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ORIGINAL ARTICLE

Effect of type of dietary non-protein energy source (starch vs. fat) on the body bile acid pool size and composition, faecal bile acid loss and bile acid synthesis in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Effects of the type of dietary non-protein energy source on the size and composition of the total body bile acid pool, on faecal bile acid loss and on bile acid synthesis were investigated in rainbow trout. Two diets were formulated (similar DP:DE ratio) that differed in the inclusion of either maize starch (Starch) or rapeseed oil (Fat) as main non-protein source. Fish were fed to satiation for 44 days. Type of non-protein energy source did not substantially affect the body bile acid pool composition. However, feeding the Starch diet resulted in a larger total body bile acid pool size compared with the Fat diet, and this despite enhanced faecal bile acid loss when feeding the Starch diet that was related to more faeces being produced. Bile acid synthesis in fish fed the Starch diet was more than two times higher compared with fish fed the Fat diet. The difference in body bile acid pool size between diets suggests upregulation of bile acid synthesis in fish fed the Starch diet beyond the level needed to compensate for the higher faecal bile acid loss and/or downregulation of bile acid synthesis in fish fed the Fat diet. The underlying mechanisms for this difference in synthesis need further investigation.

KEYWORDS
bile acid metabolism, enterohepatic circulation, glycine, *Oncorhynchus mykiss*, taurine

1 | INTRODUCTION

Synthesis of primary bile acids occurs in the liver by oxidation of cholesterol. Thereafter, bile acids are conjugated with either taurine or glycine. In fish, bile acids are predominantly conjugated with taurine (Hagey et al., 2010). Bile acids are actively secreted into bile and stored in the gallbladder. After a meal, the gallbladder receives hormonal signals (cholecystokinin), which results in its contraction and the release of bile and bile acids into the intestine (Hagey et al., 2010). In the intestine (i.e. mainly the colon in mammals), bile acids can be metabolized by bacteria which convert them into so-called secondary bile acids (Midtvedt, 1974). The importance of secondary bile acids formation in fish is not known. The majority of bile acids are actively reabsorbed within the distal part of the intestine, from where they are returned to the liver for reuse (Cai et al., 2007). Under homeostatic conditions, the total bile acid pool size and

composition is maintained relatively constant, compensating faecal bile acid loss with *de novo* synthesis (Lanzini & Lanzarotto, 2000). The process described above is known as the enterohepatic circulation of bile acids (EHC).

The replacement of fish meal and fish oil by plant ingredients in aquafeeds often results in hampered fat digestion (Førde-Skjærvik et al., 2006; Haidar et al., 2016; Olli & Krogdahl, 1994; Refstie et al., 1999). Bile acids play a key role in digestion and absorption of dietary fat (Tocher, 2010). One of the proposed mechanisms for hampered fat digestion is an altered bile acid metabolism (e.g. reduced bile acid pool size, enhanced faecal bile acid loss and/or reduced bile acid synthesis) in fish fed plant-based diets (reviewed by Romano et al., 2020). Several studies with fish showed that bile acid supplementation to plant-based diets (partly) remediates hampered fat digestion (Gu et al., 2017; Iwashita et al., 2010; Yamamoto et al., 2007). In the context of the ongoing replacement of fish meal and fish oil by plant alternatives, this highlights the need to increase our fundamental understanding of the interaction between nutritional factors, the bile acid metabolism and fat digestion in fish.

Studies investigating alterations of the bile acid metabolism in fish have been predominantly focussed on the adverse effects of antinutrients in soybean meal-based diets (e.g. saponins) and such studies consistently reported a decrease in the bile acid content of the chyme, gallbladder and/or blood compared with fish fed fish meal-based diets (Chikwati et al., 2012; Deng et al., 2013; Gu et al., 2017; Iwashita et al., 2009; Kortner et al., 2013; Krogdahl et al., 2015; Romarheim et al., 2008; Yamamoto et al., 2008). Decreased chymal bile acid content or gallbladder/blood bile acid concentration suggest a reduced total body bile acid pool size and are proposed to be related to either an increase in faecal bile acid loss or a decrease in bile acid synthesis (Romano et al., 2020).

Besides effects of specific antinutritional factors, mammalian studies show that also dietary macronutrient composition can affect the bile acid metabolism (reviewed by Chiang, 2013). Macronutrient composition can affect bile acid synthesis and the rate of EHC, which in turn were shown to affect bile acid pool size and composition. Hepner (1975) showed that the level of gallbladder contraction, which partly determines the rate of EHC, regulates the bile acid pool size in humans. In that study, lowering the dietary fat level decreased the level of gallbladder contraction and increased the bile acid pool size. Furthermore, the same study showed that also the bile acid pool composition changed with diet. Decreasing the dietary fat level resulted in a smaller share of secondary bile acids in the total bile acid pool, and this was ascribed to less exposure of primary bile acids to bacteria in the intestine as a result of decreased gallbladder contraction, and thus decreased EHC. Besides the rate of gallbladder contraction, dietary fat level and fat saturation level have also been shown to influence bile acid synthesis. Feeding a diet high in fat stimulated bile acid synthesis in rats, while a low fat diet decreased bile acid synthesis (Botham & Boyd, 1983). Furthermore, Cheema et al. (1997) found that the bile acid synthesis in mice was upregulated (measuring expression of CYP7A1 involved in bile acid synthesis) in response to a diet high in unsaturated fat. Besides dietary fat level, also dietary carbohydrate level can affect bile acid synthesis. Andersen and Hellstrom (1980) showed an increase in bile acid synthesis in humans which switched from a diet in which 60% of the energy was supplied by fat to a diet in which the energy was supplied by carbohydrates. In this study, the increase in bile acid synthesis when feeding the high carbohydrate diet was ascribed to the non-starch polysaccharide fraction. The latter increased faecal bile acid loss, which was consequently compensated by *de novo* bile acid synthesis. The effect of dietary macronutrient composition on the bile acid metabolism of fish has not been studied, but could be important to investigate since the increasing use of plant ingredients in aquafeeds translates into increasing dietary carbohydrate fractions (Maas et al., 2020).

Based on the above, this study aimed to assess whether the type of dietary non-protein energy source (Starch vs. Fat) affects the bile acid metabolism (i.e. bile acid pool size and composition, faecal bile acid loss and bile acid synthesis) of rainbow trout (*Oncorhynchus mykiss*). Two diets were formulated with a similar digestible protein to digestible energy ratio, but differing in inclusion of either maize starch (Starch) or rapeseed oil (Fat) as the main non-protein source. Alterations of the bile acid metabolism of fish in response to dietary changes have until now been shown by indirect measures (e.g. changes in the expression of genes involved in EHC or bile acid synthesis, and changes in the bile acid content of the chyme, gallbladder and blood) (Gu et al., 2014; Kortner et al., 2013, 2014; Krogdahl et al., 2015; Murashita et al., 2018; Romarheim et al., 2008). Quantitative data on changes of the bile acid metabolism in response to nutritional factors is lacking for fish. Therefore, the current study quantified body bile acid pools and faecal bile acid loss in response to the experimental diets. Furthermore, using initial and final body bile acid pool size, bile acid intake and faecal bile acid loss, bile acid synthesis was quantified for the first time in fish by means of a mass balance.

2 | MATERIALS AND METHODS

This study was conducted in accordance with the Dutch law on the use of experimental animals (Act on Animal Experiments) and was approved by the Central Animal Experiments Committee (CCD) of The Netherlands under project number 2017 W.0037. This study was performed in the experimental facilities of the Wageningen University, and fish were kept and handled in agreement with the current EU legislation.

2.1 | Feed formulation

The experimental diets were formulated to meet or exceed the minimum recommended nutrient requirements of rainbow trout (*Oncorhynchus mykiss*) according to the National Research Council (NRC, 2011).

Two diets were formulated with similar digestible protein to digestible energy ratio, but differing in the type of main non-protein

energy source (Starch vs. Fat). For this, a basal ingredient mixture was first formulated according to Table 1. Monocalciumphosphate and a vitamin/mineral premix were added to meet the requirements for phosphorus, vitamins and other minerals. Methionine and lysine were supplemented to meet amino acid recommendations. Yttrium oxide was used as inert digestibility marker. This basal ingredient mixture was combined with either maize starch (gelatinized) or rapeseed oil as shown in Table 2. The basal ingredient mixture, either with or without addition of maize starch, was pelleted by extrusion (3 mm; Research Diet Services, The Netherlands). The rapeseed oil was added to the Fat diet by vacuum coating (Wageningen University, The Netherlands). Diets were stored at 4°C throughout the experiment. The analysed nutrient content of the diets is shown in Table 2.

2.2 | Housing facilities

Fish were housed in a flow-through system consisting of 6 glass tanks (90 × 60 × 45 cm; 200 L each). Water temperature was maintained at 14 ± 1°C and water flow was maintained at 7 ± 0.5 L min^{-1} using a water flow meter (MAGFLO MAG 5000, Danfoss A/S, Denmark). Values for NH_4^+-N, NO_2^--N, NO_3^--N and conductivity of the inlet water were kept <0.5 mg L^{-1}, <0.15 mg L^{-1}, <90 mg L^{-1} and <4000 µS cm^{-1}, respectively. The pH of the inlet water was kept within the range of 7.0–8.0. The concentration of dissolved oxygen in the inlet was maintained at a level of 10 ± 0.1 mg L^{-1} by an oxygenator, which was controlled by a mass flow controller (Brooks Model 5850S; Brooks Instruments) and a microprocessor (Brooks Read Out and Control Electronics Model 0154; Brooks Instruments). The outlet of each tank was located at the lowest point of the sloping bottom and was connected to a swirl separator (44 cm in height, 24.5 cm in diameter; Aqua Optima A/S) that collected faeces and uneaten feed pellets. A photoperiod of 12-h light and 12-h darkness was maintained for the entire duration of the experiment.

TABLE 1 Composition of the basal ingredient mixture

Ingredients	Mixture (parts by weight)
Fishmeal[a]	20.00
Wheat	15.49
Wheat gluten	12.00
Soy protein concentrate	12.00
Pea protein concentrate	12.00
Fish oil	1.00
Monocalciumphosphate	1.00
Vitamin/mineral premix[b]	1.00
DL-methionine	0.40
Lysine HCl	0.10
Yttrium oxide	0.01
Total	75.00

Notes: [a] LT fishmeal – crude protein 72%, Triple Nine Fish protein, Esbjerg, Denmark.

[b] Vitamin/mineral premix. Vitamins (IU or g kg^{-1} premix): thiamin, 1 g; riboflavin, 1 g; pyridoxine, 1 g; panthotenic acid, 4 g; niacin, 2 g; biotin, 0.02 g; cyanocobalamin, 0.0015 g; folic acid, 0.2 g; ascorbic acid, 10 g; DL-alpha tocopherol acetate, 100 IU; retinyl palmitate, 3,000 IU; DL-cholecalciferol, 2,400 IU; sodium menadione bisulphate (51%), 1 g; inositol, 40 g; choline, 200 g; butylhydroxytolueen, 10 g; calcium propionate, 100 g; anti-oxidant BHT (E300-321), 10 g. Minerals (g kg^{-1} premix): iron (as $FeSO_4 \cdot 7H_2O$), 5 g; zinc (as $ZnSO_4 \cdot 7H_2O$), 3 g; cobalt (as $CoSO_4 \cdot 7H_2O$), 0.01 g; copper (as $CuSO_4 \cdot 5H_2O$), 1 g; selenium (as Na_2SeO_3), 0.05 g; manganese (as $MnSO_4 \cdot 4H_2O$), 2 g; magnesium (as $MgSO_4 \cdot 7H_2O$), 50 g; chromium (as $CrCl_3 \cdot 6H_2O$), 0.1 g; calcium (as $CaIO_3 \cdot 6H_2O$), 0.2 g.

TABLE 2 Composition and analysed nutrient content of the experimental diets

	Diet[a]	
	Starch	Fat
Ingredients (parts by weight)		
Basal ingredient mixture[b]	75	75
Maize starch (gelatinized)	25	-
Rapeseed oil	-	10
Total	100	85
Ingredients (g kg^{-1})		
Basal ingredient mixture[b]	75.0	88.2
Maize starch (gelatinized)	25.0	-
Rapeseed oil	-	11.8
Analysed nutrient content(g kg^{-1} DM)		
DM (g kg^{-1} WW)	942	971
Ash	63	73
Crude protein (N × 6.25)	474	558
Crude fat	50	178
Total carbohydrates[c]	413	191
Gross energy (kJ g^{-1} DM)	20.3	22.4
DP:DE (g MJ^{-1})	26.2	26.1
Yttrium	0.09	0.10
Total bile acids (µmol kg^{-1} DM)[d]	257	330

Abbreviations: DE: digestible energy; DM: dry matter; DP: digestible protein; N: nitrogen; WW: wet weight.

[a]Starch: diet with maize starch (gelatinized) as main non-protein energy source; Fat: Diet with rapeseed oil as main non-protein energy source.

[b]Composition of the basal ingredient mixture is given in Table 1.

[c]Total carbohydrates = 1000 - (ash + crude protein + crude fat).

[d]Dietary content of individual bile acids is given in Table 3.

2.3 | Experimental procedures and sampling

Diets were randomly assigned to the experimental units (i.e. tanks) in triplicate. Rainbow trout (*Oncorhynchus mykiss*) were obtained from the French National Institute for Agricultural Research (INRA, France). At the beginning of the experiment, fish were starved for 24 hr to allow emptying of the gastro-intestinal tract. Ten fish from

the base population were euthanized by an overdose of anaesthetic (1 ml L^{-1} 2-phenoxyethanol) and the whole body was sampled for determination of body nutrient composition and the total body bile acid pool. The remaining fish (initial body weight (BW) 202 g $fish^{-1}$ averaged over diets) were batch weighed and randomly distributed over the experimental units at a stocking density of 25 fish $tank^{-1}$. Fish were hand-fed for 44 days, twice daily (9:00 and 16:00 hr), to apparent satiation. The fish were considered to have reached apparent satiation when they stopped feeding or when the feeding time exceeded more than 1 hr. To allow accurate determination of the feed intake, uneaten feed pellets were collected using the swirl separators and by siphoning of the tanks if necessary. A feed sample of 100 g was taken weekly from each diet, pooled per diet and stored at 4°C until analysis. Tanks were checked for mortality twice daily before feeding. Faeces were collected overnight using the swirl separators, and faecal collection bottles were submerged in ice water to minimize bacterial decomposition. Faeces were collected and pooled per tank for the entire experimental period starting from week 2. The collected faeces were stored at −20°C awaiting analysis. At the end of the experiment, fish were starved again for 24 hr and batch weighed. From each tank, 10 fish were euthanized by an overdose of anaesthetic (1 ml L^{-1} 2-phenoxyethanol) and sampled for determination of final body nutrient composition and body bile acid pool. Both initial and final fish samples were stored at −20°C awaiting analysis.

2.4 | Analytical methods

Faecal samples were dried at 70°C until constant weight. Frozen fish samples were cut into small pieces using a band saw and homogenized by grinding two times in a meat mincer with a 4.5 mm die (TW-R 70, Feuma Gastromaschinen GmbH, Germany). A subsample of minced fish was freeze-dried. A mixer mill (MM 200 Retch, Brinkmann, Germany) was used to pulverize the dried faeces (12,000 RPM; 1 mm fixed screen opening), diets and freeze-dried fish samples (18,000 RPM; without fixed screen). Proximate composition and bile acids in the diets, faeces and fish was analysed in triplicate. Dry matter (DM), ash and mineral content of the fish were analysed directly on the minced fish samples, while protein, fat, energy and bile acid levels were analysed using the freeze-dried fish samples.

Dry matter (DM) was determined gravimetrically by drying samples until constant weight at 103°C (ISO 6496, 1999). Ash content was determined gravimetrically by incineration of samples until constant weight in a muffle furnace at 550°C (ISO 5984, 2002). Yttrium, phosphorus, calcium and magnesium content of the samples were measured by ICP-OES (NEN 15510, 2007). Crude protein (N x 6.25) content was measured according to Kjeldahl's method (ISO 5983–2, 2009). Crude fat was determined gravimetrically using acid hydrolysis (Hydrotherm, C. Gerhardt GmbH & Co. KG) paired with Soxhlet extraction using petroleum-ether (Soxtherm, C. Gerhardt GmbH & Co. KG) (ISO 6492, 1999). Gross energy was measured using a bomb calorimeter (C7000 IKA, IKA-Werke GmbH & Co. KG) (ISO 9831, 1998). Bile acids were extracted from the feed, faeces and fish samples according to the method described by Li et al. (2015) with some minor modifications. Samples (100 mg) were weighed directly into 2.2 ml Eppendorf Safe-Lock Tubes. Two ml of absolute ethanol was added, and samples were sonicated at 55°C for 30 min using a bath sonicator (UR-324 T Retsch, Brinkmann, Germany). The samples were subsequently heated at 80°C for 30 min using a warm water bath (SW23, Julabo GmbH). After cooling, samples were centrifuged at 11,000 ***g*** for 10 min (Centrifuge 5430, Eppendorf AG. Germany). The supernatant was aspirated and retained. The pellets were resuspended in 2 ml of absolute ethanol, heated and centrifuged as before. The supernatants were aspirated and each pooled with the first supernatant. The pellets were subsequently resuspended a third and fourth time in 2 ml methanol/chloroform (1:1, v/v), with intermediate heating, centrifuging and aspirating as before. The pooled bile acid extracts were evaporated at 40°C (Block heater SBH200D/3, Stuart, UK) under a continuous stream of air (Sample concentrator SBH CONC/1, Stuart, UK). Samples were reconstituted in 1 ml of methanol and filtered by a syringe-driven filter unit (Nylon membrane 0.2 µm, VWR®). Bile acids were measured using liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) (Institute for Clinical Chemistry and Laboratory Medicine, University of Regensburg, Germany) according to Scherer et al. (2009). In total, 21 bile acids were analysed: cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), hyocholic acid (HCA), ursodeoxycholic acid (UDCA), hyodeoxycholic acid (HDCA), and all their taurine (T-) and glycine (G-) conjugates. Detection limits for each bile acids and extraction recoveries can be found in Table S1.

2.5 | Calculations

2.5.1 | Performance indicators

Mortality (%) was calculated as $((N_0-N_t)/N_0) \times 100$, where N_0 and N_t are the initial and final number of fish per tank, respectively. Feed conversion ratio (FCR; g DM g^{-1}) was calculated as $(FI \times dmF)/(W_t-W_0)$, where FI is the total feed intake (g $fish^{-1}$), dmF is the DM content of the feed (%), and W_0 and W_t are the mean initial and final BW (g $fish^{-1}$), respectively. The geometric mean BW (W_g, g $fish^{-1}$) was calculated as $e^{((\ln Wt + \ln W0)/2)}$. Daily feed intake (% BW d^{-1}) was calculated as $((FI \times dmF)/t/W_g) \times 100$, where t is the length of the experimental period in days (d). Faeces production (RFP, % BW d^{-1}) was calculated as $((FI \times dmF \times (D_i/F_i))/t/W_g) \times 100$, where D_i is the percentage inert marker of the diet DM, and F_i is the percentage inert marker of the faeces DM. Specific growth rate (SGR; % BW d^{-1}) was calculated as $((\ln W_t - \ln W_0)/t) \times 100$.

2.5.2 | Apparent nutrient digestibility

Total carbohydrates (g kg^{-1} DM) in feed and faeces was calculated as 1000 – ash – crude protein – crude fat, with ash, crude protein, crude

fat and starch +sugars expressed in g kg^{-1} DM. The apparent digestibility coefficient (ADC; %) of each nutrient was calculated using the formula described by Bureau et al. (2003): 100 - (100 × (F/D) × (D_i/F_i)), where D is the percentage nutrient (or kJ g^{-1} gross energy) of the diet DM, and F the percentage nutrient (or kJ g^{-1} gross energy) of the faeces DM.

2.5.3 | Bile acids

Total dietary bile acid content ($Dbile_{total}$; μmol kg^{-1} DM), total body bile acid pool size ($BBAP_{total}$; μmol kg^{-1} DM) and total faecal bile acid content ($Fbile_{total}$; μmol kg^{-1} DM) were calculated by summing individually measured bile acids. Total bile acid intake (BAI_{total}), total faecal bile acid loss ($FBAL_{total}$), total body bile acid pool gain (gain $BBAP_{total}$), total bile acid synthesis (BAS_{total}), geometric mean total body bile acid pool ($BBAP_{gtotal}$) and total fractional turnover rate were calculated by summing individual bile acids. Intake of individual bile acids ($BAI_{individual}$; μmol kg^{-1} BW d^{-1}) was calculated as ((FI × dmF)/t/(W_g/1000)) × $Dbile_{individual}$, where $Dbile_{individual}$ is the content of an individual bile acid in the diet (μmol g^{-1} DM). Faecal loss of individual bile acids ($FBAL_{individual}$; μmol kg^{-1} BW d^{-1}) was calculated as (((FI × dmF) × (D_i/F_i))/t)/(W_g/1000) × $Fbile_{individual}$, where $Fbile_{individual}$ is the content of an individual bile acid in the faeces (μmol g^{-1} DM). Gain of individual bile acids in the body bile acid pool (gain $BBAP_{individual}$; μmol kg^{-1} BW d^{-1}) was calculated as ($BBAPt_{individual}$ - $BBAP0_{individual}$)/t/(W_g/1000), where $BBAPt_{individual}$ and $BBAP0_{individual}$ are, respectively, the final and initial pool size of the individual bile acid (μmol $fish^{-1}$). Synthesis of individual bile acids ($BAS_{individual}$; μmol kg^{-1} BW d^{-1}) was calculated as gain $BBAP_{individual}$ + $FBAL_{individual}$ − $BAI_{individual}$. In juvenile fish which are fed to satiation, growth is exponential. As this paper demonstrates that the size of the body bile acid pool is a function of body weight, it is best to express the bile acid pool size relative to body weight. Assuming exponential growth of the body bile acid pool size in analogy with body weight, the geometric mean body bile acid pool size is the most representative for the time interval under investigation. The geometric mean body bile acid pool size of individual bile acids ($BBAPgeometric_{individual}$; μmol kg^{-1} BW) was calculated as $e^{((\ln BBAPtindividual + \ln BBAP0individual)/2)}$/($W_g$/1000). Fractional turnover rate of individual bile acids (% $BBAPgeometric_{individual}$ d^{-1}) was calculated as ($BAS_{individual}$/$BBAPgeometric_{individual}$) × 100. The change (%) in total faecal bile acid loss between diets explained by the difference in total faecal bile acid content was calculated as (((TFBAC$_{Starch}$ − TFBAC$_{Fat}$) × ((RFP$_{Starch}$ + RFP$_{Fat}$)/2))/(TFBAL$_{Starch}$ − FBAL$_{Fat}$)) × 100, where TFBAC$_{Starch}$ and TFBAC$_{Fat}$ are, respectively, the total faecal bile acid content of fish fed the Starch and Fat diet, RFP$_{Starch}$ and RFP$_{Fat}$ are, respectively, the faeces production of fish fed the Starch and Fat diet, and TFBAL$_{Starch}$ and TFBAL$_{Fat}$ are, respectively, the total faecal bile acid loss of fish fed the Starch and Fat diet. Similarly, the change (%) in total faecal bile acid loss between diets explained by the difference in faeces production was calculated as (((RFP$_{Starch}$ − RFP$_{Fat}$) × (($F_{bStarch}$ + F_{bFat})/2))/(TFBAL$_{Starch}$ − TFBAL$_{Fat}$)) × 100.

2.6 | Statistical analysis

Tanks (n = 6) were considered as the experimental units. Data were analysed for the effect of diet (i.e. Starch vs. Fat) using one-way ANOVA. The effect of diet was tested against the variation between tanks. All data were tested for homogeneity of variance by Levene's test prior to ANOVA. Normal distribution of residuals was checked using Kolmogorov–Smirnov test. The correlations between BW and total body bile acid pool size were tested using Pearson's correlation test, and linear regression was used to model the relationship between variables. Statistical significance was tested at the .05 probability level. p-values between .1 and .05 (.1 > p ≥ .05) were defined as close to statistical significance and as indicative for tendencies in the data. All statistical tests were performed using the program SAS 9.4; SAS Institute.

3 | RESULTS

Although not the focus of the current study, data on fish performance, ADC and body nutrient composition are given in supplemental tables for completeness.

3.1 | Performance, apparent digestibility coefficient and body nutrient composition

Data on fish performance are shown in Table S2. Mortality was low and unaffected by diet (1.4% averaged over diets; p ≥ .1). Daily feed intake expressed in dry matter basis was equal for both diets (p ≥ .1) and averaged 1.57% BW d^{-1}. Despite equal feed intake, fish fed the Starch diet produced significantly more faeces expressed in dry matter basis compared with fish fed the Fat diet (0.33 vs. 0.22% BW d^{-1}; p < .01; Figure 1a; Table S2). Growth and final BW were not affected by diet (p ≥ .1).

ADC is given in Table S3. The DM ADC of the Starch diet was significantly lower compared with the Fat diet (79.5 vs. 85.5%; p < .01; Figure 1b; Table S3). The body nutrient composition at the start and end of the experiment is given in Table S4.

3.2 | Dietary bile acid content, body bile acid pool and faecal bile acid content

Of the 21 bile acids quantified, LCA, T-UDCA, G-UDCA, UDCA, T-HCA, T-HDCA, G-HDCA and HDCA were not detected in any of the samples. The terms total cholic acid (CA) and total chenodeoxycholic acid (CDCA) denote the sum of both conjugated and the unconjugated forms of the respective bile acids. Table 3 shows that the primary bile acids total CA and total CDCA were predominant in feed, body and faeces. Regardless of diet, total CA averaged over diets accounted with 87.9% for the majority of the total body bile acid pool. Second most abundant in the body pool was total CDCA (11.2%)

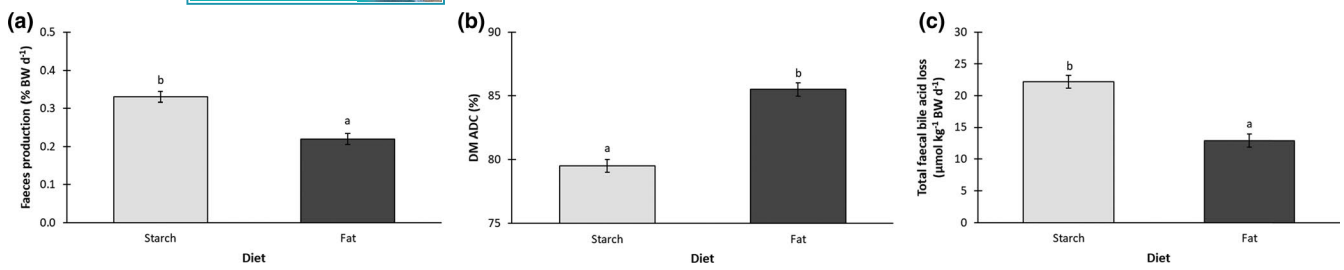

FIGURE 1 Effect of diet on faeces production (panel a), dry matter digestibility (panel b) and total faecal bile acid loss (panel c) of rainbow trout fed to satiation for 44 days; ADC: apparent digestibility coefficient; BW: body weight; DM: dry matter; Starch: diet with maize starch (gelatinized) as main non-protein energy source; Fat: diet with rapeseed oil as main non-protein energy source; Statistical data within each panel are derived from one-way ANOVA for the effect of diet, and differences between diets are denoted by different superscripts. All three parameters were affected by diet ($p < .01$). Error bars indicate standard error of means

(Table 3; Figure 2a). All other bile acids combined took a minimal share of 0.9% of the total body bile acid pool.

More than 99.0% of total CA was conjugated with taurine, while this was 93.5% of total CDCA. The remaining CDCA was unconjugated. Glycine-conjugated bile acids represented a negligible share of 0.4% of the total body bile acid pool. Regardless of diet, total CA and total CDCA accounted for 63.0% and 36.6% of the bile acids in the faeces, respectively (Table 3; Figure 2b), leaving a share of 0.4% for the secondary bile acids. Similarly to the body bile acid pool, the majority of total CA and total CDCA in the faeces was conjugated with taurine (94.8% and 95.2%, respectively) and the remaining CA and CDCA in the faeces were mostly unconjugated.

The total dietary bile acid content was similar between diets (Starch: 257 μmol kg^{-1} DM; Fat: 330 μmol kg^{-1} DM; Table 2). In contrast, the total body bile acid pool size of fish fed the Starch diet was significantly higher compared with the Fat diet (3687 vs. 2449 μmol kg^{-1} DM; $p < .05$; Figure 3a). The smaller total body bile acid pool size was mainly attributed to lower levels of T-CA and CA (summed 3304 vs. 2102 μmol kg^{-1} DM; $p < .1$; Table 3). The levels of all other measured bile acids in the body pool were unaffected by diet ($p \geq .5$). There was no significant effect of diet on total faecal bile acid content (6265 μmol kg^{-1} DM averaged over diets; $p \geq .01$; Table 3; Figure 3b). Nevertheless, a significant higher level of the bile acid T-CDCA was present in the faeces of fish fed the Starch diet compared with the Fat diet (2598 vs. 1796 μmol kg^{-1} DM; $p < .05$; Table 3). Table 3 shows that the ratios of conjugated to unconjugated and taurine to glycine-conjugated bile acids were unaffected by diet in the body bile acid pool and faecal bile acid content ($p \geq .1$). In contrast, feeding the Fat diet tended to decrease the ratio of primary to secondary bile acids in both the body and faeces ($.1 > p \geq .05$).

3.3 | Faecal bile acid loss and bile acid synthesis

Total faecal bile acid loss ($Total_{absolute}$) was more than 1.7 times higher for fish fed the Starch diet compared with fish fed the Fat diet ($p < .01$; Table 4; Figure 1c). This higher absolute faecal bile acid loss was almost entirely ascribed to higher faecal loss of the primary bile acids T-CA (12.2 vs. 8.3 μmol kg^{-1} BW d^{-1}; $p < .05$) and T-CDCA (8.5 vs. 4.0 μmol kg^{-1} BW d^{-1}; $p < .01$). The loss of secondary bile acids was mostly unaffected by diet. The effect of diet on total faecal bile acid loss became less significant when expressing it relative to the body pool size ($Total_{relative}$; $p < .05$) and was 1.97% BBAPgeometric$_{total}$ pool d^{-1} averaged over diets.

Total bile acid synthesis ($Total_{absolute}$) of fish fed the Starch diet was more than 2 times higher compared with the Fat diet ($p < .01$; Table 4). This higher total bile acid synthesis was mainly ascribed to higher synthesis of both T-CA and T-CDCA in fish fed the Starch diet compared with the Fat diet ($p < .01$). The synthesis of secondary bile acids and glycine-conjugated and glycine-unconjugated forms of the primary bile acids was low, and no clear trend in the effect of diet on synthesis was present for these bile acids. The fractional turnover rate of total bile acids was more than 1.8 times higher for fish fed the Starch diet ($p < .001$; Table 4). Data on bile acid intake and body bile acid pool gain (which were used to calculate bile acid synthesis) are given in Table S5 for completeness.

3.4 | Fractional turnover rate of cholic acid and chenodeoxycholic acid

Fractional turnover rate is bile acid synthesis expressed as a fraction of percentage of its total body pool size. Table 5 shows that the fractional turnover rate of both total CA and total CDCA were significantly higher when feeding the Starch diet compared with the Fat diet ($p < .001$). Regardless of diet, the fractional turnover rate of total CDCA was higher compared with total CA.

4 | DISCUSSION

4.1 | Composition and size of the body bile acid pool in rainbow trout

Quantitative and qualitative data on the bile acid pool in fish is scarce. The few studies that did report fish bile acid data either measured a limited number of bile acids or measured bile acids in specific tissues rather than the whole body. The main bile acids

TABLE 3 Dietary bile acid content and effect of diet on body bile acid pool and faecal bile acid content of rainbow fed to satiation for 44 days

	Dietary bile acid content		Body bile acid pool					Faecal bile acid content			
	Diet[b]			Diet[b]				Diet[b]			
	Starch	Fat	Initial[c]	Starch	Fat	SEM	*p*-value[d]	Starch	Fat	SEM	*p*-value[d]
Bile acid (μmol kg^{-1} DM)[a]											
Primary bile acids											
T-CA	119	171	3456	3273	2095	212.3	*	3716	3718	274.0	NS
G-CA	8.3	6.1	12.4	6.3	4.6	2.09	NS	3.9	5.5	1.64	NS
CA	39.7	63.4	11.7	30.7	7.0	5.83	#	267	136	96.8	NS
T-CDCA	21.4	28.3	273	326	299	46.6	NS	2598	1796	178.6	*
G-CDCA	BDL	BDL	BDL	BDL	BDL	-	-	4.1	BDL	-	-
CDCA	40.3	37.3	16.6	24.8	18.8	6.99	NS	174.1	59.1	57.13	NS
Secondary bile acids related to CA											
T-DCA	1.0	BDL	3.0	1.5	0.6	5.53	NS	9.9	11.6	1.18	NS
G-DCA	0.1	0.1	0.3	0.8	1.1	0.27	NS	0.01	0.73	0.111	*
DCA	3.5	3.3	1.5	1.8	1.5	0.62	NS	2.9	2.3	0.61	NS
Secondary bile acids related to CDCA											
T-LCA	BDL	0.3	0.2	BDL	0.4	-	-	0.8	0.8	0.15	NS
G-LCA	BDL	BDL	BDL	1.0	0.3	-	-	0.04	BDL	-	-
G-HCA	3.9	3.4	3.5	3.3	3.4	0.51	NS	1.3	1.8	0.12	#
HCA	20.0	16.3	16.6	18.2	16.8	0.58	NS	9.0	10.4	0.69	NS
Bile acid ratio											
Primary:secondary	8:1	12:1	150:1	144:1	105:1	12.3:1	#	285:1	208:1	22.3:1	#
Conjugated:unconjugated	2:1	2:1	81:1	53:1	61:1	13.7:1	NS	20:1	40:1	15.0:1	NS
Taurine:glycine conjugated	12:1	21:1	231:1	397:1	334:1	106.8:1	NS	986:1	776:1	161.5:1	NS
Total CA:total CDCA	3:1	4:1	12:1	10:1	7:1	1.5:1	NS	1:1	2:1	0.1:1	**

Abbreviations: BDL: below detection limit (detection limits are given in Table S1);DM: dry matter; SEM: standard error of means.

T-: tauro; G-: glyco; CA: cholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; LCA: lithocholic acid; HCA: hyocholic acid; total CA: sum of both conjugated and unconjugated CA; total CDCA: sum of both conjugated and unconjugated CDCA.

[a]Levels of LCA (lithocholic acid), T-UDCA (tauroursodeoxycholic acid), G-UDCA (glycoursodeoxycholic acid), UDCA (ursodeoxycholic acid), T-HCA (taurohyocholic acid), T-HDCA (taurohyodeoxycholic acid), G-HDCA (glycohyodeoxycholic acid) and HDCA (hyodeoxycholic acid) were below the detection limit in the diets, body and faeces, and are therefore not included in the table. Total dietary bile acid content is given in Table 2. Total body bile acid pool size and faecal bile acid content are given in Figure 1.

[b]Starch: diet with maize starch (gelatinized) as main non-protein energy source; Fat: diet with rapeseed oil as main non-protein energy source.

[c]Initial body bile acid pool at the start of the experiment.

[d]NS, not significant: $p \geq .1$; #: $p < .1$; *: $p < .05$; **: $p < .01$; *P*-values for body bile acid pool are derived from one-way ANOVA for the effect of diet and thus do not relate to the values reported for initial body bile acid pool.

in biliary bile of salmonids are CA and CDCA (Hagey et al., 2010). Specifically for rainbow trout, Denton et al. (1974) reported levels of biliary total CA between 85.4% and 93.6% and total CDCA between 6.0% and 14.7%. Denton et al. (1974) also reported that most bile acids were conjugated with taurine. Those findings are in line with the current study.

Several other studies investigated biliary bile acid pool size and composition of rainbow trout in the context of fishmeal replacement by soybean meal (SBM) (Iwashita et al., 2008, 2009; Murashita et al., 2013; Yamamoto, Matsunari, et al., 2012; Yamamoto, Murashita, et al., 2012; Yamamoto et al., 2007, 2008, 2010). SBM consistently resulted in a downregulation of genes involved in both bile acid transport and synthesis (Kortner et al., 2013; Murashita et al., 2018). The exact compounds in SBM that cause disturbances in the bile acid metabolism are not fully known, but saponins are likely to play a major role (Romano et al., 2020). Since diets of the current study were formulated without SBM, for comparison of body bile acid pool composition and size of the current study with literature, only data are used for rainbow trout fed animal-based protein diets (thus excluding SBM-based diets). According to literature, T-CA and T-CDCA take shares of the conjugated biliary bile acid pool of rainbow trout between 87.0–93.8% and 6.2–13.0%, respectively (Iwashita et al.,

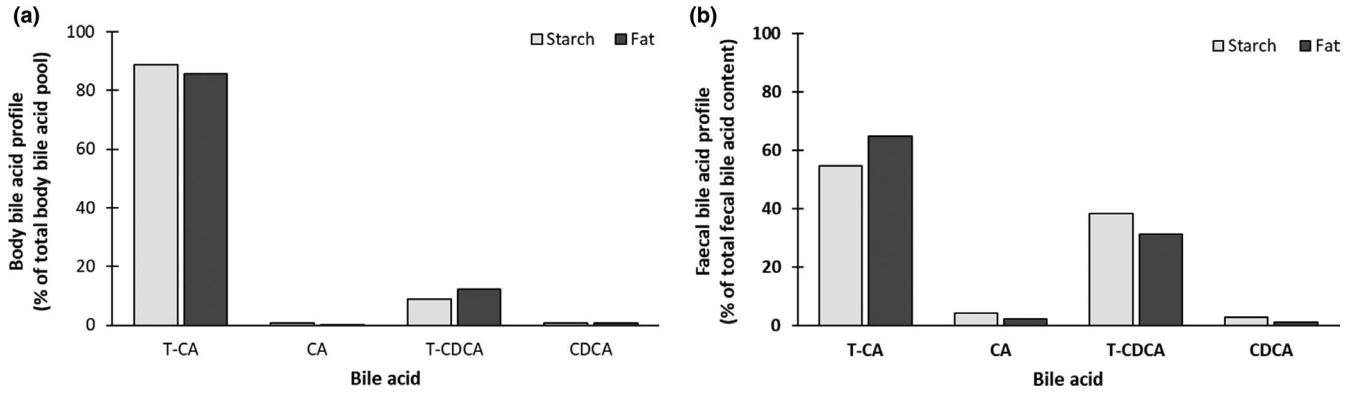

FIGURE 2 Effect of diet on the body bile acid profile (panel a) and the faecal bile acid profile (panel b) of rainbow trout fed to satiation for 44 days; T-: tauro; CA: cholic acid; CDCA: chenodeoxycholic acid; Only bile acids representing a share of more than 1% of either the total body bile acid pool or 1% of the total faecal bile acid content are shown in this figure

2008, 2009; Murashita et al., 2013; Yamamoto, Murashita, et al., 2012; Yamamoto et al., 2007, 2008, 2010). This is in line with the average value of 88.9% for T-CA and 10.7% for T-CDCA found in the current study.

Besides taurine-conjugated bile acids, Yamamoto et al. (2007), Yamamoto et al. (2008) and Yamamoto, Murashita, et al. (2012) also found glycine conjugates in the biliary bile of trout, however, only when diets were supplemented with bovine bile acids (the latter containing ±42% glycine-conjugates). The review by Romano et al. (2020) lists taurine as the only amino acid that conjugates with bile acids in several fish species, including rainbow trout. The synthesis of total glycine-conjugated bile acids in the current study was −0.13 µmol kg^{-1} BW d^{-1} averaged over the diets. This negative value indicates that the intake of glycine-conjugated bile acids via the diets (most likely originating from fishmeal and fish oil) was larger than their faecal loss and gain in the body bile acid pool combined. This suggests that the glycine-conjugated bile acids measured in the body bile acid pool most likely originated from the diets rather than from *de novo* synthesis in the fish.

The negative values for synthesis of some bile acids might also be related to re-conjugation with taurine or conversion into other bile acids (Denton et al., 1974). To our knowledge, no information about the rate of deconjugation and formation of secondary bile acids is available for fish. However, ratios of conjugated to unconjugated bile acids in the body of humans (0.9:1), and in the faeces of humans (0.8:1) and rats (0.01:1) (Chen et al., 2020; Hagio et al., 2009; Stellaard et al., 1987) are much lower compared with those found in the current study. Furthermore, those studies also reported lower ratios of primary to secondary bile acids in the body of humans (1.6:1–2.4:1) and rats (6:1), and in the faeces of humans (0.03:1–1.14:1) and rats (0.18:1) compared with this study. Both deconjugation and formation of secondary bile acids are mediated by intestinal bacteria, and in mammals, secondary bile acids are mainly formed in the colon (Midtvedt, 1974). The rate of deconjugation and formation of secondary bile acids in rainbow trout seems to be much lower compared with mammals, which most likely is related to the absence of a true colon in fish or the cold-water nature of rainbow trout. Body bile acid composition was not greatly affected by diet

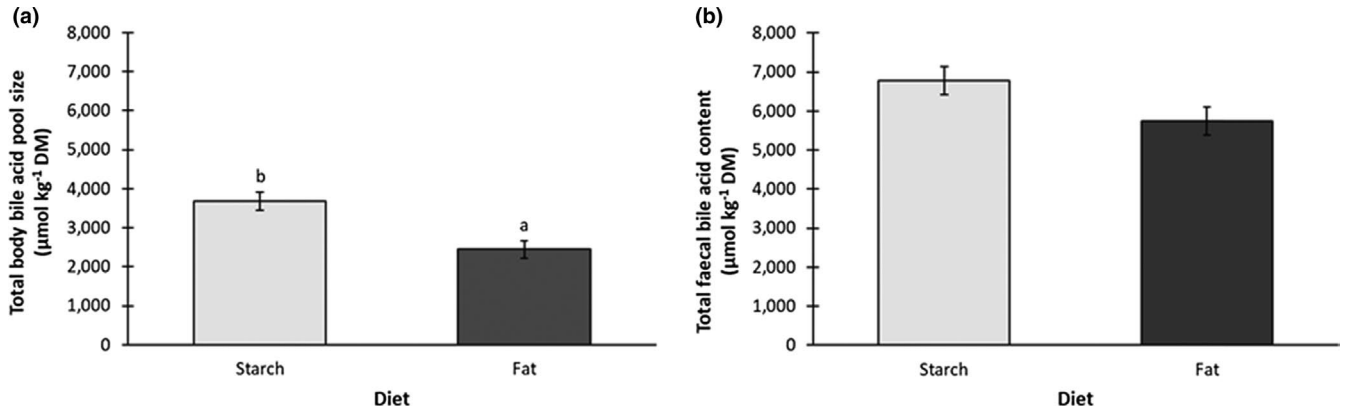

FIGURE 3 Effect of diet on total body bile acid pool size (panel a) and total faecal bile acid content (panel b) of rainbow trout fed to satiation for 44 days; DM: dry matter; Starch: diet with maize starch (gelatinized) as main non-protein energy source; Fat: diet with rapeseed oil as main non-protein energy source. Statistical data within each panel are derived from one-way ANOVA for the effect of diet, and differences between diets ($p < .05$) are denoted by different superscripts. Total body bile acid pool size was affected by diet ($p < .05$). Total faecal bile acid content was unaffected by diet ($p \geq .1$). Error bars indicate standard error of means

TABLE 4 Effect of diet on faecal bile acid loss and bile acid synthesis of rainbow trout fed to satiation for 44 days

	Faecal bile acid loss				Bile acid synthesis			
	Diet[b]				Diet[b]			
Bile acid (μmol kg^{-1} BW d^{-1})[a]	Starch	Fat	SEM	*p*-value[c]	Starch	Fat	SEM	*p*-value[c]
Primary bile acids								
T-CA	12.2	8.3	0.88	*	23.2	11.8	1.43	**
G-CA	0.01	0.01	0.004	NS	−0.12	−0.09	0.022	NS
CA	0.9	0.3	0.29	NS	0.4	−0.7	0.28	*
T-CDCA	8.5	4.0	0.54	**	9.7	5.4	0.59	**
G-CDCA	0.01	-	-	-	0.01	-	-	-
CDCA	0.6	0.1	0.18	NS	0.06	−0.33	0.125	#
Secondary bile acids related to CA								
T-DCA	0.03	0.03	0.003	NS	0.02	0.02	0.004	NS
G-DCA	0.00004	0.0020	-	*	0.001	0.006	0.004	NS
DCA	0.01	0.01	0.002	NS	−0.04	−0.04	0.007	NS
Secondary bile acids related to CDCA								
T-LCA	0.003	0.002	0.0004	NS	0.002	−0.001	0.0010	#
G-LCA	0.0001	-	-	-	0.005	0.003	0.0050	NS
G-HCA	0.004	0.004	0.0004	NS	−0.04	−0.03	0.005	#
HCA	0.03	0.02	0.001	*	−0.2	−0.1	0.01	**
Total[d]								
$Total_{absolute}$	22.2	12.9	1.02	**	33.0	16.0	1.63	**
$Total_{relative}$ (% $BBAPgeometric_{total}$ d^{-1})[e]	2.2	1.5	0.13	*	3.3	1.8	0.10	***

Abbreviations: $BBAPgeometric_{total}$, geometric mean total body bile acid pool size; BW, body weight; SEM, standard error of means.

T-: tauro; G-: glyco; CA: cholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; LCA: lithocholic acid; HCA: hyocholic acid.

[a]Faecal bile acid loss and bile acid synthesis are expressed on BW as is. The body bile acid pool in Table 3 and Figure 1 is expressed on BW DM.

[b]Starch: diet with maize starch (gelatinized) as main non-protein energy source; Fat: diet with rapeseed oil as main non-protein energy source; -: not calculated because respective bile acids were below the detection limit in feed, faeces or body.

[c]NS, not significant: $p \geq .1$; #: $p < .1$; *: $p < .05$; **: $p < .01$; ***: $p < .001$;

[d]Because of rounding, totals do not necessarily add up exactly.

[e]For bile acid synthesis, these values represent the total bile acid fractional turnover rate; the fractional turnover rates for total CA and total CDCA are given in Table 5.

composition, and none of the bile acid ratios differed significantly between diets. There is quite some variation in bile acid composition between taxonomic orders of fish; however, within orders the bile acid composition is generally stable (Hagey et al., 2010; Haslewood, 1967). Furthermore, Hagey et al. (2010) stated that within fish orders there is no apparent link between diet composition and the composition of the bile acid pool.

A comparison of total bile acid pool size of rainbow trout with literature is more difficult to make, since BW of the fish differed between studies. Figure 4a shows the reported total bile acid pool size as a function of the BW for both literature and the current study. Total bile acid pool size shows a strong positive correlation with fish weight and an increase of 100 g of BW corresponds with an increase of 85 μmol of the total bile acid pool size. For easier comparison between studies, the bile acid pool was expressed per unit of BW (Figure 4b), highlighting a large variation between studies in the measured total bile acid pool size (range 580–1729 μmol kg^{-1} BW). This variation might be ascribed to several factors including genetic differences (Lu et al., 2010), differences in the used methodology between studies (e.g. tissue sampled), differences in the time between last feeding and sampling of the fish (Romano et al., 2020), differences in the applied feeding levels (Denton et al., 1974), but also differences in diet composition (Chiang, 2009; Romano et al., 2020). That diet composition can affect the body bile acid pool was also shown by the outcome of the current study and will be elaborated upon hereafter.

4.2 | Effect of diet on body bile acid pool size

Data of this study show a smaller total body bile acid pool size of fish fed the Fat diet compared with those fed the Starch diet (Figure 3a). This smaller total body bile acid pool was ascribed to lower levels of all primary bile acids, although the difference between diets was only

significant for T-CA (Table 3). Dietary effects on the bile acid metabolism have been described in literature for both mammals and fish.

Disturbances of the bile acid metabolism of fish caused by specific antinutrients (e.g. high molecular plant proteins, (soy) saponins, non-starch polysaccharides) in ingredients replacing fishmeal (e.g. SBM) have been studied several times (Romano et al., 2020). In contrast, information about the effects of dietary carbohydrate and fat level on the bile acid metabolism of fish is lacking, this despite demonstrated effects in mammals (Chiang, 2013). Regardless of the dietary factors and their mechanism of action responsible for the altered bile acid metabolism, diet-induced changes in the bile acid metabolism (including changes in the body bile acid pool) are related to altered faecal bile acid loss and/or bile acid synthesis in the liver. The effects of diet on faecal bile acid loss and bile acid synthesis are discussed in the next paragraphs, as they might provide an explanation for the difference in the body bile acid pool size between fish fed the Starch and Fat diet of the current study.

TABLE 5 Effect of dietary main non-protein energy source on the fractional turnover rate of total cholic acid and total chenodeoxycholic acid

Fractional turnover rate (% $BBAPgeometric_{individual}$ d^{-1})[a]	Diet[b]			P-value[c]
	Starch	Fat	SEM	Diet
Total CA	2.6	1.4	0.11	**
Total CDCA	11.4	5.9	0.26	***

$BBAPgeometric_{individual}$: geometric mean body bile acid pool size of the corresponding individual bile acid (i.e., $BBAPgeometric_{totalCA}$ and $BBAPgeometric_{totalCDCA}$ respectively); SEM: standard error of means. Total CA: sum of both conjugated and unconjugated cholic acid; total CDCA: sum of both conjugated and unconjugated chenodeoxycholic acid.

[a]Total bile acid fractional turnover rates are given in Table 4 (footnote e).

[b]Starch: diet with maize starch (gelatinized) as main non-protein energy source; Fat: diet with rapeseed oil as main non-protein energy source.

[c]***: $p < .001$.

4.3 | Effect of diet on faecal bile acid loss

This study showed that the total daily bile acid loss relative to the body bile acid pool size ranged between 1.5 and 2.2% d^{-1}, depending on diet. For comparison, reported faecal bile acid loss relative to the total pool size was 1.7% d^{-1} for rats (Moundras et al., 1997) and 1–5% d^{-1} for humans (Chiang, 2013; Van Eldere et al., 1996). In the current study, absolute total faecal bile acid loss in trout fed the Fat diet was lower compared with the loss in trout fed the Starch diet. Previous studies with rainbow trout showed that DM ADC is a factor crucial to determine the level of faecal bile acid loss (Staessen et al., 2020a, 2020b). Despite the absence of a diet effect on faecal bile acid content in those studies, enhanced faeces production when feeding diets with low DM ADC resulted in an increased flush-out of bile acids with the faeces. The same mechanism explains the difference in faecal bile acid loss between diets of the current study. Although the total faecal bile acid content was not significantly different (Figure 3b), the lower DM ADC and subsequently higher faeces production of fish fed the Starch diet resulted in a total faecal bile acid loss that was 1.5 times higher compared with the Fat diet (Figure 1a–c). Total faecal bile acid loss is by definition total faecal bile acid content multiplied with faeces production. Therefore, the relative contribution of both factors to the difference in total faecal bile acid loss between diets was also calculated. Faecal bile acid content explained 31% and faeces production 69% of the difference in faecal bile acid loss. Despite a lack of a significant diet effect on faecal bile acid content, the contribution of faecal bile acid content to the difference in faecal bile acid loss between diets stems for a numerically

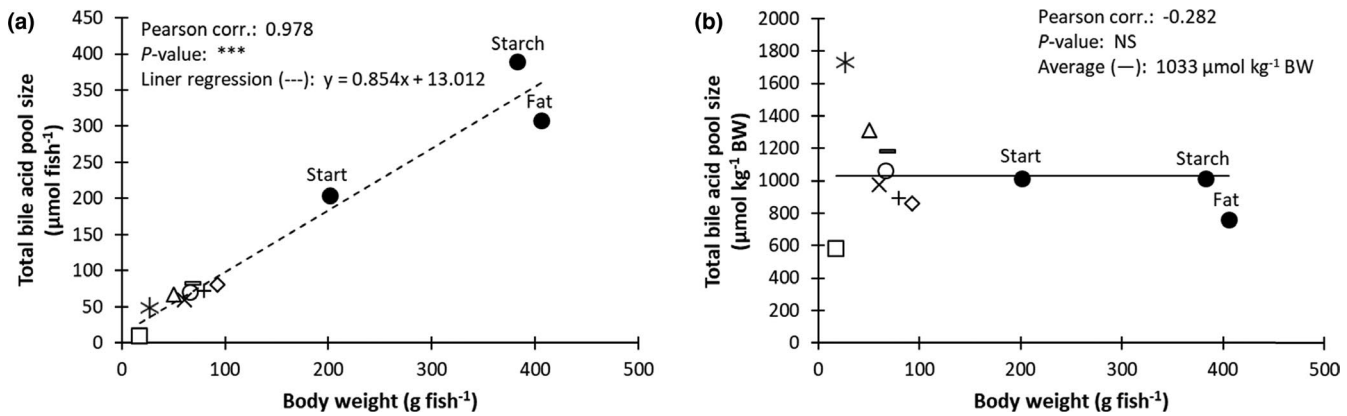

FIGURE 4 Relationship between body weight and total bile acid pool size of rainbow trout expressed per fish (panel a), and per kg body weight on as is basis (panel b); BW: body weight; Pearson corr.: Pearson correlation coefficient. Data of the current study ● (Start: fish sampled before the start of the experiment, Fat: fish fed the Fat diet sampled at the end of the experiment, Starch: fish fed the Starch diet sampled at the end of the experiment), Iwashita et al. (2009) □, Yamamoto et al. (2008) ✶, Iwashita et al. (2008) Δ, Yamamoto et al. (2007) X, Yamamoto et al. (2010) ○, Yamamoto, Murashita, et al. (2012) –, Murashita et al. (2013) + and Yamamoto, Matsunari, et al. (2012) ◊ are presented for rainbow trout fed diets containing animal-based protein as main protein source (thus excluding diets that induced soybean meal-related symptoms such as enteritis); NS, not significant: $p \geq .1$; ***: $p < .001$

higher faecal bile acid content when feeding the Starch diet. The latter is most likely related to a higher total body bile acid pool size, as was also observed in rats (Moundras et al., 1997). Numerous other dietary components, including high molecular plant protein (Iwami et al., 1990; Murashita et al., 2013), (soy) saponins (Gu et al., 2014; Kregiel et al., 2017; Krogdahl et al., 2015; Murashita et al., 2018) and non-starch polysaccharides (Ikegami et al., 1990; Moundras et al., 1997; Sinha et al., 2011) can enhance faecal bile acid loss by either directly interacting (e.g. bind, entrap, etc.) with bile acids or causing symptoms that reduce bile acid reabsorption (e.g. soybean meal-related enteritis) (Krogdahl et al., 2015; Yamamoto et al., 2007). Such components could theoretically have been present in the diets as part of the basal ingredient mixture. However, as the basal ingredient mixture was more diluted in the Starch diet (by addition of maize starch) than in the Fat diet (by addition of rapeseed oil), their effects on faecal bile acid loss, if any, is ought to have been negligible. Higher faecal bile acid loss has also been linked to higher fat intake in mammals (Reddy, 1992; Reddy et al., 1977). Especially, polyunsaturated fatty acids were suggested to inhibit reabsorption of bile acids in the intestine (Ammon & Phillips, 1974). Cummings et al. (1978) showed that an increase in faecal fat loss could explain an increase in faecal bile acid loss. This is probably because bile acids associate with fat in micelles and are in this way lost with fat excreted via faeces (Leroy et al., 1986). Despite the higher fat intake associated with the Fat diet, higher fat ADC of the Fat diet resulted in faecal fat loss not being significantly different between diets (data not shown), so also the effect of fat on faecal bile acid loss is ought to have been negligible in this study. Regardless of which mechanism(s) was responsible, the difference in faecal bile acid loss between diets of the current study does not explain the difference in total body bile acid pool size. Faecal loss removes bile acids from EHC, and under homeostasis faecal bile acid loss is compensated with *de novo* bile acid synthesis in the liver (Lanzini & Lanzarotto, 2000). Increased removal of bile acids from EHC leads to a decrease of the bile acid pool when bile acid synthesis is at its maximum (Dowling et al., 1970). Despite higher faecal bile acid loss, fish fed the Starch diet maintained a higher body bile acid pool by increasing bile acid synthesis. This increased bile acid synthesis was probably partly related to a compensation for the higher faecal bile acid loss when feeding the Starch diet. However, a compensatory mechanism does not explain a difference in body bile acid pool size at the end of the experiment. The difference in total body bile acid pool size at the end of the experiment implies an increase in bile acid synthesis beyond the level needed to compensate enhanced faecal bile acid loss of fish fed the Starch diet and/or a decrease in bile acid synthesis of fish fed the Fat diet.

4.4 | Effect of diet on bile acid synthesis

In fish, several dietary factors, including taurine (de Moura et al., 2019; Kim et al., 2015; Nguyen et al., 2015; Richard et al., 2017), cholesterol (Deng et al., 2013; Kortner et al., 2014; Yun et al., 2011) and soybean antinutrients (Gu et al., 2014; Kortner et al., 2013; Krogdahl et al., 2015; Murashita et al., 2018), were suggested to affect bile acid synthesis. However, bile acid synthesis has never been actually quantified. The presence of aforementioned dietary compounds is expected not to have differed substantially between the diets of the current study, and therefore, their effects on bile acid synthesis are ought to have been negligible. Mammalian studies showed that diet macronutrient composition can affect bile acid synthesis by altering both bile acid kinetics and expression of genes involved in bile acid synthesis. The cycling frequency of bile acids within EHC is partly determined by the extend of gallbladder contraction. The gallbladder (functioning as storage organ for bile acids between meals) contracts in response to the presence of fat and amino acids in the proximal intestine, subsequently releasing bile acids (Aldman & Holmgren, 1995). Fat diets result in a stronger contraction of the gallbladder and a higher cycling frequency of bile acids compared with carbohydrate diets (Hepner, 1975). Bisschop et al. (2004) showed a decrease in primary bile acid production of humans fed an extremely high-fat diet (83%) compared with an intermediate fat diet (41%). Those authors observed that the fractional turnover rate (% synthesis of a bile acid relative to its pool) of both primary bile acids were lower, suggesting a more efficient reabsorption of bile acids in the intestine by higher activity of bile acid transporters. Both higher cycling frequency and an increased reabsorption of bile acid when feeding fat diets were suggested to increase the flux of bile acids through the liver, resulting in decreased bile acid synthesis by downregulation of 7α-hydroxycholesterol (CYP7A1; the first rate-limiting enzyme in the classical biosynthesis pathway) through activation of the farnesoid X receptor (FXR) (Hofmann et al., 2010; Romano et al., 2020). In the current study, feeding fish the Fat diet resulted in lower fractional turnover rates of total CA and total CDCA compared with fish that were fed the Starch diet (Table 5). Together with the assumption of a stronger gallbladder contraction, an increased bile acid flux through the liver of fish fed the Fat diet would explain lower bile acid synthesis of fish fed the Fat diet through activation of FXR and inhibition of CYP7A1. To the best of our knowledge, there are no papers on the effect of gut loading (volume) on bile acid synthesis and bile acid pool size. Despite a more or less equal daily DM feed intake between diets, lower DM digestibility (Table S3) of the Starch diet should have resulted in a larger volume of intestinal content compared with the Fat diet. Furthermore assuming less gallbladder contraction in fish fed the Starch diet, the concentrations of bile acids in the intestinal content might have been lower in fish fed the Starch diet, which through positive feedback regulation to the liver, could also (partly) explain an increase in bile acid synthesis and an higher total bile acid pool size. Differences in bile acid synthesis between diets could have been further enhanced by starch and fat directly. Li et al. (2012) showed that spikes in glucose and insulin stimulate human and murine CYP7A1 and enlarge the bile acid pool size. It is not known whether glucose and insulin have the same effect in fish, but it could (partly) explain the higher bile acid synthesis of fish fed the Starch diet. High-fat diets stimulated bile acid synthesis in rats (Bertolotti et al., 1995; Botham &

Boyd, 1983). CYP7A1 was upregulated in mice fed fat-rich diets, especially when the fat source was rich in polyunsaturated fatty acids (Cheema et al., 1997). Furthermore, rapeseed oil used in the Fat diet of the current study most likely provided phytosterols. Phytosterols can compete with cholesterol for uptake in the intestine, which leads to increased faecal cholesterol loss (Miller et al., 2008). In order to conserve cholesterol, cholesterol synthesis in the liver is upregulated and bile acid synthesis downregulated as shown in Atlantic salmon (Liland et al., 2013). Perhaps, the presence of phytosterols in rapeseed oil can (partly) explain the lower bile acid synthesis of fish fed the Fat diet.

4.5 | Total bile acid pool size and fat apparent digestibility

The fat ADC of the Fat diet used in the current study was higher compared with the Starch diet, and this despite a smaller total body bile acid pool size of fish fed the Fat diet. The instinctive assumption that a larger total bile acid pool size results in better fat digestion does not necessarily hold true. Also, the dietary fatty acid profile is an important determinant for fat digestibility (Hua & Bureau, 2009). The difference in fatty acid profile between the Starch and Fat diet of the current study might explain the higher fat ADC found for the latter. Using the model of Hua and Bureau (2009) to predict digestible fat content and a theoretical fatty acid profile of fish oil (Lee & Ahn, 2003) and rapeseed oil (Zambiazi et al., 2007), the estimated fat ADC for the Starch and Fat diet was 89.6% and 91.1%, respectively, thus predicting a slightly better ADC of the fat used in the Fat diet.

4.6 | Intestinal conservation of cholic acid and chenodeoxycholic acid

Regardless of diet, the ratio of total CA to total CDCA in the faeces was lower than in the body (Table 3; Figure 2a,b). Furthermore, also the fractional turnover rate of total CA was lower compared with total CDCA for both diets (Table 5). The discrepancy between the body and faecal bile acid profile, and the lower fractional turnover rate of total CA suggest a better intestinal conservation of total CA over total CDCA in rainbow trout. Information about intestinal conservation of specific bile acids is not available for fish. In contrast to the current study, better intestinal conservation of CDCA over CA was reported for humans (Angelin et al., 1976; Einarsson et al., 1979) and rats (Schiff et al., 1972). The better intestinal conservation of CDCA (a dihydroxy bile acid) compared with CA (a trihydroxy bile acid) observed in humans and rats was ascribed to the less polar nature of CDCA which allows some passive absorption along the whole length of the small intestine (Angelin et al., 1976; Schiff et al., 1972). In rainbow trout, an apical sodium-dependent bile acid transporter has been identified (Murashita et al., 2014), but no information is available on absorption efficiency of specific bile acids.

5 | CONCLUSION

Using data from both the current study and from literature, a linear relationship was demonstrated between the bile acid pool size and the BW of rainbow trout. However, the current study demonstrates that the rate of the increase in body bile acid pool size with BW is (partly) dependent on diet composition (e.g. type of non-protein energy source). Regardless the type of non-protein energy source, taurocholic acid and taurochenodeoxycholic acid were sequentially most abundant in the total body bile acid pool, accounting for more than 90% of bile acids. In other words, dietary non-protein energy source does not seem to have a substantial impact on the body bile acid pool composition of rainbow trout. The total body bile acid pool size of fish fed the Starch diet was larger compared with the Fat diet at the end of the experiment, and this despite higher faecal bile acid loss when feeding the Starch diet. This larger bile acid pool size was possible because bile acid in fish fed the Starch diet was more than 2 times higher compared with the Fat diet. The difference in final total body bile acid pool size between diets cannot be explained by a higher bile acid synthesis that compensates for the higher faecal bile acid loss when feeding the Starch diet. Instead, results suggest a further upregulation of bile acid synthesis in fish fed the Starch diet and/or a downregulation for the Fat diet. The exact mechanism(s) responsible for the difference in bile acid synthesis between diets cannot be identified from this study, but both changes in the rate of EHC and direct effects of the type of non-protein energy source on bile acid synthesis most likely played a role. Despite a smaller body bile acid pool size, the fat ADC of the Fat diet was better compared with the Starch diet. A smaller bile acid pool size is thus not necessarily synonym for a lower fat digestibility, and other factors need to be taken into account (e.g. fatty acid profile). Furthermore, this study shows that the level of unconjugated and secondary bile acids in the body and faeces of rainbow trout are remarkably lower compared with those reported in mammalian studies. Finally, the discrepancy between the bile acid profile of the body and faeces and the lower fractional turnover rates of cholic acid, regardless of diet, suggest a better intestinal conservation of cholic acid compared with chenodeoxycholic acid in rainbow trout.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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