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ORIGINAL ARTICLE

The Digestibility and Accumulation of Dietary Phytosterols in Atlantic Salmon (*Salmo salar* L.) Smolt Fed Diets with Replacement Plant Oils

Matthew R. Miller · Peter D. Nichols · Chris G. Carter

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Abstract Phytosterols occur in high concentration in canola (*Brassica napus* L.) and other vegetable oils such as from the borage plant Echium (*Echium plantagineum* L.). We investigated if Atlantic salmon (*Salmo salar*) digest and accumulate dietary phytosterols in significant amounts in muscle and liver. Phytosterols are lipid soluble, lower cholesterol and reduce the risk of coronary heart disease in humans. We aimed to determine if fatty fish, such as salmon, can be used as a delivery source of this functional food component. Three diets containing canola oil (CO), Echium oil (EO) and fish oil (FO) were fed to Atlantic salmon smolt over 9 weeks. The digestibility of natural abundances of phytosterols by Atlantic salmon was poor compared to cholesterol. However, phytosterols accumulated in liver and muscle of fish. Significantly increased concentrations of 24-methylenecholesterol, campesterol, β-sitosterol and total phytosterol occurred in livers of EO fed fish compared to FO fed fish. Campesterol concentrations increased in CO fed fish compared to the FO fed fish. We demonstrated that natural abundances of dietary phytosterols are digested by and accumulated in liver and white muscle of Atlantic salmon smolt. However, phytosterol levels in salmon muscle will not be a major source of phytosterols in human diets and would not be expected to significantly effect human cardiovascular health.

Keywords Replacement oil · Fatty acids · Phytosterols · Canola oil · Echium oil · Sitosterol · Vegetable oil · Fish oil

Abbreviations

ADC	Apparent digestibility coefficients
ALA	α-Linolenic acid
ANOVA	One-way analysis of variance
BSFTA	*N*,*O*-Bis(trimethylsilyl)-trifluoroacetamide
CHD	Coronary heart disease
CMC	Carboxymethyl cellulose
DM	Dry matter
FA	Fatty acid(s)
GC	Gas chromatography
GC–MS	Gas chromatography–mass spectroscopy
LC	Long chain ($\geq C_{20}$)
LDL	Low-density lipoprotein
PCB	Polychlorinated biphenyls
PUFA	Polyunsaturated fatty acid(s)
SDA	Stearidonic acid
SE	Standard error
ST	Sterol(s)
TLC–FID	Thin layer chromatography–flame ionised detection
TLE	Total lipid extract
tr	Trace amounts
ω3	Omega 3
ω3 LC-PUFA	Omega 3 long chain ($\geq C_{20}$)-polyunsaturated fatty acid(s)

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M. R. Miller · C. G. Carter
School of Aquaculture,
Tasmanian Aquaculture and Fisheries Institute,
University of Tasmania, Locked Bag 1370,
Launceston, TAS 7250, Australia

M. R. Miller (✉) · P. D. Nichols
School of Aquaculture, CSIRO Food Futures Flagship
and CSIRO Marine and Atmospheric Research,
GPO Box 1538, Hobart, TAS 7001, Australia
e-mail: matthew.miller@utas.edu.au

ω6	Omega 6
WW	Wet weight

Introduction

Phytosterols, a general term applied to a large number of plant-derived sterols, are found in all tissues of higher plants and are often enriched in seeds and seed oils [1, 2]. Phytosterols are natural, organic compounds with a molecular nucleus of 17 carbon atoms and a characteristic three-dimensional arrangement of four rings. Phytosterols can act as a structural component in the plant cell membrane, a role played in mammalian cells by cholesterol [2]. Unlike cholesterol, which has an alkyl side chain of 8 carbon atoms, the side chain of phytosterols generally contains 9 or 10 carbon atoms with alkyl substitution at C_{24}. Phytosterols have wide bioactivity in humans, and in particular are considered an efficacious cholesterol-lowering agent and consequently may have a preventive role against cardiovascular disease [2, 3]. Phytosterols may also have a role in cancer prevention [2, 4]. Consequently, some margarines, butters, spreads and breakfast cereals are enriched with phytosterols and promoted as "functional foods" [5, 6].

Due to increasing demand and prices, reduced availability, the possibility of organic contaminants (such as dioxins and polychlorinated biphenyls (PCB)), and increased knowledge about fishing impacts, fish oil is being replaced in part with plant oils such as canola and soy in formulated/commercial feeds for aquaculture species [7]. Replacement oil diets have become an industry priority and over the last 20 years there has been increased scientific activity focused on the effects of substituting fish oil with plant oils, including effects on fish growth and health and on flesh quality. Limited research has been performed examining phytosterols in farmed Atlantic salmon (*Salmo salar*). The replacement of fish oil with plant oils in aquafeeds will increase dietary amounts of phytosterols. However, it has yet to be evaluated to what extent Atlantic salmon digest and accumulate phytosterols from dietary plant oils.

Pulp and paper mill effluent has been shown to affect reproductive and endocrine function in fish and this is thought to be due to the large amount of phytosterols, in particular β-sitosterol. In vivo and in vitro studies suggest that large amounts of β-sitosterol can affect fish endocrine activity and reproduction via many mechanisms/actions on numerous pathways [8].

The digestibility of phytosterols is poor in humans, and to our knowledge has yet to be evaluated in Atlantic salmon or in any other fish species. With the increased use of plant oils and meal in aquafeeds, minor components such as phytosterols will be increasingly introduced into aquaculture diets and it is necessary to examine how they are digested by salmon. Phytosterols have been shown to have potential health benefits to the human consumer, in particular as a cholesterol-lowering agent. Therefore farmed Atlantic salmon fed a replacement plant oil diet may be a novel and further delivery source of phytosterols to humans. Assessment of how they are accumulated and concentrated in tissues such as liver and muscle of Atlantic salmon fed on replacement oil diets is needed to gauge any possible advantageous affect to the consumer. This study aims to assess digestibility and accumulation in white muscle and liver of phytosterols from two experimental plant oil diets (canola and Echium) compared with a traditional fish oil diet fed to Atlantic salmon smolt.

Material and Methods

Experimental Diets

Three diets were formulated to compare canola oil (CO), Echium oil (EO) and fish oil (FO) (Table 1). Fish meal was defatted three times using a 2:1 mixture of hexane and ethanol (400 ml/100g fish meal). Full fat soybean meal (Hamlet Protein A/S, Horsens, Denmark), casein (MP Biomedicals Australasia Pty Ltd, Seven Hills, NSW, Australia), wheat gluten (Starch Australasia, Lane Cove,

Table 1 Ingredient, lipid and sterol composition (g/kg dry matter) of experimental diets

	Diet		
	CO	EO	FO
Ingredient composition (g/kg)			
Fish meal (defatted)	150	150	150
Casein	150	150	150
Wheat gluten	100	100	100
Soybean meal	180	180	180
Fish oil	0	0	200
Canola oil	200	0	0
Echium oil	0	200	0
Pre gel starch	127	127	127
Vitamin mix[a]	3	3	3
Mineral mix[b]	5	5	5
Stay C[c]	3	3	3
Chlorine chloride	2	2	2
Supernat	40	40	40
CMC	10	10	10
Sodium mono phosphate	20	20	20
Yttrium oxide	10	10	10
Chemical composition (g/kg WW)			

Table 1 continued

	Diet		
	CO	EO	FO
Dry matter	919.4	925.2	904.3
Crude protein	344.0	333.5	331.7
Crude fat	242.4	250.4	241.8
Energy (MJ/kg WW)	18.9	18.6	18.4
FAME (g/kg WW)			
Total SFA	20.4	23.9	62.9
Total MUFA	105.7	38.5	49.5
Total ω3	12.4	67.6	46.0
Total ω6	45.2	56.2	18.7
Total PUFA	58.3	123.9	71.0
Sterols and stanols (g/kg WW)			
Cholesterol[d]	1.6	2.6	6.7
Cholestanol[e]	ND	ND	0.1
24-Methylenecholesterol[f]	0.3	0.6	0.3
Phytosterols (g/kg WW)			
Brassicasterol[g]	0.6	ND	0.0
Campesterol[h]	2.1	1.8	0.4
Stigmasterol[i]	0.1	ND	ND
β-sitosterol[j]	3.4	2.4	1.2
Isofucosterol[k]	0.4	1.3	0.2
Other minor sterols[l]	0.3	0.2	0.2
Total phystosterols	6.8	5.6	1.9

EO Echium oil Crossential SA14 from Croda chemicals, *CO* canola oil, *FO* fish oil, *SFA* saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids, *CMC* carboxymethyl cellulose, *ND* not determined

[a] Vitamin mix (ASV4) supplied per kilogram of feed: 2.81 mg thiamine HCL, 1.0 mg riboflavin, 9.15 mg pyridoxine HCL, 25 mg nicotinic acid, 54.35 mg calcium D-pantothenate, 750 mg myo-inositol, 0.38 mg D-biotin, 2.5 mg folic acid, 0.03 mg cyanocobalamin, 6,350 IU retinol acetate, 2,800 IU cholecalciferol, 100 IU DL α-tocopherol acetate, 5 g menadione sodium bisulphate, 100 mg Roche rovimix E50

[b] Mineral mix (TMV4) supplied per kilogram of feed: 117 mg $CuSO_4 \cdot 5H_2O$, 7.19 mg KI, 1,815 mg $FeSO_4 \cdot 7H_2O$, 307 mg $MnSO_4 \cdot H_2O$, 659 mg $ZnSO_4 \cdot 7H_2O$, 3.29 mg Na_2SeO_3, 47.7 mg $CoSO_4 \cdot 7H_2O$

[c] L-Ascorbyl-2-polyphosphate (Stay-C, Roche Vitamins Australia, Frenchs Forest, NSW, Australia)

[d] Cholest-5-en-3β-ol

[e] 5α-Cholestan-3β-ol

[f] 24-Methylcholesta-5,24(28)-dien-3β-ol

[g] 24-Methylcholesta-5,22E-dien-3β-ol

[h] 24-Methylcholest-5-en-3β-ol

[i] 24-Ethylcholesta-5,22E-dien-3β-ol

[j] 24-Ethylcholest-5-en-3β-ol

[k] 24-Ethylcholesta-5,24(28)Z-dien-3β-ol

[l] Other minor sterols included 24-ethyl-5α-cholest-7-en-3β-ol, 4,4,14-trimethyl-5α-cholesta-8,24-dien-3β-ol (lanosterol), and other undetermined sterols

NSW, Australia) and BOIIC pre-gelatinised maize starch (Penford Australia Limited, Lane Cove, NSW, Australia) were used as ingredients. Echium oil was supplied as Crossential SA14 (Croda Chemicals, East Yorkshire, UK). Fish oil was from jack mackerel, *Trachurus symmetricus* L., (Skretting Australia, Cambridge, TAS, Australia) and a domestic refined source of pure canola oil was used (Steric Trading Pty Ltd, Villawood, NSW, Australia). Stay-C and Rovimix E50 were purchased from Roche Vitamins Australia (Frenchs Forest, NSW, Australia), and the remaining ingredients were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Yttrium oxide was used as a digestibility marker. The diets were manufactured into 3 mm diameter pellets using a California Pellet Mill (CL-2), dried and stored at 5 °C [9].

Growth Experiment

The experiment was conducted at the School of Aquaculture, University of Tasmania (Launceston, TAS, Australia). Atlantic salmon (*S. salar* L.) parr ($\approx$87.9 g) were obtained from Springfield Fisheries hatchery (Scottsdale, TAS, Australia), acclimated for 14 days in 300 l tanks and fed a commercial feed (Skretting). Prior to the experiment the fish were slowly adapted to seawater over a 21 day period. The tanks were held at a constant temperature of 12.0 °C under a natural photoperiod. Water was treated through physical, UV and biofilters. Dissolved oxygen, pH, ammonia, nitrate, nitrite, and salinity were monitored daily to ensure water quality remained within parameters recommended for Atlantic salmon [10]. The fish were held in an average of 27.4 $\pm$ 0.2 ppm saltwater. The experiment was conducted in accordance with the University of Tasmania Animal Ethics guidelines (Investigation A0008392).

At the start of the experiment fish (average weight 106.9 g) were anaesthetized (50 mg/1, benzocaine), their weight and length measured, and three fish were killed to measure initial lipid content and composition. Twenty-five fish were randomly re-allocated into each of twelve 300 l tanks, and 4 tanks were randomly allocated to each dietary treatment. The four diets were fed in triplicate at a ration of 1.8% body weight per day (% BW/d) in two equal feeds at 0900 and 1700 hours by automatic belt feeders. Every 7 days the total feed consumption (kg DM) was estimated from the amount of uneaten feed in Guelph-type sediment collectors [11, 12]. Every 3 weeks all fish were anaesthetized (50 mg/1, benzocaine) and batch-weighed. Fish were starved the day prior to weighing.

At the end of the experiment (day 84), fish were starved for 1 day prior to being anaesthetized (50 mg/1, benzocaine) and their weight and length measured. Three fish, which had doubled their initial weight, per tank were killed by a blow to the head after immersion in anaesthetic.

Samples of white muscle (approx 0.7 g), dissected from below the dorsal fin, and liver (average 2.7 g) were taken and frozen at −80 °C until analysis [7].

On days 86–90, faecal samples were collected from sediment collectors between 1100–1700 and 1900–0900 hours, freeze–dried and used in the analysis of digestibility [12]. The apparent digestibility coefficients (ADC) were calculated using the standard formula ADC (%) = 100−[100((Y_{diet}/Y_{faeces}) × ((ST_{faeces}/ST_{diet}))], where Y is percentage of yttrium oxide and ST is the % of particular sterols [13]. This calculation does not take into account possible dealkylation of phytosterols to cholesterol.

Sterol Extraction and Isolation

All samples (tissue, faeces and diets) were freeze–dried and extracted overnight using a modified Bligh and Dyer protocol [14]. This involved a single phase extraction, $CHCl_3$/MeOH/H_2O (1:1:0.9, by vol), followed by phase separation to yield a total lipid extract (TLE).

Lipid classes were analysed using an Iatroscan MK V thin-layer chromatography-flame ionization detector (TLC-FID) analyser (Iatron Laboratories, Japan). Samples of the TLE were spotted onto silica gel SIII Chromarods (5 μm particle size) and developed in a glass tank lined with pre-extracted filter paper. The solvent system used for the lipid separation was hexane:diethyl ether:acetic acid (60:17:0.1 v/v/v) [15]. After development for 25 min, the chromarods were oven-dried and analysed immediately to minimise adsorption of atmospheric contaminants. Lipid classes were quantified by DAPA software (Kalamunda, WA, Australia). The FID was calibrated for each compound class: phosphatidylcholine (retention factor 0.00), cholesterol (0.30), oleic acid (0.46); wax ester (derived from fish oil, 0.89) and triacylglycerol (derived from fish oil, 0.74).

An aliquot of the TLE was treated with 2 ml of 5% w/v KOH in 80:20 MeOH:H_2O (60 °C, 3h, milli-Q water). Following the addition of water, sterols were extracted into hexane/chloroform (4:1 v/v, 3 × 1.5 ml), transferred to vials, reduced under a stream of nitrogen and stored in chloroform. Samples were treated with *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) to form trimethylsilyl (TMS) ether derivatives (of free sterols) prior to instrument analysis.

Concentrations of plant sterols were determined by gas chromatography. Samples were made up to a known volume with a internal injection standard containing methyl nonadecanate and analysed by gas chromatography (GC) using an Agilent Technologies 6890N GC (Palo Alto, CA, USA) equipped with an Equity™-1 fused silica capillary column (15 m × 0.1 mm i.d., 0.1 μm film thickness), an FID, a split/splitless injector and an Agilent Technologies 7683 Series autosampler. Helium was the carrier gas. Samples were injected in splitless mode at an oven temperature of 120 °C. After 3 min, the oven temperature was raised to 270 °C at 10 °C per min and finally to 290 °C at 5 °C/min^{-1} holding at 290 °C for 5 min. Peaks were quantified with Agilent Technologies GC ChemStation software (Palo Alto, CA, USA).

Individual components were identified from mass spectral data and by comparing retention time data with those obtained for authentic and laboratory standards. GC–mass spectrometric (GC–MS) analyses were performed on a Finnigan Thermoquest GCQ GC–mass spectrometer fitted with an on-column injector and using Thermoquest Xcalibur software (Austin, TX, USA). The GC was equipped with an HP-5 cross-linked methyl silicone fused silica capillary column (50 m × 0.32 mm i.d.). Helium was used as the carrier gas, with operating conditions previously described [16]. Sterols were identified from comparison to known spectra and major ion fragments [17]. The stereochemistry was not determined for the C_{24} position. The retention times for the major sterols were cholesterol 20.33 min, cholestanol 20.73 min, 24-methylenecholesterol 21.51 min, brassicasterol 20.85 min, campesterol 21.63 min, stigmasterol 22.17 min, *β*-sitosterol 22.77 min and isofucosterol 22.92 min.

Statistical Analysis

Mean values are reported as plus or minus the standard error of the mean. Normality and homogeneity of variance were confirmed and percentage data were arcsin transformed prior to analysis. Comparison between means was by one-way analysis of variance (ANOVA) followed by multiple comparisons using Tukey–Kramer HSD. Significance was accepted at probabilities of 0.05 or less. Statistical analysis was performed using SPSS for Windows version 11.

Results

Growth Results

There was no significant difference in the final weight, final length or growth between fish fed the three experimental diets. Fish of an average weight (106.8 g) were grown on the three experimental diets for 12 weeks to an average sampled weight of 236.5 g.

Sterol (ST) Composition

There was no significant difference in the sterol content in the white muscle between dietary treatments as determined by TLC–FID analysis (Table 2). There was significantly

Table 2 Sterol and lipid content (wet weight) of white muscle and livers of Atlantic salmon smolt fed canola oil (CO), Echium oil (EO) and fish oil (FO) diets

Sterol content (mg/100g)	Initial (±SE)	CO (±SE)	EO (±SE)	FO (±SE)	*f*
White muscle	21.6 ± 4.8	15.0 ± 6.0	12.4 ± 6.5	13.9 ± 3.1	
Liver	139.9 ± 18.4[a]	263.8 ± 34.3[b]	144.7 ± 18.0[a]	139.7 ± 11.8[a]	7.9
Lipid content (mg/g)					
White muscle	17.9 ± 2.9	28.0 ± 3.4	30.1 ± 6.2	26.8 ± 4.9	
Liver	29.0 ± 2.2[a]	49.1 ± 3.5[b]	38.3 ± 2.4[a,b]	35.8 ± 2.7[a]	7.3

[a, b] Mean values across the row not sharing a common superscript were significantly different as determined by Tukey–Kramer HSD; $df = 3,40$, $P < 0.01$

higher total sterol content in the liver (263.8 mg/100 g) of the fish fed the CO diet. Cholesterol was the major sterol in all tissues, irrespective of diet, but the amounts of minor sterols varied. There were significantly ($P < 0.01$) higher concentrations of total phytosterols in the liver and white muscle in both the CO and EO fed fish compared to the FO or initial fish (Tables 3, 4). This equated to a fourfold increase in the white muscle and a twofold increase in the liver in total phytosterol concentrations in EO and CO fish compared to the FO fish. There were significant differences in the sterol composition of the liver (Table 4). There was a sevenfold significant ($P < 0.01$) increase in concentrations of campesterol (full chemical names for all sterols are contained in the footnote for Table 1) in the liver of both the CO and EO fish compared to the FO or initial fish. There were significant ($P < 0.01$) increases in concentrations of β-sitosterol and 24-methylenecholesterol in the liver of EO fish compared to that of the FO or initial fish. This equated to a sevenfold increase in β-sitosterol in the EO fish compared with the FO fish. There was a significantly ($P < 0.01$) higher concentration of cholestanol in the initial fish compared to the CO and EO fish. There was a significant ($P < 0.01$) five to ninefold increase in the concentrations of other minor sterols including 24-ethyl-5α-cholest-7-en-3β-ol, lanosterol, and other undetermined sterols in the liver of CO and EO fish.

There was no significant difference in the sterol composition (%) between the white muscle for fish fed the three diet treatments (Table 3). However, although not significant, the same trends in increased concentration of individual phytosterols that occurred in the liver were observed in the white muscle. There was a three to fivefold increase in the amount of β-sitosterol and a twofold increase in campesterol in the white muscle of fish fed EO and CO diets compared with the FO fish.

Digestibility of Phytosterols

There was no significant difference between the ADC of cholesterol between the three diet treatments (Table 5).

Table 3 Sterol composition (mg/100g wet weight) of white muscle of Atlantic salmon smolt fed canola oil (CO), Echium oil (EO) and fish oil (FO) diets

Sterol	Initial (±SE)	CO (±SE)	EO (±SE)	FO (±SE)	*f*
Cholesterol	25.7 ± 2.2	15.4 ± 2.0	15.2 ± 2.0	13.6 ± 2.8	
Cholestanol	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	
Brassicasterol	0.0 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	
24-Methylenecholesterol	0.2 ± 0.1	0.1 ± 0.1	0.4 ± 0.1	0.1 ± 0.0	
Campesterol	0.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	
Stigmasterol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	
β-sitosterol	0.0 ± 0.0	0.3 ± 0.2	0.5 ± 0.3	0.0 ± 0.0	
Isofucosterol	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	
Other minor sterols[c]	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	
Total phytosterols[d]	0.2 ± 0.1[a]	0.9 ± 0.2[b]	0.8 ± 0.3[b]	0.2 ± 0.1[a]	8.5

Systematic names for all sterols are contained in the footnote for Table 1

[a, b] Mean values across the row not sharing a common superscript were significantly different as determined by Tukey–Kramer HSD; $df = 3,36$, $P < 0.01$

[c] Other minor sterols including 24-ethyl-5α-cholest-7-en-3β-ol, lanosterol, and other undetermined sterols

[d] Includes all C_{28} and C_{29} sterols excluding 24-methylenecholesterol

Table 4 Sterol composition (mg/100g wet weight) of liver of Atlantic salmon smolt fed canola oil (CO), Echium oil (EO) and fish oil (FO) diets

Sterol	Initial (±SE)	CO (±SE)	EO (±SE)	FO (± SE)	*f*
Cholesterol	99.2 ± 7.0	222.5 ± 21.8	180.1 ± 16.8	151.4 ± 12.5	
Cholestanol	0.7 ± 0.2[c]	0.1 ± 0.0[a]	0.2 ± 0.1[a, b]	0.4 ± 0.1[b, c]	9.2
Brassicasterol	0.0 ± 0.0	0.5 ± 0.1	0.3 ± 0.1	0.4 ± 0.0	
24-Methylenecholesterol	0.3 ± 0.1[a]	1.7 ± 0.1[a, b]	3.2 ± 0.8[b]	0.9 ± 0.1[a]	5.8
Campesterol	0.2 ± 0.1[a]	4.6 ± 0.7[b]	4.9 ± 0.9[b]	0.7 ± 0.1[a]	6.5
Stigmasterol	0.0 ± 0.0	0.1 ± 0.0	0.8 ± 0.3	0.3 ± 0.1	
β-sitosterol	0.0 ± 0.0[a]	0.1 ± 0.1[a, b]	0.7 ± 0.2[b]	0.0 ± 0.0[a]	6.5
Isofucosterol	0.0 ± 0.0	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	
Other minor sterols[d]	0.3 ± 0.0[a, b]	0.5 ± 0.0[b]	0.9 ± 0.0[c]	0.1 ± 0.0[a]	6.4
Total phytosterols[e]	0.5 ± 0.2[a]	6.1 ± 1.1[b,c]	7.5 ± 1.0[c]	3.3 ± 0.4[a, b]	6.8

Systematic names for all sterols are contained in the footnote for Table 1

[a, b, c] Mean values across the row not sharing a common superscript were significantly different as determined by Tukey–Kramer HSD; $df = 3,36$, $P < 0.01$

[d] Other minor sterols including 24-ethyl-5α-cholest-7-en-3β-ol, lanosterol, and other undetermined sterols

[e] Includes all C_{28} and C_{29} sterols excluding 24-methylenecholesterol

There were no significant differences between the ADC of any phytosterol between the three diet treatments. Cholesterol has an ADC of 46.7–65.8% across the three diets. In general phytosterols had a lower ADC than cholesterol. Specifically, C_{28} phytosterols such as brassicasterol and campesterol had a relative absorption of 9.2–18.1%, three to sixfold lower than that of cholesterol. 24-Methylenecholesterol was excluded from the calculation as it is a metabolite of cholesterol and is usually not associated with plant phytosterols. C_{29} phytosterols including β-sitosterol, stigmasterol, and isofucosterol have a relative absorption of 1.1–7.1%, 7–60-fold lower than that of cholesterol.

Table 5 Apparent digestibility coefficients (ADC) (%) for the different sterols in canola oil (CO), Echium oil (EO) and fish oil (FO) diets fed to Atlantic salmon

ADC (%)	CO (±SE)	EO (±SE)	FO (±SE)
Cholesterol	57.5 ± 4.4	46.7 ± 5.4	65.8 ± 7.2
Cholestanol	ND	ND	52.7 ± 4.1
Brassicasterol	9.2 ± 8.0	ND	ND
24-Methylenecholesterol	12.4 ± 3.5	27.3 ± 5.2	17.0 ± 4.1
Campesterol	14.4 ± 2.2	15.2 ± 2.1	18.1 ± 1.6
Stigmasterol	4.7 ± 3.2	ND	ND
β-sitosterol	7.1 ± 2.1	4.1 ± 4.3	1.1 ± 2.0
Isofucosterol	7.6 ± 1.1	2.4 ± 2.7	8.5 ± 2.6
Other minor sterols[a]	3.4 ± 3.1	7.5 ± 3.3	5.2 ± 3.1

Systematic names for all sterols are contained in the footnote for Table 1

ND Not determined

[a] Other minor sterols including 24-ethyl-5α-cholest-7-en-3β-ol, lanosterol, and other undetermined sterols

Discussion

Due to the decreasing availability and increasing price of fish oil, renewable land plant based oils have been increasingly incorporated into aquafeeds, and the replacement of fish oil has become an industry priority. Plant based oils contain phytosterols which are not part of the natural diet of Atlantic salmon. Therefore it is important to investigate how natural abundances of phytosterols in replacement oils are digested and incorporated in different tissues of salmon. We demonstrated that both the liver and the white muscle accumulated significantly increased amounts of total phytosterols with both plant oil diets (CO and EO) compared to FO fed fish. Differences in the absolute abundances and relative levels of individual phytosterols were demonstrated in the liver and white muscle of fish fed plant oil diets compared to the FO and initial fish. This study indicates that small amounts of phytosterols can be digested and accumulated in both the white muscle and liver of fish when fed replacement plant oil over a 12 week period.

Digestibility of Phytosterols in Atlantic Salmon

Although cholesterol and phytosterols have similar structures, phytosterols have significantly reduced absorption compared to cholesterol and it has been shown that negligible amounts of phytosterol are absorbed by humans [18, 19]. There have been numerous studies looking at cholesterol and phytosterol adsorption in humans [20–23]. From these studies cholesterol has an absorption range of 33–60%, with phytosterols having reduced absorption, 2–10% for campesterol and 0.5–5% for β-sitosterol [20–23]. The

digestibility of sterols and phytosterols in Atlantic salmon show similar patterns to those in humans. Our study demonstrates that cholesterol has an ADC of 46.7–65.8%. A reduced digestibility was demonstrated with phytosterols. An ADC of 14–18.1% for campesterol and 1.1–7.1% for β-sitosterol was observed across the diets. C_{29} phytosterols, such as β-sitosterol and isofucosterol, had reduced digestibility compared with that of C_{28} phytosterols such as 24-methylcholesterol. Phytosterols may be dealkylated to cholesterol which will reduce their ADC. Phytosterols and cholesterol are also likely to be metabolized by either internal and/or external microorganisms, or possibly by the smolt themselves. Both dealkylation and metabolism will also reduce the ADC, but the extent of these processes is yet to be assessed in Atlantic salmon. Phytosterols can be esterified with a mixture of fatty acids which are naturally occurring in small quantities in plants. The concentration of phytosterol esters can change rapidly in response to various types of stimuli. In this study, phytosterol esters were not detected in fish tissues by TLC–FID, however, small quantities occur in plant based replacement diets. It has been shown that in canola oil, 65% of the total phytosterols are present as free sterols and 35% as phytosterol esters [24]. Total phytosterols were measured in this trial and used in the calculation of digestibility. We have not defined the proportions of free phytosterols and phytosterol esters in the diets. However, the digestibility of total phytosterols was measured from saponified total solvent extract (TSE), which include both esters and free phytosterols. As no phytosterol esters were detected by TLC–FID in the fish muscle or liver this indicates that dietary phytosterol esters are hydrolyzed in the intestine or not digested [25]. Our study showed no difference in the digestibility of individual phytosterols across diets, which suggests that dietary concentration does not affect their digestibility by Atlantic salmon.

Accumulation of Phytosterols in Atlantic Salmon

We determined that 0.8–0.9 mg of total phytosterols per 100 g of tissue were accumulated in the white muscle and 6.1–7.5 mg/100 g in the liver of fish fed plant oil replacement diets over a 12 week period. Our experimental diets contained 100% replacement vegetable oil. This level of replacement would not be the case in commercial aquafeeds. Phytosterol accumulation is dependant on dietary concentration as they are unable to be in vivo biosynthesized. As phytosterols are lipid soluble, the higher the fat content of the fish or tissue will increase the amount of phytosterols it contains. The major phytosterols accumulated in the liver and white muscle were campesterol and β-sitosterol. Generally the relative concentrations of individual phytosterols in the liver and white muscle reflected that of their diet. However, C_{29} phytosterols had a reduced digestibility compared with C_{28} phytosterols and therefore lower amounts accumulated in the liver or white muscle in both the CO and EO fish.

Phytosterols are very expensive ingredients and therefore it would not be economically feasible for these ingredients to be added to aquafeeds. However, replacement plant oils, such a canola, contain phytosterols as minor components. Canola is a prime candidate for fish oil replacement as it contains high levels of ω3 PUFA, largely as α-linolenic acid (ALA, 18:3ω3) and is grown in sufficient quantities to meet future aquaculture demands. A recent study suggested that Echium oil also may be a candidate as it contains an unusual ω3 PUFA profile containing stearidonic acid (SDA, 18:4ω3), which was shown to maintain concentrations of important omega 3 long chain PUFA (ω3 LC-PUFA) in Atlantic salmon parr [7]. Both these plant oils have phytosterol compositions which are dependent on genotype, planting location and temperature [26–28].

In our study all the experimental diets had minor concentrations of phytosterols from the meal and oil sources. In the CO and EO diets the major source of phytosterols was the plant oil. All the diets contained plant based proteins, which consisted of soybean meal, casein and wheat gluten. As phytosterols occur in membranes and perhaps other parts of plant cells, the plant meals also will have residual concentrations of phytosterols. The FO diet contained 1.9 g/kg WW phytosterols, while the CO (6.8 g/kg WW) and the EO (5.6 g/kg WW) had 3.5 and 2.9 times the amount of total phytosterols (Table 1).

In the present study sterols represented only about 1% of the total lipid of salmon, and 92–99% of that 1% is present as cholesterol. Salmon smolt in this study had an average total white muscle lipid content of 28.3 and 39.6 mg/g in the liver. Therefore the amount of phytosterols in Atlantic salmon smolt compared to other lipids including cholesterol is relatively low. Phytosterols cannot be synthesized by humans or fish, therefore they are supplied in the diet. Phytosterols, due to their lipophilic nature, have been added to margarines and spreads which are known as "functional foods". Oily fish, such as Atlantic salmon, may provide another possible delivery source. Phytosterols in combination with the high levels of omega 3 long chain ($\geq C_{20}$) polyunsaturated fatty acids (ω3-LC PUFA) found in salmon may act in unison to deliver an enhanced benefit against coronary heart disease (CHD). Additionally and unlike ω3-LC PUFA, phytosterols are more stable compounds and are not as readily oxidised under severe conditions, such as deep frying.

Most human nutrition studies with phytosterols assessed an intake of 0.8–4.0 g of phytosterol as a daily dose which reduced the lower density lipoprotein (LDL) cholesterol

between 6 and 15% which reduces the risk of CHD [2, 29]. Dietary intake of phytosterols ranges from 150 to 400 mg/day, with a typical composition of 65% β-sitosterol, 30% campesterol and 5% stigmasterol [2, 19]. The content of phytosterol from this experiment in white muscle of Atlantic salmon smolt is 0.8–0.9 mg/100 g which is well below the concentration range of dietary phytosterol generally being supplied in functional foods for CHD prevention. This result will be elevated in larger commercial cultured fish, where oil content (7–20%) of white muscle is markedly higher than observed in the smolt (2–3%) examined here. Due to use of extrusion technologies commercial salmon aquafeeds have an oil content of 30–40%. Our study used an inclusion of 20% oil which will underestimate the phytosterol content compared to commercial diet. It was not possible for this experiment to obtain the high lipid content of commercial aquafeeds. However, even at this lower inclusion, it was possible to demonstrate that phytosterols significantly accumulated in the tissues of Atlantic salmon. Our experiment was for a 12 week period. It is plausible to suggest that over a life time of replacement oil diets that Atlantic salmon will accumulate considerably more phytosterols. However, at this point it is yet to be determined to what concentrations Atlantic salmon will accumulate phytosterols. The phytosterol concentrations in the white muscle (0.8–0.9 mg/100 g) of plant oil fed fish is several orders of magnitude less than the recommended daily serving size of functional foods such as spreads and margarines (phytosterols total $\approx$ 200 mg/day) [30]. However, in a balanced diet, this additional amount of phytosterols (we estimated to be $\approx$20–50 mg/serve in a large commercial size fish) is a 10–25% increase in the average adult human dietary intake of phytosterols.

Environmental Effects of Phytosterols

Several studies have shown that phytosterols in effluent, in particular β-sitosterol, produced estrogenic effects in maturing fish as well as a reduced cholesterol level [8, 31, 32]. Cholesterol is a precursor of steroid hormones and therefore the presence of structurally similar molecules or the reduction of cholesterol may effect maturation in fish. Phytosterols, in particular β-sitosterol, are present in pulp and paper mill effluents and have been associated with different reproductive responses in fish including reduction in gonad size, delayed sexual maturation, and reduced expression of secondary sexual characteristics [31, 33, 34]. A recent study strongly suggested that cholesterol in Atlantic cod (*Gadus morhua*) liver is lowered by phytosterol rich soy based diet and is possibly involved in changes observed in gonad development [32]. It has also been suggested that increased sterol-like compounds may have contributed in the postponed spawning of Baltic cod in the Baltic Sea [32]. However, most farmed fish are harvested prior to maturation and a postponing of maturation may be advantageous to the aquaculture industry. Research has focused on the effect of phytosterols as a waterborne contaminant on fish health, reproduction and endocrine function [31, 33, 34]. The effects of dietary sources of phytosterols on fish have yet to be assessed, however, dietary concentrations are significantly lower than that of the effluent experiments.

There have been many well designed and executed experimental aquaculture trials with replacement oil, which have all shown no difference in growth, performance or health of fish [7, 35–40]. Further research looking at dietary phytosterol accumulation over a longer period and in particular reproductive and endocrine function such as the production of vitellogenin will provide insight on the accumulation and function of these ingredients. The use of vegetable oils in aquafeeds is now commonplace throughout the aquaculture industry and the small accumulation of phytosterols in Atlantic salmon that we have demonstrated will be advantageous to their continued use. However, Atlantic salmon require a dietary source of ω3 LC-PUFA which can presently only be provided by fish oil. Therefore, oil bends of plant and fish oils are commonly used throughout the aquaculture industry. While it has been shown that phytosterols in pulp mill effluent can be detrimental to fish health, low concentrations that occur in plant oils most likely will not affect health and performance of fish, and ultimately may provide health benefits to the consumer. Although the digestibility of phytosterols is low and their accumulation in fish of commercial size is likely to be at a level lower than used in functional food such as spreads and margarines, a small enhancement of dietary phytosterols along with the high content of ω3 LC-PUFA may give increased CHD protection to consumers.

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