

United Soybean Board Domestic Programs Report Form

Project # and Title	USB #1730-352-0504 Effects of soy proteins on bile acid and taurine status in fish
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1. Introduction

Despite decades of research and millions of dollars spent on soybean use in fish feeds, the anti-nutrients in soy protein products that cause enteritis and alter bile acid metabolism in the liver have not been fully identified. Most research has focused on isolating and evaluating specific anti-nutrients that also affect terrestrial animals, such as saponins because they cause enteritis in fish. However, marine fish, such as yellowtail (*Seriola spp.*) and red sea bream (*Pagrus major*), develop “green liver condition” characterized by patchy areas of green pigment on the liver visible at necropsy when fed high-soy diets, unlike terrestrial animals and freshwater fish (Watanabe et al., 1998; Goto et al., 2001a; Takagi et al., 2005; Takagi et al., 2008). The green liver is associated with impaired bile acid metabolism, increased production of hemolytic biliverdin and reduced excretion of bile pigments (ditaurobilirubin) from the liver into bile (Goto et al., 2001a; Takagi et al., 2006). This condition is corrected by dietary supplementation of taurine or bile salts, or by increasing the levels of taurine-rich animal proteins in diets. Plant-derived feed ingredients, including plant proteins, do not contain taurine. Most vertebrates have the capacity to synthesize taurine from sulfur-amino acids such as methionine and cysteine in sufficient quantities to supply physiological needs via the actions of cysteine dioxygenase (CDO), cysteine sulfinate decarboxylase (CSD) and putative but yet uncharacterized hypotaurine dehydrogenase (Sumizu, 1962; Griffith, 1987; Huxtable, 1989). However, this capacity is limited in most marine fish species due to low or no CSD activity (Goto et al., 2001b; Yokoyama et al., 2001). As a result, marine fish species require a dietary source of taurine, provided either by fishmeal or animal protein meals or by direct supplementation. The essentiality of taurine and its role in the fish growth and health has been reviewed in detail by El-Sayed (2014) and Salze and Davis (2015).

Bile acids are synthesized in the liver from cholesterol, which is conjugated with taurine or glycine, and stored in the gallbladder (Danielsson and Sjoval, 1975). They are released into the small intestine to mix with digesta coming from the stomach after a meal. Conjugated bile acids solubilize dietary lipids, sterols and fat-soluble vitamins by forming chylomicrons which are readily absorbed by enterocytes in the lumen. Bile acids are reabsorbed in the ileum and returned to the liver via the hepatic portal vein, a process called enterohepatic recycling. This recycling is the main source of cholesterol and taurine used in the liver to produce conjugated bile acids, and the recovery is about 95%

in humans. Bile acid recycling in fish is thought to be equally important for normal digestion and metabolism of lipids and fat-soluble vitamins.

In vitro studies indicate that soybean seeds contain bile acid-binding proteins that potentially sequester bile acids in the intestine, removing them from the body (Makino et al., 1988; Nagaoka et al., 1997; Choi et al., 2002). Most studies determining the essentiality of taurine in fish utilize diets that contain very low levels of animal proteins and moderate to high levels (20%-60%) of soybean meal or soy protein concentrate. Substantial evidence suggests that the high dietary need for taurine reported for many marine fish is an artifact of the effect of high levels of soy protein on bile acid metabolism. In fish feeding trials where the effect of varied taurine levels was evaluated by measuring plasma cholesterol, bile acid, and taurine levels, these compounds were significantly reduced in fish fed soy-based diets, indicating bile acid binding in the intestine and reduction of intestinal reabsorption of bile acids via enterohepatic recycling (Nguyen et al., 2011; Kortner et al., 2013; Murashita et al., 2013). A high molecular-weight fraction (HMF) in soy protein was hypothesized to be responsible for impaired bile acid metabolism. In fact, a HMF derived from soy protein isolate increased fecal excretion of bile and nitrogen in rats; feces contained indigestible protein/peptide which bound to bile acids by hydrophobic amino acids (Higaki et al., 2006). Choi et al. (2002) showed that a six-amino acid residue (VAWWMY: Val-Ala-Trp-Trp-Met- Tyr) present in soy glycinin had high bile acid-binding capacity in vitro. In the first ever in vivo study, Nagaoka et al. (2010) showed that the peptide VAWWMY, termed "soystatin", was responsible for bile acid-binding and inhibition of cholesterol absorption in rats. The bile acid-binding ability of soystatin was roughly equivalent to that of cholestyramine, a drug that sequesters bile acids in the intestine and lowers plasma cholesterol in humans suffering from high plasma cholesterol.

There has little need to investigate dietary taurine on growth performance or bile acid metabolism in livestock and poultry because these species have the capacity to synthesize taurine to meet their physiological needs. Similarly, the need for dietary taurine by farmed fish was not recognized for decades because freshwater species can synthesize taurine de novo and most fish feeds contained fishmeal, a rich source of taurine. It was only with the expansion of marine fish culture and a shift in diet formulation toward higher soy product use that the dietary need for taurine became apparent. The major sign of taurine deficiency in marine fish, green liver condition, first appeared after the Japanese yellowtail industry switched from high fishmeal to high soy feeds in the early 1990s. Although taurine deficiency signs were alleviated by supplementing high soy diets with taurine, much higher levels of taurine were needed to overcome taurine deficiency in marine species, mainly yellowtail, than more recent studies indicate is necessary. This may be related to the bile acid binding properties of soy proteins. The specific fractions of soy antigens responsible for altering cholesterol and bile acid metabolism in marine fish have not been determined. Research to identify these fractions may now be possible with identification of the soy peptide soystatin. We hypothesize that taurine reserves in fish fed high soy diets and bile acid-binding compounds such as cholestyramine and soystatin will be depleted even if taurine is being

synthesized from precursor sulfur-amino acids, resulting in reduced growth and altered bile acid metabolism. The goal of this project is to determine the effects of soystatin on bile acid and taurine metabolism.

Rainbow trout are a commercially important species in USA and abroad, and most of their nutrient requirements have been established (NRC, 2011). Repeated studies in our laboratory show that trout can tolerate a moderate level of soy proteins in the diet for several months without developing enteritis and have high taurine biosynthetic ability compared to marine fish. Moreover, many of the genes associated with cholesterol and bile acid metabolism have been identified and measured in trout in our laboratory. Therefore, rainbow trout is an excellent model species to test the interference of soy protein fractions in the bile acid and taurine metabolism. The project will develop a model for bile acid metabolism-interference and taurine deficiency using cholestyramine, determine if soystatin exerts the same effect, and assess their effects when added to trout diets on cholesterol, taurine and sulfur-amino acid status of rainbow trout using physiological and genomic tools. The marine aquaculture sector is a rapidly developing industry in the USA and abroad. It has the potential to become a very large and high value global market for USA soy products, provided that soy processing methods or other means to overcome the deleterious effects of soy protein antigens can be developed.

1. Materials and methods

Experimental diets: Soystatin (SS) peptide of 95% purity was purchased from Thermo Fisher Scientific Inc., Rockford, IL. Cholestyramine (CHOL) was purchased from a local pharmacy. Experimental diets were formulated as follows:

1. Diet 1 (FM): Fishmeal-based control diet
2. Diet 2 (FM+SS): Diet 1 with 0.03% soystatin corresponding to 30% soybean meal
3. Diet 3 (FM+CHOL): Diet 1 with 3.5% cholestyramine
4. Diet 4 (SBM): Diet containing 30% soybean meal (negative control)
5. Diet 5 (SBM+T): Diet 4 supplemented with taurine equivalent to levels in Diet 1

Diets were formulated to contain 38% digestible protein and 17.5 MJ/kg digestible energy, and meet the nutrient requirements of rainbow trout (NRC, 2011). Pollock oil was increased in Diets 4 and 5 to increase the cholesterol levels of these diets to those of diets containing fishmeal. Diet mixtures were cold pelleted at the University of Idaho's Hagerman Fish Culture Experiment Station (HFCES) using a laboratory-scale California pellet mill fitted with a 2.4-mm die. Feeds were dried and stored at ambient temperature (20-22 °C). Samples of the diets were collected for chemical analyses.

Fish, feeding and sampling: Rainbow trout eggs (TroutLodge, Sumner, WA) were hatched and reared using commercial diets for three months at the HFCES. Thirty fish (initial body weight: 15- 20 g) was into each of 15, 145-L tanks. Each tank was supplied with 8-10 L/min of constant temperature (15 °C) spring water fed by gravity to the fish rearing laboratory. Each diet was assigned randomly to three tanks in a completely randomized design. Fish were hand-fed to apparent satiation three times per day, six days per week for eight weeks. Photoperiod were maintained at 14 h light: 10 h dark with fluorescent lights controlled by electric timers. At the end of eight weeks, 46-hour postprandial, four fish per tank were anesthetized with tricaine methanesulfonate (MS-222, 100 mg/L, buffered to pH 7.0). Blood was collected from the caudal vessels of fish with 1-ml syringes fitted with 25G 3/4-inch needle and allowed to clot for 30 min, then centrifuged at 1000 g for 8 minutes to collect serum for cholesterol and taurine analysis. Upon euthanizing those fish with MS-222 (200 mg/L, buffered to pH 7.0), liver was excised to measure levels of cholesterol, bile acid and bilirubin. Intestine were removed for analysis of cholesterol and bile acid. Intestinal digesta were collected for cholesterol, bile acid and bilirubin analysis. Gall bladder were excised to measure bile acid, bilirubin and biliverdin. A sample of white muscle was collected for amino acids including taurine analysis. Another four fish per tank were euthanized to remove liver and distal intestine for gene expression analysis. Tissue samples were snap-frozen in liquid nitrogen and stored at -80 °C until analysis. All fish handling and sampling, plus the experimental protocols used in this project were approved in advance by the University of Idaho's Institutional Animal Care and Use Committee (IACUC).

Chemical analyses: Frozen whole-body fish were partially thawed, chopped and made into a puree by a food processor. Samples of soybean meal feed, and whole-body were analyzed for proximate composition and energy using modified AOAC (2002) (Villasante et al. (2015). Bile acids in liver, gallbladder, and digesta were determined using total bile acids and bilirubin assay kits (Cell Biolabs, Inc., San Diego, CA). Total cholesterol in serum was analyzed with total cholesterol assay kit (Cayman Chemical IncAnn Arbor, MI). Liver and digesta samples were extracted with the mixture of chloroform: Isopropanol: NP-40 and the lipid solution were analyzed for total cholesterol as for serum. Serum samples were deproteinized with a centrifugal filter unit (Amicon Ultra 0.5ml 10K) for taurine analysis. Taurine from tissue samples was extracted using 70% ethanol (Spitze et al., 2003; Gormley et al., 2007). The extracted material was centrifuged, and the liquid fraction then was derivatized and analyzed by a Biochrom 30+ amino acid analyzer (Biochrom US, Holliston, MA).

RNA isolation and real-time quantitative PCR: RNA from each tissue was isolated by homogenizing the tissue in TRIzol (Invitrogen, Carlsbad, CA). The protocol recommended by Qiagen was followed for the rest of the RNA isolation. Extracted RNA was quantified and treated with DNase, and 1 µg were the reverse- transcribed following the methods of the manufacturer (BioRad, Hercules, CA).

Real-time quantitative PCR was carried out using the CFX96 Real-Time System (BioRad Laboratories, Hercules, CA), in a 10µl total volume, using iTaq SYBR Green Supermix

(BioRad), and with 500nmol primers according to the protocol provided by the manufacturer. PCR cycling conditions for all genes were as follows: 95 °C for 5s followed by 55 °C for the 30s over 40 cycles with an initial denaturation step of 95 °C for 3min. For each fish, PCR reactions were run in duplicate on RNA samples.

Relative expression values for genes involved in bile acid metabolism (*cyp7a1*, *fxr*, and *hmgcr*) and taurine metabolism (*cdo* and *csd*) were determined by including five serial dilutions of a standard (pooled from each experimental sample for a given tissue).

Mean cycle threshold values (Ct values) for each target gene were normalized with mean GAPDH Ct values. Primer PCR efficiencies were calculated and utilized for PCR correction for all primer pairs (Pfaffl, 2001). Normalized data were analyzed using the relative quantification method described by Pfaffl (2001).

Calculations: Growth performance and feed utilization of fish were evaluated using conventional indices (Hardy and Barrows, 2002), such as specific growth rate, thermal growth unit coefficient, feed conversion ratio, protein efficiency ratio, nutrient retention efficiency.

Data Analysis: Tank means were used for statistical analysis. Fish growth and feed utilization indices, physiological parameters, and gene expression data were tested for normality and homogeneity of variance prior to one-way Analysis of Variance (ANOVA). If significant differences were found, data were subjected to Tukey's HSD test to separate the means at a significance level of $P < 0.05$. All the statistical analyses were conducted with SAS Version 9.3 software.

2. Results

Diets: The proximate composition and selected nutrient composition of the five experimental diets used in the growth trial is presented in Table 1. All diets were formulated to contain similar amounts of cholesterol. Soystatin was added to diet 2 at a level calculated to be similar to the amount present in diets containing soybean meal. The amount of cholestyramine added to diet 3 was approximately double that used to treat hypercholesterolemia in human medicine, expressed on a mg/kg body weight, to ensure a treatment effect would occur. Analyzed proximate composition and energy levels of the diets was similar to expected values and exhibited relatively minor variation among diets. Taurine levels in diets ranged from 0.19 % (Diet 4) to 2.21 % (Diet 5). Analyzed cholesterol levels in diets were similar, ranging from 1251 mg/kg (Diet 4) to 1352 mg/kg (Diet2). Minor variation in composition of experimental diets is common and reflects variability in results of chemical analysis as much as actual differences among diets.

Growth trial: Rainbow trout juveniles were fed diets containing different protein sources (fishmeal and soybean meal), soystatin, cholestyramine and taurine for eight weeks. The growth performance and feed utilization of the fish are presented in Table 3. Fish fed the

Fishmeal + Cholestyramine diet (Diet 3) had significantly lower ($P < 0.05$) final weight (49.7 g/fish) than the fish fed all other diets (69.2 -71.7 g/fish), but there was no significant difference ($P > 0.05$) among the other diets. The performance of the fish for mean weight gain, SGR and PER followed the same trend as that for final weight. FCR of diets 1, 2, 4 and 5 was significantly better than that of diet 3. It did not vary among the diets 1, 2, 4 and 5. Survival of fish varied from 97.8 % (Diet 5) to 100% (Diet 2, 3 and 4).

Whole-body proximate composition and nutrient retention: Whole-body proximate composition and nutrient retention of rainbow trout juveniles fed the experimental diets are presented in Table 4. Dry matter of fish whole-body varied from 23.8% (Diet 3) to 29.9% (Diet 4). Crude protein of fish whole-body ranged from 52.9% (Diet 4) to 72.2% (Diet 3) on dry-matter basis. Crude fat of fish whole-body ranged from 18.3% (Diet 3) to 39.1% (Diet 4) on dry-matter basis. Ash content of fish whole-body ranged from 7.15% (Diet 4) to 10.04% (Diet 3) on dry-matter basis. Gross energy of fish whole-body ranged from 23.5 cal/g (Diet 2) to 27.7 cal/g (Diet 4 and 5) on dry-matter basis. Whole-body protein, ash and muscle taurine level were significantly higher in fish fed the diet 3 than in fish fed other diets whereas whole-body dry matter, crude fat and gross energy were significantly lower in fish fed diet 3 than in fish fed other diets. However, it was not different among the fish fed rest of the diets ($P > 0.05$). Protein retention in fish ranged from 24.7% (Diet 3) to 33.9% (Diet 2). Lipid retention ranged from 11.4% (Diet 3) to 59.2% (Diet 4) whereas energy retention varied from 15.9% (Diet 3) to 37.7% (Diet 2 and 4). Protein, lipid and energy retention of fish fed diet 3 was significantly lower than those of fish fed other diets. However, it was not different among the fish fed rest of the diets ($P > 0.05$).

Chemical analysis:

The results of the chemical assessment of serum, tissues, and digesta are presented in Table 5. Total cholesterol in serum, liver, and digesta of fish fed diet ranged from 2.77 mmol/L (Diet 3) to 4.87 mmol/L (Diet 4 and 5), 203 $\mu\text{mole/g}$ (Diet 2 and 5) to 230 $\mu\text{mole/g}$ (Diet 3) and 89.8 $\mu\text{mole/g}$ (Diet 4) to 228 $\mu\text{mole/g}$ (Diet 3) respectively on wet-matter basis. Serum cholesterol of fish fed diet 3 was significantly lower than fish fed other diets and cholesterol in digesta of fish fed diet 4 was significantly lower than those of fish fed diet 1, 2 and 3. However, it was not different among the fish fed rest of the diets. Total cholesterol in the liver of the experimental fish did not vary significantly among the dietary treatments ($P > 0.05$). The taurine level in serum and muscle of fish fed diet ranged from 107 $\mu\text{mole/L}$ (Diet 4) to 174 $\mu\text{mole/L}$ (Diet 5), and 0.21% (Diet 4) to 0.33% in Diets 3 and 4, respectively. Serum taurine level was significantly lower in fish fed Diet 4 than in fish fed Diet 5. However, it was not different among the fish fed rest of the diets. Total bile acid of digesta was significantly lower in fish fed Diet 3 than in fish fed other diets. However, it was not different among the fish fed the other diets. Total bile acid in the liver ranged from 0.77 $\mu\text{mole/g}$ (Diets 1 and 3) to 0.83 $\mu\text{mole/g}$ (Diet 2), in the gall bladder from 52.3 mmole/g (Diet 3) to 66.8 mmol/g (Diet 4), and in digesta from 4.76 $\mu\text{mole/g}$ (Diet 3) to 11.4 $\mu\text{mole/g}$ (Diet 2) on wet-matter basis. Digesta bile acid of fish fed diet 3 was significantly lower than fish fed other diets. However, it was not different

among the fish fed the rest of the diets. Total bile acid in liver and gall bladder of the experimental fish did not vary significantly among the dietary treatments ($P>0.05$). Total bilirubin in the liver, gall bladder and digesta of the experimental fish did not vary significantly among the dietary treatments ($P>0.05$). Total bilirubin levels in liver, gall bladder and digesta varied from 129 nmol/g to 146 nmol/g, 203 nmol/g to 239 nmol/g and 131 nmol/g to 156 nmol/g on a wet-matter basis.

Whole body and muscle amino acid analysis revealed minor but interesting differences among dietary treatment groups (Tables 6 and 7). Whole body taurine level was increased by dietary taurine supplementation of the soybean meal-based diet (Diet 5) but it was not reduced by cholestyramine supplementation or by feeding the soy-based diet without taurine supplementation. No other notable differences were measured, other than a general elevation of all amino acids in the whole body samples associated with reduced fat levels in fish fed Diet 3, the fishmeal plus cholestyramine diet. Muscle taurine increased when taurine was supplemented to the soybean meal diet, as expected. However, in contrast to the results of whole body analysis, fish fed the soybean meal diet (Diet 4) had lower muscle taurine levels.

Gene expression:

Relative gene expression in the liver and intestine of rainbow trout fed experimental diets is presented in table 6. Cyp7a1 expression in liver varied from 0.90 (Diet 5) to 1.00 (Diet 1). It was significantly lower in fish fed Diet 5 than Diets 1 and 3. However, no significant difference was observed among the other diets. Hmgcr expression in liver from 0.95 (Diet 2) to 1.19 (Diet 4). Hmgcr of fish fed Diet 4 was significantly higher than that of fish fed Diet 2. However, it was not different among the fish fed rest of the diets. Cdo and csd expression in liver varied from 0.78 (Diet 3) to 1.00 (Diet 1), 0.91 (Diet 3) to 1.00 (Diet 1). Cdo and csd expression of fish fed diet 3 were significantly lower than fish fed diet 1. However, it was not different among the fish fed rest of the diets. Fxr expression in intestine varied from 0.97 (Diet 2) to 1.01 (Diet 5). There were no significant differences among fish fed all experimental diets in Fxr expression.

3. Discussion

The study was designed to assess effects of diet on aspects of bile acid metabolism, with the expectation that soy-based trout diets would result in alterations of taurine status associated with disruption of enterohepatic recycling of bile acids. A novel experimental approach used in the study involved supplementing a fishmeal-based diet with the serum-lowering drug cholestyramine and with the soy protein soystatin. The goal of the project was to determine if this model could provide insight into alterations of bile acid metabolism in fish similar to observations in dogs and cats fed diets containing high levels of plant-based feed ingredients. Cholestyramine binds with bile acids in the digesta in the small intestine in humans, preventing bile acids from being absorbed by intestinal cells (enterocytes) and being returned to the liver by the hepatic portal vein for reprocessing and secretion into the gall bladder. Enterohepatic bile acid recycling

recovers over 95% of cholesterol secreted as bile acids into the intestine. If this recycling pathway is blocked with cholestyramine, the liver compensates by removing cholesterol from the blood to synthesize new bile acids. Since bile acids contain taurine, it was expected that adding cholestyramine would interfere with taurine recycling and this would be detected by assaying serum taurine levels, whole body taurine levels, bile acid and bilirubin levels in the liver and gall bladder. In addition, since rainbow trout possess the capacity to synthesize taurine from cysteine, gene expression of enzymes in the pathway of taurine synthesis would be up-regulated and therefore show higher expression levels. Soystatin was added to Diet 2 at a level matching the amount present in Diets 4 and 5 associated with soybean meal.

Adding cholestyramine to the fishmeal diet containing adequate levels of cholesterol and taurine caused significant reductions in weight gain, whole body fat level and protein efficiency ratios in rainbow trout. Reduced fish growth and whole body fat levels are likely associated with interference with digestion of dietary fat through interference with production of micelles by bile acids in the distal intestine. Similar effects have been noted in other species of fish fed high soy diets. Serum cholesterol levels and digested cholesterol levels were also significantly reduced when cholestyramine was added to the diet. However, liver and intestinal cholesterol levels were not affected. Cholestyramine did not affect total bile acid or bilirubin levels in liver or gall bladder. Accumulation of these compounds in the liver of fish when bile acid production is affected by taurine deficiency causes 'green liver' condition in some species of marine fish, a visible manifestation that is easily detected upon gross physical examination. Green liver condition is a marker for taurine deficiency in marine fish species that, as a whole, have limited capacity to synthesize taurine from the amino acid cysteine, which in turn is synthesized from methionine. Rainbow trout, in contrast, have active taurine biosynthesis capacity and do not exhibit green liver or any other specific sign of taurine deficiency when taurine levels in the diet are low. Adding soystatin to the fishmeal diet had no effect on any measured parameter, demonstrating that the effects of soybean meal on cholesterol and taurine status in trout at normal levels used in commercial feeds are not associated with soystatin. It is possible that at higher pharmacological doses, soystatin has an effect. Further research using higher levels of soystatin will be required to answer this question.

The role of soybean meal in taurine status of fish is complicated and controversial. Soy proteins, like all plant-derived feed ingredients, do not contain taurine whereas animal or fish-derived feed ingredients are rich sources of taurine. Formulating feeds with 30% fishmeal or a combination of fishmeal and land animal protein sources, such as poultry byproduct meal, will provide $>2\text{mg taurine kg}^{-1}$ diet, a level shown in previous studies to be sufficient to support normal trout growth (Gaylord et al., 2006). All-plant protein trout feeds do not contain taurine and several studies have shown that supplementing $5\text{ mg taurine kg}^{-1}$ diet restored trout growth rates (Gaylord et al., 2006; 2007). This finding contradicts results of Yokoyama and Nakazoe (1992) who reported that muscle taurine in rainbow trout increased when fish were fed a very low taurine diet based upon casein without plant proteins and supplemented with methionine or cysteine. A later study by

Yamamoto et al. (1996) confirmed that rainbow trout fed a plant-based diet supplemented with amino acids resulted in increased whole body taurine, supporting the contention that rainbow trout possessed sufficient biosynthetic capacity to meet their needs for taurine when fed a taurine-deficient diet. The main differences that could explain the contradictory findings of Gaylord et al. (2006; 2007) and Yokoyama and Nakazoe (1992) and Yokoyama et al. (1996) are diet formulation and the fish used in the studies. Gaylord et al. used small juvenile trout while Yokoyama's studies used larger fish.

Gene expression results yielded inconclusive results of the effects of soystatin and soybean meal on bile acid recycling and bile acid synthesis, although *hmgcr* expression was elevated in livers of fish fed the soybean meal diet. This gene is involved in bile acid synthesis and its elevated expression suggests that bile acid recycling was impacted by this diet, leading to higher bile acid synthesis (Kortner et al., 2013). However, expression results for *fxr* were not affected by diet. This gene is associated with bile acid metabolism and recycling in the intestine. Thus, these two genes gave conflicting results. Cholestyramine reduced expression levels of *cdo* and *csd* in the liver. Both genes code for enzymes in the pathway of taurine biosynthesis from cysteine. If taurine levels in the liver were reduced by diet, these enzymes would be expected to increase in activity. Gene expression levels are affected by time of sampling after a meal, generally rising and falling back to baseline levels over a period of time after feeding. Fish in this study were sampled 46 hours after feeding and it is possible that expression levels of the genes measured in this study had returned to baseline levels after having been up-regulated or down-regulated in the 12-24 hours after feeding in response to enterohepatic recycling and other mechanism.

Muscle tissue in fish represents the largest taurine pool in the body and in the present study, muscle taurine levels were reduced in fish fed the soybean meal diet. This suggests that taurine reserves were being utilized to supply taurine for bile acid synthesis to compensate for reduced enterohepatic recycling of bile acids in this dietary treatment, thereby supporting the hypothesis that the taurine level in the soybean meal diet (0.19%) was insufficient to maintain body stores. Muscle levels of taurine in fish fed the fishmeal diets were equivalent among dietary treatment groups. Dietary taurine in these treatment groups was 0.3%, indicating that this dietary level was sufficient to maintain body stores. These results do not, however, support the conclusion that that rainbow trout require 0.3% taurine in the diet because lower taurine levels in a fishmeal-based diet were not tested. Until dietary levels below 0.3% are tested in a diet without higher soy protein levels, any conclusion about a dietary taurine requirement of rainbow trout is premature. Further, only a single level of taurine was supplemented to the soy-based diet. Based on these results, we can conclude that rainbow trout fed a high-soy diet without fishmeal or animal protein meals require more than 0.19% taurine and less than 0.5% taurine to maintain whole body and/or muscle taurine levels. Further research is needed to refine this estimate of the dietary taurine needed by rainbow trout in low and high soy diets and to determine if soy interferes with enterohepatic recycling of bile acids (and cholesterol + taurine) in fish to an extent requiring dietary intervention.

Conclusions

The results of this study demonstrated that rainbow trout bile acid responses to a high soy diet are complex. The study failed to demonstrate an effect of soystatin on serum cholesterol or taurine levels, whereas cholestyramine had a profound effect on serum cholesterol levels, fish growth, bile acid levels in digesta and whole body fat levels. This demonstrates that feed constituents that interfere with bile acid recycling can cause major disruption of bile acid metabolism. Results were inconclusive as to the potential confounding effect of dietary soybean meal on taurine metabolism in fish.

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Table 1. Ingredient and nutrient composition of the experimental diets fed to rainbow trout juveniles over an 8-week growth trial (% , as-fed basis)

Ingredients	Diet 1	Diet 2	Diet 3	Diet 4
	Fishmeal	Fishmeal + Soy statin	Fishmeal + Cholestyramine	Soybean meal
Fishmeal, sardine	30.00	30.00	30.00	10.00
Poultry by product meal	8.00	8.00	8.00	8.00
Blood meal, spray dried	2.00	2.00	2.00	2.00
Corn protein concentrate (Empyreal 75)	10.97	10.97	10.97	12.00
Soy protein concentrate (Profine VF)	5.00	5.00	5.00	5.00
Soybean meal, dehulled and solvent extracted	0.00	0.00	0.00	30.00
Wheat gluten meal	5.00	5.00	5.00	5.00
Wheat flour	20.05	20.02	16.55	5.00
L-aspartic acid	2.12	2.12	2.12	2.12
DL-methionine	0.00	0.00	0.00	0.00
L-lysine HCl	0.37	0.37	0.37	0.37
Taurine	0.00	0.00	0.00	0.00
Soystatin (peptide)	0.00	0.03	0.00	0.00
Cholestyramine (70%)	0.00	0.00	3.50	0.00
Dicalcium phosphate	0.90	0.90	0.90	2.00
Trace mineral mix, Trouw Nutrition ¹	0.10	0.10	0.10	0.10
Vitamin Premix, ARS 702 ²	1.00	1.00	1.00	1.00
Choline chloride (60%)	0.60	0.60	0.60	0.60
Vitamin C (Stay C, 35%)	0.20	0.20	0.20	0.20
Pollock oil	5.69	5.69	5.69	14.00
Canola oil	8.00	8.00	8.00	0.00
<i>Nutrients (analyzed values, DM basis)</i>				
Dry Matter (%)	93.12	92.88	91.99	92.00
Crude protein (%)	48.46	48.24	48.99	48.00
Crude fat (%)	20.46	20.85	20.82	21.00
Ash (%)	9.37	9.96	9.70	8.00
Gross energy (MJ/kg)	22.9	22.9	23.0	22.00
Taurine (%)	0.30	0.30	0.29	0.30
Lysine (%)	3.00	2.99	2.93	3.00
Methionine (%)	1.06	1.06	1.04	1.00
Cholesterol (mg/kg)	1322	1352	1318	1200

¹Trace mineral premix supply the following to the diet (mg/kg diet): Zn (as ZnSO₄ 7H₂O), 50; Mn (as MnSO₄), 7.5; Cu (as CuSO₄ 5H₂O), 2.5; I (as KIO₃), 1; selenium, 0.05.

² Vitamin premix supply the following to the diet (mg/kg diet): D calcium pantothenate, 46.47; pyridoxine (pyridoxine HCl), 13.68; riboflavin, 9.58; niacinamide, 21.78; folic acid, 2.49; thiamine (thiamine mononitrate), 9.1; inositol, 599; biotin, 0.33; vitamin B₁₂, 0.03; menadione sodium bisulfite complex, 1.1; vitamin E (DL α -tocopherol acetate), 131.9 IU; vitamin D₃ (stabilized), 6594 IU; vitamin A (vitamin A palmitate, stabilized), 9641 IU; ethoxyquin, 198.

Table 3. Growth performance and feed utilization of rainbow trout juveniles fed experimental diets for 8 weeks^{1,2}

	Diet 1	Diet 2	Diet 3	Diet 4
	Fishmeal	Fishmeal + Soy statin	Fishmeal + Cholestyramine	Soybean
Initial weight (g/fish) ³	8.31±0.15	8.35±0.15	8.39±0.05	8.35±0.08
Final weight (g/fish)	70.8±1.53 ^a	69.2±1.54 ^a	49.7±1.42^b	71.7±0.93 ^a
Weight gain (g/fish)	62.5±1.38 ^a	60.9±1.42 ^a	41.3±1.47^b	63.4±0.85 ^a
Mean weight gain (%)	752±3.69 ^a	729±11.8 ^a	492±20.3^b	759±3.02 ^a
Specific growth rate (%/day)	3.82±0.01 ^a	3.78±0.03 ^a	3.17±0.06^b	3.84±0.01 ^a
Survival (%)	98.9±1.11	100	100	100
Average feed intake (g, DM/fish)	65.9±1.02	63.0±1.59	61.3±0.91	65.9±1.07
Feed conversion ratio	1.06±0.01 ^b	1.03±0.03 ^b	1.49±0.06^a	1.04±0.02 ^b
Protein efficiency ratio	2.01±0.03 ^a	2.12±0.01 ^a	1.42±0.05^b	2.07±0.07 ^a

¹Mean±SE (n=3) in the same row that share the same superscript are not statistically different ($P>0.05$; Completely Randomized Design, One-factor ANOVA; Tukey's HSD test).

²All calculations were performed on an average fish weight basis.

³ Each treatment group consisted of 90 fish (3 tanks, 30 fish per tank).

Table 4. Whole-body proximate composition (% wet basis), taurine and nutrient retention (%) of rainbow trout juveniles fed experimental diets for 8 weeks^{1,2}

	Initial fish ³	Diet 1 Fishmeal	Diet 2 Fishmeal + Soy statin	Diet 3 Fishmeal + Cholestyramine	Diet 4 Soybean
<i>Proximate composition</i>					
Dry matter (%)	24.5±0.13	28.9±0.16 ^b	29.5±0.23 ^{ab}	23.8±0.32^c	29.9±0.21 ^a
Crude protein (%)	14.4±0.08	16.0±0.09 ^b	15.8±0.16 ^{bc}	16.8±0.07^a	15.8±0.19 ^{bc}
Crude fat (%)	7.39±0.04	10.6±0.20 ^b	11.4±0.17 ^{ab}	4.27±0.37^c	11.7±0.32 ^{ab}
Ash (%)	2.06±0.01	2.24±0.06 ^{ab}	2.12±0.07 ^b	2.33±0.02^a	2.13±0.04 ^b
Gross energy (MJ/kg)	6.27±0.05	7.85±0.08 ^b	8.13±0.08 ^{ab}	5.48±0.14^c	8.29±0.10 ^a
<i>Taurine (%)</i>					
	0.48±0.01	0.45±0.01 ^a	0.44±0.01 ^a	0.57±0.01^b	0.45±0.003 ^a
<i>Nutrient retention</i>					
Protein (%)	-	32.7±0.19 ^a	33.9±0.52 ^a	24.7±0.96^b	33.1±1.48 ^a
Lipid (%)	-	55.2±1.70 ^a	58.9±1.31 ^a	11.4±1.94^b	59.2±3.03 ^a
Energy (%)	-	35.2±0.39 ^a	37.7±0.53 ^a	15.9±0.34^b	37.7±1.32 ^a
Taurine (%)	-	42.4±0.35 ^b	42.6±1.45 ^b	31.3±2.22 ^c	66.5±1.79 ^a

¹Mean±SE (n=3) in the same row that share the same superscript are not statistically different ($P>0.05$; Completely Randomized Design, One-factor ANOVA; Tukey's HSD test).

²Five fish from each tank were used for whole-body analysis.

³Values for initial fish are for reference only; they were not used for statistical analysis.

Table 5. Chemical parameters of serum, liver, gall bladder, digesta and muscle of rainbow trout juveniles fed experimental diets for 8 weeks^{1,2}

	Diet 1	Diet 2	Diet 3	Diet 4
	Fishmeal	Fishmeal + Soy statin	Fishmeal + Cholestyramine	Soybean
<i>Total cholesterol</i>				
Serum (mmol/L)	4.74±0.23 ^a	4.77±0.24 ^a	2.77±0.23^b	4.87±0.10 ^a
Liver (µmole/g wet)	224±17.2	203±7.55	230±16.3	228±29.0
Intestine (µmole/g wet)	291±10.9	295±7.66	258±7.54	293±7.70
Digesta (µmole/g wet)	203±10.7 ^a	199±27.8 ^a	228±22.1 ^a	89.8±6.81^b
<i>Taurine</i>				
Serum (µmol/L)	142±10.6 ^{ab}	144±17.8 ^{ab}	137±12.5 ^{ab}	107±7.27^b
Muscle (% dry weight)	0.26±0.01 ^{bc}	0.28±0.01 ^{bc}	0.33±0.02 ^b	0.21±0.03 ^c
Muscle (% wet weight)	0.07	0.07	0.08	0.05
<i>Total bile acid</i>				
Liver (µmol/g wet)	0.77±0.09	0.83±0.13	0.77±0.06	0.78±0.10
Gall bladder (mmol/g wet)	64.3±2.22	65.6±3.02	52.3±6.88	66.8±5.32
Digesta (µmol/g wet)	10.7±0.26 ^a	11.4±1.88 ^a	4.76±1.29^b	9.66±1.49 ^a
<i>Total bilirubin</i>				
Liver (nmol/g wet)	146±7.62	134±11.4	136±13.0	136±8.89
Gall bladder (nmol/g wet)	211±27.4	216±14.1	239±20.5	209±13.8
Digesta (nmol/g wet)	156±13.3	143±13.0	150±6.65	135±6.89

¹Mean±SE (n=12 fish per treatment) in the same row that share the same superscript are not statistically different ($P>0.05$; Completely Randomized Design, One-factor ANOVA; Tukey's HSD test).

²Four fish from each tank were used for chemical analysis.

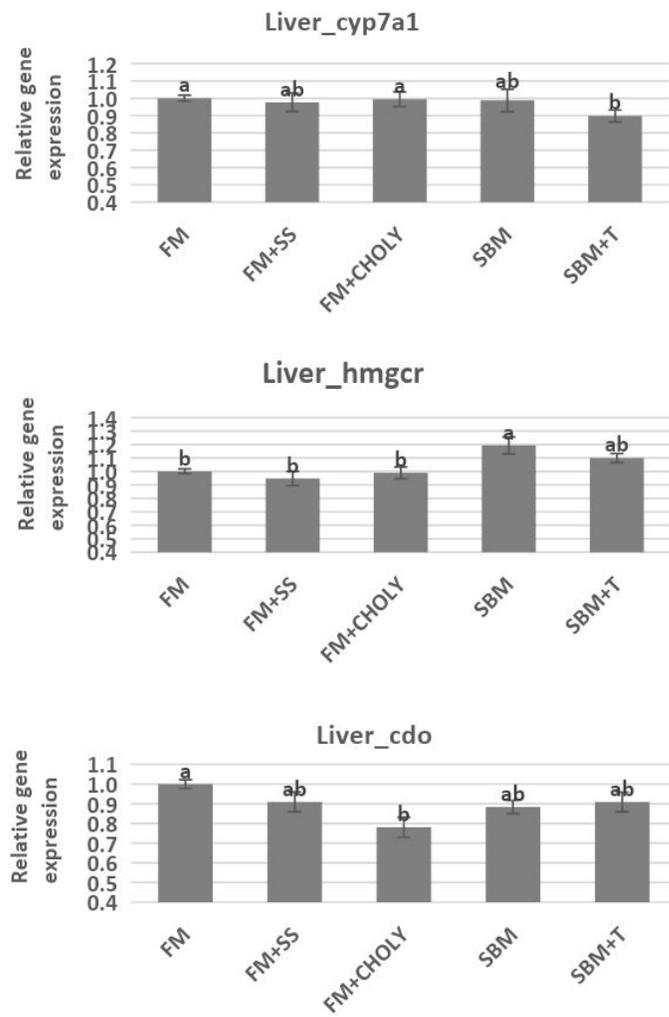
Table 6. Whole-body amino acid profile (exception for Trp.) of rainbow trout experimental diets for 8 weeks (% wet basis)

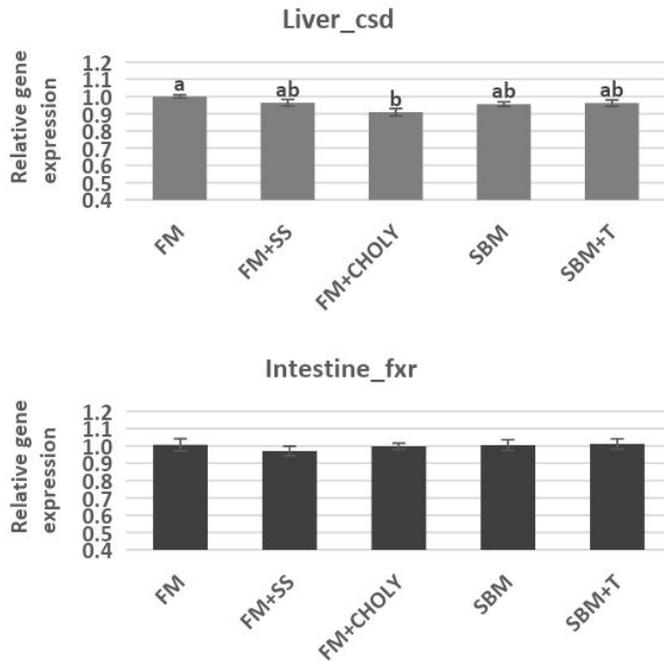
		Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
	Initial fish	Fishmeal	Fishmeal + Soy statin	Fishmeal + Cholestyramine	Soybean	Soybean + Taurine
<i>EAA</i>						
Arginine	0.80	0.93	0.92	1.00	0.92	0.88
Histidine	0.34	0.37	0.36	0.41	0.38	0.37
Isoleucine	0.60	0.67	0.64	0.71	0.67	0.66
Leucine	0.96	1.09	1.05	1.16	1.08	1.07
Lysine	1.07	1.26	1.21	1.34	1.23	1.21
Methionine	0.38	0.44	0.43	0.45	0.44	0.44
Cysteine	0.12	0.14	0.13	0.15	0.14	0.14
Phenylalanine	0.55	0.63	0.60	0.67	0.62	0.61
Tyrosine	0.46	0.57	0.55	0.60	0.58	0.43
Threonine	0.57	0.65	0.64	0.69	0.65	0.66
Valine	0.69	0.78	0.74	0.83	0.77	0.74
<i>NEAA</i>						
Alanine	0.79	0.92	0.93	1.00	0.92	0.87
Aspartic Acid	1.25	1.43	1.41	1.53	1.44	1.39
Glutamic Acid	1.64	1.87	1.89	2.07	1.89	1.86
Glycine	0.98	1.10	1.17	1.20	1.10	0.94
Hydroxyproline	0.13	0.16	0.21	0.19	0.18	0.17
Ornithine	0.02	0.02	0.01	0.02	0.02	0.01
Proline	0.55	0.62	0.66	0.70	0.63	0.57
Serine	0.47	0.53	0.56	0.57	0.55	0.57
Taurine	0.14	0.13	0.13	0.14	0.13	0.17
Total AA	12.53	14.33	14.28	15.45	14.37	13.81

Table 7. Muscle amino acid profile (exception for Trp.) of rainbow trout experimental diets for 8 weeks (% wet basis)

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
	Fishmeal	Fishmeal + Soy statin	Fishmeal + Cholestyramine	Soybean	Soybean + Taurine
<i>EAA</i>					
Arginine	1.13	1.12	1.22	1.08	1.07
Histidine	0.51	0.51	0.57	0.50	0.51
Isoleucine	0.92	0.91	0.98	0.88	0.88
Leucine	1.49	1.48	1.60	1.44	1.43
Lysine	1.74	1.72	1.88	1.66	1.65
Methionine	0.59	0.59	0.64	0.56	0.57
Cysteine	0.19	0.19	0.21	0.18	0.18
Phenylalanine	0.82	0.82	0.88	0.80	0.80
Tyrosine	0.76	0.76	0.84	0.75	0.58
Threonine	0.83	0.83	0.90	0.81	0.82
Valine	1.03	1.02	1.11	1.00	0.96
<i>NEAA</i>					
Alanine	1.10	1.09	1.18	1.06	1.04
Aspartic Acid	1.91	1.90	2.06	1.84	1.81
Glutamic Acid	2.63	2.56	2.84	2.49	2.48
Glycine	0.90	0.90	0.95	0.85	0.80
Hydroxyproline	0.04	0.04	0.04	0.03	0.04
Ornithine	0.01	0.02	0.02	0.02	0.01
Proline	0.63	0.63	0.70	0.62	0.60
Serine	0.63	0.62	0.68	0.61	0.64
Taurine	0.07	0.07	0.08	0.05	0.15
Total AA	17.95	17.76	19.39	17.24	17.06

Figure1. Relative mRNA expression of genes involved in bile acid metabolism (*cyp7a1* and *hmgcr*) and taurine metabolism (*cdo* and *csd*) of liver, and bile acid metabolism (*fxr*) of distal intestine of rainbow trout juveniles fed experimental diets for 8 weeks^{1,2}





¹Mean±SE (n=12 fish per treatment) in the same row that share the same superscript are not statistically different ($P>0.05$; Completely Randomized Design, One-factor ANOVA; Tukey's HSD test).

²Four fish from each tank were used for gene expression.

*cyp7a1: Cholesterol 7 α -hydroxylase - commits cholesterol to the neutral bile acid biosynthesis pathway

hmgcr: 3-Hydroxy-3-methylglutaryl-CoA reductase - rate-limiting enzyme in cholesterol biosynthesis

cdo: cysteine dioxygenase

csd: cysteine sulfinic acid decarboxylase

fxr: farnesoid X receptor - bile acid receptor and biological sensor for the regulation of bile acid biosynthesis