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The polycyclic aromatic hydrocarbons benzo[*a*]pyrene and phenanthrene inhibit intestinal lipase activity in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Elevated levels of polycyclic aromatic hydrocarbons (PAHs) are detected in aquafeeds where fish oils are (partially) replaced by vegetable oils. The highly lipophilic PAHs solubilize readily in oil droplets and micelles in the intestinal lumen that can affect enzymatic lipid digestion by altering lipase activity. We therefore investigated the effect of two PAHs, benzo[*a*]pyrene (BaP) and phenanthrene (PHE), on bile salt-activated lipase (BAL) activity in desalted luminal extracts of the proximal intestine of rainbow trout (*Oncorhynchus mykiss*) using the triacylglycerides rapeseed oil and fish oil as substrates.

The hydrolysis of rapeseed oil and fish oil measured at a calculated substrate concentration of 2.2 mM, increased linearly up to 30 min at 15 °C. Substrate dependency under initial velocity conditions was described by simple Michaelis-Menten kinetics with a K_m value of 1.2 mM for rapeseed and fish oil. Rapeseed oil hydrolysis was inhibited by 1 nM BaP and 10 nM PHE. The hydrolysis of fish oil was only inhibited by 10 μM BaP. The *in vitro* lipase activity data were corroborated by TLC/HPLC analysis of the reaction products, showing that in the presence of BaP and PHE, 46–80% less free fatty acids (FFA) were hydrolysed from rapeseed and fish oil triacylglycerides.

The presence of low concentrations of BaP and PHE decreased rapeseed oil hydrolysis by BAL whereas fish oil hydrolysis was not affected. The replacement of fish oil by rapeseed oil in aquafeeds introduces PAHs that could affect lipid digestion.

1. Introduction

Traditionally, marine fish oils and fishmeal have been used as main fish ingredients in aquafeeds. However, the rapidly growing aquaculture sector cannot continue to rely on the limited supply of fish ingredients. Hence, fish oil and fishmeal in aquafeeds are more and more replaced with ingredients from plant origin (FAO, 2014; Pickova and Mørkøre, 2007; Tacon and Metian, 2008). The inclusion of vegetable ingredients in aquafeeds, however, introduced polycyclic aromatic hydrocarbon (PAH) congeners, including benzo[*a*]pyrene (BaP) and phenanthrene (PHE) in Atlantic salmon (*Salmo salar*) tissue (Berntssen et al., 2005, 2010, 2015). PAHs are ubiquitous lipophilic organic contaminants composed of two or more fused aromatic rings. These contaminants are mostly formed due to incomplete combustion or pyrolysis of organic matter (Moret and Conte, 2002). In aquafeeds, PAHs are formed by thermal processing of oil-containing seeds and grains during toasting or gas drying (Moret et al., 2005; Phillips, 1999; Teixeira et al., 2007). The diet contributes substantially to PAH exposure with cereals, vegetable fats and oils being the principal culprits (Phillips, 1999). Concern about these contaminants has been due to the carcinogenic, mutagenic/genotoxic and other toxic effects induced by PAHs (EFSA, 2008).

After oral ingestion, the lipophilic nature of PAHs promotes their solubilization in oil droplets and mixed micelles in the intestinal lumen (Jandacek and Genuis, 2013; Kelly et al., 2004; Porter et al., 2007) where it can potentially interfere with lipase activity and lipid digestion. Luminal entry of emulsified lipids stimulates the exocrine pancreas and gall bladder to secrete digestive lipases and bile juice, respectively, in the intestinal lumen (Olsen and Ringø, 1997; Tocher, 2003). In many teleost species, the exocrine pancreas is distributed diffusely around the gastrointestinal tract and secretes its enzymes into the lumen of the pyloric caeca and/or proximal intestine (Bakke et al., 2010). Biliary components (*e.g.* bile salts and cholesterol) spontaneously form mixed micelles with free fatty acids (FFA) and, to a lesser

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extent, with mono-, di- and triacylglycerides (Bakke et al., 2010; Phan and Tso, 2001; Wang et al., 2013; Yeap et al., 2013).

Two key lipolytic enzymes are secreted by the exocrine pancreas in mammals, *viz.* bile salt-activated lipase (BAL) and pancreatic lipase (Wang and Hartsuck, 1993). Lipases hydrolyze ester bonds in triacylglycerides (TAGs), phospholipids, cholesteryl esters and fat-soluble vitamins. In mammals, pancreatic lipase is the most important digestive lipase. In fish, however, BAL is considered to be the most important digestive lipase (Bogevik et al., 2008; Bogevik, 2011; Gjellesvik et al., 1992; Olsen and Ringø, 1997; Rønnestad et al., 2013; Sæle et al., 2010; Tocher, 2003). BAL has a broad substrate specificity, is highly dependent on bile salts to be catalytically active and is more efficient in hydrolyzing polyunsaturated fatty acids (PUFAs), which are abundant in the diet of marine and freshwater fish (Chen et al., 1990; Gjellesvik, 1991; Wang and Hartsuck, 1993).

The lipid composition of micelles can affect the solubility of PAHs. Indeed, PAHs have a higher solubility in micelles composed of unsaturated long-chain fatty acids compared to saturated short-chain fatty acids (Doi et al., 2000; Laher and Barrowman, 1983) whereas solubility in micelles composed of long-chain triacylglycerides is often low (Porter et al., 2007). Fish oil is an important source of $n-3$ unsaturated long-chain fatty acids such as eicosapentaenoic acid (EPA; 20:5$n-3$) and docosahexaenoic acid (DHA; 22:6$n-3$) (Bell and Waagbø, 2009; Sioen et al., 2008). Rapeseed oil is mainly used as a substitute for fish oils and contains high levels of oleic acid (*ca.* 60%; 18:1$n-9$) and moderate levels of linoleic acid (*ca.* 20%; 18:2$n-6$) and saturated short-chain fatty acids (Table 1). The different lipid compositions of rapeseed oil and fish oil are thus likely to affect PAH levels in micelles and, hence, influence lipase activity.

The objective of the present study was to investigate the effects of BaP and PHE on the lipolytic activity of lipase in rainbow trout (*Oncorhynchus mykiss)* using rapeseed oil and fish oil as substrates.

2. Materials and methods

2.1. Animals

Rainbow trout (*Oncorhynchus mykiss*) with a body weight of 429 ± 78 g (mean ± SD) were obtained from a commercial hatchery 'Keijzersberg' in Blitterswijck, the Netherlands. Fish were kept at 15.0 ± 0.5 °C (mean ± SD) in an indoor recirculating system containing 575 L (input of 1 L fresh tap water per minute) of biofiltered and UV-treated Nijmegen tap water. Trout were fed a commercial fish feed (Optiline trout, 3.0 mm, Skretting, Utah, USA) with an automated feeder at 9.30 and 16.30 h at a ration of 2% of the estimated body weight per day.

Previous studies showed that 6 h post-feeding feed was mainly located in the proximal intestine in Atlantic salmon weighing 200 g (de Gelder et al., 2016). Therefore, to ensure access to intestinal luminal contents, rainbow trout were anaesthetized 6 h post-feeding in 0.1% (v/v) 2-phenoxyethanol (Sigma, St. Louis, USA) and euthanized by spinal transection caudal of the opercula. Experimental design obeyed Dutch legislation and was approved by the ethical review committee of Radboud University (RU-DEC 2012-315).

Table 1
Fatty acid composition (area percentage of total fatty acids) of various diets containing 100% of the different oil sources. Table was adapted from [1]Bell et al. (1999) and [2]Torstensen et al. (2004).

	Olive oil[1]	Fish oil[2]	Rapeseed oil[2]
14:0	0.3	6.7	0.4
16:0	9.7	11.7	5.7
18:0	3.2	1.0	1.7
Sum saturates	13.7	20.4	9.1
16:1$n-7$	0.7	8.0	0.6
18:1$n-7$	1.5	3.4	3.2
18:1$n-9$	73.9	11.2	53.6
20:1$n-9$	0.7	17.1	2.1
22:1$n-11$	0.7	13.3	1.0
Sum monoenes	77.5	57.1	61.1
18:2$n-6$	5.9	3.5	19.5
20:2$n-6$	–	0.2	–
20:4$n-6$	–	0.3	–
Sum $n-6$	5.9	4.1	19.5
18:3$n-3$	0.5	1.1	8.6
18:4$n-3$	–	2.8	0.2
20:4$n-3$	–	0.4	–
20:5$n-3$	0.3	5.9	0.7
22:5$n-3$	–	0.4	–
22:6$n-3$	0.6	4.6	1.0
Sum $n-3$	1.4	15.8	10.6

2.2. Preparation of BAL extracts

As the exocrine pancreas is made up of diffuse tissue in between the pyloric caeca, the best way to collect pancreatic enzymes such as BAL is by collection of luminal contents. The peritoneal cavity was opened and the proximal intestine, defined as the section directly posterior of the pyloric caeca to the beginning of the distal intestine, recognized by its darker appearance and annulo-spiral septa, was dissected and placed in a Petri dish on ice. All subsequent steps were performed at 0 – 4 °C. Visceral fat was removed, the proximal intestine was opened longitudinally and the luminal contents were gently extruded and suspended in four volumes (w/v) ice-cold 100 mM phosphate buffer containing 2.5% aprotinin and 0.1 mM PMSF, pH 7.5. All chemicals were purchased from Sigma-Aldrich Co, St Louis, U.S.A. unless mentioned otherwise. Samples were centrifuged at 10.000 *g* for 10 min at 4 °C. The lipid layer was aspirated from the surface of the supernatant after which the supernatant was collected and designated as crude BAL extract.

Crude BAL extracts were desalted overnight at 4 °C by dialysis (Tube-O-Dialyzer, MWCO 4000 Da, G-Biosciences St Louis, U.S.A.) against 100 mM phosphate buffer to remove endogenous bile salts. Protein concentrations of the desalted BAL extracts were measured by spectrophotometry with a Coomassie Brilliant Blue reagent kit (Bio-Rad, München, Germany) using bovine serum albumin as a reference, and diluted to 1.0 mg protein·mL^{-1} unless mentioned otherwise.

2.3. Validation of the modified titrimetric assay

Lipase activity was assessed with a modified titrimetric assay (Gotthilf, 1974) by measuring the decline in pH following lipid hydrolysis. Lipase activity can be assessed in a volumetric assay as described by Gotthilf (1974), in which the fatty acids liberated from the triacylglycerol substrate are titrated with NaOH. To prevent dilution of substrate and enzyme concentrations in the incubate by the addition of NaOH titrant, we have chosen to measure the initial decrease in pH of the incubate with a sensitive pH electrode (GK2401C Radiometer Analytical, Villeurbanne Cedex, France) connected to a pH meter (CG-842 Schott Geräte GmbH, Mainz, Germany).

All assay media were mechanically stirred, pre-warmed for 10 min and maintained at the designated temperature. The modified titrimetric assay was validated with a substrate emulsion containing 11% (final assay concentration: 35 mM) commercially available olive oil and 89% gum Arabic (10% w/v) that was added to assay medium containing 30 mM sodium taurocholate and 32 mM NaCl. Olive oil is a generally used substrate to measure lipase activity and known for its high levels of monounsaturated fatty acids and deficiency in $n-3$ unsaturated long-chain fatty acids (Gupta et al., 2003). The assay medium was completed with substrate emulsion, assay medium and dH_2O (3:3:2.9 v/v). The medium was adjusted to exactly pH 9.0 with 0.1 or 0.01 M NaOH and maintained at pH 9.0 for 8 min to stabilize gum Arabic. After 8 min, porcine pancreatin (4 × United States Pharmacopeia (U.S.P.) specifications; 8.0 units lipase·mg pancreatin^{-1}), dis-

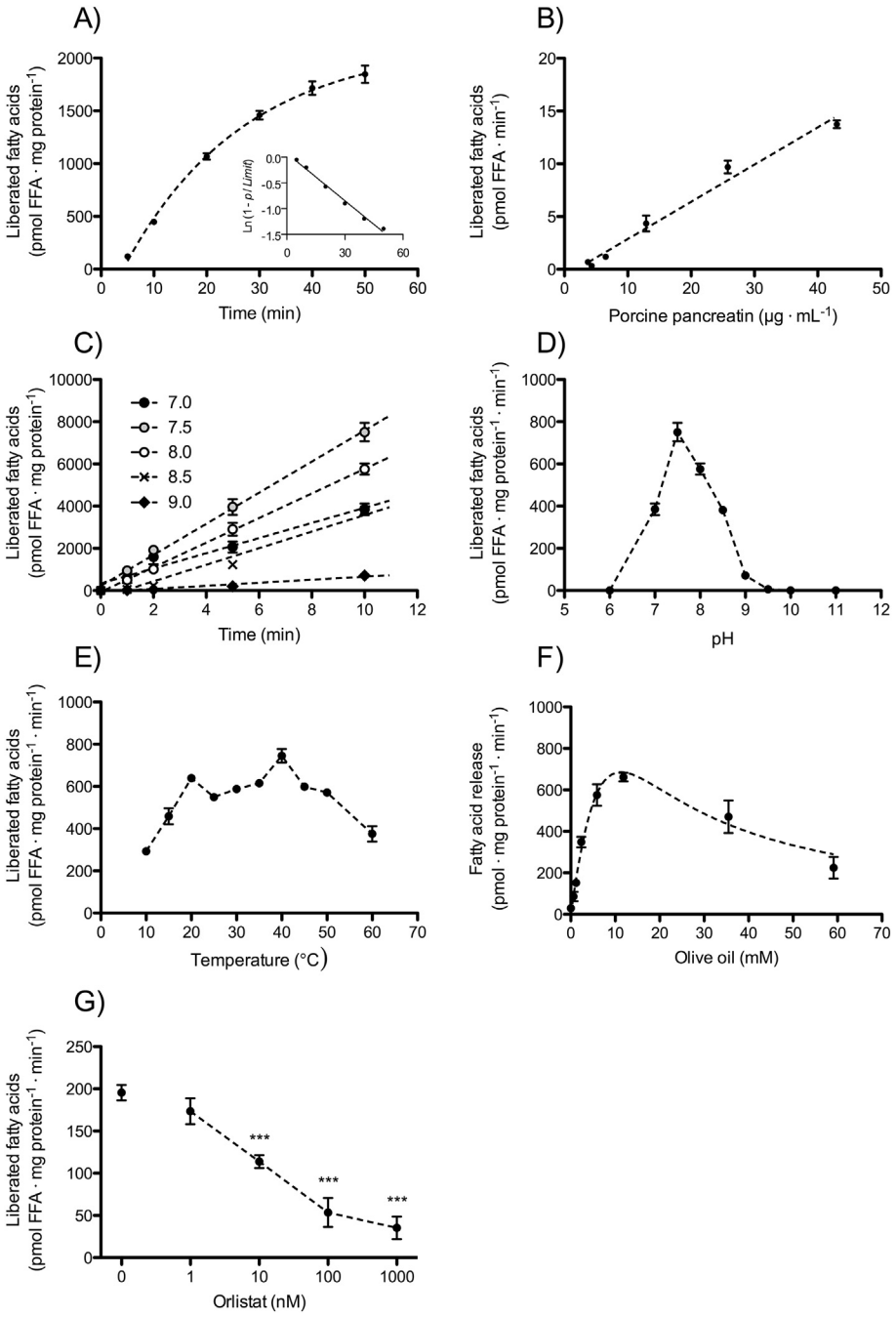

Fig. 1. Optimization of the pH-drop assay with olive oil as substrate and porcine pancreatin as lipase source. A) Time course of lipase activity with 36 mM olive oil as substrate, 25 µg porcine pancreatin·mL^{-1} at pH 9.0. Data were fitted to a single exponential equation with a calculated limit and a rate constant of 2567 pmol FFA·mg protein^{-1} and k = 0.044 min^{-1}, respectively. The inset shows linearization of the single exponential equation, indicative of a single site substrate. B) Effect of increasing porcine pancreatin concentrations ranging from 4 to 40 µg pancreatin·mL^{-1} at t = 5 min. C) Effect of pH on enzyme activity. D) Lipase activity at pH values from 6 to 11. E) Temperature dependence at temperature values from 10 to 60 °C. F) Substrate dependent hydrolysis. The kinetic parameters derived from this curve are: maximum hydrolysis rate V_{max} is 3402 pmol FFA·mg protein^{-1}·min^{-1}, K_m is 22.4 mM olive oil and a substrate inhibition constant (K_i) of 5.7 mM olive oil. G) Inhibition of lipase activity with 2.4 mM olive oil by Orlistat. Orlistat significantly decreased olive oil hydrolysis by 40% (ANOVA with Dunnett's multiple comparison test). Points represent experimental data (mean ± SEM; n = 3 and n = 5 for the orlistat experiment). Due to the small sample size (n = 3) and the relatively large standard errors no sensible 95% CIs could be calculated.

solved in 300 µL assay medium was added as a source of lipase. The pH was readjusted to 8.0 with NaOH, the volume was adjusted with dH_2O to 9.3 mL and the change in pH was measured. Samples were measured in triplicate, measured pH values were recalculated to a H^+ concentration, corrected for non-enzymatic fatty acid formation by subtracting measurements on heat-inactivated samples (boiled for 10 min at 100 °C) and normalized for the total assay volume and protein content of the incubate. The recalculated H^+ activity was interpreted to be equivalent to one released proton (H^+) per liberated FFA by lipase (Gotthilf, 1974). The liberation of FFA by lipase activity was expressed as pmol FFA per minute per mg protein.

The time course of lipase activity by porcine pancreatin with 35 mM olive oil as substrate is well described by a single first-order exponential rate equation (Fig. 1A). Data points converged well on a linearization of the single exponential, indicative of one active component in pancreatin lipase activity. Olive oil hydrolysis was linear up to 40 µg pancreatin·mL^{-1} (Fig. 1B). All further validation experiments were performed with 6.5 µg pancreatin·mL^{-1}. Linearity of olive oil hydrolysis was

observed at pHs 7.5, 8 and 8.5 up to 10 min (Fig. 1C), hence we chose an incubation time of 2 min to measure initial hydrolysis rates in all further experiments with porcine pancreatin. Optimal pH for porcine pancreatic lipase was 7.5 (Fig. 1D). However, as the time course was no longer linear at pH 7.0, pH 8.0 was chosen for all further experiments to allow a wider pH range over which pH could decrease. Optimal lipase activity was observed around 40 °C (Fig. 1E). The initial hydrolysis rate with olive oil was best described by a substrate inhibition model with a substrate inhibition constant (K_i) of 5.7 mM olive oil (Fig. 1F). Pre-incubation of porcine pancreatin for 1 h with 10 nM of the lipase inhibitor orlistat decreased olive oil hydrolysis by 40%.

2.4. Trout intestinal lipase activity

Lipase activity was assayed as previously described with some small modifications. Briefly, all media were cooled to 15 °C and maintained at this temperature during the assay as this resembles the ambient temperature of our laboratory stock and that of feral rainbow trout (11–18 °C). A volume of 0.1 mL substrate emulsion containing 11% (final assay concentration: 2.2 mM) olive oil, fish feed graded rapeseed oil (Emmelev AS, Denmark) or fish oil (Norsildmel, Norway) and 89% gum Arabic was added to 4.4 mL assay medium (final concentration: 20 mM sodium taurocholate and 32 mM NaCl). Assay conditions were 100 μg desalted BAL extracts of *O. mykiss* and 20 mM bile salt (taurocholate) (Bogevik et al., 2008). The solution was adjusted to exactly pH 9.0 and after 8 min 100 μL (1 mg protein·mL^{-1}) desalted BAL extract was added. The pH was readjusted with NaOH, the volume was adjusted to 5.0 mL with dH_2O and the decline in pH was measured. The assay with desalted BAL extracts was optimized for time and substrate dependency with olive oil, rapeseed oil and fish oil. Initial hydrolysis rates were measured in a 30 min incubation time unless mentioned otherwise. Under these assay conditions < 1% of the substrate was hydrolyzed. Olive oil was used as substrate to validate lipase activity in BAL extracts. The time course of lipase activity in desalted BAL extracts with 2.2 mM olive oil was well described by a single first-order exponential rate equation and the logarithmic transformed data points converged well on a linearization of the single exponential. The calculated rate constant and limit were 0.015 min^{-1} and 1352 pmol·mg protein^{-1}, respectively. Substrate dependency at t = 30 min showed characteristic single-site Michaelis-Menten kinetics with calculated Michaelis constant (K_m) value of 0.5 mM and a V_{max} of 11.8 pmol·mg protein^{-1}·min^{-1}.

2.5. Lipase activity in the presence of PAHs

Stock solutions of 100 mM BaP and PHE were prepared in acetone (propan-2-one). A preliminary test confirmed that a final assay concentration of 0.1% acetone had no effect on lipid hydrolysis in desalted BAL extracts of the proximal intestine of *O. mykiss* (data not shown). Due to their non-polar, lipophilic nature, PAHs adsorb strongly to labware surfaces. Previous studies have shown that untreated glass vials had the highest recovery and lowest adsorption of PAHs (de Gelder et al., 2017); we therefore used glass vials for all experiments. The substrate emulsion was pre-incubated for 2 min with BaP or PHE prior to addition to the assay medium. The effect of BaP and PHE on rapeseed and fish oil hydrolysis was assessed at the established K_m values of the oil substrates (*i.e.* 1.2 mM). Final assay conditions were: 100 μg BAL extract, 1.2 mM rapeseed or fish oil, pH 8.0 at 15 °C and measured at t = 30 min at initial rate. At t = 30 a sample of 1 mL was immediately frozen in liquid nitrogen and stored at − 80 °C for lipid class analysis.

2.6. Lipid class analysis

Lipids were extracted from the samples by adding 750 μL of a chloroform–methanol mixture (2:1 v/v) to 60 μL of the assay medium after which the solution was vortexed. Then, 250 μL chloroform and 250 μL ddH_2O were added and again vortexed thoroughly (Bligh and Dyer, 1959). The samples were then filtered on a vacuum block in 10 mL reservoirs with a paper filter. The reservoirs are rinsed twice with a chloroform:methanol mix (2:1 v/v). The filtered samples were collected and dried in a RapidVap (Labconco, MO, USA). Lipid class composition was determined using high-performance thin layer chromatography analysis as described by Henderson and Tocher (1992). Briefly, samples were re-suspended in chloroform to an approximate lipid concentration of 0.1 mg·mL^{-1} based on the weight of the dried sample. To silica plates (20 × 10 cm), 1 μL chloroform was applied 1 cm from the bottom and polar lipids were separated in methyl acetate:isopropanol:chloroform: methanol and 0.25% (w/v) aqueous KCl (25:25:25:10:9, v/v). After drying the TLC plates, neutral lipids were separated in isohexane:diethyl ether:acetic acid (80:20:1.5, v/v). Lipid classes were visualised by charring at 160 °C for 15 min after spraying with 3% copper acetate (w/v) in 8% phosphoric acid (v/v) and identified by comparison with commercially available standards. Lipid classes were quantified by scanning densitometry (CAMAG TLC Scanner 3) and calculated using an integrator (WinCATS-Planar Chromatography manager, Version 1.2.0). Mono- and diacylglycerides were below the limit of quantification (< 0.1 mg·g sample^{-1}), hence all data are given as triacylglycerides or FFA (as area percentage of total lipid content).

2.7. Analysis and statistics

The molarities of olive oil, rapeseed oil and fish oil were calculated based on the molecular weight of the most abundant FFA covalently bound to glycerol per oil. The density of olive oil, rapeseed oil and fish oil was calculated from the weights of 50 mL oil that were 0.911, 0.914 and 0.918 kg·L^{-1}, respectively. The most prevalent FFA (*i.e.* oleic acid) esterified to glycerol in olive oil gave an average triacylglyceride molecular weight of 885.4 g·mol^{-1}. The average triacylglyceride molecular weight in rapeseed and fish oil was 882.8 and 903.5 g·mol^{-1}, respectively. With the average molecular weights and density, the molarity of olive oil, rapeseed oil and fish oil was calculated to be 1.03, 1.04 and 1.02 M, respectively.

Enzyme kinetic data were analyzed using weighted non-linear regression procedures in the statistical programming language R (version 3.3.1) (R Development Core Team, 2013) where the Gauss-Newton algorithm for least squares estimation of parameters was employed. For each data point the SEM was used as an explicit weighting value. A Kolmogorov-Smirnov normality test revealed that data points in all groups followed a normal distribution. Time courses were fitted to a single first-order rate equation:

$$p = Limit \cdot (1 - e^{-kt}) \quad (1)$$

where p represents the liberated FFA (pmol FFA·mg protein^{-1}) by lipase, *Limit* (pmol·mg protein^{-1}) represents the uptake at time (t) approaching ∞, and k is a first-order rate constant (min^{-1}). The FFA liberated by lipase was log-transformed to check for a systematic deviation from a single first-order rate equation by:

$$-kt = \ln\left(1 - \frac{p}{Limit}\right) \quad (2)$$

Initial hydrolysis rates approaching t = 0 min were calculated from the slope of the tangents of the time course by solving dp/dt at t = 0 min.

Initial hydrolysis rates of lipase activity were fitted to a simple Michaelis-Menten equation:

$$v_0 = \frac{V_{max} + [S]}{K_m + [S]} \quad (3)$$

where v_0 is enzyme initial rate (pmol FFA·mg protein^{-1}·min^{-1}), [S] is the substrate concentration (mM), V_{max} is the maximum rate (pmol·mg

Table 2
The effects of bile salt (taurocholate) on the hydrolysis of olive oil (pmol FFA·mg protein^{-1}·min^{-1}) in crude and desalted BAL extracts from the proximal intestine of *O. mykiss*. Measurements were corrected for rising acidity by subtracting control incubations (heat inactivated samples). Mean values ± SD; n = 5. Different superscript letters indicate significant differences (Student's *t*-test with Bonferroni's post-hoc test; $p < 0.001$).

BAL extract	Bile salt	FFA release (pmol FFA·mg protein^{-1}·min^{-1}) Average SEM
Control	20 mM	0.2 ± 0.1
Crude	–	2.5 ± 0.5[a]
Desalted	0 mM	3.7 ± 0.5[a]
Desalted	20 mM	15.5 ± 1.5[b]

protein^{-1}), and K_m is the Michaelis constant (mM). When a simple Michaelis-Menten equation did not describe the data adequately, a substrate inhibition function containing the Michaelis-Menten equation plus an inhibition constant (K_i; mM) was used (Cornish-Bowden, 1995). Calculated model parameters were compared using Akaike's information criterion (AIC) and the best fitting (ANOVA, $p \leq 0.05$) and the most parsimonious model was chosen. Statistical significance was therefore evaluated with a one-way ANOVA followed by Bonferroni's or Dunnett's multiple comparison test, where appropriate. Results were considered statistically significant when $p < 0.05$ (two-tailed) and indicated with; *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$).

3. Results

Addition of 20 mM bile salt (taurocholate) to desalted BAL extracts from luminal trout contents significantly increased lipid hydrolysis by 300% compared to crude and desalted BAL extracts (ANOVA; $p < 0.001$). Heat inactivated samples had no hydrolytic activity (Table 2).

The time course of rapeseed and fish oil hydrolysis by BAL extracts proceeded linearly up to 30 min (Fig. 2A). The hydrolysis of rapeseed and fish oil is well described by a single first-order exponential rate equation (Fig. 2B) with calculated rate constants of 0.01 and 0.008 min^{-1}, respectively. The calculated limit for lipid hydrolysis at a substrate concentration of 2.2 mM was practically similar for rapeseed and fish oil (*i.e.* 918 and 1186 pmol FFA·mg protein^{-1}). The initial hydrolysis rates calculated at t = 0 min were 9.2 and 9.0 pmol FFA·min^{-1}·mg protein^{-1} for rapeseed and fish oil, respectively. Data points converged on a linearization of the single exponential, indicative of a single substrate site reflecting a single lipase entity.

Substrate dependency at initial hydrolysis rates showed characteristic single-site Michaelis-Menten kinetics for rapeseed and fish oil (Fig. 3). The calculated Michaelis constant (K_m) value was 1.2 mM for rapeseed as well as for fish oil. Proceeding from these observations, a concentration of 1.2 mM rapeseed and fish oil was chosen to assess the effects of BaP and PHE on lipase activity.

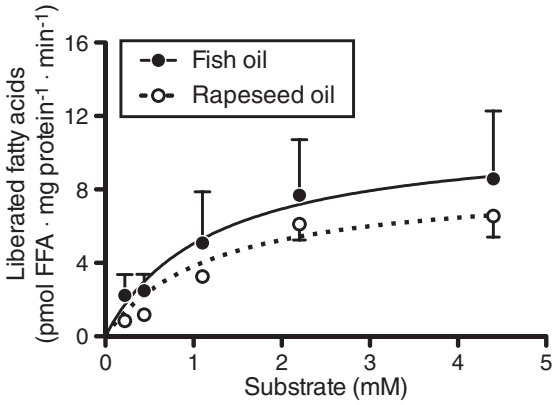

Fig. 3. Substrate dependence of lipase activity measured at initial rate in BAL extracts of the proximal intestine of *O. mykiss* (n = 5, mean ± SEM). The hydrolysis of rapeseed and fish oil are described by Michaelis-Menten kinetics. Calculated values for rapeseed oil hydrolysis were K_m = 1.2 mM (95% CI [0.5, 1.9]) and V_{max} = 8.4 pmol·mg protein^{-1} [7.3, 9.5]. Calculated values for fish oil hydrolysis were K_m = 1.2 mM [0.6, 1.7] and V_{max} = 11.1 pmol·mg protein^{-1} [9.4, 12.8].

Both PAHs inhibited the hydrolysis of rapeseed oil where BaP was 10-fold more potent than PHE (Fig. 4). Rapeseed oil hydrolysis had significantly decreased by 40% in the presence of 0.001 μM BaP ($p < 0.01$) and by 55% with 0.01 μM PHE ($p < 0.01$) compared to the control incubation (0 μM BaP or PHE). Interestingly, only the highest BaP concentration significantly inhibited fish oil hydrolysis by 50% ($p < 0.05$). Unfortunately, since BaP has a low solubility in an aqueous environment, the effect of BaP on lipase activity with concentrations higher than 10 μM could not be measured.

TLC/HPLC analyses of the reaction products corroborate the *in vitro* lipase activity data. Without PAHs, approximately 85% of the total lipid content consisted of FFA after rapeseed and fish oil hydrolysis (Table 3). TLC/HPLC analyses showed that the hydrolysis of rapeseed as well as fish oil was inhibited by 0.001 μM BaP and 0.01 μM PHE whereas the hydrolysis of fish oil measured with the *in vitro* lipase assay was only significantly inhibited by 10 μM BaP. In the presence of 0.001 μM BaP, 50% of the lipid content consisted of triacylglycerides and FFA after fish oil hydrolysis. Rapeseed oil hydrolysis was inhibited by 80% by 0.001 μM BaP as only 17% FFA were liberated by lipase activity. Similar to BaP, 0.01 μM PHE inhibited FFA liberation from triacylglycerides in rapeseed and fish oil by 55%.

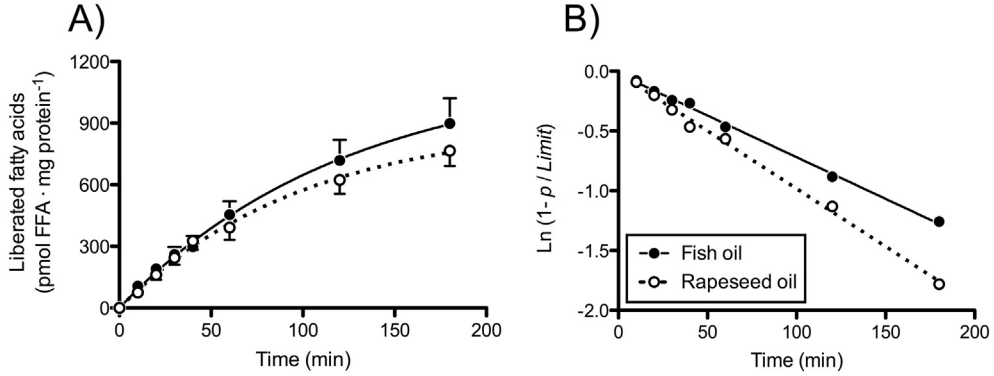

Fig. 2. Time course of rapeseed oil (open symbols) and fish oil (closed symbols) hydrolysis by BAL extracts of the proximal intestine of *O. mykiss* (n = 5, mean ± SEM). Time kinetics are well described by a single exponential equation. Calculated parameters for rapeseed oil hydrolysis were: *Limit* = 918 pmol·mg protein^{-1} (95% CI [842, 994]), k = 0.010 min^{-1} [0.008, 0.011] and for fish oil hydrolysis: *Limit* = 1186 pmol·mg protein^{-1} [1106, 1265], k = 0.008 min^{-1} [0.007, 0.009]. B) Linear logarithmic transformation, indicative of one active component in lipid hydrolysis.

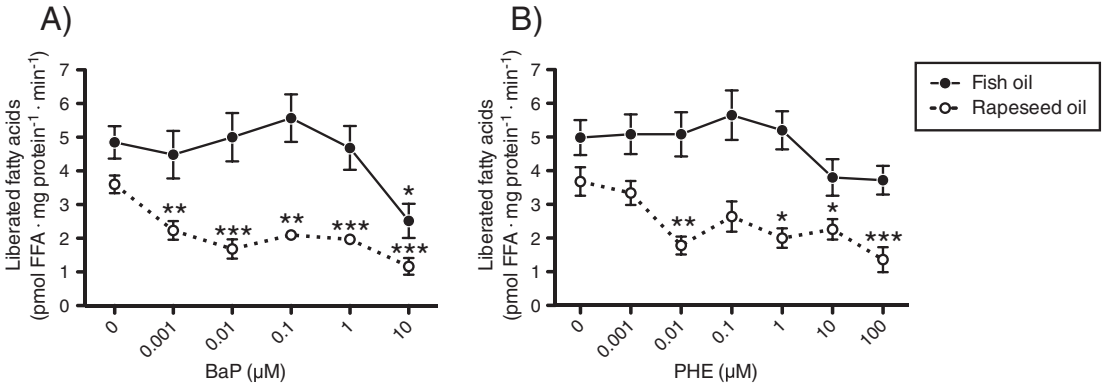

Fig. 4. Hydrolysis of rapeseed oil (open symbols) and fish oil (closed symbols) in the presence of BaP (A) and PHE (B) by desalted BAL extracts of the proximal intestine of *O. mykiss* (n = 6, mean ± SEM). Experimental incubations are compared to the control incubation (0 μM PAHs) with an ANOVA with Dunnett's multiple comparison test.

4. Discussion

The lipophilic PAHs, BaP and PHE inhibit rapeseed oil hydrolysis by BAL in rainbow trout. This effect depends on the substrate's oil composition, as the hydrolysis of fish oil was largely insensitive to PAHs compared to rapeseed oil. The established inhibitory concentrations of BaP and PHE are in the range of 0.3 to 2 $\mu g \cdot L^{-1}$, and are comparable with detected BaP and PHE concentrations of 1.0 and 17 $\mu g \cdot kg^{-1}$ in aquafeeds (Berntssen et al., 2010). Therefore, we speculate that the occurrence of PAHs in aquafeeds by partial replacement of fish ingredients by vegetable ingredients could decrease lipid digestion *in vivo*. This could result in decreased intestinal fatty acid uptake and have a negative impact on organismal energy metabolism.

This study provides support for the notion that BAL is the prominent lipase in rainbow trout as the addition of bile salts (*i.e.* taurocholate) considerably increased lipase activity in desalted BAL extracts (Howles et al., 2000; Lowe, 1997; Wang and Hartsuck, 1993). Linearization of the data points of the single exponential from rapeseed and fish oil hydrolysis illustrated a single catalytic component in the BAL extracts, reflecting a single lipase entity. This indicates that there was no contamination by other lipases (*e.g.* bacterial lipases) than BAL. In Atlantic salmon, rainbow trout and Atlantic cod (*Gadus morhua*) bile salts also stimulated lipase activity (Bogevik et al., 2008; Gjellesvik et al., 1992; Lie and Lambertsen, 1985; Tocher and Sargent, 1984). BAL is able to hydrolyze water-soluble lipid substrates composed of short-chain fatty acids. However, BAL requires activation by bile salts to hydrolyze mono-, di- and triacylglycerides, cholesteryl esters, phospholipids, lysophospholipids, ceramides and fat-soluble vitamins (Hui and Howles, 2002; Moore et al., 2001; Wang and Hartsuck, 1993). It is presumed that bile salts interact with two sites on BAL's tertiary structure to activate the protein. One site protects BAL from proteolysis and promotes binding to the surface of lipid emulsions while the other site causes a conformational change after bile salt binding that exposes the active site of the enzyme (Hui and Howles, 2002; Kurtovic et al., 2009; Moore et al., 2001). Crude luminal extracts prepared 6 h post-feeding displayed similar lipase activities as desalted extracts without bile salts. The capability of BAL to hydrolyze short-chain fatty acids without bile salts might explain the low lipase activity in the desalted extracts (Hui and Howles, 2002; Wang and Hartsuck, 1993).

To validate whether the initial decrease in pH of the incubate coincides with an increased FFA concentration, TLC/HPLC was used to quantitatively measure FFA liberation from triacylglycerides. The TLC/HPLC analysis confirms the yield of FFA following the incubation of a lipid substrate with a BAL extract and the inhibitory effect of BaP and PHE on rapeseed oil hydrolysis. The data also showed inhibition of fish oil hydrolysis by both PAHs, an effect that was not detected with the *in vitro* pH assay. We have to note that TLC/HPLC measures the total lipid content, including the 6–12% FFA already present in native fish oil before hydrolysis, whereas the *in vitro* pH assay measures the decline in pH resulting from FFA newly liberated from the lipid substrate. Since the BAL extracts were prepared in a phosphate buffer, some hydronium ions will be buffered and not detected with the pH electrode. These technicalities likely result in some overestimation of the TLC/HPLC outcome that might explain the different results obtained from TLC/HPLC and the *in vitro* pH assay.

Dietary lipids and their digestion products, such as FFA, interact with lipophilic contaminants in their intestinal uptake and luminal transfer (de Gelder et al., 2016; Doi et al., 2000; Dulfer et al., 1998; Gobas et al., 1993; Laher and Barrowman, 1983; Vasiluk et al., 2008; Vetter et al., 1985). When PAHs enter the gastrointestinal tract, micelles can facilitate luminal transfer across the unstirred water layer towards the brush border membrane of enterocytes (Doi et al., 2000; Laher and Barrowman, 1983). The solubility of lipophilic contaminants is higher in micelles composed of unsaturated long-chain fatty acids compared to saturated short-chain fatty acids (Doi et al., 2000; Laher and Barrowman, 1983). Fish oil contains more PUFAs that have longer fatty acid chains and a higher degree of unsaturated bonds than rapeseed oil (Table 1). This fact coincides well with the more potent inhibition of rapeseed oil hydrolysis by both PAHs. Since micelles composed of rapeseed oil have a lower partitioning of PAHs compared to fish oil-composed micelles, more PAHs would be freely available to inhibit lipase activity. The relative resistance of fish oil hydrolysis to PAHs is likely due to the increased partitioning of PAHs in fish oil-composed micelles leaving less PAHs free to directly interact with lipases.

Triacylglyceride hydrolysis in the intestinal lumen is necessary as

Table 3
Lipid class composition (area percentage of total lipid content) of triacylglycerides and FFA after rapeseed and fish oil hydrolysis in the presence of 0.001 μM BaP and 0.01 μM PHE by desalted BAL extracts of the proximal intestine of *O. mykiss* (n = 2, mean ± SD).

	Rapeseed oil			Fish oil		
	0 μM PAH	1 nM BaP	10 nM PHE	0 μM PAH	1 nM BaP	10 nM PHE
Triacylglycerides (%)	16 ± 5	83 ± 6	65 ± 6	8 ± 2	50 ± 5	56 ± 1
FFA (%)	85 ± 5	17 ± 6	35 ± 6	92 ± 2	50 ± 5	44 ± 1

intact triacylglycerides cannot cross the intestinal brush border membrane (Gilham et al., 2007; Gilham and Lehner, 2005). Double knockout C57BL/6 mice lacking pancreatic lipase and BAL displayed a 40% reduction in dietary triacylglyceride absorption compared with wild type C57BL/6 mice under high fat/high cholesterol dietary conditions (Gilham et al., 2007). The inhibition of lipase activity by BaP and PHE also results in a functional lipase deficiency, and would likely reduce lipid digestion leading to higher luminal lipid contents as triacylglycerides cannot cross the intestinal brush border membrane. In a lipid-rich diet less lipophilic chemicals are absorbed by the intestinal epithelium, probably because higher luminal lipid contents reduce their bioavailability (de Gelder et al., 2017; Kania-Korwel et al., 2008). Oral administration of PCBs and chlorobenzenes in goldfish (*Carassius auratus*) or PCB 136 in female C57BL/6 mice decreased intestinal uptake of these lipophilic contaminants with increased dietary lipid content (Gobas et al., 1993; Kania-Korwel et al., 2008). Furthermore, increasing the lipid content of a diet with a mixture of non-absorbable long-chain fatty acid bonded by ester links to sucrose (Olestra) also increased fecal excretion of lipophilic chemicals (Geusau et al., 1999; Jandacek et al., 2005; Moser and McLachlan, 1999; Mutter et al., 1988). Although both BaP and PHE are absorbed along the entire teleost intestinal tract into the systemic circulation following gavage (Bakke et al., 2015; de Gelder et al., 2016; Solbakken et al., 1984), transmembrane uptake of BaP, in intestinal brush border membrane vesicle preparations of rainbow trout, decreases in the presence of high EPA and oleic acid concentrations (de Gelder et al., 2017). Therefore, the inhibited hydrolysis of rapeseed oil by BaP and PHE could aid in decreasing intestinal PAH uptake from vegetable based aquafeeds as a decreased lipid digestion elevates luminal lipid contents. In a previous study, we have observed that a vegetable (rapeseed) oil based diet decreased intestinal BaP and PHE concentrations *in vivo* in Atlantic salmon compared to a fish oil based diet (de Gelder et al., 2016). This decrease in intestinal PAH uptake might be an effect initiated by the more potent inhibition of rapeseed oil hydrolysis by BaP and PHE. Since a decreased lipase activity could reduce intestinal lipid absorption and therefore maintain high luminal lipid contents, this could lead to a decreased PAH bioavailability (de Gelder et al., 2017; Gilham et al., 2007; Kania-Korwel et al., 2008). In contrast, BaP and PHE did not inhibit fish oil hydrolysis. Therefore, luminal lipid contents shall decrease following lipid digestion and intestinal FFA uptake, which increases intestinal PAH uptake (de Gelder et al., 2017).

5. Conclusion

This study has shown that BaP and PHE inhibit rapeseed oil hydrolysis *in-vitro*. Substitution of fish oils by vegetable oils in aquaculture increases aquafeed levels of BaP and PHE. Therefore, the inclusion of vegetable oils could result in a decreased lipase digestion and subsequently a decreased intestinal fatty acid uptake which ultimately could negatively impact organismal energy metabolism. In vegetable-based aquafeeds more PAHs would be freely available to inhibit lipase activity as PAHs have a lower solubility in micelles that consist of shorter vegetable oil fatty acid chains lengths and have a lower degree of unsaturated bonds compared to fish oil fatty acids. Contrary, micelles composed of fish oil have a higher PAH solubility and increase PAH partitioning in the intestinal lumen, leaving less PAHs free to directly interact with lipases and maintain lipase activity. Therefore, the fatty acid and lipid composition of an alternative aquafeed is an important determinant for lipid digestion and should be a considered in the development of novel sustainable aquafeeds.

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References

Bakke, A.M., Glover, C.N., Krogdahl, Å., 2010. Feeding, digestion and absorption of nutrients. In: Grosell, M., Farrell, A.P., Brauner, J.P. (Eds.), The Multifunctional Gut of Fish. Academic Press, Oxford, pp. 57–110.

Bakke, M.J., Nahrgang, J., Ingebrigtsen, K., 2015. Comparative absorption and tissue distribution of ^{14}C-benzo(*a*)pyrene and ^{14}C-phenanthrene in the polar cod (*Boreogadus saida*) following oral administration. Polar Biol. 39, 1165–1173.

Bell, J.G., Waagbø, R., 2009. Safe and nutritious aquaculture produce: benefits and risks of alternative sustainable aquafeeds. In: Holmer, M. (Ed.), Aquaculture in the Ecosystem – Benefits and Risks for Future Development. Springer Science, New York, pp. 185–212.

Bell, J.G., Tocher, D.R., Farndale, B.M., McVicar, A.H., 1999. Effects of essential fatty acid-deficient diets on growth, mortality, tissue histopathology and fatty acid compositions in juvenile turbot (*Scophthalmus maximus*). Fish Physiol. Biochem. 20, 263–277.

Berntssen, M.H.G., Lundebye, A.-K., Torstensen, B.E., 2005. Reducing the levels of dioxins and dioxin-like PCBs in farmed Atlantic salmon by substitution of fish oil with vegetable oil in the feed. Aquac. Nutr. 11, 219–231.

Berntssen, M.H.G., Julshamn, K., Lundebye, A.-K., 2010. Chemical contaminants in aquafeeds and Atlantic salmon (*Salmo salar*) following the use of traditional - versus alternative feed ingredients. Chemosphere 78, 637–646.

Berntssen, M.H.G., Ornsrud, R., Hamre, K., Lie, K.K., 2015. Polyaromatic hydrocarbons in aquafeeds, source, effects and potential implications for vitamin status of farmed fish species: a review. Aquac. Nutr. 21, 257–273.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.

Bogevik, A.S., 2011. Marine wax ester digestion in salmonid fish: a review. Aquac. Res. 42, 1577–1593.

Bogevik, A.S., Tocher, D.R., Waagbø, R., Olsen, R.E., 2008. Triacylglycerol-, wax ester- and sterol ester-hydrolases in midgut of Atlantic salmon (*Salmo salar*). Aquac. Nutr. 14, 93–98.

Chen, Q., Sternby, B., Akesson, B., Nilsson, Å., 1990. Effects of human pancreatic lipase-colipase and carboxyl ester lipase on eicosapentaenoic and arachidonic acid ester bonds of triacylglycerols rich in fish oil fatty acids. Biochim. Biophys. Acta 1044, 111–117.

Cornish-Bowden, A., 1995. Reactions of more than one substrate. In: Cornish-Bowden, M. (Ed.), Fundamentals of Enzyme Kinetics. Portland Press Ltd., London, pp. 129–158.

Doi, A.M., Lou, Z., Holmes, E., Li, C.-L., Venugopal, C.S., James, M.O., Kleinow, K.M., 2000. Effect of micelle fatty acid composition and 3, 4, 3′, 4′-tetrachlorobiphenyl (TCB) exposure on intestinal [^{14}C]-TCB bioavailability and biotransformation in channel catfish in situ preparations. Toxicol. Sci. 55, 85–96.

Dulfer, W.J., Govers, H.A.J., Groten, J.P., 1998. Kinetics and conductivity parameters of uptake and transport of polychlorinated biphenyls in the Caco-2 intestinal cell line model. Environ. Toxicol. Chem. 17, 493–501.

EFSA, 2008. Scientific opinion of the panel on contaminants in the food chain on a request from the European commission on polycyclic aromatic hydrocarbons in food. EFSA J. 724, 1–114.

FAO, 2014. The State of World Fisheries and Aquaculture 2014. The Food and Agriculture Organization of the United Nations, Italy, Rome.

de Gelder, S., Bakke, M.J., Vos, J., Rasinger, J.D., Ingebrigtsen, K., Grung, M., Ruus, A., Flik, G., Klaren, P.H.M., Berntssen, M.H.G., 2016. The effect of dietary lipid composition on the intestinal uptake and tissue distribution of benzo[*a*]pyrene and phenanthrene in Atlantic salmon (*Salmo salar*). Comp. Biochem. Physiol. C Toxicol. Pharmacol. 185-186, 65–76.

de Gelder, S., van Och, L., Zethof, J., Pelgrim, T.N.M., Rasinger, J.D., Flik, G., Berntssen, M.H.G., Klaren, P.H.M., 2017. Uptake of benzo[*a*]pyrene, but not of phenanthrene, is inhibited by fatty acids in intestinal brush border membrane vesicles of rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. C Toxicol. Pharmacol. 195, 1–8.

Geusau, A., Tschachler, E., Meixner, M., Sandermann, S., Päpke, O., Wolf, C., Valic, E., Stingl, G., McLachlan, M., 1999. Olestra increases faecal excretion of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Lancet 354, 1266–1267.

Gilham, D., Lehner, R., 2005. Techniques to measure lipase and esterase activity in vitro. Methods 36, 139–147.

Gilham, D., Labonté, E.D., Rojas, J.C., Jandacek, R.J., Howles, P.N., Hui, D.Y., 2007. Carboxyl ester lipase deficiency exacerbates dietary lipid absorption abnormalities and resistance to diet-induced obesity in pancreatic triglyceride lipase knockout mice. J. Biol. Chem. 282, 24642–24649.

Gjellesvik, D.R., 1991. Fatty acid specificity of bile salt-dependent lipase: enzyme recognition and super-substrate effects. Biochim. Biophys. Acta 1086, 167–172.

Gjellesvik, D.R., Lombardo, D., Walther, B.T., 1992. Pancreatic bile salt dependent lipase from cod (*Gadus morhua*): purification and properties. Biochim. Biophys. Acta 1124, 123–134.

Gobas, F.A.P.C., McCorquodale, J.R., Haffner, G.D., 1993. Intestinal absorption and biomagnification of organochlorines. Environ. Toxicol. Chem. 12, 567–576.

Gotthilf, N., 1974. Lipase: titrimetric assay. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis. Academic Press Inc., New York, pp. 814–818.

Gupta, R., Rathi, P., Gupta, N., Bradoo, S., 2003. Lipase assays for conventional and molecular screening: an overview. Biotechnol. Appl. Biochem. 37, 63–71.

Henderson, R.J., Tocher, D.R., 1992. Thin-layer chromatography. In: Hamilton, R.J., Hamilton, S. (Eds.), Lipid Analysis: A Practical Approach. IRL Press, Oxford, pp. 65–111.

Howles, P.N., Hui, D.Y., 2000. Cholesterol esterase. In: Mansbach IIC.M., Tso, P., Kuksis,

A. (Eds.), Intestinal lipid metabolism. Springer Science + Business Media, New York, pp. 119–134.
Hui, D.Y., Howles, P.N., 2002. Carboxyl ester lipase: structure-function relationship and physiological role in lipoprotein metabolism and atherosclerosis. J. Lipid Res. 43, 2017–2030.
Jandacek, R.J., Genuis, S.J., 2013. An assessment of the intestinal lumen as a site for intervention in reducing body burdens of organochlorine compounds. Sci. World J. 2013, 1–10.
Jandacek, R.J., Anderson, N., Liu, M., Zheng, S., Yang, Q., Tso, P., 2005. Effects of yo-yo diet, caloric restriction, and olestra on tissue distribution of hexachlorobenzene. Am. J. Physiol. Gastrointest. Liver Physiol. 288, G292–G299.
Kania-Korwel, I., Hornbuckle, K.C., Robertson, L.W., Lehmler, H.-J., 2008. Influence of dietary fat on the enantioselective disposition of 2,2′,3,3′,6,6′-hexachlorobiphenyl (PCB 136) in female mice. Food Chem. Toxicol. 46, 637–644.
Kelly, B.C., Gobas, F.A.P.C., McLachlan, M.S., 2004. Intestinal absorption and biomagnification of organic contaminants in fish, wildlife, and humans. Environ. Toxicol. Chem. 23, 2324–2336.
Kurtovic, I., Marshall, S.N., Zhao, X., Simpson, B.K., 2009. Lipases from mammals and fishes. Rev. Fish. Sci. 17, 18–40.
Laher, J.M., Barrowman, J.A., 1983. Polycyclic hydrocarbon and polychlorinated biphenyl solubilization in aqueous solutions of mixed micelles. Lipids 18, 216–222.
Lie, Ø., Lambertsen, G., 1985. Digestive lipolytic enzymes in cod (*Gadus morrhua*): fatty acid specificity. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 80B, 447–450.
Lowe, M.E., 1997. Structure and function of pancreatic lipase and colipase. Annu. Rev. Nutr. 17, 141–158.
Moore, S.A., Kingston, R.L., Loomes, K.M., Hernell, O., Bläckberg, L., Baker, H.M., Baker, E.N., 2001. The structure of truncated recombinant human bile salt-stimulated lipase reveals bile salt-independent conformational flexibility at the active-site loop and provides insights into heparin binding. J. Mol. Biol. 312, 511–523.
Moret, S., Conte, L.S., 2002. A rapid method for polycyclic aromatic hydrocarbon determination in vegetable oils. J. Sep. Sci. 25, 96–100.
Moret, S., Purcaro, G., Conte, L.S., 2005. Polycyclic aromatic hydrocarbons in vegetable oils from canned foods. Eur. J. Lipid Sci. Technol. 107, 488–496.
Moser, G.A., McLachlan, M.S., 1999. A non-absorbable dietary fat substitute enhances elimination of persistent lipophilic contaminants in humans. Chemosphere 39, 1513–1521.
Mutter, L.C., Blanke, R.V., Jandacek, R.J., Guzelian, P.S., 1988. Reduction in the body content of DDE in the Mongolian gerbil treated with sucrose polyester and caloric restriction. Toxicol. Appl. Pharmacol. 92, 428–435.
Olsen, R.E., Ringø, E., 1997. Lipid digestibility in fish: a review. Rec. Res. Dev. Lipid Res. 1, 199–264.
Phan, C.T., Tso, P., 2001. Intestinal lipid absorption and transport. Front. Biosci. 6, 299–319.
Phillips, D.H., 1999. Polycyclic aromatic hydrocarbons in the diet. Mutat. Res. 443, 139–147.
Pickova, J., Mørkøre, T., 2007. Alternate oils in fish feeds. Eur. J. Lipid Sci. Technol. 109, 256–263.
Porter, C.J.H., Trevaskis, N.L., Charman, W.N., 2007. Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs. Nat. Rev. Drug Discov. 6, 231–248.
R Development Core Team, 2013. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing.
Rønnestad, I., Yúfera, M., Ueberschär, B., Ribeiro, L., Sæle, Ø., Boglione, C., 2013. Feeding behaviour and digestive physiology in larval fish: current knowledge, and gaps and bottlenecks in research. Rev. Aquac. 5, S59–S98.
Sæle, Ø., Nordgreen, A., Olsvik, P.A., Hamre, K., 2010. Characterization and expression of digestive neutral lipases during ontogeny of Atlantic cod (*Gadus morhua*). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 157, 252–259.
Sioen, I., De Henauw, S., Verbeke, W., Verdonck, F., Willems, J.L., Van Camp, J., 2008. Fish consumption is a safe solution to increase the intake of long-chain n-3 fatty acids. Public Health Nutr. 11, 1107–1116.
Solbakken, J.E., Solberg, M., Palmork, K.H., 1984. A comparative study on the disposition of three aromatic hydrocarbons in flounder (*Platichthys flesus*). FiskDir. Skr. Ser. Havunders. 17, 473–481.
Tacon, A.G.J., Metian, M., 2008. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: trends and future prospects. Aquaculture 285, 146–158.
Teixeira, V.H., Casal, S., Oliveira, M.B.P.P., 2007. PAHs content in sunflower, soybean and virgin olive oils: evaluation in commercial samples and during refining process. Food Chem. 104, 106–112.
Tocher, D.R., 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Rev. Fish. Sci. 11, 107–184.
Tocher, D.R., Sargent, J.R., 1984. Studies on triacylglycerol, wax ester and sterol ester hydrolases in intestinal ceca of rainbow trout (*Salmo gairdneri*) fed diets rich in triacylglycerols and wax esters. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 77, 561–671.
Torstensen, B.E., Frøyland, L., Lie, Ø., 2004. Replacing dietary fish oil with increasing levels of rapeseed oil and olive oil - effects on Atlantic salmon (*Salmo salar* L.) tissue and lipoprotein lipid composition and lipogenic enzyme activities. Aquac. Nutr. 10, 175–192.
Vasiluk, L., Pinto, L.J., Tsang, W.S., Gobas, F.A.P.C., Eickhoff, C., Moore, M.M., 2008. The uptake and metabolism of benzo[*a*]pyrene from a sample food substrate in an in vitro model of digestion. Food Chem. Toxicol. 46, 610–618.
Vetter, R.D., Carey, M.C., Patton, J.S., 1985. Coassimilation of dietary fat and benzo(*a*) pyrene in the small intestine: an absorption model using the killifish. J. Lipid Res. 26, 428–434.
Wang, C.S., Hartsuck, J.A., 1993. Bile salt-activated lipase. A multiple function lipolytic enzyme. Biochim. Biophys. Acta 1166, 1–19.
Wang, T.Y., Liu, M., Portincasa, P., Wang, D.Q.H., 2013. New insights into the molecular mechanism of intestinal fatty acid absorption. Eur. J. Clin. Investig. 43, 1203–1223.
Yeap, Y.Y., Trevaskis, N.L., Porter, C.J.H., 2013. Lipid absorption triggers drug supersaturation at the intestinal unstirred water layer and promotes drug absorption from mixed micelles. Pharm. Res. 30, 3045–3058.