



# 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  $429 \pm 78$  g (mean  $\pm$  SD) were obtained from a commercial hatchery 'Keijzersberg' in Blitterswijk, the Netherlands. Fish were kept at  $15.0 \pm 0.5$  °C (mean  $\pm$  SD) in an indoor recirculating system contain-

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 <sup>1</sup>Bell et al. (1999) and <sup>2</sup>Torstensen et al. (2004).

|               | Olive oil <sup>1</sup> | Fish oil <sup>2</sup> | Rapeseed oil <sup>2</sup> |
|---------------|------------------------|-----------------------|---------------------------|
| 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                      |

ing 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).

### 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<sup>-1</sup> 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<sub>2</sub>O (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<sup>-1</sup>), dis-

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