higher average ER and significantly

Table 4. Energy budget and oxygen uptake of carp fed the experimental diets

Experimental group	C[1]		S150[2]		S150b[3]	
	Mean	SD	Mean	SD	Mean	SD
Initial Gross energy (GE) of carcass (kJ)	80.2	6.5	82.4	3.1	82.5	6.1
Final GE content of carcass (kJ)	318.5	19.9	416.8	50.0	376.0	46.6
Feed GE uptake (kJ)	1052	65	1169	84	1123	60
EE[4]; % of GE fed	48.2^{b}	3.2	41.7^{a}	4.2	45.3^{ab}	3.9
ER[5]; % of GE fed	22.7^{b}	0.7	28.5^{a}	2.3	26.1^{a}	2.7
AUE[6]; % of GE fed	29.1	2.9	29.8	4.2	28.6	3.4
O_2 consumption ($mgO_2\ h^{-1}\ kg^{-0.08}$)	318.7	23.6	269.1	28.6	300.1	27.2
O_2/bodymass gain ($g\ g^{-1}$)	0.63^{b}	0.05	0.49^{a}	0.07	0.56^{ab}	0.05

Mean values which do not share the same superscripts differ significantly at $p < 0.05$
[1]C = fish fed control feed
[2]S150 = fish fed control feed containing 150mg/ kg Quillaja bark saponin
[3]S150b = fish fed control feed and control feed containing 150mg/ kg Quillaja bark saponin during alternate weeks
[4]EE = oxygen uptake (mg) x 14.85
[5]ER = energy retention
[6]AUE = energy fed – energy expenditure - energy retention.

lower average EE values, while the corresponding average values of the S150b group were in between those of the C and S150 groups. The average AUE values were similar in all groups.

The weight of internal organs

The average LSI and ISI values of the experimental fish groups (Table 5) were not significantly different. The absolute values of the saponin fed groups were however lower than that of the control group for both parameters.

Table 5. Liver-Somatic Index (LSI) and Intestine-Somatic Index (ISI)

Experimental group	C[1]		S150[2]		S150b[3]	
	Mean	SD	Mean	SD	Mean	SD
LSI[4]	1.85	0.28	1.62	0.21	1.62	0.16
ISI[5]	3.19	0.35	2.94	0.38	2.96	0.25

[1]C = fish fed control feed
[2]S150 = fish fed control feed containing 150mg/ kg Quillaja bark saponin
[3]S150b = fish fed control feed and control feed containing 150mg/ kg Quillaja bark saponin during alternate weeks
[4]LSI =wet liver weight/ wet body weight x 100
[5]ISI = wet intestine weight/ wet body weight x 100

4. Discussion

The results obtained from the feeding experiments indicated that supplementation with QS at a level of 150 mg kg^{-1} caused a significant increase in MGR, FCE, PPV and ALC of carp with initial weight of about 20 g. The efficiency of energy utilisation indicated by parameters such as EE, ER and OPM were also significantly better when the fish were fed continuously with the S150 diet. On the whole, these results are in agreement with what we reported in our previous manuscript (Francis et al. 2001a). The fish that received the S150 feed intermittently did not perform as well as the S150 group in this experiment, showing that the growth promoting effect was more pronounced when there was a continuous dietary supply of QS. The performance of the S150b group was however better than the C group.

The possible mechanisms of action of the dietary QS are yet to be elucidated. According to the current information, dietary saponins or their breakdown product in the intestine of animals, the sapogenins, are not absorbed into the blood stream (Gestener et al. 1968; Yoshikoshi et al. 1995). A tempting explanation for higher growth in the saponin fed groups would be the better absorption of nutrients across the intestinal membranes that are permeabilized by saponins. Saponins are known to influence the permeability of biological membranes (Seeman et al. 1973; Seeman 1974) and rat intestinal mucosal cells (Johnson et al. 1986) *in vitro*. An increase in the apparent permeability of the brush border may have important implications for uptake of macromolecules (Gee et al. 1996). ISCOMS (immune stimulating complexes-formed by the combination of cholesterol, saponin, phospholipid and amphiphatic proteins) containing partially purified QS, when fed to tilapia increased the absorption of the antigens into the intestinal tissues (Jenkins et al. 1991). The calculated average values of AUE, which were similar in the control and saponin fed groups, however indicate similar amounts of feed energy excretion by fish in the different experimental groups in the current experiment. If there was a saponin influenced actual increase in intestinal absorption, it was at the micronutrient level.

Carp in the S150 group had significantly higher carcass dry matter, lipid and energy contents. This contrasted with results obtained in our previous experiment were carcass chemical composition of the experimental groups were similar (Francis et al. 2001a). Protein and fat assimilation efficiency were significantly higher in the S150 group as evidenced by the significantly higher PPV and ALC values and could have

been caused by increased uptake of nutrients. The higher carcass lipid could result from increased conversion of nutrients into lipid depots in the body.

The differences in the standard metabolic rate before the start of the experiment and the average metabolic rates after the experiment, even though considerable, were not statistically significant, because of high variability within groups. High levels of variation in the metabolic rates of individual fish have been reported in other studies (Becker and Fishelson 1990; Becker et al. 1992) and could be considered typical for fish. It is notable that the increased growth in the S150 group did not lead to an increase in the average metabolic rates in fish of this group. The metabolic rates of the S150b group were lower than that of the C group, even though they grew better during the experimental period, and had a higher average SMR before the start of the experiment. The average metabolic rate values of the S150b group during the course of the experiment (Fig. 2) demonstrate the capacity of the dietary saponins to lower metabolic rate, even while growth was stimulated compared to control. Similar observations of growth stimulation along with lowered metabolic rate, have been made in carp (Francis et al. 2001a), and tilapia (Francis et al., 2001b) fed diets containing 150 mg kg^{-1} QS. The reasons for this effect of QS could not be clearly explained using information currently available in literature. The significantly lower amount of oxygen consumed per unit body mass gained, energy expenditure, and the significantly higher energy retention in the S150 group compared to the C group, point to higher metabolic efficiency of carp in the S150 group. It has been previously reported that changes of functional membrane proteins in rank and structure, induced by fluidity of membrane caused by saponins, could lead to changes in protein activities (Ma and Xiao 1998). The QS mixture that was used in the current study, when present in the diet of carp has been found to stimulate some gut and liver enzymes (Serrano jr. et al. 1998). It was found that the activity of the gut enzymes (trypsin and amylase) was significantly stimulated at the 300 mg kg^{-1} and 450 mg kg^{-1} levels. The activity of two liver enzymes, cytochrtome *c*-oxidase (CO) and lactate dehydrogenase (LDH) were significantly higher in carp fed a diet containing 150 mg kg^{-1} QS. These results indicate the capability of dietary QS mixture to act both at the intestinal and general metabolic levels. It is quite possible that the saponins or their breakdown products may have been absorbed into the blood stream and their presence in the body might have influenced the activity of liver enzymes, CO and LDH. The presence of saponins in fish blood could not be experimentally established because of the difficulty in detecting low levels of saponins. It has been

previously observed that active principles in the ethanol or chloroform extracts of some plants (containing saponins) administered orally can cause profound effects such as upsetting the hormonal balance in human beings (Düker et al. 1991; Quin and Xu 1998) thereby indirectly indicating that they could be absorbed into animal bodies. Saponins are known to have influence on the amounts of circulating hormones in the blood stream (Düker et al. 1991; Quin and Xu 1998; Tamura et al. 1997).

The organ weights indicate that feeding QS did not exert any xenobiotic stress on the fish livers. Fish have been shown to respond to xenobiotics present in their environment by increasing their liver sizes (McFarland et al. 1999). There were also no obvious differences in external appearance between the liver and the intestine of the fish feeding the different diets.

It could be concluded that QS at a dietary level of 150 mg kg^{-1}, is harmless to common carp and could promote growth and metabloic efficiency. More experiments are required to identify the active principle/s responsible for the observed effects and it's/ their mode of action.

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Chapter 4

Effects of *Quillaja* saponins on growth, metabolism, egg production and muscle cholesterol in individually reared Nile tilapia (*Oreochromis niloticus* (L.))

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Abstract

The effects of supplementation of a *Quillaja* saponin (QS) mixture in the diets of tilapia have been studied using a respirometer system that allowed feeding and continuous measurement of oxygen consumption of individual fish. Five fish each were given control diet (C group), or control diet supplemented with 150 mg kg^{-1} (S150 group) or 300 mg kg^{-1} (S300 group) QS. At the end of 14 weeks the body mass gain of the S300 group was significantly higher than control ($P< 0.05$) whereas that of the S150 group had an intermediate value. The S150 group had a higher growth rate ($P = 0.05$) after the first three weeks of feeding the experimental diets, compared to the other two groups. At the end of the experiment the S300 group had significantly higher ($P< 0.05$) average values for energy retention, apparent lipid conversion, carcass fat, energy and significantly lower ($P< 0.05$) average values for apparently unutilised energy and carcass ash content compared to the C group. The corresponding values of the S150 group were intermediate between the C and S300 groups. One out of two female fish in the S150 group and both the female fish in the S300 group never produced eggs during the entire fourteen-week experimental period. Contrarily, all the three female fish in the control group and one out of the two female fish in the S150 group regularly produced eggs, at a rate of approximately once in every 14 days. The muscle cholesterol level in the S300 group was significantly higher than that of the C group. Possible mechanisms of action of the dietary saponins are discussed.

Key words: energy, growth, lipid, metabolism, muscle cholesterol, *Oreochromis niloticus*, protein, *Quillaja* saponins, reproduction, respirometry

1. **Introduction**

A wide variety of plant species contain saponins in leaves, stem, seeds, bark, blossoms, fruit and roots (Price et al., 1987). Although their basic function in plants is unknown, they have been shown to inhibit mould and protect plants from insect attack. In addition, saponins are antimicrobial and can also serve as a source of monosaccharides for the plant (see review by Barr et al., 1998).

When present in the diet of animals they are believed to have several negative effects. For example dietary saponins derived from different plants have been held responsible for depression of feed intake, reduction in weight gain, accentuation of ruminant bloat, photosensitization (Cheeke, 1996), inhibition of the active uptake of nutrients (Johnson et al., 1986) including vitamins (Jenkins and Atwal, 1994) and minerals (Southon et al., 1988) in the intestine, lowering of protein digestibility (Shimoyamada et al., 1998), and for inducing infertility (Tewary et al., 1973; Quin and Xu, 1998). On the other hand saponins have also long been known to possess properties useful to man as they are the active components in a large number of traditional 'herbal medicine' preparations. There have been reports of antiviral activity of saponins from *Glycyrrhiza radix*, cholesterol lowering activity of saponins from soybean, immunostimulant activity of saponins from *Quillaja saponaria* Molina, and hypo-glyceamic and anti-diabetic activity of saponins from fenugreek (Kensil, 1996; Petit et al., 1993). Partially purified *Quillaja saponaria* Molina saponin preparations such as Quil A, have found wide-spread use in veterinary vaccines (Kensil, 1996).

Saponins are of relevance in human nutrition because of their presence in several human food items and 'health products'. For example crude *Quillaja* extracts, typically containing 9-10% saponin, are commercially used as flavourings and foam producers for beverage production (Kensil, 1996). Saponin (glycyrrhizin)-containing liquorice extracts are widely used in confectionery and its average consumption per person per day was reported to be 3, 4, and 2 mg for US, Belgium and UK respectively (Spinks and Fenwick, 1990). Ridout et al. (1988) estimated the mean daily intake of saponins arising from baked beans, lentils and peas to be approximately 15 mg/person, in the UK population as a whole, and the mean daily intake of saponins by vegetarians in the UK was 110 mg/person. In comparison, Asian vegetarians consumed 214 mg/person. Purified *Quillaja* saponins are also being considered for use as adjuvants in human oral vaccines.

Newinger (1994) identified triterpenoid saponins as the main active compounds present in the most effective fish poisoning plants in Africa. However, not all saponins are antinutritional and some of them may even have positive effects in animals (Liener, 1994). In two previous

experiments we have shown that inclusion of *Quillaja* saponins (QS), which are predominantly triterpenoid, at low levels (150 mg kg^{-1}) in the diet of common carp resulted in increased growth and metabolic efficiency (Francis et al., 2000a, b).

Tilapia has become the most commercially cultured fish in the relatively short period since its introduction because it is robust and adaptable to changing environments. One disadvantage of tilapia pond culture is that when male and female fish are reared together they produce large amounts of fry many of which survive because of the parental care exhibited by the adult fish. This results in overproduction of small fish but very few reach marketable size due to competition for food. In many countries tilapia sex-reversed by testosterone treatment are currently used to avoid this problem. However, this practice may have undesired effects on the human consumers of treated fish and the environment. An alternative solution may be the addition of saponins to the diet since these compounds have previously been reported to alter the plasma levels of the hormones that regulate reproductive activity (Tamura et al., 1997).

An experiment was therefore designed to determine the effects of inclusion of QS at levels of 150 mg kg^{-1} and 300 mg kg^{-1} in the diet of Nile tilapia (*Oreochromis niloticus*) on growth, energy metabolism, body composition and muscle cholesterol. The frequency of mouth brooding in the female fish was also monitored as differences between treatments were observed during the course of the experiment.

2. Materials and methods

Experimental fish

Tilapia (*Oreochromis niloticus*) were obtained from the South East Asian Fisheries Research Institute, Philippines. They were brought to Hohenheim when they weighed about 1 g and grown in 200 litre aquaria at 27 ± 1°C. They were fed a diet containing approximately 42 % protein, 10 % lipid, 10 % ash and an energy content of 20 kJ g^{-1} on dry matter basis, until they reached about 50 g. Once this weight was attained they were fed the same diet at a level that has been found to be approximately enough for maintaining body weight (3.2 g $kg^{-0.8}$ day^{-1}). Five days prior to the start of the experiment, 25 tilapia of comparable body weight (50 ± 8.2 g) were selected from a large population, weighed and placed individually in respiration chambers. The remaining ten fish were killed and preserved for determining the initial chemical composition. During accommodation (5 days), the fish continued to receive maintenance levels of feed. The fish were starved for one day prior to the start of experiment.

At the start of the experiment three experimental diets, namely C, S150 and S300 (see below for details of preparation) were assigned to 5 fish each in a random manner.

At the end of 14 weeks the experiment was terminated, the fish were weighed, killed and dissected carefully to isolate and weigh the liver, intestine and gonads. About 2 g of muscle was taken from below the dorsal fin of each fish for muscle cholesterol determination. This was taken into account while making energy balance calculations. The remaining fish and the organs removed from it were immediately deep-frozen together, without any further loss of material. Prior to analysis, the carcasses were autoclaved for 30 minutes at 120°C, homogenised, refrozen and freeze dried.

Feeds and feeding regime

The three experimental diets were prepared from the same basal diet to ensure uniformity of composition. The ingredients and chemical composition of the basal diet are shown in Table 1. This basal diet was ground and pelleted (about 2 mm diammeter) without any additions in the case of the control feed (C). Saponin (QS; Sigma no. 2149; Sigma, St. Louis, USA) was first dissolved in demineralised water and mixed thoroughly at the rate of 150 mg kg^{-1} (S150) and 300 mg kg^{-1} (S300). The moist pellets were frozen, freeze-dried and stored in a freezer (-18°C) until use.

Table 1. Ingredients and chemical omposition of the basal diet

Ingredients (%)	
Fish Meal[a]	50
Whole wheat meal	42
Sunflower oil	4
Vitamin mixture[b]	2
Mineral mixture[c]	2

Chemical compostion (% in DM)	
Crude protein	37.0
Lipid	9.4
Ash	10.8
Gross energy (MJ kg^{-1} DM)	19.4

[a] fish meal obtained from Württembergische Zentralgenossenschaft, Germany
[b] and [c] prepared after Meyer-Burgdorff et al. (1989)

In the respirometer, the fish were fed individually at 20 g per kg metabolic body mass ($kg^{0.8}$) per day initially, in 6 instalments using automatic feeders which emptied feed into a feeding tube attached to the respiration boxes. During the fourth week the feed ration per day was reduced to 16 g $kg^{-0.8}$ and from the fifth week onwards further to 9.6 g $kg^{-0.8}$ metabolic body mass. Once a week the fish were weighed and the ration for each fish was adjusted according to its body mass. There was no feeding on the weighing day.

After the random distribution of 5 fish each to the respective treatments 3 fish belonging to the control group and two others, each belonging to the S150 and S300 group, were identified to be female fish, based on the form of their genital papilla: in the female this was broad with a rounded tip, whereas in the male fish it was narrow with a pointed tip. The female fish that were observed mouth-brooding during the course of experiment were immediately taken out from the respiration chamber and the eggs

liberated using a smooth stainless steel spatula. After removing the eggs the fish were given a day to recover during which feeding was avoided. The feeding rate was adjusted during the succeeding days so that the total feed amount for the week was fully provided.

The experimental set-up

During the experiment the fish were kept in respiration chambers (5 litre capacity) of the computer controlled fish respirometer system (for a description of the system see Focken et al., 1994) at the University of Hohenheim, Department of Animal Nutrition and Aquaculture. Thirty two measurements of oxygen consumption per individual fish were made every 24 hours, and recorded on the hard disk of a computer controlling the system. The system was lit with fluorescent tubes to give a day length of 12 hours. The water-flow rate was adjusted (0.4 to 0.65 l min^{-1}) to keep the oxygen saturation above 75 %. This water passed continuously through an attached bio-filter to facilitate microbial degradation of the metabolites. In addition, about a tenth of the water in the system was replaced every day. Once a week, when the fish were weighed, the system was cleaned and the oxygen probe calibrated. This process took approximately 3 hours. During this time the fish were kept in containers with water taken out from the system. The water temperature in the experimental system was maintained at 27 ± 0.1°C. All water quality parameters were maintained at acceptable levels (pH: 7-7.9; total NH_3: 0.1-0.2 mg l^{-1}; nitrite: 0.07-0.1 mg l^{-1}, and nitrate: 1-3 mg l^{-1}) throughout the experiment.

Chemical analyses

The chemical composition of the experimental diets and the fish were analysed according to recognised methods (Neumann and Basler, 1983) i.e. dry matter was measured by drying to constant weight at 105°C, crude protein as macro-Kjeldahl (N x 6.25), lipids by extraction with petroleum ether, and gross energy by bomb calorimetry (IKA C 7000), with a benzoic acid standard. Muscle cholesterol was estimated using a commercial cholesterol estimation kit (Catalogue No. 139050, Boehringer Mannheim, Germany).

Calculations and statistical analysis

The calculations were performed for each fish individually. Feed Conversion (FCR) was calculated as feed consumption (dry matter)/ live weight gain and Metabolic Growth Rate (MGR) as live weight gain per day (g)/ average metabolic live weight ($kg^{0.8}$) per day over the experimental period. The Standard Metabolic Rate (SMR) was taken as the lowest metabolic rate sustained for at least 1.5 h by the undisturbed animal that has not been fed for the past 24 hours (Ultsch et al., 1980). To calculate SMR the relevant oxygen consumption values for

were obtained before the start of experimental feeding, during the 24-hour period when the fish were not fed after a 5-day acclimatisation period in the respiration chambers. Oxygen uptake (g) x 14.85 gave the energy expenditure in kJ g^{-1} (Huisman, 1976) during the whole growth experiment, and the energy apparently not metabolised (AUE) was calculated by subtracting energy expenditure and energy retention (ER; gross energy gain of the carcass) from the gross energy of the feed consumed. Protein Productive Value (PPV) and Apparent Lipid Conversion (ALC) were calculated as protein gain x 100/ feed protein and lipid gain x 100/ feed lipid and Protein Efficiency Ratio (PER) was calculated as live weight gain(g)/ protein fed (g).

The Gonado-Somatic Index (GSI), Liver-Somatic Index (LSI), and Intestine-Somatic Index (ISI) were calculated by dividing the respective fresh organ weight by the total fresh body weight and multiplying by 100.

The data were subjected to ANOVA and statistical comparisons between the feeding groups were made using the Duncan's Multiple Range test (Statistica for Windows, release 5.1 H, '97 edition.). The significance of observed differences was tested at $p < 0.05$. The values reported are Mean ± SD.

3. Results

Feed intake and behaviour of tilapia

During the first three weeks the fish completely consumed the feed provided. During the fourth week the fish were observed not to consume their daily ration completely and hence this was reduced to 16 g per kg metabolic body mass ($kg^{0.8}$). As even after this reduction feed remained for longer period in many aquaria the ration was further reduced to 9.6 g per kg metabolic body mass. This ration was then continued until the end of the experiment. There was no abnormal behaviour in any of the treatment groups during the whole experimental period.

Growth rate

The average body mass gain during the 14-week experimental period of the S300 group was 245 % over the initial body mass, significantly ($P< 0.05$) higher than that of the control group

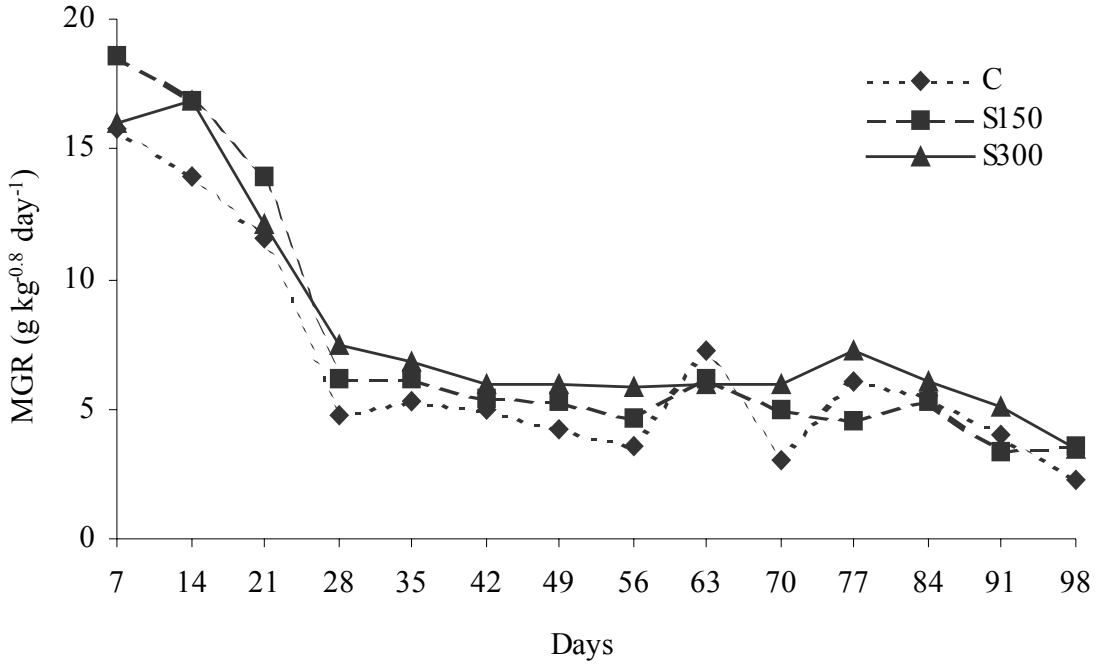

Fig. 1. Average weekly Metabolic Growth Rates (MGR) of the experimental tilapia

(188 %), but not that of the S150 group (226 %). There was a 26 % difference between the final average body mass of the C and S300 groups and a 16 % difference between the C and S150 groups. The average MGR was the highest in the S300 group at the end of the experiment, and the lowest in the C group. The growth rate however did not follow a uniform pattern during the experimental period (Fig 1). The highest average MGR was in the S150 group (16.1) after three weeks of feeding the experimental feeds, and lowest in the C group (13.6; *P* value of the difference between the two

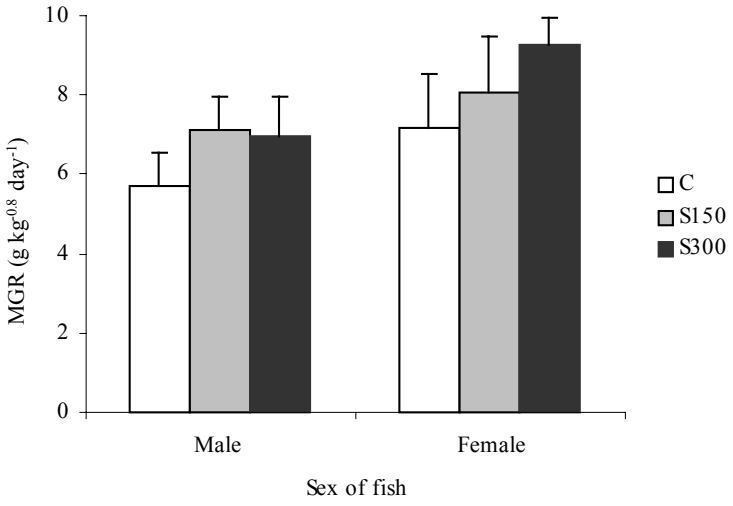

Fig. 2. Sex-related average Metabolic Growth Rates (MGR) of the experimental tilapia

Table 2. Spawning pattern of female tilapia on a weekly basis

Fish group	No. of spawnings during the 14 week experimental period
C[1]	8
C[1]	3
C[1]	7
S150[2]	6
S150[2]	nil
S300[3]	nil
S300[3]	nil

[1]C = fish fed control feed
[2]S150 = fish fed control feed containing 150mg/ kg *Quillaja* bark saponin
[3]S300 = fish fed control feed containing 300mg/ kg *Quillaja* bark saponin

was exactly 0.05), with the figure for S300 group lying in between (14.7). The feed intake and growth of the fish of all groups declined drastically after this initial period.

Reproductive activity in female tilapia

The three female fish in the control group and one out of the two female fish in spawned at predictable time intervals. The remaining female fish, i.e. one out of two of the S150 group and both fish of the S300 group, never spawned during the entire fourteen-week experimental period. The number of spawnings per fish is given in Table 2. The ovaries of the non-spawning fish were full of apparently normal eggs when the fish were dissected at the end of the experiment. The quantity and morphology of the eggs produced by the S150 fish were similar to those of the control.

At the end of the experiment the female fish had grown significantly better than the male fish ($P< 0.05$). The growth of the female fish was also better among treatment groups as compared to the males. The sex related MGR in the treatment groups is presented in Fig 2.

Size and nature of the internal organs

Table 3. Liver-Somatic Index, Intestine-Somatic Index, and sex-related Gonado-Somatic Index of experimental tilapia

LSI[1] and ISI[2]				
Fish group	LSI		ISI	
	Mean	SD	Mean	SD
C[3]	2.70	0.35	1.59	0.19
S150[4]	2.23	0.69	1.52	0.35
S300[5]	2.00	0.37	1.55	0.31

Sex-related GSI[6]				
	Male		Female	
	Mean	SD	Mean	SD
C	1.1	0.2	2.2	1.9
S150	0.9	0.2	3.2	1.0
S300	0.9	0.6	3.7	0.9

[1]LSI =wet liver weight/ wet body weight x 100
[2]ISI = wet intestine weight/ wet body weight x 100
[3]C = fish fed control feed
[4]S150 = fish fed control feed containing 150mg/ kg *Quillaja* bark saponin
[5]S300 = fish fed control feed containing 300mg/ kg *Quillaja* bark saponin
[6]GSI = wet gonad weight/ wet body weight x100

There were no noticeable differences in the appearance of the liver, intestine and gonads. No significant differences occurred in LSI and ISI, or GSI values between the experimental groups (Table 3) even though there was a declining trend in all these indices with increasing saponin concentration in the diet.

Carcass composition

The average lipid and gross energy content of the S300 group was significantly higher as compared to the C group with the value for S150 group lying in between (Table 4). The ash content was significantly higher in the C group as compared to the S300 group. There were no significant differences in the other carcass composition parameters of the experimental groups.

Table 4. Initial and final chemical composition of the experimental fish

	Initial		**C**[1]		**S150**[2]		**S300**[3]	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Dry matter (DM), % of fresh matter	25.4	1.3	30.4	0.9	31.0	0.3	31.1	0.6
Crude protein(N x 6.25), % of DM	56.9	3.1	56.1	2.1	53.5	1.5	53.6	2.1
Petroleum ether extract, %	12.9	2.6	20.4 [b]	2.0	22.4 [ab]	1.4	24.7 [a]	2.3
Ash, %	20.9	1.9	16.4 [a]	0.9	15.9 [ab]	1.0	14.5 [b]	1.5
Gross energy, kJ g^{-1}	19.4	1.0	21.0 [b]	0.5	21.1 [b]	0.5	23.0 [a]	1.2

Mean values which do not share the same superscripts differ significantly at $p < 0.05$
[1]C = fish fed control feed
[2]S150 = fish fed control feed containing 150mg/ kg *Quillaja* bark saponin
[3]S300 = fish fed control feed containing 300mg/ kg *Quillaja* bark saponin

Metabolic rates and oxygen consumption

The SMR were 65.5 ± 13.3, 63.1 ± 15. and 65.9 ± 11.2 respectively for the C, S150 and S300

Table 5. Average body mass increase, food conversion and protein and fat utilisation parameters of the treatment groups

Experimental group	C[1]		S150[2]		**S300**[3]	
	Mean	SD	Mean	SD	Mean	SD
Initial live mass (g)	48.5	10.2	49.3	9.0	51.0	6.9
Final live mass (g)	139.3 [b]	15.0	161.1 [ab]	16.4	175.9 [a]	22.4
Body mass gain (g)	90.9[b]	18.6	111.7[ab]	17.9	124.9[a]	21.9
Food conversion ratio, FCR[4] (g g-1)	1.61	0.34	1.41	0.19	1.32	0.26
Protein efficiency ratio, PER[5]	1.7	0.4	2.0	0.3	2.1	0.4
Protein productive value, PPV[6] (%)	32.0	5.4	34.1	4.3	37.2	6.8
Apparent lipid conversion, ALC[7] (%)	53.4[b]	11.3	65.8[ab]	6.8	79.9 [a]	17.7

Mean values which do not share the same superscripts differ significantly at $p < 0.05$
[1]C = fish fed control feed
[2]S150 = fish fed control feed containing 150mg/ kg *Quillaja* bark saponin
[3]S300 = fish fed control feed containing 300mg/ kg *Quillaja* bark saponin
[4]FCR = feed consumed (dry matter)/ live weight gain
[5]PER = wet weight gain/ protein fed
[6]PPV = total protein gain/ total protein fed x 100
[7]ALC = total lipid gain/ total lipid fed x 100

groups. The highest average metabolic rate ($mgO_2\ kg^{-0.8}\ h^{-1}$) at the end of the experiment was in the S300 group (168 ± 15). The average metabolic rate of the C and S150 groups (159 ± 9 and 158 ± 15 respectively) was similar at the end of the experiment. The average oxygen consumption per unit body mass gained was the highest in the C group (0.63 ± 0.12 g g^{-1}) compared to the two treatment groups (S150 and S300 had similar average values 0.55 ± 0.05 g g^{-1}), but the differences were not statistically significant.

Feed conversion and assimilation

The calculated average values of food conversion ratio, and protein and lipid assimilation values are presented in Table 5. The FCR values in the S150 group were significantly lower than those of control after the first three weeks of feeding the experimental diets. The S300 group, however, had the highest average values for PPV and PER at the end of the experiment. Average ALC value of the S300 group was significantly higher than the corresponding value of the C group. The S150 group had an intermediate ALC value compared to the other two groups.

Energy utilisation

The complete energy budget of the fish in the three groups is presented in Table 6. There were significant differences ($P< 0.05$) between the average values of ER and AUE between the C and S300 groups. The other energy utilisation parameters were not statistically different.

Table 6. Complete energy budget of the different experimental groups

	C[1]		**S150**[2]		**S300**[3]	
	Mean	SD	Mean	SD	Mean	SD
Initial GE content of carcass, kJ	239.7	50.4	244.1	44.7	252.3	34.0
Final GE content of carcass, kJ	887.7	94.9	1052.8	90.5	1265.2	212.1
Feed GE uptake, kJ	2756	286	3012	297	3084	116
EE[4], % of GE fed	30.3	1.5	30.3	2.7	32.5	3.0
ER[5], % of GE fed	23.7 [b]	4.4	27.0 [ab]	2.5	32.7 [a]	6.9
AUE[6], % of GE fed	45.9 [a]	4.8	42.8 [ab]	4.6	34.8 [b]	9.5

Mean values which do not share the same superscripts differ significantly at $p < 0.05$
[1]C = fish fed control feed
[2]S150 = fish fed control feed containing 150mg/ kg *Quillaja* bark saponin
[3]S300 = fish fed control feed containing 300mg/ kg *Quillaja* bark saponin
[4]EE = oxygen uptake (mg) x 14.85
[5]ER = energy retention
[6]AUE = energy fed – energy expenditure - energy retention.

Muscle cholesterol content

The average cholesterol content in the muscles of the male and female fish belonging to the S300 group fish was highest compared to the other two groups (Table 7).

4. Discussion

Tilapia kept in partially closed systems and fed artificial diets alone, have been previously been reported to have lowered feed intake, lowered feed conversion efficiency and decreased growth after a certain period of time (Jackson et al., 1982). This may be due to the fact that tilapia need a continuous supply of their natural food or because of a build-up of growth inhibiting factors in the system. In the present study the average MGR (7.3) and the average FCR (1.5) could be considered satisfactory for the purpose of assessing the influence of dietary saponins, even though a profound reduction occurred in both feed intake and growth after three weeks of feeding the experimental feeds.

Table 7. Muscle cholesterol content (g/100g tissue) of the experimental groups

All fish		
Diet	Means	Std.Dev.
C[1]	0.077[b]	0.013
S150[2]	0.084[b]	0.014
S300[3]	0.112[a]	0.027
Males		
Diet	Means	Std.Dev.
C	0.068	0.01
S150	0.084	0.02
S300	0.096	0.01
Females		
Diet	Means	Std.Dev.
C	0.083[b]	0.01
S150	0.084[b]	0.00
S300	0.137[a]	0.02

Mean values in columns that do not share the same superscripts differ significantly at $p < 0.05$
[1]C = control diet
[2]S150 = diet containing 150mg/ kg *Quillaja* bark saponin
[3]S300 = diet containing 300mg/ kg *Quillaja* bark saponin

The average metabolic growth rate was highest in the S150 group during the first three weeks ($P = 0.05$). The effect of dietary saponins however, seemed to decrease as the experiment progressed, probably due to adaptation by the fish. The growth rate in the S300 group increased compared to the control and S150 groups during the remaining nine weeks of the study. The growth stimulating potential of dietary QS was similar to that observed in common carp (Francis et al., 2000a, b). A possible hypothesis to explain the initial increase in growth of the saponin fed fish would be that the nutrient absorption from the intestine was increased because the intestinal walls were permeabilized by the dietary saponin. Cell membranes treated with saponins have been found to develop through holes of about 40 to 50 A° diameter. Such membranes are then permeable to large molecules like ferritin (Seeman et al., 1973; Seeman, 1974). ISCOMS (immune stimulating complexes-

formed by the combination of cholesterol, saponin, phospholipid and amphiphatic proteins) containing partially purified QS, when fed to tilapia increased the absorption of antigens into the intestinal tissues (Jenkins et al., 1991). This hypothesis was supported by the lower AUE and the higher ER values in the S150 and S300 groups indicating lower excretion and higher assimilation of feed energy in these groups. It is also worth mentioning that the average FCR value was lower and the PPV values tended to be higher in the treatment groups indicating that these fish were using their food more efficiently than the controls. Reduced active transport in the gut brush border might have resulted in lower relative oxygen consumption in the saponin fed groups.

The less pronounced growth promoting effect of saponins in the S300 group during the initial experimental period probably occurred because the higher saponin dose caused excessive defoliation of the intestinal mucosa. Saponins have previously been shown to damage the intestinal mucosa of rats, but they were able to compensate for defoliation by increasing the production of new mucosal cells up to a dietary saponin level of 1.5 % (Gee and Johnson, 1988).

At the start of the experiment we did not expect that dietary QS would suppress reproductive activity in female tilapia in the way that it appeared to do. Because of this, we did not take any particular care to ensure the numbers of female fish in the treatment groups were equal. In any case the numbers were too small for valid conclusions to be reached. Consequently, the following observations should be treated as preliminary. Dietary saponins have previously been reported to alter the plasma levels of hormones that regulate reproductive activity. Orally administered ethanolic extracts of the rhizome of *Cimicifuga racemosa* (8 mg extract per day), significantly reduced the levels of leutinizing hormone (LH) but not follicle stimulating hormone (FSH) in the plasma of menopausal women after 8 weeks of treatment (Düker et al., 1991). Release of LH from the pituitary is important for triggering the process of ovulation. The steroidal saponin OSW-1 administered to rats in doses of 4.5 and 9 μg kg^{-1} significantly reduced oestradiol (E2) levels in the serum (Tamura et al., 1997). In the present study, the tendency for lower GSI values in the males of the S150 and S300 groups (Table 3) could also have been an effect of dietary saponins since GSI values are correlated to the circulating levels of estrogen, testosterone and 11 ketotestosterone, and active spematogenesis (Melamed et al., 2000) (The lower average GSI value in females of the C group could be explained by the fact that fish belonging to this group spawned shortly before the termination of the experiment).

There is little concrete information as to the mechanism of action of the dietary saponins that caused the effects observed in the S150 and S300 groups. It is tempting to attribute the higher growth rates occurring in the S300 group during the later part of the experiment to a redistribution of nutrients caused by the suppression of spawning in the female fish since it was the higher growth in the females that was mainly responsible for the improved performance of the group as a whole (Fig 2).

Results of previous experiments indicate that saponins can lower cholesterol in serum (Malinow et al., 1977; Oakenfull and Sidhu, 1983; Sauvaire et al., 1991). We were unable to find any studies on the effects of saponins on muscle cholesterol levels in the literature. In the present experiment the fish in the S300 group, especially the females, had significantly higher levels of muscle cholesterol (Table 7). The higher muscle cholesterol content could be related to the higher carcass lipid content in the saponin-fed groups (Table 4). Chavali et al. (1987) mention the possibility that saponins may cause increased *de novo* synthsesis of cholesterol in mouse spleen cells *in vitro*.

Many of the observed effects of QS on tilapia indicate a systemic or physiological mode of action. These include the partial and complete suppression of spawning in the S150 and S300 groups respectively, the tendency for lower average consumption of oxygen per unit gain of body mass, the higher values of ER in the saponin fed fish compared to the control, and the significant differences in the carcass chemical composition between the treatment groups. According to current knowledge, dietary saponins or sapogenins, their breakdown product in the intestine of animals, are not absorbed into the blood stream (Gestener et al. 1968; Yoshikoshi et al. 1995). However, several reports from immunological studies, where QS are used as vaccine adjuvants, indicate that saponins delivered orally can have a systemic immunomodulatory effect (Sjolander and Cox, 1998). Possible effects of non-saponin components in the crude saponin mixture used in this study can also not be ruled out. Kensil (1996) mentions that commercially available non-fractionated saponin extracts have been reported to contain about 20% saponins and 80% tannins and polyphenolics.

Further studies are required to identify the active principles present in QS that are responsible for the observed effects and to elucidate their mechanism of action in biological systems.

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Chapter 5

Dietary *Quillaja* saponins may suppress egg laying in tilapia

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Abstract

Fifteen female tilapia were reared individually in a system that allows feeding and continuous measurement of oxygen consumption, They were fed a control diet (C) and a diet containing 300 mg kg^{-1} *Quillaja* saponins (S300) in an experiment that lasted thirteen weeks. All fish produced eggs and were observed to mouth brood during an initial 3 week period when they were fed the control feed. During the next seven weeks 8 randomly selected tilapia were given the S300 diet while the remaining 7 fish continued to receive the C diet. During this period there were 15 occurrences of spawning among the C fed group (7 fish) and 10 occurrences of spawning in the S300 fed group (8 fish). Three fish in the S300 group and one fish in the C group did not spawn during this period. During the following three weeks, the feeds of the two groups were interchanged, and out of seven fish, which were now fed the S300 feed, four fish spawned once, while in the eight fish which now received the C diet, one fish spawned once. There were no significant differences in the metabolic growth rate or metabolic rate of fish in the two different groups. The ovarian morphology of the fish showed differences at the end of the experiment. The observed effects were hypothesised to be due to an upsetting effect of dietary saponins on the balance of reproductive hormones such as tilapia gonadotropic hormone (taGtH) and estrogen. It was concluded that dietary QS has potential to prevent the widespread problem of overproduction of small fish in tilapia culture.

Key words: *Quillaja* saponins, spawning, mouth brooding, *Oreochromis niloticus*

Introduction.

Nile tilapia are among the most important species used in aquaculture. A major drawback of tilapia as a culture fish is their prolific reproduction resulting in the harvested fish not attaining body weights acceptable to consumers in many countries. Production of all-male populations by treatment of tilapia fry with synthetic steroid hormones have been used as a solution to this problem. Because of environmental and consumer health concerns, this treatment is prohibited in most countries practising tilapia culture. It would therefore be helpful if natural substances that could prevent excessive reproduction of tilapia in culture ponds were identified. In a previous experiment we observed that female Nile tilapia fed a diet containing 300 ppm *Quillaja* saponins did not produce eggs during a 14 week experimental period (Francis et al., 2000). Here we present observations on growth, mouth brooding, and metabolic rate made in a succeeding

experiment where fifteen female Nile tilapia were reared individually and fed a diet containing 300 ppm *Quillaja* saponins (QS).

Materials and methods.

Fish

Tilapia (*Oreochromis niloticus*) were obtained from the South East Asian Fisheries Research Institute, Philippines. They were brought to Hohenheim when they weighed about 1 g and were grown in 200 litre aquaria at 27 ± 1°C. They were fed a diet containing approximately 42 % protein, 10 % lipid, 10 % ash and an energy content of 20 kJ g-1 on dry matter basis. Five days prior to the start of the experiment, fifteen mature female tilapia of comparable body weights (80.5 ± 6.4 g) were randomly selected from a large population and transferred to the experimental set up. During acclimatisation (5 days), the fish were provided with the control feed at levels observed to ensure maintenance of energy and nutrient balance (3.2 g $kg^{-0.8}$ day^{-1}).

Experimental set up

The fish were placed in 15 aquaria which were part of a warm water re-circulating system where feeding and continuous measurement of oxygen consumption of individual fish could be measured (technical details described in Focken et al., 1994). Thirty two measurements of oxygen consumption per individual fish were made every 24 h and recorded on the hard disk of the computer, which controls the system. The system was lit with fluorescent tubes to give a day length of 12 h. The water flow rate through the respirometer chambers, which was controlled by electronic flow meters connected to each chamber, was adjusted (0.4 to 0.65 l min^{-1}) to keep the oxygen saturation in water above 75 % in the chambers. Every day about a tenth of the water in the system was replaced. The water temperature was maintained at 27 ± 0.1°C. Once a week, when the fish were weighed, the chambers were cleaned and the oxygen probe calibrated. During cleaning of chambers the fish were kept in plastic buckets containing water taken out from the system. The entire process of weighing and cleaning the system took approximately 3 h. During the experimental period all water quality parameters were maintained at acceptable levels (pH – 7-7.9; total NH_3 – 0.1-0.2 mg l^{-1}; nitrite – 0.07-0.1 mg l^{-1}, and nitrate – 1-3 mg l^{-1}).

The fish were divided in a random manner into 2 groups, group 1 with 7 fish (84.3 ± 5.5 g) and group 2 with 8 fish (77.2 ± 5.5 g). Both groups were fed the control feed initially for three weeks (phase 1), until all of them had produced eggs. From the fourth week onwards, fish of group 1 continued to receive the control feed and those of group 2 were given the S300 diet (phase 2).

After continuing this diet regime for 7 weeks the diets were reversed, that is group 1 now received the S300 diet and group 2 the C diet. This phase (phase 3) extended for a period of 3 weeks. The total duration of the experiment was 13 weeks.

Feeds and feeding

The diets were prepared from the same basal diet to ensure uniformity of composition (chemical composition as previously mentioned). The basal diet was ground and pelleted (about 2 mm diameter) without any additions in the case of the control feed (C). Saponin (QS; Sigma no. 2149; Sigma, St. Louis, USA), first dissolved in de-mineralised water, was mixed thoroughly (at the rate of 300 mg kg^{-1} in the case of the S300 diet) with the powdered feed using a mixer before pelleting. The moist pellets were frozen, freeze-dried and stored in a freezer (-18°C) until use.

The fish were fed individually at 20 g per kg metabolic body mass ($kg^{0.8}$) per day, in 6 equal instalments using automatic feeders which emptied feed into a feeding tube attached to the aquaria. The fish were weighed once every week and the ration for each fish was adjusted according to its body mass. There was no feeding on the weighing day. The fish were carefully observed three times a day. Tilapia that were found to mouth-brood during the course of the experiments were taken out from the aquaria immediately and the eggs emptied from the mouth using a smooth stainless steel spatula. After removing the eggs the fish were given a day's time to recover. Feeding was avoided during that day. The feeding rate during the next two days was so adjusted that the total feeding quota for the week was fully provided. At the end of the experiment the fish were weighed and dissected to make observations on the ovarian morphology.

Calculations

The Metabolic Growth Rate (MGR) was calculated as live weight gain (g)/ average metabolic live weight ($kg^{0.8}$)/ day. The average Metabolic Rate (MR) was calculated as mg oxygen consumed $kg^{-0.8}$ h^{-1} on a weekly basis. The Gonado-Somatic Index (GSI) was calculated as (weight of the ovary/ weight of the fish) x 100.

Results

Growth and metabolism

All fish consumed the allotted feed ration completely and there was no mortality during the experiment. The growth of all the fish during the experimental period was normal. The average weight of the fish increased from 80.5 ± 6.4 to 228.6 ± 29.0 over the 13 week experimental period (data not shown). The MGR and MR of groups 1 and 2 were statistically similar throughout the experiment (Figs 1 and 2). The absolute values of MGR of group 2 tended to rise and the RMR tended to be lower during phase 2 of the experiment when these fish were fed the S300 diet.

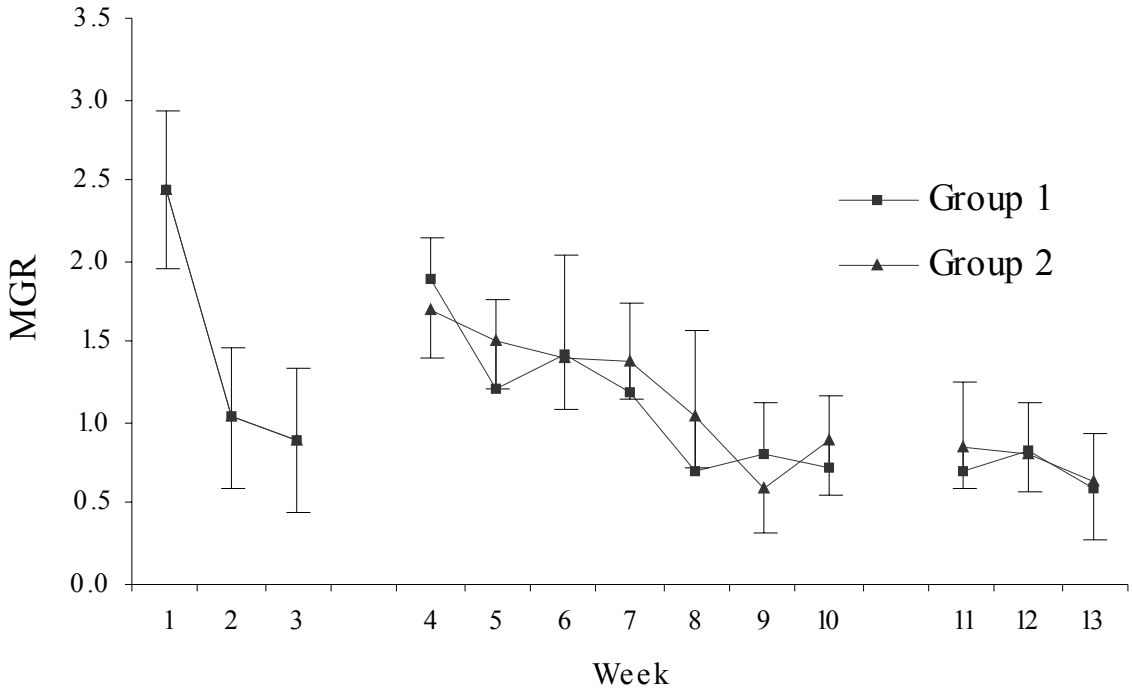

Figure 1. The Metabolic Growth Rate (MGR) of tilapia in the two treatment groups (Group 1 included tilapia fed diet C during phase 1, C during phase 2 and S300 during phase 3 and Group 2 included fish fed diet C during phase 1, S300 during phase 2 and C during phase 3; gaps are shown between the three phases of feeding)

Spawning and mouth brooding

Occurrences of spawning in the individual fish are shown in Table 1. During the initial phase of the experiment (3 weeks) all the 15 fish spawned and were observed to brood the eggs in the mouth cavity. Two tilapia spawned 2 times during this time period.

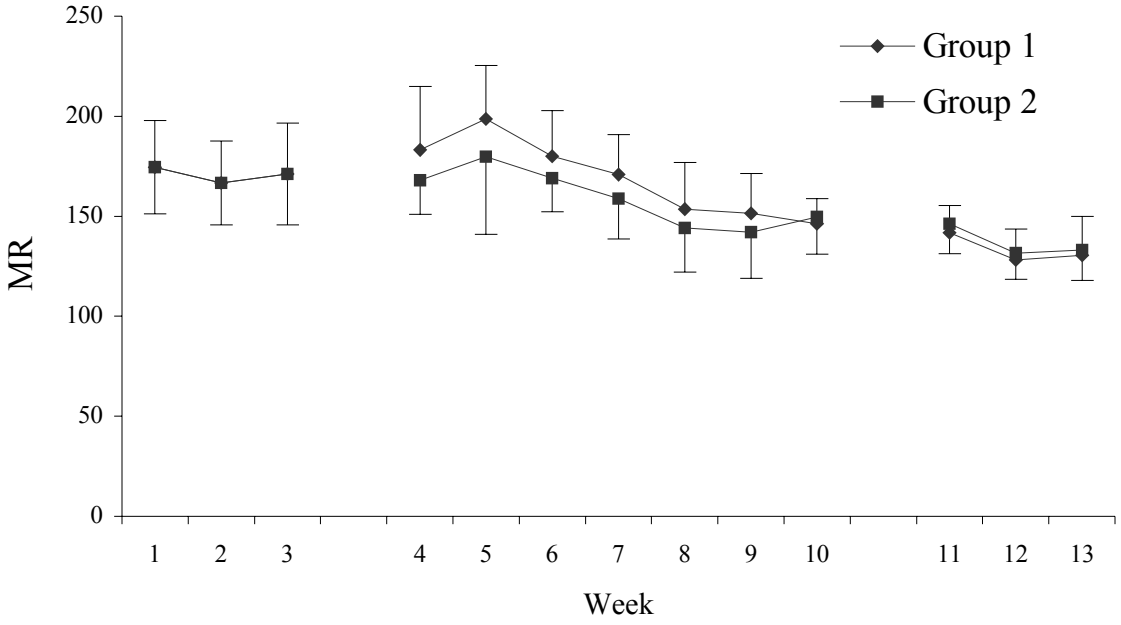

Figure 2. The Average Metabolic Rate (MR; mg O_2 consumed/$kg^{-0.8}$/h) of tilapia in the two treatment groups (Group 1 included tilapia fed diet C during phase 1, C during phase 2 and S300 during phase 3 and Group 2 included fish fed diet C during phase 1, S300 during phase 2 and C during phase 3; gaps are shown between the three phases of feeding)

During the second phase of the experiment, out of 7 tilapia fed the control diet, 5 were regular spawners. Four fish of this group spawned three times each during this seven week phase and one fish two times. Out of the remaining two, one spawned only once and the other one did not spawn at all. In the S300 group 3 out of 8 fish were regular spawners. Two fish spawned 3 times and one 2 times. Two out of eight fish spawned only once; one of them during the first day of phase 2. Three fish belonging to this group did not spawn at all during the 7 week phase. In total, there were 15 occurrences of spawning in the 7 fish of the C group and 10 occurrences of spawning in 8 fish in the S300 group. This is a 70 % reduction in occurrence of spawning in the S300 group. The average mass and morphological appearance of the eggs produced in both the treatment groups seemed to be similar.

Table 1. Record of egg production and mouth brooding in female tilapia fed control (C) and S300 diets

	Phase 1				**Phase 2**								**Phase 3**			
Fish no.	**Feed**	Week			**Feed[1]**	Week							**Feed[2]**	Week		
		1	2	3		4	5	6	7	8	9	10		11	12	13
1	**c**			x	**c**		x			x		x	**S300**			x
2	**c**		x		**c**	x			x			x	**S300**			x
3	**c**		x		**c**		x		x			x	**S300**			
4	**c**	x		x	**c**		x		x			x	**S300**		x	
5	**c**			x	**c**			x		x			**S300**	x		
6	**c**		x		**c**			x					**S300**			
7	**c**		x		**c**	No egg production							**S300**			
8	**c**	x			**S300**	No egg production							**c**			
9	**c**	x		x	**S300**	No egg production							**c**			
10	**c**		x		**S300**	x		x		x			**c**			
11	**c**		x		**S300**	x							**c**			
12	**c**		x		**S300**		x		x		x		**c**		x	
13	**c**		x		**S300**		x			x			**c**			
14	**c**		x		**S300**	No egg production							**c**			
15	**c**		x		**S300**						x		**c**			

x mark indicates occurrence of mouth brooding
[1] 8 fish put on a diet containing 300ppm *Quillaja* saponins (S300)
[2] the feeds reversed at this point, ie fish fed control were given S300 feed and those fed S300 feed were given control feed

During the third phase of the experiment of three weeks, out of seven fish which were now fed the S300 feed, four fish spawned once, while in the eight fish which now received the C diet one fish spawned once.

The ovarian morphology of the fish showed differences at the end of the experiment. Based on the morphological appearance of the ovaries, the fish could be grouped into four classes (Table 2). The ovaries where considered to be normal when they were filled with normal eggs (eggs which had the characteristic shape, size - a diameter of >1.5 mm was considered to be the normal dimension of the eggs present in ripe ovaries of fish of the size used in the experiment - , and colour).

2 out of 7 fish fed control diet during the second phase of the experiment, and which spawned only once or not at all, had comparatively small ovaries (GSI of 1.7 and 3.1 respectively) with a lot of fat deposits and very small eggs. These two fish had more fat deposits in the viscera when compared to the other fish.

1 out of 8 fish which was fed the S300 feed during the second phase of the experiment and was spawning normally during this phase and stopped spawning when their feed was switched to control feed, and one fish that spawned once during phase two, had big and fatty ovaries (the

Table 2. Classification of tilapia based on differences in ovarian morphology at the end of the experimental period

Group No.	Description of ovarian morphology	No. of fish fed the C diet#	No. of fish fed the S300 diet#	Apparent ovarian abnormality
1	Normal, filled with apparently healthy eggs, most of which were more than 1.5 mm in diameter (except for the 2 fish that spawned during the last week, which had smaller eggs and ovaries)	5	1	--
2	Big fatty ovaries containing a large no. of morphologically normal eggs (>1.5 mm diameter size), abnormally high fat content in the ovary, ovarian sheath with a tougher than normal consistency	--	2	++*
3	Relatively small ovaries with high fat deposition, large no. of small eggs (<1 mm diameter)	2	--	+++*
4	Ovaries with a large no. of irregular shaped and small eggs, fat deposits and intermittent dark coloured tissue, ovarian membrane tougher than normal	--	5	+++++*

diets fed during the second and longest phase of the experiment
* number of + signs indicate the relative morphological abnormality of the respective ovaries

GSI values were 4.8 and 5.4 respectively) that contained morphologically normal eggs, but the ovarian membranes appeared to be thicker than in the normal fish.

3 out of 8 fish fed the S300 diet during the second phase and which never spawned during the second and third phases of the experiment and two fish in this group that spawned only one or two times, had ovaries that were morphologically abnormal with black spots, some obviously normal mature eggs and some abnormally shaped eggs, and fat deposits in the ovary. The ovaries in these fish were having normal size (GSI = 3.5 ± 0.43) but had a tougher ovarian membrane.

Discussion.

The growth and metabolic rate of both groups of fish were similar and could be considered normal for tilapia reared in tanks. Decline in growth of tilapia reared in partially closed systems observed during a previous experiment (Francis et al., 2001) was in evidence in this experiment as well. The stimulation of growth in tilapia fed a diet containing 300 mg kg^{-1} QS reported in the previous experiment (Francis et al., 2001) was not in evidence to that extend during the current experiment. The differences during the current experiment were the higher average body weight

of the fish used and the fact that tilapia were fed at high intensity with the standard diet for three weeks before the S300 diet was administered. In the previous experiment mentioned above the average initial weight of the fish were about 37 % lower and the fish that were kept on a maintenance diet for a long time were fed the experimental diets right from the start of the experiment. The average MGR values were interestingly higher in group 2 fed the S300 diet during phase 2 of the experiment. In a pattern similar to observations during the previous experiment the MR values also tended to be lower in group 2 when they were fed the S300 diet.

The observations in this experiment indicate that the QS mixture had a suppressing or even preventing effect on egg production by female tilapia. However, at a level of 300 mg kg^{-1} in the diet, all the female tilapia were not affected. Also, it could not be ascertained if the suppression of egg production would have been reversible because the third phase of the experiment could not be continued beyond 3 weeks. Because of increasing size, the fish were no longer able to move freely in their aquaria and hence the experiment had to be terminated. In these three weeks the fish that were not spawning during phase 2 of the experiment did not show any indication of being ready for spawning. No satisfactory conclusions could be drawn regarding this phase because of its shortness and the possibility of fish feeling constrained by lack of space, even though it was observed that tilapia that were fed the S300 diet in phase 2 showed a much lower frequency of egg production compared to those fed the C diet in phase 2.

How dietary saponins influence egg production in female tilapia is not clear at this stage. There are no reports thus far on the effects of dietary saponins on tilapia reproduction. Previous reports indicate that dietary saponins or their breakdown product formed in the intestine of animals, the sapogenins, have not been detected in the blood stream of these animals (Gestener et al., 1968; Yoshikoshi et al., 1995). Absorption of traces of these substances into the body of the fish cannot, however, be ruled out as it is difficult to detect low levels saponin in the serum.

Reports of *in vivo* effects of saponins on reproductive function in other animals are isolated and scattered. The mass of testes of mice fed 0.5 or 1.5 % *Quillaja* extract were significantly lower, but the differences were not statistically significant when the weights were expressed relative to body weight (Philips et al., 1979). Saponins from *Gleditischia horrida, Costus speciosus* Sm, and *Phytolacca dodecandra* exhibited antifertility effect in female mice (Chou et al., 1971; Tewary et al., 1973; Stolzenberg and Parkhurst, 1976). Quin and Xu (1998) found that the butanol extract of *Mussaenda pubescens* was active for terminating pregnancy in rats. Extracts of this plant are used as a contraceptive in the Fujian province of China.

Saponins have also been shown to influence the release of reproductive hormones *in vitro*. Saponins from *Harpullia cupanoides* Roxb. and *Majidea fosteri* Radlk (Fam Sapindaceae) from

African plants even at levels as low as 10 µg/ml were found to be extremely strong stimulators of luteinising hormone (LH) release from cultured hypophysial cells (El Izzi et al., 1989). Extracted *Petersianthus macrocarpus* triterpenoid saponins stimulated LH release in a dose dependant manner from 10 µg ml^{-1} to 300 µg ml^{-1} *in vitro* on rat pituitary cells (El Izzi et al., 1992).

Administration of saponins into the blood stream of rats also had been shown to influence the production of reproductory hormones. Ethanol extracts of *Petersianthus macrocarpus* injected into female rats stimulate uterine growth, lowered LH release, and blocked the oestrous cycle (see El Izzi et al., 1992). The steroidal saponin OSW-1 isolated from *Ornithogalum saundersiae* injected into rats at 9 µg kg^{-1} on the morning of proestrous inhibited the onset of the next proestrous (a dose of 4.5 µg kg^{-1} had no effect). OSW-1 inhibited ovarian oestrogen secretion which may be the cause of the effects on oestrous cycle (Tamura et al., 1997). These results indicate that once the saponins enter the blood stream they are able to influence the production and release of hormones such as taGtH (LH analogue in tilapia), and estrogen from endocrine glands which might upset the hormonal equilibrium necessary for the normal functioning of the reproductive system. They might also have a direct effect on the ovarian membranes.

The morphological differences seen in the ovaries of the tilapia may have been caused by the blockage of their normal functioning. These may therefore be a secondary effect of hormonal imbalance. Also there is no satisfactory explanation for non-production of eggs by one fish in the control group. One possibility is an oversensitivity of this fish to QS from the feeds, or either QS or its derivatives from the faeces of the other treatment group that might have leached into the water.

The mechanism by which QS mediate suppression egg production in female tilapia need to be clearly elucidated. Once this is done adding QS to the diet has potential to become a valuable tool in tilapia culture. Studies would also be needed to investigate the possibility of saponins affecting reproduction in fish feeding diets that contain these substances.

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Chapter 6

Effects of long term feeding of *Quillaja* saponins on sex ratio, muscle and serum cholesterol and LH level in Nile tilapia (*Oreochromis niloticus* (L.))

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Revised version accepted for publication in Comparative Biochemistry and Physiology - C

Abstract

Seventeen-day-old Nile tilapia fry were fed a standard diet (C) or diets containing 50 to 700 mg kg^{-1} *Quillaja* saponin (QS) extract (groups S50, S150, S300, S500 and S700). After the first 8 weeks, 30 randomly selected tilapia from each of the treatments were placed in separate aquaria and fed the standard diet witout saponins from then on (these were designated S50/C, S150/C, S300/C, S500/C and S700/C). The fish grew from an initial average weight of about 30 mg to a final average weight of 79 g during the 6-month feeding period. The difference between the average weight of C-fed tilapia and the treatment with the highest average weight after 6 months was 53.5%. The sex ratio of tilapia in the saponin-fed groups deviated from the normal 50:50 male:female ratio, with the S700 group showing a significantly higher number of males. QS stimulated LH release from dispersed tilapia pituitary cells. This effect was abolished in the presence of serum. Serum LH values did not show any diet-dependent trend in either male or female tilapia *in vivo*. In both continuously saponin-fed and only-initially saponin-fed groups, the average serum (but not muscle) cholesterol levels in males showed an increasing trend increasing trend (r^2 values of 0.62 and 0.69) with increasing dietary saponin level. Carcass composition analysis of male tilapia showed that the saponin-fed groups starting from a dietary level of 150 mg kg^{-1} had higher dry matter, crude protein, and crude lipid, and lesser ash content compared to controls. It was concluded that dietary QS has the potential to change the sex-ratio in favour of males, and to improve growth and body composition parameters in tilapia fry. More investigations are required to determine the optimum dietary level of QS for maximum positive effects.

Keywords: tilapia, *Quillaja* saponins, sex-ratio, growth, leutinising hormone, cholesterol

1. Introduction.

Nile tilapia is an important culture fish. According to FAO figures (FAO, 2001) it is among the fastest growing aquaculture species in the world, with an average growth rate of above 13% per annum since 1984. It can tolerate and sustain reasonable growth under the unstable environmental conditions prevailing in many small and marginal farms in the tropics, where temperature, dissolved oxygen and sometimes salinity can fluctuate widely in the ambient water. Tilapia aquaculture is and will continue to be important, particularly for the lesser-

developed countries in the tropics. About 1,067,019 m.t. or 97% of farmed tilapia produced in developing countries in 1999 with Asia alone producing about 82% of this amount (FAO, 2001). A major drawback of tilapia as a culture fish is their prolific reproduction, resulting in a harvested fish size, which is unacceptable in many markets. Treatment with steroid hormones has been applied to produce all-male populations, in order to circumvent this problem. Because of environmental and consumer health concerns (over 99% of hormones administered through the diet are released into the water in less than 24 h; see review by Pandian and Sheela, 1995), this treatment is prohibited in all but a few countries. Tilapia are also otherwise valuable as an important experimental vertebrate model (Anken et al., 1993) and as a potential source of pancreatic islets for possible transplantation into diabetics (MacKenzie, 1996). Studies on the physiology of these fish are therefore of high scientific interest. In previous experiments, we observed that dietary *Quillaja* saponins (QS) stimulate growth and inhibit egg production by female Nile tilapia (Francis et al., 2001, Francis et al., unpublished). Negative effects of saponins on fertility have been reported for other species (Benie et al, 1990; El-Izzi et al., 1992). The objectives of the current experiment were to study the effects of QS on reproductive development when added to the diet, and when tested on LH secretion from the pituitary cells of Nile tilapia.

2. Materials and Methods

2. 1. *Experimental feeds*

There were six experimental diets designated C (control), S50, S150, S300, S500 and S700 prepared from the same basal diet to ensure uniform composition. The basal diet contained approximately 40% protein, 10% lipid, 10% ash and had 20 kJ g^{-1} gross energy on a dry matter basis. The ingredients were as previously reported (Francis et al., 2001). The basal diet was pelleted (to about 2 mm diam.) with no additions in the case of the control feed (C). Saponin (QS; Sigma no. S-2149, Sigma, St. Louis, MO. USA), dissolved in demineralised water, was mixed thoroughly (at 50, 150, 300, 500 and 700 mg kg^{-1} in the S50, S150, S300, S500 and S700 diets, respectively) with the powdered feed using a mixer before pelleting. The moist pellets were frozen, freeze-dried and stored in a freezer (-18°C) until use. For the *in vitro* LH production experiments from dispersed tilapia cells, a more purified form of QS (QS-P, Sigma no. S-4521) was used in addition.

2. 2. *Experimental fish and feeding regime*

About 600 14-day-old tilapia fry were obtained from the 'Institut fur Tierzucht und Haustiergenetik, Georg-August Universität, Göttingen, Germany. They were fed a commercial ('Tetra mini') flaked feed for 3 days, to allow them to recover and acclimatise to conditions in our laboratory. They were then split into seven treatment groups of 70 fish each. Two of the seven groups were fed control (C) feed and each of the five remaining tanks were provided the S50, S150, S300, S500 and S700 diets respectively. The fish were fed at the rate of 20% of body mass till they reached 0.8 g, then 12% of body mass till they weighed on average 3.5 g each, followed by 6% of body mass till the average weight was about 12 g. Afterwards, they were fed at a level of 20 g per kg metabolic body mass (kg $^{0.8}$) and was further reduced, when they reached about 25 g body mass, to 10 g per kg $^{0.8}$. After 8 weeks of feeding the experimental diets, 30 tilapia from each of the five treatment groups were placed in separate tanks and fed the C diet. These groups were designated S50/C, S150/C, S300/C, S500/C and S700/C, respectively, based on the diet of their original group. The two control groups were later each split into two separate groups to equalize tank-density.

During the experiment the fish in each treatment were kept in 75 l aquaria that were part of a main recirculating system, under a 12 h light/12 h darkness regime. The water in the aquaria with saponin-containing feed was allowed to flow through to avoid possible contamination in the recirculating system. The fish were weighed once every two weeks and the feed ration adjusted according to body mass. Oxygen saturation was constantly kept above the 75% level. The water temperature in the experimental system was maintained at 27 ± 0.1°C. All water-quality parameters were maintained at acceptable levels (pH: 7-7.9; total NH3: 0.1-0.2 mg l^{-1}; nitrite: 0.07-0.1 mg l^{-1}, and nitrate: 1-3 mg l^{-1}) throughout the experiment.

2. 3. *Measurements and analyses*

Three male and three female fish from each group were taken out after 5 months to draw blood for hormone analysis. They were weighed and blood was drawn from the haemal arch of each using disposable syringes. The blood was stored on ice until the serum was separated from the formed elements by centrifugation (10000g for 5 min). The serum was stored at –20°C until LH radioimmunoassay (RIA). The fish were then dissected, and the gonads taken out and weighed. The gonado-somatic index (GSI) was calculated by dividing the fresh gonad weight by the total wet body weight and then expressed as a percentage.

After about 6 months (Jun 2000 to Nov 2000) of feeding the experimental diets, the fish in each treatment group were first collectively weighed and the number of males and females in

each group counted. Sexing was based on the shape of the genital papilla, which is broad in females and pointed in males. The male fish also produced milt upon gentle squeezing of the abdomen. The Specific Growth Rate (SGR) was calculated using the formula {[LN(W_0)-LN(Wt)] / t}*100, where LN(W_0) and LN(Wt) are natural logarithms of initial and final weights and t is the time interval in days.

Eleven males (eight from groups that were fed saponins only initially because of the lower number of fish) and five females (only three phenotypic females each from the S700 and S700/C groups were taken because of the lower number of female fish) were again randomly selected from each group. From these, six male fish were immediately killed with a sharp blow to the head and stored at –18°C until carcass chemical composition analysis. Only male fish were taken for proximate analysis because the female fish were needed for breeding experiments. The chemical composition of the experimental diets and the fish was analyzed using srandard methods (Neumann and Basler, 1983), i.e. dry matter was measured by drying to constant weight at 105°C, crude protein as macro-Kjeldahl (N x 6.25), lipids by extraction with petroleum ether and gross energy by bomb calorimetry (IKA C 7000) with benzoic acid as a standard.

The remaining sampled fish were weighed individually and then blood was drawn as previously described. The serum was stored at –20°C until RIA and cholesterol assay. Serum cholesterol was measured using a cholesterol estimation kit (Roche Diagnostic, Mannheim, Germany). The GSI was determined after dissecting the testes from the male fish. Five grams of muscle tissue were then taken from each fish and stored at –18°C for determining muscle cholesterol content (using a cholesterol estimation kit; Boehringer Mannheim, Germany).

2. 4. *In-vitro stimulation of LH secretion*

Pituitary cells were dispersed according to Levavi-Sivan and Yaron (1992) and Levavi-Sivan et al., (1995). Briefly, pituitary cells from 80 to100 fish were collectively dispersed, counted and then plated at 100,000 cells per well of 96-well plate, in 0.2 ml medium (M199,10% fetal calf serum (FCS), 10 mM HEPES, 1% antibiotics [Pen-strep-nystatin suspension]; Biological Industries, Beit Ha'emek, Israel), and cultured for 4 days at 28^{o}C under 5% CO_2. On the fourth day, the cells were rinsed twice with medium before addition of the test substances in stimulation medium (M199, 1% antibiotics, 0.1% BSA), for 5 h. Salmon GnRH (Trp^7,Leu^8-LHRH; sGnRH) and QS were dissolved directly in the medium and were prepared fresh. The level of sGnRH used was 100 nM, the most effective dose in raising taGtH levels in dispersed

tilapia pituitary cells (Levavi-Sivan et al., 1995). When the effect of QS was tested in the presence of serum 20% FCS was added to the stimulation medium instead of the BSA.

2. 5. *Radioimmunoassay*

The gonadotropin of tilapia (taGtH), possibly equivalent to mammalian LH was determined in the medium by specific RIA according to Levavi-Sivan and Yaron (1992). The second antibody employed was donkey anti-rabbit IgG bound to magnetizable compound (Amerlex-M, Amersham, UK). The sensitivity of the assay was 0.5 ng/tube; the intra- and interassay coefficients of variation were 7.3% and 14%, respectively. The taGtH was measured by homologous RIA, according to Bogomolnaya et al., (1989), using taGtH purified from pituitaries of adult fish during the spawning season. It is presumed, therefore, that this RIA measures the maturational GtH II (=LH) as defined for salmon (Suzuki et al., 1988a,b). Levels of hormone were expressed as the ratio between the basal secretion and the secretion after stimulation.

2. 6. *Breeding experiment*

From the remaining fish, one male and three female fish from each treatment were kept in breeding aquaria and observed for 3 months. During this period, they were maintained on the same diets as previously. All other conditions, such as water-quality parameters, light-darkness regime, etc. were also kept identical.

2. 7. *Statistical treatment*

The deviation of the proportion of males and females from the expected ratio was tested by chi-square test. All other data were subjected to ANOVA and statistical comparisons between treatments were made using Duncan's multiple range test (Statistica for Windows, release 5.1 H, 1997 edition.). The significance of observed differences was tested at $p< 0.05$.

3. Results

3. 1. *Feeding and growth*

Fish in all groups ate all of the feed provided and did not show any abnormal behavior during the experimental period. In the S700/C tank, accidental blockage of the water inlet tube resulted in the death of some fish. In other tanks, there were scattered deaths of one or two

fish when they either jumped out of the aquaria or were killed by other fish. Dead fish were removed as soon as they were noticed.

Table 1. Total number (N) and average body weight of tilapia in the various treatments after 6 months

Treatment	N	Average body weight (g)
C	94	63.9
S50/C	23	73.1
S150/C	20	91.2
S300/C	24	86.1
S500/C	23	64.4
S700/C	19	75.2
S50	33	78.9
S150	25	98.1
S300	28	96.4
S500	29	98.0
S700	26	94.3

C-tilapia fed control feed; S50/C to S700/C-tilapia fed diets containing 50, 150....up to 700 ppm *Quillaja* saponins for 8 weeks (week 3 to week 11) followed by control; S50 to S700-tilapia fed diets with 50, 150...upto 700 ppm *Quillaja* saponins throughout

The average final body weight of fish in each treatment group is shown in Table I. The average body weights of the treatment groups tended to be higher than controls. Among the treatment groups, the fish that received the saponin-containing diet throughout grew better than those that were put on the control diet after 8 weeks. In both of these groups, the fish, which had once received or continued to receive the S150 diet had the highest specific growth rate (Fig. 1). Whether the difference in growth between different treatments was statistically significant could not be ascertained since all the fish in the saponin treated groups were kept in the same aquaria.

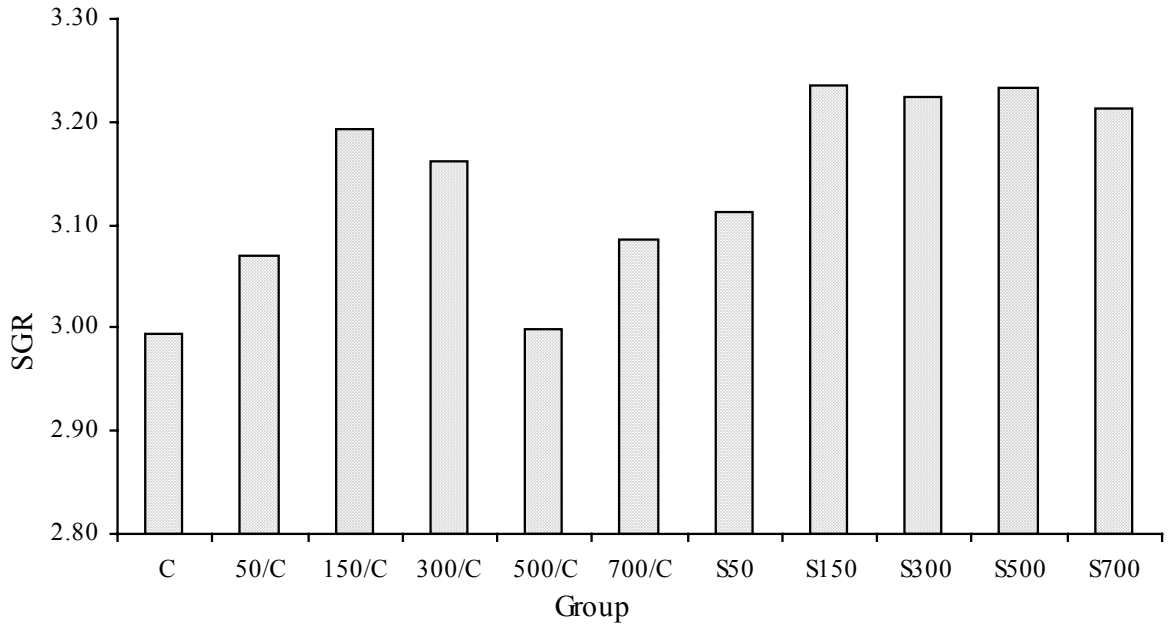

Figure 1. Average Specific Growth Rate (SGR, % per day) among treatment groups

3. 2. *Sex ratio and GSI*

The proportion of males and females in the treatment groups is given in Fig. 2. There were uneven sex ratios in the treatment groups compared to controls. The number of males was higher in some of the groups that received the saponin-containing diets. A chi-square test of goodness of fit showed that the S700 group deviated significantly from the expected 50:50 ratio. The control group had close to the typical gender ratio. The gonado-somatic index of the male fish calculated from the samplings after 5 and 6 months are shown in Table II. A notable decline in GSI values occurred during the sixth month of fish growth. There were differences in average GSI values among the treatment groups. Generally, the GSI of the male fish in groups fed with saponin diets throughout tended to be lower than in controls, whereas male tilapia in groups fed the saponin diets for only 8 weeks had higher average GSI values than controls. This trend was clearer in the fish that were sampled after 5 months.

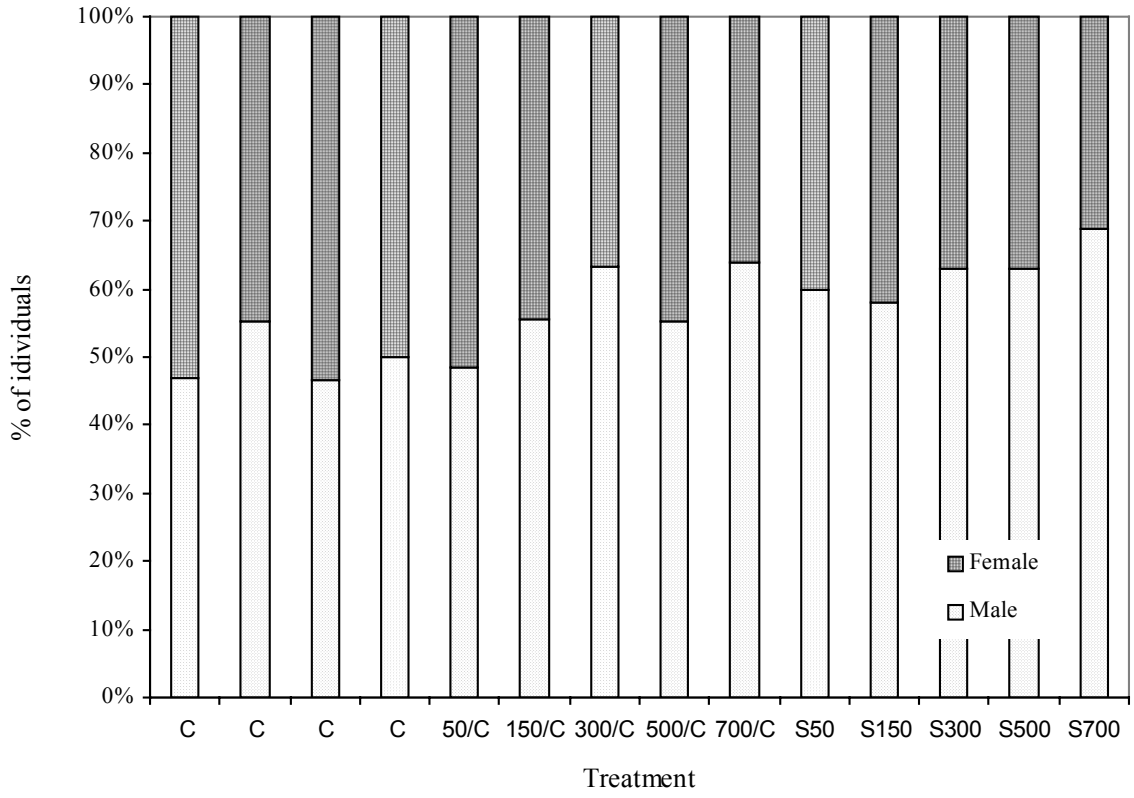

Figure 2. Proportion of males and females (phenotypic) among the treatment groups at the end of 6 months

Table 2. Gonado-Somatic Index (GSI) of tilapia in various treatments at 5 (n = 3) and 6 (n = 5) month samplings

Treatment	GSI after 5 months		GSI after 6 months	
	Average	SD	Average	SD
C	1.03^{ab}	0.42	0.24^{cd}	0.09
S50/C	1.44^{a}	0.52	0.19^{cd}	0.01
S150/C	1.35^{a}	0.02	0.16^{d}	0.02
S300/C	1.21^{a}	0.33	0.32^{abcd}	0.02
S500/C	1.49^{a}	0.45	0.44^{ab}	0.25
S700/C	1.21^{a}	0.38	0.19^{cd}	0.02
S50	1.15^{a}	0.09	0.34^{bc}	0.17
S150	0.41^{b}	0.28	0.24^{cd}	0.11
S300	0.80^{ab}	0.16	0.18^{cd}	0.06
S500	0.42^{b}	0.28	0.32^{abcd}	0.15
S700	0.91^{ab}	0.53	0.22^{cd}	0.05

Averages not sharing common superscripts are significantly different (P<0.05)
C-tilapia fed control feed; S50/C to S700/C-tilapia fed diets containing 50, 150....up to 700 ppm *Quillaja* saponins for 8 weeks (week 3 to week 11) followed by control; S50 to S700-tilapia fed diets with 50, 150...upto 700 ppm *Quillaja* saponins throughou

3. 3. *Carcass chemical composition of male fish*

There were differences in the carcass chemical compositions of the treatment groups (Table III). The data, however, could not be statistically treated because all the fish in each treatment were from the same aquarium. The groups that received the saponin diets throughout tended to have higher carcass protein and gross energy, and lower ash content. The highest carcass dry matter and carcass crude lipid contents were recorded in the S150/C and S300/C groups.

3. 4. *Serum LH level*

The measurements of LH level in the serum of 5- and 6-month-old male and female tilapia did not give uniform results. The deviation in values in the individual groups (3 or 5 fish) were very high, but there were still some differences among treatment groups (Table IV). However, no dietary saponin-dependent trend or pattern of change in LH titters could be noticed. The hormone levels were higher in males than in females at the age of 5 months. The titter in males increased two- to three fold from the level at 5 months to that at 6 months. In the 6-month old female fish, the LH titter increased several-fold during this interval, reaching levels approximately double those of the 6-month-old males.

Table 3. Average carcass chemical composition parameters of male fish (n = 6) in treatment groups

Treatment	Dry matter (%)	Crude Protein (N x 6.4) (%)	Crude ash (%)	Crude lipid (%)	Gross energy (kJ/g)
C	26.7	54.6	18.9	22.1	21.4
S50/C	25.8	56.5	17.9	21.4	21.6
S150/C	29.3	53.1	16.5	24.7	22.5
S300/C	27.0	54.9	16.8	24.0	22.9
S500/C	26.0	54.8	18.1	20.9	22.1
S700/C	27.5	54.5	17.8	23.1	21.9
S50	26.5	55.8	18.3	21.3	21.7
S150	27.4	55.2	16.6	23.4	22.3
S300	25.7	57.1	17.2	21.1	22.4
S500	27.0	55.5	16.6	23.2	22.9
S700	27.0	54.4	16.9	23.8	22.9

C-tilapia fed control feed; S50/C to S700/C-tilapia fed diets containing 50, 150....up to 700 ppm *Quillaja* saponins for 8 weeks (week 3 to week 11) followed by control; S50 to S700-tilapia fed diets with 50, 150...upto 700 ppm *Quillaja* saponins throughout

3. 5. *In vitro stimulation of LH secretion by QS saponin*

The effect of QS on taGtH release was examined using tilapia pituitary cells in primary culture. Exposuring the cells, for 5 h, to QS resulted in a moderate increase in LH release, similar to that evoked by sGnRHa. However, the more purified QS (QS-P; 10, 100 or 200 μg/ml) evoked a dose-dependent increase in LH output, which was significantly higher than that evoked by sGnRHa (10 nM; Fig. 3A). To determine whether QS can alter the cell response to GnRH, both substances were added concomitantly. The selected saponin concentration was 10 μg/ml to allow for an additive effect. When QS and sGnRHa were added simultaneously, LH release was not significantly different from basal, while when QS-P and sGnRHa were added simultaneously, gonadotropin release was equal to the sum of the releases promoted by each component alone (Fig. 3B). We also tested the effect of saponins on cultured cells in the presence of serum. Exposure of the cells to 10 μg/ml saponin, in the presence of 15% FCS, abolished the stimulatory effect (Fig. 3C).

3. 6. *Serum and muscle cholesterol*

There were no significant differences between serum cholesterol levels of the treatment groups. The cholesterol content in the serum of female fish was on average approximately 50% higher than that of males. Serum cholesterol levels showed some interesting trends in the

Table 4. Average serum LH levels (ng/ml) of tilapia in treatment groups after 5 (n = 3 for males and females) and 6 (n = 5 for males and females) months

Treatment	Males				Females			
	After 5 months		After 6 months		After 5 months		After 6 months	
	Average	SEM	Average	SEM	Average	SEM	Average	SEM
C	18.3^{abc}	5.4	49.8^{abc}	13.9	3.4^{bcd}	1.3	142.6	55.6
S50/C	29.6^{abc}	0.4	60.7^{abc}	54.2	16.1^{abc}	10.4	107.4	39.4
S150/C	9.4^{bc}	5.2	36.7^{abc}	21.3	3.1^{bcd}	0.3	148.1	71.0
S300/C	36.4^{abc}	8.6	35.3^{abc}	11.7	8.2^{abcd}	2.4	70.7	19.1
S500/C	51.7^{ab}	14.6	36.0^{abc}	11.2	3.8^{bcd}	1.4	162.8	55.4
S700/C	34.9^{abc}	5.8	30.3^{abc}	11.9	1.3^{cd}	0.3	71.0	19.1
S50	31.8^{abc}	3.7	83.6^{abc}	36.8	4.6^{bcd}	1.8	201.1	63.3
S150	15.3^{abc}	12.8	85.0^{abc}	25.2	20.6^{ab}	9.1	148.4	81.0
S300	34.9^{abc}	24.8	36.1^{abc}	24.0	6.0^{bcd}	4.2	201.6	111.2
S500	20.1^{abc}	7.6	127.1^{ab}	88.0	5.3^{bcd}	2.3	111.8	63.7
S700	32.2^{abc}	22.7	15.1^{bc}	5.8	3.5^{bcd}	0.1	54.9	33.3

Averages not sharing common superscripts are significantly different ($P<0.05$)
C-tilapia fed control feed; S50/C to S700/C-tilapia fed diets containing 50, 150....up to 700 ppm *Quillaja* saponins for 8 weeks (week 3 to week 11) followed by control; S50 to S700-tilapia fed diets with 50, 150...upto 700 ppm *Quillaja* saponins throughout

male tilapia. In both continuously saponin-fed and only-initially saponin-fed groups, the average serum cholesterol levels in males showed a steadily increasing trend, from C to S700/C or C to S700 (Fig. 4). The coefficient of determination values of regression lines fitted to the average values were 0.62 and 0.69, respectively. No such trend existed in the average cholesterol values among the feeding classes of the female fish.

The muscle cholesterol values did not differ significantly among the treatment groups. The average muscle cholesterol values in the females were also higher than those of males, even though the difference was not as big as in the case of serum cholesterol. There were no apparent dietary treatment-dependent trends among muscle cholesterol levels.

3. 7. *Breeding trials*

The dominant males in the S150/C, S500/C and S700/C groups were extremely aggressive and killed all other fish within 1 day in the breeding tanks, and hence no further observations could be made in these groups. One female each in the S300 and S500 groups spawned once immediately afterbeing placed for breeding, but the eggs did not hatch and were spit out by

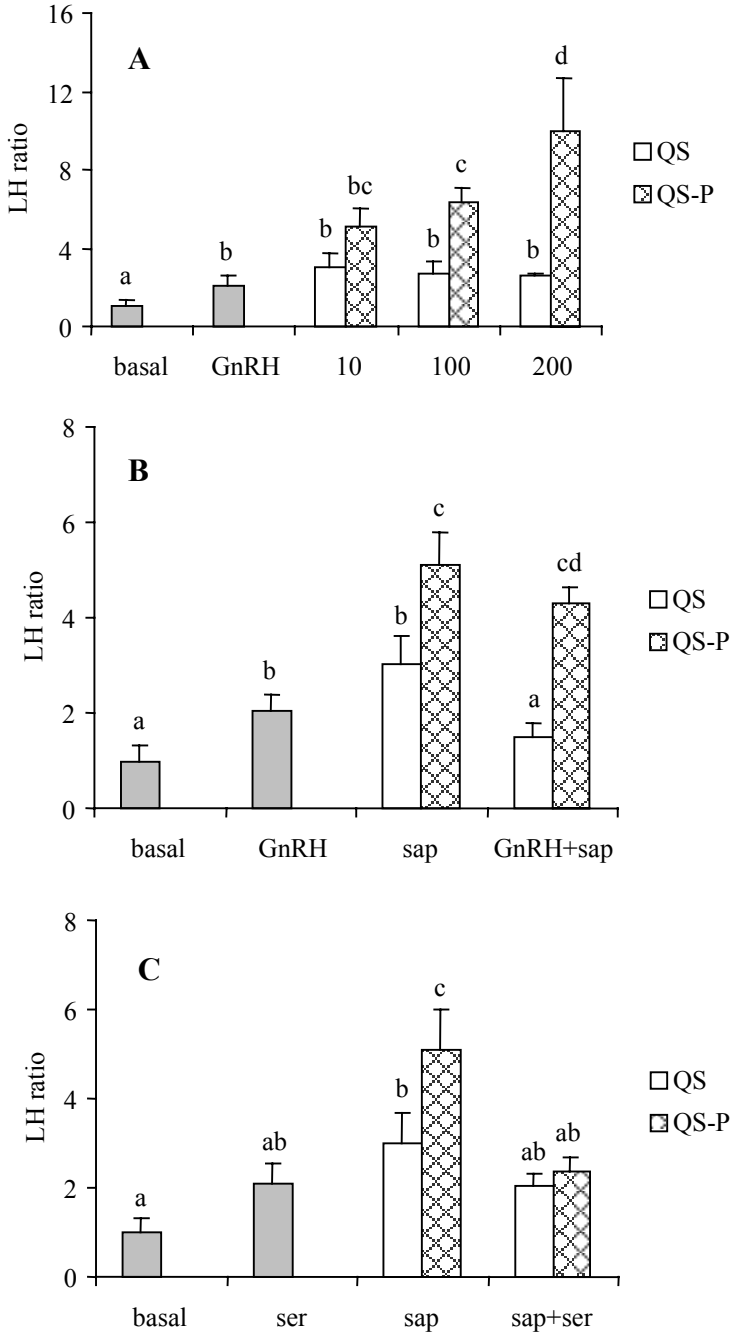

Figure 3. The in vitro effect of *Quillaja* saponin (QS) or purified QS (QS-P) on LH release from dispersed tilapia pituitary cells. A. Cells were treated for 5 h with either 100 nM salmon GnRH (GnRH) or with various concentrations of the saponin (10, 100 or 200 µg/ml). **B**. Release of LH by cultured tilapia pituitary cells stimulated by 100 nM salmon GnRH (GnRH), 10 µg/ml QS, or a mixture of both compounds. **C**. Dispersed tilapia pituitary cells were cultured in the absence (basal) or presence of 20% fetal calf serum (ser), or with 10 µg/ml QS (sap) or QS in the presence of serum (sap + ser). Each value is the mean ± SEM of three different wells from three to four independent experiments.

the females after 2 days; there was no further spawning for the following 3 months. Three female tilapia in the control group and one in the S300/C spawned after ~ 4 weeks and

produced fry. The mouth-brooding or killed tilapia were removed from the breeding aquaria and were replaced in the case of treatments in which female fish were available.

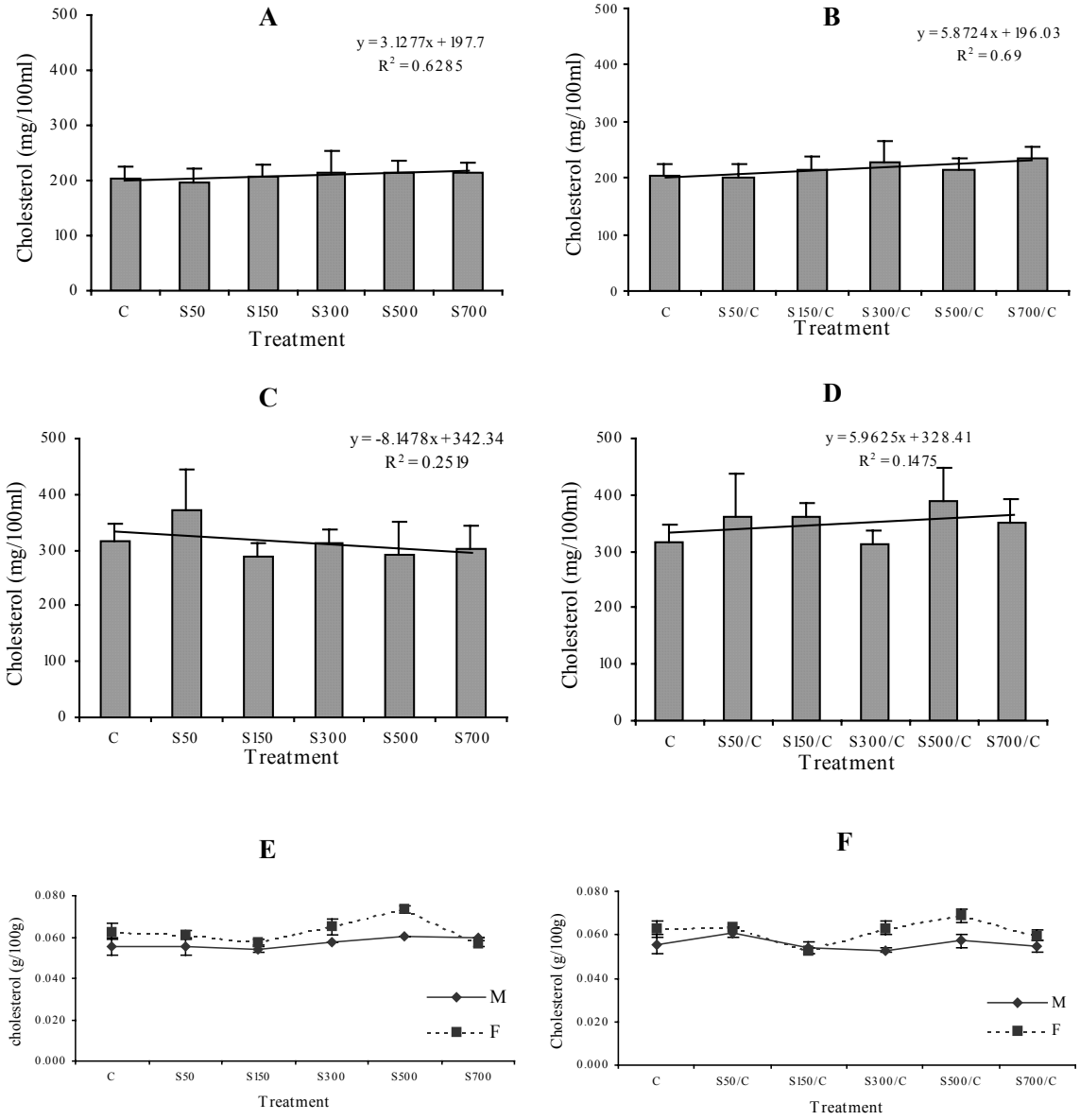

Figure 4. Average cholesterol levels in the serum (mg/100ml) and muscle (g/100g) of male and female fish in the treatment groups. A and B, serum cholesterol levels of males; C and D serum cholesterol level of females; E and F, muscle cholesterol levels of males and females respectively in different treatments.

4. Discussion.

Dietary QS at levels higher than those used here (> 1500 mg kg^{-1}) have previously been reported to depress feed intake in Chinook salmon (Bureau et al., 1998). However, up to a dietary level of 700 mg kg^{-1}, QS did not cause suppression of feeding or any other apparent abnormal behavior, in the current experiment where the body weight of tilapia increased from 30 mg to ~100 g. The feeding rate of androgen-fortified feed, particularly during the initial period, has been reported to be crucial for inducing sex inversion in tilapia (Shelton et al., 1981). The initial feeding rate of 20% of body weight was higher than the 12-15 % recommended by Shelton et al. (1981). The fish in the saponin-fed groups had higher absolute specific growth rate (SGR) than controls. This was in agreement with our previous observation of a growth promoting effect of dietary QS in tilapia (Francis et al., 2001). Oral application of other androgenic substances has also been previously found to have a positive anabolic effect in tilapia (Tayamen and Shelton, 1978; Macintosh et al., 1988; also see review by Pandian and Sheela, 1995), particularly at steroid doses that were pre-optimal for sex inversion. However, growth depression occurred invariably over the long term (see review by Pandian and Sheela, 1995). The average SGR values of tilapia that received a saponin-supplemented diet throughout were higher than those receiving saponins only for the first 8 weeks. In both groups, maximum growth was achieved at the 150 mg kg^{-1} level of supplementation. It could not be ascertained whether the difference in growth between different treatments was statistically significant. In our previous experiment (Francis et al., 2001) tilapia fed 150 mg kg^{-1} saponins had significantly higher growth rates 3 weeks after feeding started. Tilapia fed 300 mg kg^{-1} QS showed a significantly higher body weight gain after 14 weeks of feeding in that experiment. Results of the current experiment further evidence the growth-promoting effects of dietary QS.

The sex ratio of tilapia in the saponin-fed groups deviated from the normal 50:50 ratio, with the S700 group showing a significant deviation from this pattern in favour of males. Deviation from the normal sex ratio was also evident in the treatment groups that were fed saponins only for the first 8 weeks, suggesting that sex-inversion occurrs during this initial period. The first 21 days have been judged the most effective period for sex reversal in tilapia fry (Clemens and Inslee, 1968). The percentage of males in any of the treatments was not as high in the current experiment as in experiments that made use of synthetic androgens (Cruz and Mair, 1994; Gale et al., 1999). Even though the concentration of the saponin mixture added to the diet was much higher than the 60 mg kg^{-1} of synthetic androgens used in most

studies, the concentration of saponins actually present was lower because the mixture used is known to have only about 20% of these compounds (approximate content of 10% sapogenin), the remaining 80% being made up of tannins and polyphenolics (Kensil, 1996). Among the saponins themselves, there may be differences in effectiveness of the different compounds present. The highest proportion of males was in the S700 and S700/C groups. However, the growth-promoting effect of dietary QS was pronounced at the much lower supplementation level of 150 mg kg^{-1} indicating potentially different mechanisms effecting the two actions of dietary QS.

All the dissected male fish had apparently normal testes, and almost all had running sperm. The GSI was higher in 5-month old male tilapia fed saponins only for the first 8 weeks. The lowest values were recorded in the fish fed saponins throughout. Previous studies with synthetic androgen-fed tilapia also reported lower GSI values in hormone-treated fish (Cruz and Mair, 1994). It has been postulated that the GSI values in male tilapia are correlated with the levels of testosterone, 11 keto-testosterone and estradiol in the plasma (Melamed et al., 2000). We cannot satisfactorily explain the higher GSI value of the fish that received saponins only for the first 8 weeks, compared to the ones that received saponins throughout. There was a notable decline in GSI values from 5 to 6 months of age. The decline was more pronounced in the groups fed saponins only at the beginning. There were still differences in GSI values between treatments, but there was no pronounced diet-dependent trend. The highest absolute GSI value at the end of 6 months was recorded in the S500/C group.

Saponins have been previously reported to affect the release of hormones, such as LH, from the pituitary (Benie et al., 1990). LH is considered to regulate all aspects of teleost reproduction (Suzuki et al., 1998a) and is particularly important for final oocyte maturation and ovulation (Suzuki et al., 1998b). We previously observed the inhibitory effect of dietary QS on ovulation in female tilapia having apparently normal eggs in their ovaries (Francis et al., 2001 and unpublished observations). It was therefore hypothesised that changes in LH levels would give an indication of the mechanism of action of QS on tilapia at the pituitary level.

We were able to demonstrate that QS promotes LH release from cultured pituitary cells of tilapia. The more purified saponin was more efficient at increasing the rate of hormone release indicating that saponins are the responsible active principles. Saponins from *Petersianthus macrocarpus* have been shown to stimulate both FSH and LH release by cultured pituitary cells of rat. Moreover, the saponin promoted an increase in uterine weight and blocked the estrous cycle in the luteal phase (Benie et al., 1990). Although there is some evidence that

saponin can act through permeabilization of cell membranes, this seems at least not to be the only mode of action in tilapia pituitary cells, since in the range of concentrations used, we found a dose-dependant response of LH to QS-P (Fig. 3). We also studied its effect on cultured cells in the presence of serum. Cells incubated in the usual BSA-containing medium released LH in a dose-dependent manner. In contrast, when cells were incubated with the same amount of saponin in the presence of 15% FCS, the LH release was significantly lower, and similar to the basal level. Since it is commonly accepted that saponins have a high affinity for cholesterol and other serum lipids, the serum protection may be due to saponin's absorption by serum lipids, prevailing its activity. This effect of serum protection on gonadotropin release from rat pituitary cells has also been seen with another saponin from *P. macrocarpus* (El Izzi et al., 1992).

The average LH values *in vivo*, however, did not show any trends with respect to the effect of dietary QS because of the extremely high variability within treatments. What was notable in these measurements were the different values for the 5- and 6-month old tilapia. The increase in serum LH in females was particularly pronounced. Since dietary QS was shown to affect the sex ratio, it can be concluded that this change is not exerted via LH levels but rather via interference in either the FSH at the pituitary level or the steroids (probably androgens) at the gonad level. The protective effect of the serum on the gonadotropin release *in vitro* may not be as strong *in vivo* due to the low lipid concentration in tilapia serum compared to 20% FCS.

It has become increasingly clear that the lipid environment of membrane proteins, including ion channels, transporters, and receptors, plays an important role in their function (Bastiaanse et al., 1997). In biological membranes, cholesterol is organized into structural and kinetic domains or pools. Saponins may interact with the polar heads of membrane phospholipids and the OH of cholesterol through their OH groups. Moreover, their hydrophobic steroid backbone could intercalate into the hydrophobic interior of the bilayer. Both of these effects may contribute to altering the lipid environment around membrane proteins and activate specific receptors, channels or enzymes (Attele et al., 1999). The saponin-induced dose-dependent LH release from tilapia pituitary cells observed provide evidence for this mode of action. Moreover, the combination of natural GnRH and saponin, both at sub-maximal doses, did not elicit a higher response than each substance alone, suggesting that its mechanism of action may involve indirect activation of the receptor. Based on their structure, another suggested mode of action for saponins is through binding to the estradiol receptor in mammals (Martin et al., 1978) and fish (Latonnelle et al., 2000). After binding to the receptor, the saponin can elicit either agonistic or antagonistic effects *in vivo* and *in vitro* (Attele et al.,

1999). The androgenic potency of QS in tilapia can b explained by antagonistic effects on the estradiol receptor.

The increasing trend in serum cholesterol content in male tilapia belonging to both saponin-fed groups is in contrast to previous findings in which a saponin-induced decline in serum cholesterol was observed in gerbils and humans (Potter et al., 1993; Harris et al., 1997). There was also no decline in the serum cholesterol content of female tilapia in the saponin-fed groups relative to controls, but no dietary saponin-dependent trend was in evidence. Moreover, muscle cholesterol levels were not lower in the saponin groups compared to controls. The differences in serum and muscle cholesterol contents between males and females were also noteworthy. Cholesterol is a key compound in the biosynthesis of steroid hormones.

During the breeding trials, three female tilapia belonging to the control group and one female fish belonging to the S300/C group produced fry over a 3-month period. In the S500 and S700 groups, one female each produced eggs and were observed to mouth -brood immediately after they were placed together for breeding in the breeding aquaria. But they spit out the eggs after 2 days (similar to females having unfertilized eggs). The dominant males (identifiable by their external reddish coloration) in the S150/C, S500/C and S700/C were especially aggressive and killed all other fish placed in the same aquaria. Since the GSI of these males tended to be higher, particularly at the after 5- month sampling, their aggressive behaviour could have been due to higher androgen levels. Similar results have been obtained in trout fed dietary genistein (a phytoestrogen structurally similar to saponin): testicular development was accelerated in males, spawning was delayed and gamete quality was impaired, leading to a low percentage of ovulating females, a lower fertilization rate and a lower viability of fry in females fed a diet with 500 ppm genistein (Bennetau-Pelissero et al., 2001). The results from the breeding experiments need to be treated as preliminary because of the low number of females available in some of the saponin-fed groups. Detailed trials, including artificial fertilization methods, need to be carried out to further study the effects of dietary QS on the reproduction of Nile tilapia.

The chemical composition of the male fish in the different treatment groups also did not evidence a negative influence of saponins up to a level of 700 mg kg^{-1}. On the other hand, there were indications of higher carcass nutrient parameters in the saponin-treated groups, particularly at dietary levels higher than 150 mg kg^{-1} in both only-initially saponin-fed and continuously saponin-fed tilapia. These results are also in agreement with our previous observations in tilapia fed dietary QS (Francis et al., 2001). Whether these effects are caused

by a saponin-influenced increase in nutrient absorption in the intestine or by systemic effects of saponins remains to be clarified. Because of the low number of female tilapia in the treatment groups at the end of the experiment, their carcass chemical composition could not be determined.

In conclusion, dietary QS could potentially replace hazardous synthetic androgens in producing all-male populations or reducing female's fertility if fed to tilapia at an early age, as is practiced with synthetic androgen treatment. *Quillaja* extracts containing saponins are used commercially as flavorings and foam producers in a variety of human food preparations (Kensil, 1996) and have been classified as 'generally recognised as safe' (GRAS) in the US (Fenwick et al., 1992). The optimal concentration of QS for the production of all-male/infertile female populations needs to be determined through further feeding trials. This optimal dosage could then be fed to tilapia during the gonadal differentiation stage (up to 30 dpf for *O. niloticus*, Hines et al., 1999) of their life cycle.

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Chapter 7

Effects of butanol extract from *Yucca schidigera* powder on growth and metabolism in common carp (*Cyprinus carpio* L.)

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Abstract

The effects of inclusion of *Yucca schidigera* butanol extract, containing steroidal saponins, at 50 and 100 mg kg^{-1} in common carp feed were studied during a 10 week feeding experiment. The experiment was conducted in a respirometer system which allowed feeding and continuous measurement of oxygen consumption of individual fish. The resultant data along with carcass composition values were used to set up complete energy budgets for each individual fish. At the end of the experiment there were no significant differences between the experimental groups with regard to growth rates, food conversion efficiency or feed energy utilisation parameters. However, during the initial 7 weeks, carp fed 100 mg kg^{-1} dietary *Yucca* saponin (YS100 group) tended to have higher growth and feed conversion ratio compared to control. The metabolic rate of the YS100 group during this initial 7 week period (measured as average oxygen consumption per kg metabolic body mass per hour) tended to be lower even with the higher growth. Carp fed 50 mg kg^{-1} *Yucca* saponins had the lowest growth rates and highest average oxygen consumption during the whole experimental period. *Yucca* saponins, at levels used in this experiment, had no positive or negative effects on the growth or feed energy utilisation in common carp, but it may be worthwhile to try out other levels.

Keywords: *Yucca schidigera*, butanol extract, *Cyprinus carpio*, growth, metabolic efficiency

1. Introduction

Quillaja saponins included at very low dietary levels were found to increase metabolic efficiency and stimulate growth in common carp and tilapia in previous experiments (Francis et al. 2001a, b and c). The predominant variety of saponins present in the *Quillaja* extract are triterpenoid saponins. *Yucca schidigera* powder, on the other hand contains steroid saponins and is a major commercial source of these saponins (Cheeke, 1999). The saponin-rich extracts of *Y. schidigera* have been of extensive interest in livestock and poultry production, mainly because of their ammonia binding properties (Cheeke, 1996). Recently *Yucca* saponins have also been found to be an anti-food-deteriorating agent because of their toxicity against certain yeasts (Miyakoshi et al., 2000). Dietary *Y. schidigera* extract has been found to improve growth, feed efficiency and health in ruminants, poultry and pigs (Mader and Brown, 1987; Johnston et al., 1981, 1982; Anthony et al., 1994; Wang et al., 2000). The mechanism by which the positive effects of *Yucca* saponins in livestock are mediated is not clearly understood. Proposed modes of action include stimulation of intestinal microfloral growth

(Peestock, 1979), direct binding of ammonia (Headon et al., 1991) and inhibition of selected gut microbes (Hussain and Cheeke, 1995; Wang et al., 2000) particularly rumen protozoa (Wallace et al., 1994).

The current experiment was aimed at testing whether the growth promoting effects of low dietary levels of triterpenoid *Quillaja* saponins in common carp are repeated when steroidal *Yucca schidigera* saponins are used.

2. Materials and methods

Extraction of saponins

An aqueous suspension of 4 g of finely ground *Y. schidigera* powder (DK sarsaponin 30^{TM}, Desert King International, Chula Vista, CA 91911, USA) in 120 ml double distilled water was kept for 2 hours with gentle stirring, centrifuged at approximately 3000 g for 15 min and the supernatant collected. An equal volume of n-butanol was added to this supernatant and gently stirred for 30 min and allowed to stand overnight. The butanol fraction was collected. The interphase between the water and butanol layers was centrifuged for 5 min at approximately 3800 g to recover butanol completely. The procedure was repeated with fresh butanol (same volume as previously) and the butanol fraction recovered again. The butanol fractions were pooled and the butanol removed by flushing with nitrogen along with stirring at 50°C. The solid butanol extract was then mixed with a small amount of double distilled water, frozen, and freeze dried to obtain solid powder. The aqueous portion remaining after butanol extraction was also processed in the same way and obtained in the powder form. Both these were then stored at –18°C until use.

Identification with TLC

The presence and haemolytic potential of the saponins present in the butanol extract, and the aqueous portion remaining after butanol extraction were then detected using Thin Layer Chromatography (TLC). TLC was carried out on silica gel 60F 254 (Merck) with $CHCl_3$:MeOH:H_2O (18:11:2.7) as solvent. Detection of saponins was with anisaldehyde spray reagent (anisaldehyde: HOAc:MeOH: con. H_2SO_4 at 0.5:10:85:5, the acid being added drop by drop after keeping the mixture of the other three on ice). The saponins appear as coloured spots. The haemolytic activity of the spots was detected using blood spray. Cattle blood diluted 1:15 with PBS (PBS: 25ml KH_2PO_4 prepared by mixing 27.281g in 1 litre double distilled water is mixed with 39.34 ml 0.1N NaOH and made up to 100ml with double distilled H_2O) was used for the spray.

Experimental fish

Carp (*Cyprinus carpio* L.) of about 1.0 g weight were obtained from the Federal Fishery Research Agency, Institute for Coastal and Inland Fishery, Ahrensberg, Germany. They were brought to Hohenheim and grown in 200 l aquaria maintained at 23 ± 1°C and were fed a diet containing approximately 420 g kg^{-1} protein, 100 g kg^{-1} lipid, 100 g kg^{-1} ash and an energy content of 20 kJ g^{-1} on a dry matter basis, until they reached about 20 g. They were then fed the same diet at a level that ensured maintenance of weight (3.2 g $kg^{-0.8}$ day^{-1}; Meyer-Burgdorff et al., 1989). Five days prior to the start of the experiment, 25 carp of comparable body mass (~17.5 g) were selected from a large population, weighed and 15 of them were placed in 15 individual respiration chambers of a device previously described by Focken et al. (1994). The remaining ten fish were killed by a sharp blow to the forehead and preserved for determination of the initial carcass chemical composition. During acclimatisation in the chambers (5 days), the fish continued to receive maintenance levels of feed. The fish were starved for one day prior to the start of the experiment. At the start of the experiment the three experimental diets namely C, Y50 and Y100, were assigned each to 5 fish in a random manner.

Feeds

Table I. Ingredients and chemical composition of the basal diet

Ingredients	g kg^{-1}
Fish Meal[a]	500
Whole wheat meal	420
Sunflower oil	40
Vitamin mixture[b]	20
Mineral mixture[c]	20

Chemical compostion	g kg^{-1} in DM
Crude protein	409
Lipid	104
Ash	104
Gross energy (MJ kg^{-1} DM^{-1})	20.5

[a] Norwegian fish meal obtained from Württembergische Zentralgenossenschaft, Germany.
[b] and [c] prepared after Meyer-Burgdorff et al. (1989)

Three experimental diets, one control named C, and two with *Yucca schidigera* butanol extract (YBE) named Y50 and Y100 were prepared from the same basal diet available in the pelleted form, to ensure uniformity of composition. The ingredients and chemical composition of the basal diet is shown in Table 1. This was ground and then pelletted (about 2 mm diameter) without any additions forming the feed for the control group (C). The YBE was first dissolved in demineralised water and then mixed thoroughly with the powdered basal feed before pelletting the remaining two feeds: in one at a level of 50 mg kg^{-1} (Y50) and in the second at 100 mg kg^{-1} (Y100). The moist pellets were frozen, freeze-dried and stored at -18°C until use.

The experimental set-up

During the experiment, the fish were kept in respiration chambers (5 litres capacity) of the fully automated, computer controlled fish respirometer system (Focken et al., 1994) at the University of Hohenheim, Department of Animal Nutrition and Aquaculture. Thirty two measurements of oxygen consumption per individual fish were made every 24 hours and recorded on the hard disk of the computer which controls the system. The system was lit with fluorescent tubes to give a day length of 12 hours. The water flow rate through the respirometer chambers, which was controlled by electronic flow meters connected to each chamber, was adjusted (0.4 to 0.65 L min^{-1}) to keep the oxygen saturation above 75 % in the chambers. Every day about a tenth of the water in the system was replaced with fresh water. The water temperature was maintained at 27 ± 0.1°C. Once a week, when the fish were weighed, the chambers were cleaned and the oxygen probe calibrated. While cleaning the chamber the fish were kept in plastic buckets containing water taken out from the system. The entire process of weighing and cleaning the system took approximately 3 hours. During the experimental period all water quality parameters were maintained at acceptable levels (pH – 7-7.9; total NH_3 – 0.1-0.2 mg l^{-1}; nitrite – 0.07-0.1 mg l^{-1}, and nitrate – 1-3 mg l^{-1}).

Feeding regime

In the respirometer, the fish were fed individually at a level of 16 g $kg^{-0.8}$ day^{-1} (this level was found to produce maximum growth and feed efficiency in previous experiments in our laboratory) in 6 equal instalments using automatic feeders attached to the respiration boxes. The fish were weighed once a week and the feed ration for each fish was adjusted according to its body mass. Feed was not given on the day when weight was taken.

At the end of ten weeks the experiment was terminated and the fish were weighed, killed as described previously and immediately deep frozen. Prior to analysis, the carcasses were individually autoclaved for 30 min at 120°C, homogenised, refrozen and freeze dried.

Chemical analyses

The chemical composition of the experimental diets and the freeze-dried fish was analysed according to the official methods (Neumann and Basler, 1983) i.e. dry matter by drying to a constant weight at 105°C, crude protein by macro-Kjeldahl (N x 6.25), lipids by extraction with petroleum ether, and gross energy by bomb calorimetry (IKA C 7000).

Calculations and statistical analysis

All calculations were performed for each fish individually. Feed Conversion (FCR) was calculated as feed consumption (dry matter)/ live weight gain, and Metabolic Growth Rate (MGR) as live weight gain (g)/ average metabolic live weight ($kg^{0.8}$)/ day. The average oxygen consumption was represented as mg oxygen consumed/ $kg^{0.8}$/ hour. The Standard Metabolic Rate (SMR) was taken as the lowest metabolic rate sustained for 3 hours by the undisturbed animal that had not been fed for the preceding 24 hours. The relevant oxygen consumption values for calculating SMR were obtained during the 24 hour fasting period after a 5 day acclimatisation period in the respirometer chambers on the maintenance ration prior to feeding the experimental diets. Oxygen uptake (g) x 14.85 gave the energy expenditure in kJ during the whole experiment (Huisman, 1976), and the energy apparently not metabolised (AUE) was calculated by subtracting energy expenditure and energy retention (ER; gross energy gain of the carcass) from the gross energy of the feed consumed. Productive Protein Value (PPV) and Apparent Lipid Conversion (ALC) were calculated as protein gain x 100/ feed protein and lipid gain x 100/ feed lipid.

The data were subjected to ANOVA and statistical comparisons between the feeding groups were made using the Duncan's Multiple Range test (Statistica for Windows, release 5.1 H, '97 edition). The significance of observed differences was tested at $P < 0.05$.

3. Results

Extraction of saponins from Yucca schidigera powder

The average weight of freeze dried butanol extract per g raw *Yucca* powder was 0.09 (± 0.00) g and that of the aqueous portion remaining after butanol extraction was 0.27 (± 0.01) g. TLC showed that the butanol extract had 11 spots with Rf values of 0.21, 0.24, 0.30, 0.35, 0.37, 0.41, 0.44, 0.45, 0.47, 0.51, and 0.58. The fastest moving six spots on TLC (Rf values between 0.407 to 0.581) were haemolytic, with the one having Rf value of 0.581 showing the strongest haemolytic activity. On the other hand, the aqueous extract remaining after butanol extraction did not develop any spot typical of saponins on the TLC plate and did not show any haemolytic activity. Only butanol extracts were used in the feeding experiment.

Feed intake and behaviour of carp

All fish consumed the feed provided and no mortality was observed during the experimental period. Since the fish ate the diet as it dropped into the respirometer box there was little

chance for soluble saponins to leach into water. There was no abnormal behaviour of fish in any of the treatment groups during the whole experimental period.

Growth rates

The growth rates of the different groups did not differ significantly at the end of the experiment. The MGR of the Y100 group was higher than that of the C group until the seventh week of the experiment, the difference between the two widening till this time. Thereafter the MGR of the C group started to increase compared to the Y100 and continued to do so till the end of the experiment. The Y50 group had the lowest growth rate throughout the experiment, except for the tenth week when average MGR in the Y100 group dropped even lower (Fig 1).

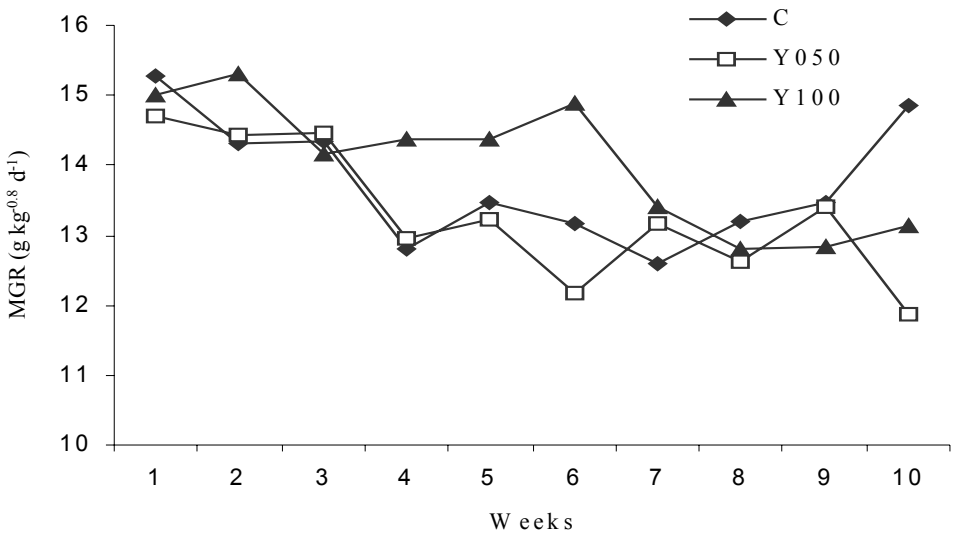

Fig. 1. Average weekly Metabolic Growth Rate (MGR) of carp fed the experimental diets

Carcass composition

The chemical composition of the experimental fish in the three groups at the end of the experiment was statistically similar (Table 2). The average carcass lipid content in the Y100 group was about 16 % higher than that of the C group. Average ash content was also lowest in the Y100 group.

Table 2. Initial and final body mass and chemical composition of experimental fish

	Initial		C[1]		Y50[2]		Y100[2]	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Number of fish	10		5		5		5	
Initial live mass, g	17.5	2.1	17.4	2.1	17.4	2.0	17.4	2.0
Final live mass, g			108.8	20.1	102.8	11.3	111.6	15.6
Dry matter (DM), %	21.1	1.1	21.4	0.2	22.0	1.3	22.2	0.9
Crude protein(N x 6.25), %	64.2	2.6	68.4	0.7	67.9	2.7	67.3	1.1
Crude lipid, %	13.9	3.2	14.1	1.5	14.7	3.1	16.5	1.0
Ash, %	13.1	0.7	11.3	0.8	11.6	0.6	10.8	0.3
Gross energy, kJ g^{-1}	19.8	0.6	21.7^{ab}	0.6	21.3^{b}	0.6	22.1^{a}	0.1

[1]C = fish fed control feed
[2]Y50 = fish fed control feed containing 50mg/ kg butanol extract of *Yucca* powder
[3]Y100 = fish fed control feed containing 100mg/ kg butanol extract of *Yucca* powder

Oxygen consumption

The average oxygen consumption per unit body mass gained (OPMG) and MR values of the treatment groups were statistically similar at the end of the experiment. The OPMG was highest (13.7 % higher than control) in the Y50 group. The OPMG values of the C and the Y100 group were similar at the end of the experiment. The weekly average metabolic rate (MR, calculated as oxygen consumption in mg $kg^{-0.8}$ h^{-1}; average of 168 hours) values for the whole experimental period are given in Table 4. The average metabolic rate values during the first 5 weeks of the experiment were lower in the Y100 group compared to control (Fig 2).

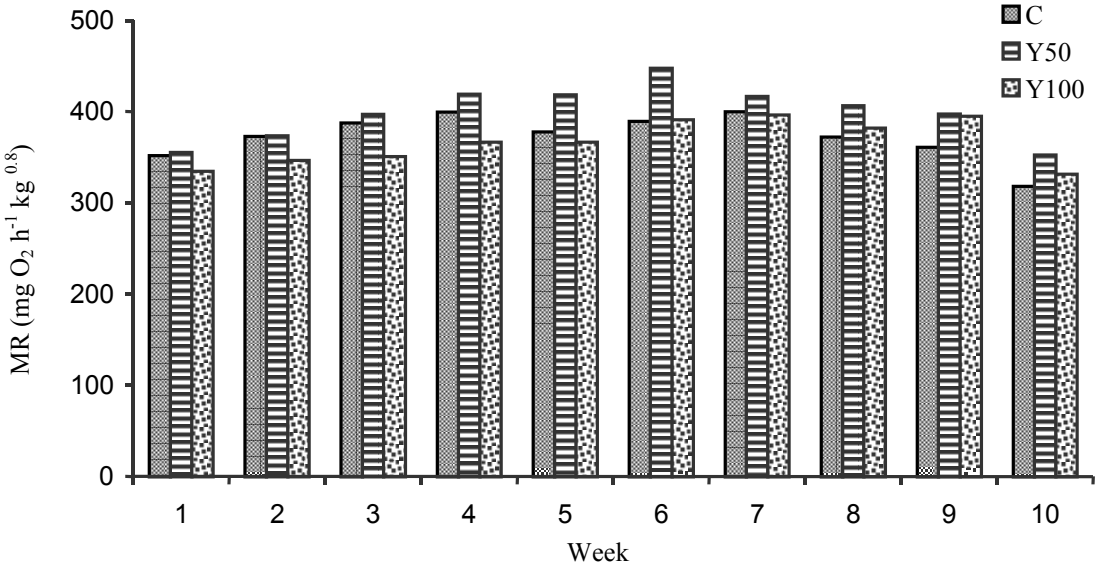

Figure 2. Weekly average Metabolic Rate (MR) of carp fed the experimental diets

Feed conversion and assimilation

There were no significant differences in feed conversion ratio and protein and lipid assimilation values between the treatment groups at the end of the experiment (Table 3). Through the course of the experiment, the FCR followed a pattern similar to that of MGR, i.e. initially lower in the Y100 group but later levelling off, and towards the end of the experiment the C group becoming better than the Y100 group. The Y50 group consistently had the lowest value for FCR. The PPV and PER values were similar in the Y100 and C groups and lower in the Y50 group. The average ALC value was higher in the Y100 group as compared to the others.

Table 3. Feed consumption and feed utilisation parameters of experimental fish

Experimental group	C[1]		Y50[2]		Y100[3]	
	Mean	SD	Mean	SD	Mean	SD
Feed consumption (g dry matter)	82.6	11.7	81.8	7.0	85.3	11.3
Metabolic growth rate, MGR[4] (g kg-0.8 d-1)	13.8	1.1	13.3	0.6	14.0	0.8
Food conversion ratio, FCR[5] (g g-1)	0.91	0.07	0.96	0.06	0.91	0.04
Productive protein value ,PPV[6] (%)	40.0	2.9	38.7	2.4	40.9	1.5
Protein efficiency ratio, PER[7]	2.7	0.2	2.6	0.2	2.7	0.1
Apparent lipid conversion, ALC[8] (%)	32.5	6.3	33.6	10.8	40.6	3.6

[1]C = fish fed control feed
[2]Y50 = fish fed control feed containing 50mg/ kg butanol extract of *Yucca* powder
[3]Y100 = fish fed control feed containing 100mg/ kg butanol extract of *Yucca* powder
[4]MGR, metabolic growth rate = g live weight gain/ kg0.8/day
[5]FCR, feed conversion ratio = feed consumed/ live weight gain
[6]PER, protein efficiency ratio = wet weight gain/ protein fed
[7]PPV, protein productive value = total protein gain/ total protein fed x 100
[8]ALC, apparent lipid conversion = total lipid gain/ total lipid fed x 100

Energy utilisation

The complete energy budget of the fish in the three groups is given in Table 4. There were no statistically significant differences in the energy retained and excreted between the groups. The Y100 group had the highest energy retention value. The AUE value (a measure of energy excreted) was lowest in this group. The energy expenditure per unit feed energy was similar in the C and Y100 groups, but higher in the Y50 group. The average oxygen uptake per unit body mass gain was lowest in the Y100 group followed by the C group. The Y50 group had the highest value for this parameter.

Table 4. Energy budget, SMR, MR and oxygen uptake per unit body mass gain of carp fed experimental diets

Experimental group		C[1]		Y50[2]		Y100[3]	
		Mean	SD	Mean	SD	Mean	SD
Initial GE content of carcass	kJ	72.5	8.6	72.8	8.3	72.5	8.2
Final GE content of carcass	kJ	507	102	485	81	546	60
Feed GE uptake	kJ	1692	239	1674	144	1746	232
EE[4]	% of GE fed	51.3	1.5	55.4	3.9	51.5	3.9
ER[5]	% of GE fed	25.5	2.5	24.5	2.8	27.2	0.8
AUE[6]	% of GE fed	23.2	3.4	20.1	1.6	21.4	3.2
SMR[8]	$mgO_2\ h^{-1}kg^{-0.8}$	85.6	14.6	77.4	8.7	76.2	9.0
MR[7]	$mgO_2\ h^{-1}kg^{-0.8}$	373	19	398	32	366	29
O_2 uptake/bodymass gain	$g\ g^{-1}$	0.65	0.05	0.73	0.08	0.64	0.05

[1]C = fish fed control feed
[2]Y50 = fish fed control feed containing 50mg/ kg butanol extract of *Yucca* powder
[3]Y100 = fish fed control feed containing 100mg/ kg butanol extract of *Yucca* powder
[4]EE, energy expenditure = oxygen uptake (mg) x 14.85
[5]ER, energy retention = energy retention
[6]AUE, apparently unutilized energy = energy fed - energy expenditure - energy retention.
[7]MR, average metabolic rate = mg O_2 consumed/ $kg^{0.8}$/ hour
[8]SMR, standard metabolic rate = minimum O_2 consumption (mg; after 24 hrs of starving) / $kg^{0.8}$/ hour

4. Discussion

The saponin content of the *Yucca* powder were fully extracted by the procedure described above, into the butanol extract in the current experiment. Colour development on TLC, and hemolytic activity typical of saponins were restricted to this fraction and were not evident when the aqueous fraction remaining after butanol extraction was tested. Biologically active steroidal substances such as 5β-spirostan- β-ol, and sarsasapogenin and smilagenin monodesmoside saponins present in *Y. schidigera*, have all been previously found to be completely butanol extractable (Killeen et al., 1998).

Dietary YBE did not have any negative effect on carp at both the levels tested. At a level of 100 mg kg^{-1} YBE apparently promoted growth and feed conversion efficiency during the first seven weeks of the feeding period. Thereafter, the control fish had higher growth rate compared to the Y100 group. At the end of the experiment, the Y100 group tended to have higher values for carcass lipid content, feed lipid assimilation, energy retention and lower feed energy excretion. The differences were however not statistically significant. The only significant differences between the experimental groups occurred for the carcass gross energy content where the Y100 group had significantly higher values than the Y50 group, but not the

control group. It is difficult to explain the apparently more deleterious effect exerted by YBE at the 50 mg kg^{-1} dietary level compared to the 100 mg kg^{-1} level. Such erratic responses of animals to different levels of dietary *Yucca* saponins have also been previously reported. For example, chicken fed 125 mg kg^{-1} *Yucca* saponins had significantly lower haematocrit, haemoglobin, and red blood cell counts, compared to birds fed 250 mg kg^{-1} *Yucca* saponins which showed no significant differences in these blood parameters (Balog et al., 1994).

In our previous experiments, carp fed *Quillaja* saponin at 150 mg kg^{-1}, had significantly higher body mass gain at the end of 8 weeks (Francis et al., 2001a, b). Dietary *Yucca* saponins at levels tried in the current experiment did not have any significant growth promoting effect in carp. This has perhaps to do with the difference in chemical nature of the of the saponins used in the current experiment. The level of saponin in the diet in the current experiment was kept lower compared to our previous experiments, as it was felt that the butanol extract prepared in this experiment had higher saponin content compared to the commercial *Quillaja* saponin powder previously used (where the saponin level is about 20 %; Kensil, 1996).

The role of *Yucca* saponins as growth promoters and feed efficiency enhancers in ruminants and monogastrics has been controversial with some authors reporting positive effects (Goodall, 1979; Johnston et al., 1981; Foster, 1983; Mader and Brumm, 1987; Hussain and Cheeke, 1995) and others, no effects (Preston et al., 1985, 1987; Dziuk et al., 1985; Wu et al., 1994).

The tendency for higher MGR, ER, and carcass lipid and lower FCR (during the initial period of the experiment) and AUE levels in the Y100 group, are generally in agreement with the results of our previous feeding experiments where triterpenoid *Quillaja* saponins in carp and tilapia (Francis et al., 2001a, b, c). At least initially, the steroidal saponins at the 100 mg kg^{-1} level increased metabolic efficiency in carp (as seen from the higher growth and lower metabolic rate during the initial period), similar to the triterpenoid saponins. In the later stages of the experiment, however, their influence seemed to decline. The triterpenoid *Quillaja* saponins was hypothesised to promote growth in carp and tilapia by increasing the uptake of nutrients from the intestine, and stimulating gut and liver enzymes in addition to other possible systemic effects (Francis et al., 2001 a, b, c.). The lower feed energy excretion (evidenced by lower AUE) in both Y50 and Y100 groups (Table 4) lends support to the hypothesis that dietary saponins increased the absorption of nutrients from the intestine. The average feed energy retention (ER) values are, however, higher only in Y100 group compared to control, indicating that the absorbed feed energy was somehow not available for growth in the Y50 group. The lowering of average metabolic rates observed in previous experiments

where carp were fed triterpenoid saponins were also not in evidence when average values for the whole experimental period are considered (Fig. 2). The metabolic rate of the Y50 group was consistently higher, compared to control, even though the SMR of this group was lower at the start of the experiment (Table 4).

In conclusion *Yucca* saponins present in the butanol extract at the dietary levels tested did not have any effect in common carp. It would however be interesting to test the effects of other levels considering the initial, apparently positive effect in this experiment.

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Chapter 8

Preliminary investigations into the effects of purified hemolytic *Gypsophila* saponins on growth and nutrient assimilation efficiency in common carp, *Cyprinus carpio*.

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Abstract

Hemolytic saponins were purified from a raw commercial *Gypsophila paniculata* saponin mixture (containing mainly triterpenoid saponins). These were then fed to *Cyprinus carpio* at various dietary levels (5, 17, 150, and 250 mg kg^{-1}). The purified haemolytic *Gypsophila* saponins had no negative effect on the growth performance of carp at the tested levels over an 8 week experimental period. There appeared to be a trend of higher growth and feed utilisation efficiency in the saponin fed groups as compared to control. Dose-dependent effects were however, not observed. The effects of purified haemolytic saponins were not different from that of dietary raw *Gypsophila* saponin mixture at the same level of supplementation. In fish fed the haemolytic saponins, growth promoting effects could be observed at very low level of 5 mg kg^{-1} of the diet. Further experiments are required to ascertain weather purified haemolytic triterpenoid saponins are more potent in promoting growth in common carp compared to raw saponin extracts.

Keywords: *Gypsophila*, Haemolytic saponins, *Cyprinus carpio*, growth, protein, lipid efficiency.

1. Introduction

Saponins have been considered to be highly toxic to fish (Newinger, 1994). Fish bioassay has been used as an effective procedure for detection of saponins. Commercial *Quillaja* saponins included at very low levels (150 ppm) in the diet were, however, found to stimulate growth and increase metabolic efficiency in common carp and tilapia in previous experiments (Francis et al. 2000 a, b and c). The predominant variety of saponin present in the *Quillaja* extract are triterpenoid saponins. Several triterpenoid saponins have been isolated and identified in the genus *Gypsophila* (Elagamal et al., 1996; Frechet et al., 1991). The saponins present in this genus have well known applications and form a product of industrial interest (Acebes et al., 1998). The *Gypsophila* root extract consists of a mixture of acidic triterpenoid saponins similar in structure to those found in a variety of foods consumed by humans (Price et al., 1987). Also, saponins from the root of *G. paniculata* and *G. arrostii* have been used as detergent and expectorant (Hostettmann and Marston, 1995). *G. struthium* is known as a source of saponins since antiquity and is also used in gastronomy in Arabic countries (del Castillo et al., 1986). The roots of *G. oldhamiana* have been used as a remedy for the treatment of fever and infantile malnutrition in China, and a triterpenoid saponin from *G.*

pacifica has been shown to reduce the concentration of serum cholesterol in atherosclerotic rabbits (see Liu et al., 1995).

The aim of the current experiment was to separate out the haemolytic fraction from the commercial raw *Gypsophila* saponin mixture and to test whether the positive effects of dietary *Quillaja* saponin mixture (Francis et al. 2000 a, b) were repeated when purified haemolytic *Gypsophila* saponins were fed to common carp at various levels. One set of fish were also fed a diet containing the commercial raw *Gypsophila* saponin mixture for comparative purposes.

2. Materials and methods

Identification of presence and haemolytic potential of saponins in commercial Gypsophila saponin mixture with TLC

Gypsophila paniculata saponins were obtained from a commercial source (Serva Electrophoresis GmbH, D-69115 Heidelberg, Germany; special order). The presence and haemolytic potential of the saponins present in the commercial preparation were detected using Thin Layer Chromatography (TLC). TLC was carried out on Silica gel 60F 254 (Merck) with $CHCl_3$:MeOH:H_2O (60:55:15) as solvent. Detection of saponins was with sulphuric acid spray reagent (Ethanol:Ethyl acetate: con. H_2SO_4 at 18:10:10, the acid being added drop by drop after keeping the mixture of the other two on ice). The sprayed TLC plate was then heated at 105°C for 5min. The saponins appear as differently coloured spots. The haemolytic activity of the spots were detected using blood spray on the TLC plate. Blood diluted at 2:30 with a phosphate buffer (25ml KH_2PO_4, prepared by mixing 27.281 g in 1 litre double distilled water, was mixed with 39.34ml 0.1N NaOH and made up to 100ml with double distilled H_2O) was used.

Purification of haemolytic Gypsophila saponins by column chromatography

A silica gel column was prepared by the following procedure. 145 g silica gel 60 (0.063-0.200mm, No. 1.07734.2500, Merck, Darmstadt, Germany) was made into a slurry with the solvent ($CHCl_3$:MeOH:H_2O at 60:55:15) and poured into a column (1m with a radius of 1.5 cm) and allowed to settle overnight so that a column of 50 cm height is obtained. The next day the solvent level was brought down to just above the upper end of the silica column. Then 600 mg of *Gypsophila* was dissolved in 2 ml distilled water and poured carefully over of the column, without disturbing the upper level of the column. 2 ml of the solvent was then slowly eluted. This was repeated 3 times successively after adding 2 ml solvent ($CHCl_3$:MeOH:H_2O at 60:55:15) each time. After the saponin solution had formed a compact band at the top of the

column, the solvent was carefully added to the top of the column so that a solvent level of about 8 cm was continuously maintained at the top The solvent was then eluted from the column at the rate of 1.6 ml per minute. The first 120 ml was discarded and then the eluted solvent was collected in 5 ml samples and stored. Aliquots from fractions were then tested for the presence and haemolytic activity of saponins using TLC as described above (20µl from each of the alternate tubes were applied to the TLC).

The samples which contained the haemolytic saponins were then evaporated, mixed with a small quantity of water, frozen, freeze dried and tested again on TLC. The purified haemolytic *Gypsophila* saponins were then stored at –18°C until use.

Feeds and feeding regime

The experimental diets for each experiment namely C, GS5, GS17, GS150, GS250 and GSR150 were prepared from the same basal diet to ensure uniformity of composition. The ingredients and chemical composition of the basal diet used for the experiments are shown in Table I. The basal diet was ground and then repelleted (pellets of about 2 mm diammeter) without any additions in the case of the control feed (C). The purified haemolytic *Gypsophila* saponins (GS) at the rate of 0, 5, 17, 150, and 250 mg kg^{-1}, and raw *Gypsophila* saponin mixture (GSR) at the rate of 150 mg kg^{-1}, dissolved in de-mineralised water was mixed thoroughly with the powdered feed using a mixer before pelleting the C, GS5, GS17, GS150, GS250 and GSR150 feeds respectively. The moist pellets were frozen, freeze-dried and stored at -18°C until use.

Table 1. Chemical composition of experimental feeds

Composition of experimental diets	
Basal composition	%
Fish meal[a]	50
Wheat meal	42
Sun flower oil	4
Mineral premix[b]	2
Vitamin premix[c]	2

Chemical composition (dry matter)	%
Crude protein (N x 6.25)	40.9
Petroleum ether extract	8.6
Ash	11.2
Gross energy, kJ g^{-1}	19.6

[a] Fish meal obtained from Württembergische Zentralgenossenschaft, Germany
[b] and [c] prepared after Meyer-Burgdorff et al. (1989)

The fish were fed as a group at a level of 16 g $kg^{-0.8}day^{-1}$ (a level found to produce maximal growth and feed conversion values in previous experiments in our lab) in 6 equal instalments using automatic feeders attached to the aquaria.. The fish were weighed individually once a week and the ration for each aquarium was adjusted according to its body mass. The feed

ration was calculated individually and the ration for each tank was the total of the individual feed rations. There was no feeding on the weighing day.

Experimental fish

Carp (*Cyprinus carpio* L.) were obtained from the Federal Fishery Research Agency, Institute for Coastal and Inland Fishery, Ahrensberg, Germany. They were brought to Hohenheim when they weighed about 1 g and grown in 200 litre aquaria at 23 ± 1°C. They were fed a diet containing approximately 42 % protein, 10 % lipid, 10 % ash and an energy content of 20 kJ g^{-1} on a dry matter basis. Five days prior to the start of experiment, carp weighing approximately 14 g were selected from a large population. They were then placed in groups of 5 in 6 aquaria of 50 l capacity. The fish were marked by cutting fins so that the chemical composition and growth rate of individual fish could be calculated. Ten fish each of comparable body weights as the respective experimental fish, were killed and preserved for determining the initial chemical composition. During acclimatisation (5 days) in the experimental set up, the fish continued to receive maintenance levels of feed (3.2 g $kg^{-0.8}$ day^{-1}; Meyer-Burgdorff et al., 1989). The fish were starved for one day immediately previous to the start of the experiment. Thereafter feeds named C, GS5, GS17, GS150, GS250 and GSR150 were assigned to one box each in a random manner.

At the end of eight weeks the experiment was terminated. The fish were weighed, killed and immediately deep frozen. Prior to analysis, the carcasses were autoclaved for 30 minutes at 120°C, homogenised, refrozen and freeze dried.

The experimental room was lit with fluorescent tubes to give a day length of 12 hours. The water temperature was maintained at 27 ± 0.1°C. Every day about a tenth of the water in the system was replaced with fresh water. The water quality parameters were maintained within a critical range during the experimental period (pH – 7-7.9; total NH_3 – 0.1-0.2 mg l^{-1}; nitrite – 0.07-0.1 mg l^{-1}, and nitrate – 1-3 mg l^{-1}).

Chemical analyses

The chemical composition of the experimental diets and the freeze dried fish were analysed for each fish individually according to official methods (Neumann and Basler, 1983) i.e. dry matter was measured by drying to constant weight at 105°C, crude protein as macro-Kjeldahl (N x 6.25), lipids by extraction with petroleum ether and gross energy by bomb calorimetry (IKA C 7000) with benzoic acid standard.

Calculations and statistical analysis

Feed Conversion (FCR) was calculated as feed consumption (dry matter)/ live weight gain, and Metabolic Growth Rate (MGR) as live weight gain (g)/ average metabolic live weight ($kg^{0.8}$) per day. The Productive Protein Value (PPV, %) and Apparent Lipid Conversion (ALC, %) were calculated as protein gain x 100/ feed protein and lipid gain x 100/ feed lipid and Protein Efficiency Ratio (PER) was calculated as live weight gain(g)/ protein fed (g). Parameters such MGR and carcass composition was determined on an individual basis in each treatment group. FCR, PPV and ALC were calculated for each treatment as a group.

3. Results

Identification and purification of the hemolytic saponins.

The commercial *Gypsophila* saponin mixture showed 10 spots on TLC. The Rf values were 0.03, 0.07, 0.10, 0.13, 0.22, 0.39, 0.42, 0.52, 0.54, and 0.58. Of these only two spots (with Rf values, 0.52 and 0.54) were haemolytic. These two haemolytic saponins (with similar polarities) eluted along with the 38^{th} to 64^{th} 5 ml samples (135 ml). TLC confirmed the presence of only these two haemolytic saponins in this portion of the eluted solution. About 88 mg of the haemolytic saponins were obtained from the 600 mg applied to the column.

Feed intake and behaviour of carp

All fish ate the feed provided completely. Since the fish ate the feed pellets as they dropped into the respirometer box there was little chance that the soluble saponins might have leached into the water. There was no mortality or abnormal behaviour of fish in any of the treatment groups during the whole experimental period.

Growth rates and feed conversion

At the end of the experimental period (8 weeks) the growth rate, calculated on an individual basis in each treatment group, in the different groups were statistically similar (Table 2) even though absolute growth was higher in all the saponin fed groups compared to control. The absolute FCR value was lowest in the GS5 group and highest in the C group. The value tended to be lower in all the saponin fed groups (1.0 to 1.08) than the C group (1.3).

Table 2. Initial and final average body weights, MGR, and FCR of carp fed experimental diets

	Initial weight		Final weight		MGR[1]		FCR[2]
Fish group	Average	SD	Average	SD	Average	SD	
C[3]	13.1	1.8	43.6	8.1	10.1	1.2	1.30
GS5[4]	14.7	2.1	60.4	7.8	12.5	1.1	1.00
GS17[5]	14.6	1.0	59.5	12.3	12.3	2.4	1.01
GS150[6]	14.1	1.1	53.8	15.9	11.3	3.1	1.08
GS250[7]	14.9	2.5	58.7	9.4	12.1	1.7	1.06
GSR150[8]	13.5	1.5	52.3	10.6	11.6	2.4	1.08

[1]MGR = g live weight gain/ $kg^{0.8}$/day
[2]FCR = feed consumed/ live weight gain
[3]C= control
[4]GS5: Carp fed 5 mg kg^{-1} purified haemolytic *Gypsophila* saponin
[5]GS17: Carp fed 17 mg kg^{-1} purified haemolytic *Gypsophila* saponin
[6]GS150: Carp fed 150 mg kg^{-1} purified haemolytic *Gypsophila* saponin
[7]GS250: Carp fed 250 mg kg^{-1} purified haemolytic *Gypsophila* saponin
[8]GSR150: Carp fed 150 mg kg^{-1} raw *Gypsophila* saponin mixture

Carcass composition

The chemical composition of the experimental fish in the three groups at the end of the experiment showed significant differences (Table 3). The average carcass dry matter, lipid and energy content tended to be higher in the saponin fed groups. The GSR150 group had the highest value for all these parameters. The average protein and ash content was higher in the C group.

Table 3. Initial and final carcass composition (dry matter basis) of carp fed the experimental feeds

Fish group	DM in fish[1]		Protein[2]		Fat[3]		Gross Energy[4]		Ash[5]	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Initial	20.7	0.60	60.5	1.54	13.0	2.60	20.0	1.72	12.3	0.75
C[6]	20.5^{b}	0.50	67.1^{a}	1.38	14.3^{b}	1.21	21.1^{b}	0.50	13.6^{a}	0.54
GS5[7]	21.0^{b}	0.79	63.5^{ab}	2.96	17.7^{ab}	2.90	22.3^{a}	0.38	12.1^{b}	0.58
GS17[8]	21.4^{ab}	1.67	63.4^{ab}	3.14	14.3^{b}	2.59	22.0^{ab}	0.28	12.3^{ab}	0.56
GS150[9]	21.3^{ab}	0.56	65.6^{ab}	3.21	13.9^{b}	4.00	21.7^{ab}	1.16	12.5^{ab}	1.46
GS250[10]	20.6^{b}	0.89	64.8^{ab}	3.00	15.3^{ab}	2.97	21.9^{ab}	0.84	12.5^{ab}	0.30
GSR150[11]	22.7^{a}	1.62	62.0^{b}	2.06	18.3^{a}	1.54	22.6^{a}	0.62	12.6^{ab}	1.40

[1]Dry matter (DM), % of fresh matter
[2]Crude protein(N x 6.25), % of DM
[3]Petroleum ether extract, %
[4]Gross energy, kJ g-1
[5]Ash, %
[6]C= control
[7]GS5: Carp fed 5 mg kg^{-1} purified haemolytic *Gypsophila* saponin
[8]GS17: Carp fed 17 mg kg^{-1} purified haemolytic *Gypsophila* saponin
[9]GS150: Carp fed 150 mg kg^{-1} purified haemolytic *Gypsophila* saponin
[10]GS250: Carp fed 250 mg kg^{-1} purified haemolytic *Gypsophila* saponin
[11]GSR150: Carp fed 150 mg kg^{-1} raw *Gypsophila* saponin mixture

Table 4. Feed utilisation parameters of carp fed experimental diets

Fish group	PPV %[1]	PER[2]	ALC %[3]
C[4]	24.7	2.00	22.8
GS5[5]	35.3	2.60	47.2
GS17[6]	35.8	2.57	36.4
GS150[7]	35.0	2.41	32.7
GS250[8]	33.4	2.46	36.1
GSR150[9]	35.4	2.42	50.7

[1]PPV = total protein gain/ total protein fed x 100
[2]PER = wet weight gain/ protein fed x 100
[3]ALC = total lipid gain/ total lipid fed x 100
[4]C= control
[5]GS5: Carp fed 5 mg kg^{-1} purified haemolytic *Gypsophila* saponin
[6]GS17: Carp fed 17 mg kg^{-1} purified haemolytic *Gypsophila* saponin
[7]GS150: Carp fed 150 mg kg^{-1} purified haemolytic *Gypsophila* saponin
[8]GS250: Carp fed 250 mg kg^{-1} purified haemolytic *Gypsophila* saponin
[9]GSR150: Carp fed 150 mg kg^{-1} raw *Gypsophila* saponin mixture

Feed conversion and assimilation

The absolute PPV, PER, and ALC values were higher in the saponin fed groups compared to the C group (Table 4).

Discussion

The method used in the current method was simple and effective in separating out haemolytic saponins from the raw *Gypsophila* saponin mixture. Since one of the aims of the experiment was to test whether the haemolytic saponins were more biologically active when present in the diet compared to the crude saponin extract, the two haemolytic saponins (that have similar polarities) were taken together for the feeding experiment. Changes in the composition of the solvent could yield purified individual compounds, by altering their relative speeds of migration in the column.

Very few *in vivo* trials have been carried out to assess the various effects of purified saponins (Lacaille-Dubois and Wagner, 2000). This is in spite of the fact that saponins are already present in many veterinary vaccines. The first saponin-based human vaccine could be soon expected on the market (Lacaille-Dubois and Wagner, 2000). More investigations at a biological level are required to explain mechanisms of action, which still remain in the dark.

In the current experiment there were no differences in growth rate between the GS150 and the GSR150 group indicating that when present in the diet the purified haemolytic *Gypsophila* saponins did not have a higher negative or positive effect compared to the raw *Gypsophila* saponin extract, at a level of 150 mg kg^{-1}. The lack of replicates makes it difficult to make a conclusive statement regarding the ability of dietary *Gypsophila* saponins to stimulate growth and protein and energy assimilation efficiencies in common carp. The number of treatments was not increased at this stage because of the difficulty in purifying saponins and the preliminary nature of the trial. There was, however, a trend towards increased growth and feed utilisation efficiency in saponin fed groups. The observed effects of saponins in the current experiment such higher growth rate, lower FCR, higher PPV and ALC values and

differences in carcass composition parameters between C and GS groups were generally in agreement with that of the results of our previous experiments where crude *Quillaja* saponins were fed at a dietary level of 150 mg kg^{-1} to carp and tilapia (Francis et al., 2000a, b, c). These results show that the effects were equally felt when the raw or purified haemolytic saponins were fed to carp at higher levels. Haemolytic saponin concentrates were, however, effective at very low levels such as 5 mg kg^{-1}. More experiments are required to determine whether concentration of haemolytic fractions increases the potency of saponins as growth enhancers in carp. It was seen that feeding at a level of 5 mg kg^{-1} to 250 mg kg^{-1} did not have any dose dependant effects on the experimental carp showing that beyond a certain level there were no incremental effects of haemolytic saponin concentrates. It could be concluded that, the growth promoting activity of dietary saponins that may have been mediated by their action on membranes (Jenkins et al., 1991; Onning et al., 1996) and/or stimulation of intestinal and liver enzymes (Serrano et al., 1998), is not dose dependent.

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General Discussion

In the preceding chapters some biological effects of low dietary levels of saponins on common carp and Nile tilapia was discussed. In Chapter 1, published literature on the effects that saponins have on animal systems have been reviewed. In chapters 2 and 3 it was shown that at a dietary level of 150 mg kg^{-1}, *Quillaja* saponins (QS) fed continuously produced a significant increase in body weight gain in common carp. QS also had a metabolic effect in that it reduced the amount of oxygen consumed per gram body weight gain. The efficiency of feed utilisation indicated in parameters such as feed conversion ratio and protein productive value was also higher in carp feeding QS. In Chapter 4, the effects of dietary QS in Nile tilapia are described. The initial growth promoting effects of a dietary level of 150 mg kg^{-1} QS were also evident here. It was, however, the level of 300 mg kg^{-1} QS that produced sustained weight gain in Nile tilapia which was significantly higher than that of controls at the end of 14 weeks. As previously observed in carp, QS lowered the metabolic demand for oxygen and increased the efficiency of feed nutrient utilisation. The female fish fed the 300 mg kg^{-1} QS diet grew better than male fish and did not produce eggs. In chapter 5 the results of an experiment in which the inhibitory effect of QS on egg production in female tilapia was demonstrated is explained. Results presented in Chapter 6 show that 17-day-old tilapia fry fed QS at 700 mg kg^{-1} demonstrated a significant deviation from the typical 1:1 male female ratio in favour of males. Growth promoting effects were not noticed when carp were fed steroid saponins from *Y. schidigera* as explained in Chapter 7. In Chapter 8 the results of a preliminary feeding trial with purified haemolytic saponins on growth and body composition in common carp are presented.

The intention of this discussion is to analyse the results of our studies in general, and suggest directions for future research. Some results that could not be presented in the previous chapters are included here for this purpose. The effects of QS that were observed could be broadly classified into the following categories:

a. Effects on growth
b. Effects on carcass composition and assimilation of feed nutrients
c. Effects on metabolism
d. Effects on tilapia reproduction

1. Effects on growth

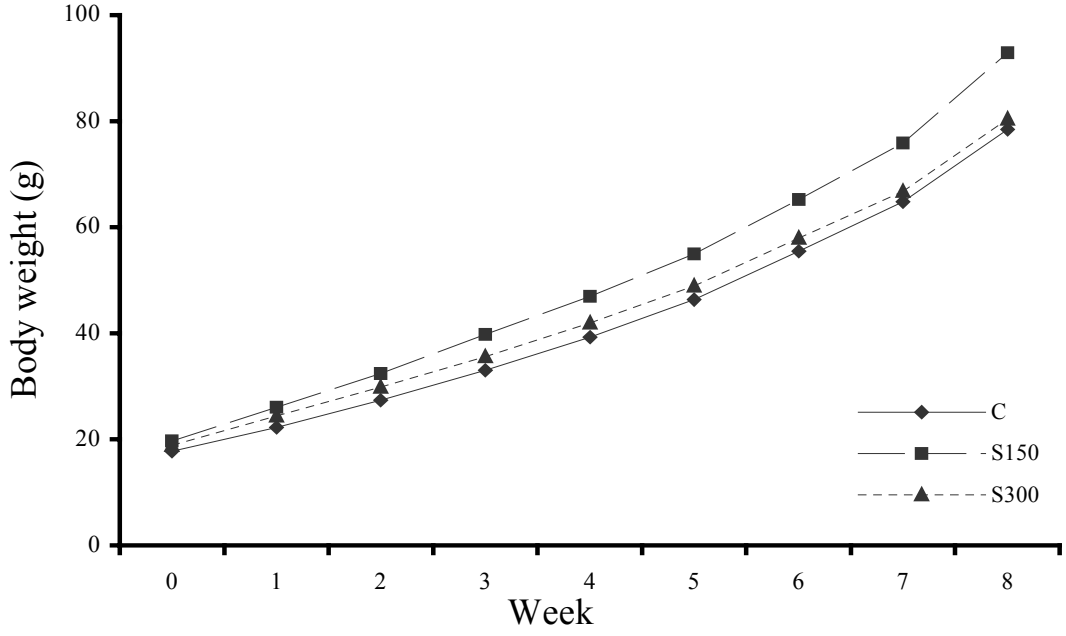

Fig. 1. Body weight increase of common carp fed control diet (C) or a diet containing either 150 mg kg^{-1} (S150), or 300 mg kg^{-1} (S300) QS

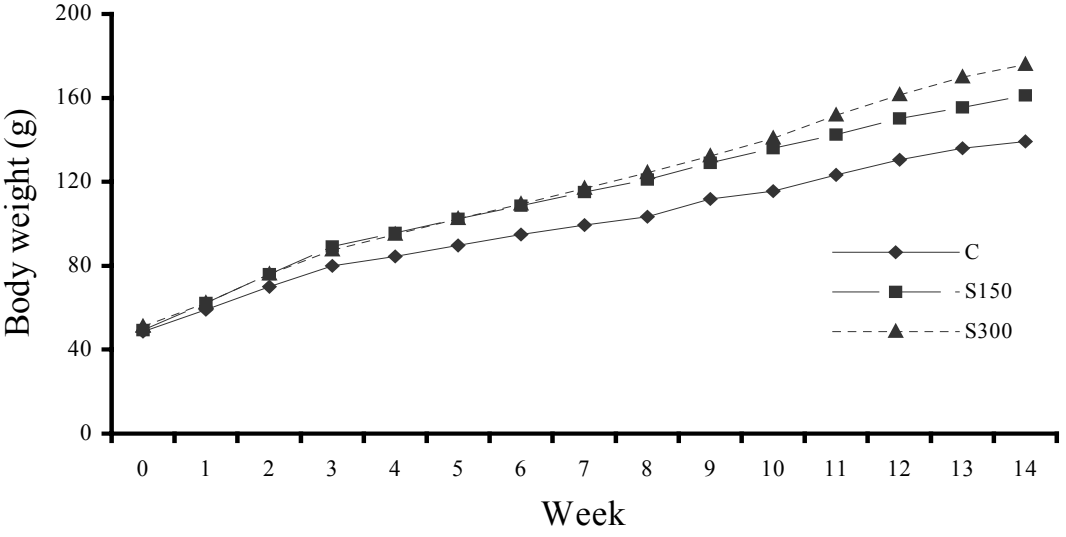

Fig. 2. Body weight increase of Nile tilapia fed control diet (C) or a diet containing either 150 mg kg^{-1} (S150), or 300 mg kg^{-1} (S300) QS

Triterpenoid saponins promoted the growth of both common carp (chapters 2, 3 and 8) and Nile tilapia (chapters 4 and 6) whereas the steroid *Yucca* saponins had a less pronounced effect in common carp (chapter 7). The average final body weight of carp fed QS was about

18 % higher and that of tilapia and was more than 20 % higher than that of fish that had similar average weights at the start of the respective experiments but which did not receive QS (Fig 1 and 2). The differences in metabolic growth rate and specific growth rate of QS fed fish was not always significantly higher probably because of the limited number of fish that could be accommodated in the respirometer chambers where most of the experiments were conducted. There was also a high variability among groups. The use of individual fish in the respirometer in our experiments, however gives a much deeper insight into the way in which food is utilised compared with group feeding
experiments.

The effects of saponins at the level of 150 mg kg^{-1} was most pronounced during the initial few weeks of the respective experiments. There was also some evidence that the age and initial body size of the fish may have an effect on the action of dietary saponins in carp. In both experiments described in Chapters 2 and 3, carp weighing approximately 18-20 g were used. In two later experiments that used either lighter (experiment 1) or heavier (experiment 2) fish the effects of saponins at 150 mg kg^{-1} were not as pronounced as shown in Table 1.

Table 1. Initial and final body weights, Metabolic Growth Rate (MGR) and Feed Conversion Ratio (FCR) of carp in experiments 1 and 2

	Experiment 1				Experiment 2			
	C[1]		S150[2]		C		S150	
	M	SD	M	SD	M	SD	M	SD
Initial body weight (g)	11.4	1.0	11.6	1.7	29.9	2.5	29.0	2.2
Final body weight (g)	64.1	8.2	60.9	14.7	112.9	18.7	115.1	13.8
MGR[3]	11.5	0.7	10.9	1.6	13.8	3.8	13.9	3.2
FCR[4]	1.01	0.04	1.05	0.04	0.95	0.17	0.90	0.08

[1] C =carp fed control diet
[2] S150 = carp fed a diet containing 150 mg kg^{-1} *Quillaja* saponin
[3] MGR = average weight gain/ average metabolic body weight ($kg^{0.8}$)/days
[4] FCR = average feed consumed (dry matter)/ live weight gain
M and SD represent means and standard deviations

The mechanisms contributing to the growth promoting effects of QS are yet to be fully clarified as indicated in the respective chapters. It is also not clear why supplementation with the same QS mixture produced non-uniform results in the different experiments.

One factor that makes it more difficult to interpret the effect of dietary saponins was the presence of a number of individual compounds with different properties in the QS saponin mixture used in our feeding trials. As a preliminary step towards identifying the nature of saponins that have growth promoting effects in fish, a feeding experiment in carp using diets

supplemented with haemolytic saponins was conducted (described in chapter 8). It was observed that the average values of growth performance parameters were higher in carp that consumed the supplemented feed compared to control, results that are generally in agreement with the results from fish feeding experiments using triterpenoid QS as a dietary supplement. However, there were no differences between carp fed the raw *Gypsophila* saponin mixture or any of the levels of haemolytic saponins as regards their growth or any of their nutrient assimilation parameters even though haemolytic saponins were effective at very low levels such as 5 mg kg^{-1}. More experiments are required to determine whether increasing the concentration of haemolytic fractions increases the potency of saponins as growth enhancers in carp.

Future research in this area should concentrate on understanding the physiological mechanisms by which dietary saponins increase growth and feed conversion efficiency in carp and tilapia. Diverse effects of dietary saponins in animal systems are discussed in chapter 1. They include an increase in the permeability of intestinal membranes to dietary nutrients and/or a stimulation of the activity of digestive enzymes (Serrano et al., 1998), which increases the efficiency of feed nutrient utilisation. It has been observed by us in an ongoing study that QS does not cause any obvious damage to the intestinal membranes in tilapia fry when present up to a level of 700 mg kg^{-1} in the diet. It also remains to be determined whether the saponins themselves or their breakdown products produced in the intestines enter the blood of the fish and cause their effects systemically. Recent reports suggest that even without actually entering the body, substances could produce systemic effects. It has been reported that hormones such as ghrelin synthesised primarily in the stomach wall could act as intermediaries between stomach, hypothalamus and pituitary and may be involved in energy balance (Tschöp et al., 2000). The ability of saponins to influence serum hormone levels has been discussed in chapter 1. More insights into these aspects need to be gained through further experiments.

2. Effects on carcass composition and nutrient assimilation

There were no obvious patterns in the effects of dietary saponins on carcass chemical composition. A general increase in the percentage composition of carcass dry matter and crude lipid content were however observed, as explained in chapters 2, 3, 4, 6, 7 and 8.

In the experiments previously mentioned in this chapter, protein and fat assimilation were higher in the saponin fed fish (table 2) indicating a positive influence of dietary QS even in cases where growth and body weight gain were not significantly influenced.

Saponins increased absorption and hence availability of dietary nutrients which might have been the reason for their improved assimilation into the fish carcass. The future research suggested in the previous section may also help to clarify the mechanism of saponin action here.

Muscle cholesterol levels also tended to be higher in tilapia fed saponins. The increase in muscle cholesterol content in experiment 4 might have been due to the general increase in fat content. But the tendency in experiment 6 for serum cholesterol content to increase with increasing levels of dietary QS independently of carcass cholestrol and fat content points to a specific influence of dietary QS on this parameter. The biochemical reason for this increase would be worth investigating, considering the key role of cholesterol in sex steroid synthesis.

Table 2. Protein Productive Value (PPV) and Apparent Lipid Conversion (ALC) of carp in experiments 1 and 2

	Experiment 1				Experiment 2			
	C[1]		S150[2]		C		S150	
	M	SD	M	SD	M	SD	M	SD
PPV[3]	31.7	0.8	34.8	1.6	34.9	14.9	39.1	13.6
ALC[4]	40.6	8.3	40.5	21.8	40.0	28.9	44.1	19.2

[1] C =carp fed control diet
[2] S150 = carp fed a diet containing 150 mg kg^{-1} *Quillaja* saponin
[3] PPV = average total protein gain/ total protein fed x 100
[4] ALC = average total lipid gain/ total lipid fed x100
M and SD represent mean and standard deviations

3. Effects on respiratory metabolism

A comparative study of the metabolic rate and oxygen consumption among carp and tilapia fed saponins shows a shift of emphasis in the metabolic activity in saponin fed fish. The consumption of oxygen per unit body weight gain was lower in these fish and the retention of feed energy was higher and feed energy excretion lower as explained in chapters 2, 3, 4 and 7. The physiological mechanisms of these effects are not entirely clear. Some possibilities are discussed in these chapters based on the literature available to date. Most interesting from a physiological point of view is the lowering of fish oxygen demand for growth. This would have positive implications in tropical aquacultural systems where dissolved oxygen is often a limiting factor. The higher efficiency of oxygen utilisation may point to a higher synthetic efficiency of proteins and lipids leading to a reduction in synthetic heat production. Increased retention of feed energy for growth and lower nutrient excretion are also beneficial from both the production and environmental points of view.

4. Effects on tilapia reproduction

Observations made on the dietary saponin-induced changes in reproductive behaviour are explained and discussed in chapters 4, 5 and 6. The retarding effects on egg production in adult females and the capacity for sex inversion in tilapia fry indicate effects at the hormonal level. Data from gonado-somatic index measurements also support this contention. Our efforts to identify any saponin-induced change in the level of one of the key hormones in reproductive functioning, the leutinizing hormone (LH), did not reveal any dose dependent patterns. Once the optimum dietary level of saponins that produces complete sex inversion in tilapia fry or prevents egg production in female tilapia is determined, this effect of saponin will have considerable potential in tilapia aquaculture where one of the major problems is overproduction of fry that do not grow to marketable size. The effect of saponins on levels of reproductive hormones should be further studied by closer monitoring of hormones such as estrogen, testosterone and gonadotropic hormones *in vivo* and by using cultured tilapia pituitary cells. *In vitro* studies have the advantage of requiring only small quantities of material for the identification of the individual compounds responsible.

Conclusions and implications

The main conclusions were

1. Triterpenoid QS at 150 mg kg^{-1} have a growth promoting effect in common carp when given continuously along with the diet (chapters 2 and 3). Purified haemolytic *Gypsophila* saponins tend to increase growth in carp at levels as low as 5 mg kg^{-1} (chapter 8).
2. In Nile tilapia QS at levels ranging between 150 and 700 mg kg^{-1} in the diet have a growth promoting effect (chapters 4 and 6)
3. QS at 300 mg kg^{-1}inhibited egg production in female tilapia (chapter 5)
4. QS at 700 mg kg^{-1} fed to tilapia fry caused a significant deviation from the 1:1 male female ratio (chapter 6).
5. *Yucca schidigera* butanol extracts containing steroid saponins did not have growth promoting effects in common carp (chapter 7)
6. Saponins generally tended to reduce the metabolic rate in carp and tilapia and tended to lower the oxygen demand for growth in both fish species (chapters 2, 3, 4 and 7)

The results obtained have practical as well as scientific implications.

The practical implications include the potential for increasing the efficiency of feed utilisation of culture fish. The lowering of the demand of oxygen for growth is also important, especially in tropical aquaculture where the level of dissolved oxygen is often a limiting factor. The importance of preventing overcrowding in tilapia culture has been explained in the relevant chapter. Similarly important is the potential for producing all-male populations by feeding QS to tilapia fry.

As explained in chapter 1, saponins have been found to have diverse effects in the animal and human systems and are the active principles in several medicinal and health products. They are also being considered for inclusion in human vaccine preparations. Any additions to our knowledge of their effects in the animal body are therefore of high interest and relevance.

It is hoped that the findings presented in this thesis that bring to light hitherto unknown effects of dietary saponins will stimulate more research into the biochemical and physiological aspects of these effects in animal systems.

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Summary

Aquaculture is currently the fastest growing food production sector of the world. Its share in total world food fish production is set to increase from 29 % in 1996 to 38 % by the year 2010. Consequently the use of supplementary feeds in fish culture is also expected to increase. The use of synthetic substances such as antibiotics and steroid hormones for increasing feed utilisation efficiency are currently prohibited in several countries. In this context, the search for natural, biologically active, renewable, and biodegradable plant products that could be used to replace hazardous synthetic growth stimulants is highly relevant. Saponins are a group of compounds present in many wild plants and cultivated crops and are commonly found in traditional medicinal preparations. During the course of this research work these substances were also discovered to promote the growth of fish. Feeding experiments using saponin-supplemented (commercial *Quillaja* saponin) feeds were conducted in two commonly cultured fish species, namely common carp (*Cyprinus carpio* L.) and tilapia (*Oreochromis niloticus* (L.)).

Most of the experiments were conducted in a computer controlled respirometer system that allowed feeding and continuous measurement of oxygen consumption of up to 15 individual fish simultaneously. The respiration data obtained, together with chemical analyses of the feed and the fish were used to construct complete energy budgets for individual fish. Preliminary tests and experiments with tilapia fry were conducted in aquaria that were part of a warm-water re-circulating system.

Quillaja saponins (QS) at a dietary level of 150 mg kg^{-1} (S150 diet) significantly increased body weight gain in common carp compared to controls (C). Up to four weeks, the average feed conversion ratio (FCR) and metabolic growth rate (MGR) values of the S150 group were significantly better than those of the C group. However, the differences narrowed later on and by the end of the experiment were no longer significant. Giving the S150 feed only intermittently did not sustain the growth promoting effect of dietary QS over the whole experimental period. Growth was significantly higher in carp fed QS continuously (MGR of the S150 group was highest during the initial weeks as previously) as were FCR, productive protein value (PPV) and apparent lipid conversion (ALC). Compared with the control group however the metabolic rate was lower. The efficiency of energy utilisation indicated by

parameters such as energy expenditure (EE), energy retention (ER) and amount of oxygen consumed per unit body mass gain (OPM) were also significantly better in the S150 group. Dietary QS at 300 mg kg^{-1} (S300 diet) also significantly increased body weight gain in tilapia compared to a control group. The S150 group had a higher growth rate after the first three weeks of feeding, but this effect decreased as the experiment progressed. At the end of the experiment the S300 group also had significantly higher average values for ER, ALC, carcass fat, energy, and significantly lower average values for AUE and carcass ash content than the C group. The corresponding values for the S150 group were intermediate between the C and S300 groups. QS also had an inhibitory effect on egg production by tilapia. When fed to tilapia fry QS altered the normal 1:1 sex ratio. The treatment group fed a diet containing 700 mg kg^{-1} QS had a significantly higher number of males than the control after a period of 6 months. In separate, *in vitro* measurements it was found that QS stimulated the release of leutinising hormone (LH) from cultured tilapia pituitary cells, but apparently this did not happen *in vivo* since serum levels of LH were not found to be elevated in the saponin fed fish

In breeding trials where males and females from different treatments were put together, normal fry production occurred only in the control. The growth promoting properties of dietary QS were also evident in this experiment. Dietary saponins were found to affect serum and muscle cholesterol levels. Adult tilapia - but not tilapia fry - fed a dietary level of 300 mg kg^{-1} QS were found to have significantly higher muscle cholesterol levels than fish fed the control diet. In tilapia fry, the average serum cholesterol levels in males showed a steady increase from dietary QS levels of 0 to 700 mg kg^{-1}, while no such trend existed among the females.

Supplementation with 50 and 100 mg kg^{-1} of *Yucca schidigera* butanol extract (containing steroidal saponins) did not significantly promote the growth of carp despite showing initial promise. The purified haemolytic fraction of triterpenoid *Gypsophila* saponin extract (isolated using column chromatography) did not produce any toxic effects in carp at dietary levels up to 250 mg kg^{-1} in a preliminary feeding trial. A growth promoting effect was however evident at the very low level of about 5 mg kg^{-1} when the purified fraction was used.

Studies currently underway show that dietary QS up to a level of 700 mg kg^{-1} does not cause any structural alterations in the intestinal mucosa of tilapia fry. QS (which have been classified as ‘generally recognised as safe’ (GRAS) in the US) therefore has potential

application as a growth promoter in carp and tilapia diets. Dietary supplementation with QS might also help mitigate the problem of overproduction of small fish in tilapia culture. More research needs to be done to determine the mechanisms of action of QS and also to test whether saponins from other sources have similar effects. Once relevant data have been obtained, biochemical models could be established to determine the efficacy of purified individual compounds. This would help to predict the action of dietary saponins more accurately and to improve their standardisation. This knowledge is essential if firm recommendations are to be made concerning the use of saponins as feed additives.

Zusammenfassung

Effekte von niedrig dosierten Saponinen in Diäten auf das Wachstum von zwei häufig kultivierten Fischarten - Karpfen (*Cyprinus carpio* L.) und Tilapien (*Oreochromis niloticus* (L.))

Die Aquakultur ist weltweit der am schnellsten wachsende Sektor der Nahrungsmittelproduktion. 1996 betrug ihr Anteil an der weltweiten Fischproduktion 29% und für 2010 wird eine Zunahme auf 38% erwartet. Die Steigerung der Produktion durch verstärkten Einsatz von Supplementfuttermitteln führt zu höheren Kosten und zusätzlichen Umweltbelastungen. Die Menge des eingesetzten Futters läßt sich reduzieren, indem man die Effizienz der Futterverwertung verbessert. Der Einsatz synthetischer Substanzen wie Fütterungsantibiotika oder Steroide zur Steigerung der Futterverwertung ist jedoch in den meisten Ländern verboten. In diesem Zusammenhang gewinnt die Suche nach natürlichen, biologisch aktiven Substanzen als Ersatz für gefährliche synthetische Wachstumsförderer zunehmend an Bedeutung. Saponine, eine Gruppe von Substanzen, die in vielen Wild- und Kulturpflanzen vorkommen, sind bisher vor allem als Wirkstoff in der traditionellen Medizin bekannt. Im Rahmen der vorliegenden Arbeit konnte die wachstumsfördernde Wirkung dieser Substanz bei Fischen erstmals nachgewiesen werden. Dazu wurden Experimente an Karpfen und Tilapien mit einem kommerziell erhältlichen Saponinextrakt aus *Quillaja saponaria* als Futterzusatz durchgeführt.

In einigen Vorversuchen und Experimenten mit sehr kleinen Fischen wurden die Untersuchungen nicht in der computergesteuerten Respirationsanlage durchgeführt, Ansonsten wurde der Sauerstoffverbrauch von 15 Fischen individuell parallel gemessen. Aus den Respirationsdaten sowie der chemischen Zusammensetzung des Futters und der Fischkörper zu Beginn und nach Beendigung der Experimente wurde für jeden Fisch eine Energie- und Stoffbilanz erstellt, um die Effizienz der Energie- und Proteinverwertung zu bestimmen.

Im ersten Experiment wies die Saponingruppe die mit 150 mg kg^{-1} *Quillaja* saponinen supplementiert (S150) wurde, ein signifikant höheres Wachstum auf als die Kontrollgruppe, während die Gruppe die mit 300 mg kg^{-1} supplementiert wurde (S300) kein höheres Wachstum aufwies. Innerhalb der ersten 4 Wochen waren die Futterverwertung und die metabolische Wachstumsrate der S150 Gruppe gegenüber der Kontrolle signifikant erhöht. Diese Unterschiede waren jedoch innerhalb der folgenden 4 Wochen nicht mehr nachweisbar. Die Proteinverwertung war in der S150 Gruppe ebenfalls am höchsten. Die S150 Gruppe wies

auch die höchste Energieretention auf, jedoch war der Anteil der scheinbar nicht nutzbaren Engergie in der S300 Gruppe am geringsten. Da die Unterschiede im Wachstum innerhalb der letzten vier Wochen verschwunden waren lag die Vermutung nahe, dass es sich um eine Anpassung an die Saponine handelte. Deshalb wurde in einem zweiten Experiment überprüft, ob Eine regelmäßige Unterbrechung der Saponinfütterung (zyklisches Angebot) diesen Anpasungseffekt verhindern konnte. Das beste Wachstum zeigte jedoch die S150 Gruppe (durchgehend gefüttert), die sowohl zu einer höheren metabolischen Wachstumsrate, Futter- und Proteinverwertung sowie zu einer besseren scheinbaren Fettverdaulichkeit führte, obgleich die metabolische rate geringer war als in der Kontrollgruppe. Energieverwertung, Energieansatz und Sauestoffverbrauch bezogen auf die Körpermasse waren ebenfalls gegenüber der Kontrollgruppe signifikant verbessert.

Diätetisches QS bei 300 mg kg^{-1} (S300) zeigte auch, dass das Körpergewicht der Tilapien, verglichen mit der Kontrollgruppe, signifikant erhöht wurde. Die Gruppe wies auch eine höhere Energieretention, scheinbare Fettverdaulichkeit und eine geringere scheinbar ungenutzte, Energiegröße auf. Körperfettgehalt und Energiegehalt waren ebenfalls höher, als in der Kontrollgruppe. Die S150 Gruppe wies nur während der ersten 3 Wochen des Experiments ein höheres Wachstum auf. Im Bezug auf die weiteren Parameter lag diese Gruppe zwischen der S300 und der Kontrolle. Ein negativer Effekt auf die Eiproduktion von Tilapien bei Saponin Supplementierung konnte beobachtet werden. Dies wurde in einem weiteren Experiment mit in Einzelhaltung gefütterten Tilapien bei einer Saponin Zulage von 300 mg kg^{-1} bestätigt. Einen weiteren Beleg für diesen androgenen Effekt von *Quillaja* saponinen – mehr männliche Fische - zeigte ein Experiment mit 17 Tage alten Tilapien die mit 50 bis 700 mg kg^{-1} (S50, S150, S300, S500 und S700 Gruppe) gefüttert wurden. In der S700 Gruppe wurden nach 6 Monaten signifikant mehr männliche Tiere beobachtet. Am Ende des Experimentes wiesen die Saponin-behandelten Fische höhere Körpermassen und spezifische Wachstumsraten auf. In Brutexperimenten konnten nur die Tiere der Kontrollgruppe innerhalb von 3 Monaten normal reproduzieren. QS erhöhte die Ausschüttung von luteinisierenden Hormonen (LH) aus kultivierten Hypophyse-Zellen der Tilapien *in vitro*. Die Hypothese, dass das Level des LH eine Auswirkung auf das Verhältnis der Geschlechter hat, konnte nicht bestätigt werden. Es wurde herausgefunden, dass Saponine in der Nahrung den Cholesterinspiegel im Serum und im Muskel beeinflussen. Unsere Ergebnisse stehen im Widerspruch zu vorangegangenen Studien mit anderen Tieren, bei denen von einer durch Saponin-verursachten Abnahme von Cholesterin im Serum berichtet wurde. Ausgewachsene Tilapien - aber nicht junge Tilapien, die mit einem Saponingehalt von 300 mg kg^{-1} im Futter gefüttert wurden, zeigten signifikant höhere Cholesterinwerte im Muskel als die Kontrolltiere. In den jungen männlichen Tilapien stiegen bei Saponingehalten von 0 bis 700 mg kg^{-1} die

durchschnittlichen Cholesterinwerte im Serum an. Bei jungen weiblichen Tilapien wurde dieser Trend nicht festgestellt.

Ein Experiment mit einem Butanolextract von *Yucca schidigera* zeigte, daß 50 und 100 mg kg^{-1} dieser steroidalen Saponine zu keiner verbesserten Futterverwertung, keiner höheren Wachstumsrate und keiner erhöhten Energieverwertung bei Karpfen führte. Während der ersten vier Wochen zeigte die 100 mg kg^{-1} Gruppe jedoch ein schnelleres Wachstum und eine geringere metabolische Rate im Vergleich zur Kontrollgruppe. Es wurde versucht die chemische Struktur der Saponine zu untersuchen, welche die beobachteten Effekte verursachte. Die aufgereinigte (durch Säulenchromatographie) hämolytische Fraktion der triterpenoiden *Gypsophila* Saponine zeigte in einem Vorversuch bis zu einem Level von 250 mg kg^{-1} keine toxischen Effekte. Das Wachstum von Karpfen war normal. Sehr geringe Konzentrationen dieser aufgereinigten Fraktion (5 mg kg^{-1}) zeigten jedoch eine wachstumsfördernde Wirkung.

Zur Zeit noch andauernde Studien zeigen, dass QS keine strukturellen Veränderungen der intestinalen Mucosa von Tilapien verursachen. Daraus wurde geschlossen, daß QS (die als ‚Generally Recognised As Safe' (GRAS) in den USA anerkannt sind) wachstumsfördernde Effekte auf Karpfen und Tilapien ausüben. Diätetische Ergänzung mit QS könnte auch dazu beitragen, das Problem der Überproduktion von Jungfischen in der Tilapienkultur abzumildern. Weitere Arbeiten sind jedoch notwendig, um die biologischen Effekte der QS im Detail zu untersuchen sowie Tests, ob Saponine aus anderen Quellen ähnliche Effekte haben. Sobald diese ermittelt wurden, können biochemische Modelle erstellt werden, um die Wirksamkeit von aufgereinigten Saponinen abzuschätzen und Empfehlungen für die Praxis abzuleiten.

Erklärung

Hiermit versichere ich, diese Arbeit selbständig angefertigt und keine anderen, als die angegebenen Hilfsmittel verwendet zu haben. Zitate sind im Text kenntlich gemacht. Diese Arbeit ist noch nicht in dieser oder anderer Form einer Prüfungsbehörde vorgelegt worden.

Stuttgart – Hohenheim, den 15 Juni 2001 George Francis