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Carnivores on a vegetable dish

Interactions of fatty acids on diet-introduced toxicants in salmonids

Proefschrift

ter verkrijging van de graad van doctor aan de Radboud Universiteit Nijmegen op gezag van de rector magnificus prof. dr. J.H.J.M. van Krieken, volgens besluit van het college van decanen in het openbaar te verdedigen op vrijdag 19 januari 2018 om 14.30 uur precies

door

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geboren op 26 oktober 1985 te Eindhoven
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‘When you have eliminated the impossible, whatever remains, however improbable, must be the truth.’

*Sherlock Holmes*  
*The Sign of Four*
Chapter 1

General introduction
Current aquaculture practice is not sustainable

In 2013 the aquaculture production for human consumption has been estimated to be approximately 60 million tonnes of seafood (finfishes, crustaceans, molluscs, amphibians, freshwater turtles, sea cucumbers, sea urchins, sea squirts and edible jellyfish). With an expected annual growth rate of 2.5%, aquaculture production will reach 85 million tonnes in 2022. Clearly, the aquaculture industry is one of the fastest-growing global food-producing sectors [59]. Finfish production is a very important part of aquaculture practices and will continue to be [198, 200, 232].

Traditionally, commercial aquafeeds for marine carnivorous species such as Atlantic salmon (*Salmo salar*) and gilthead seabream (*Sparus aurata*) consist of fishmeal and fish oil that are derived from so-called forage, reduction or feed fisheries (predominantly pelagic species) and from by-product trimmings (e.g., skeletons, viscera and skin) that result from the processing of fish [178, 207]. Fishmeal is produced by cooking and pressing of fresh raw fishes and fish by-products and finalized by drying and milling of the meal. A mixture of fish oil, water and dissolved and suspended proteins, vitamins and minerals is obtained from pressing cooked fish that is further refined and purified into fish oil [178]. Around 60% and 80% of the annual global fishmeal and fish oil supply, respectively, is used in the manufacture of aquafeeds [24, 178, 199].

The steadily growing aquaculture leads to an increased pressure on the limited supply of highly valuable marine aquafeed ingredients [178, 198, 207]. Besides rising global prices of finite marine ingredients, ecological sustainability and the ethical issue of using fishery resources for animal feeding rather than for direct human consumption are important incentives in the development of alternative ingredients for aquafeeds [12, 24, 59, 166, 212, 214]. The challenge faced by the rapidly growing industry is to relieve pressure on ‘traditional’ feed ingredients through substitution of fishmeal and fish oil by alternative aquafeed ingredients. Over the last two decades extensive research is carried out to evaluate potential alternative ingredients in commercial aquafeeds [12, 199-201, 214]. Fishmeal is a preferred aquafeed ingredient because of its high protein content, essential amino acid profile, required micronutrient concentrations, high digestibility and absence of so-called anti-nutrients (e.g., lectins in plant material) that can interfere with nutrient absorption [12, 59, 65]. Fish oil is mainly included as oil source in aquafeeds because it provides $n$-3 polyunsaturated fatty acids (PUFA) that are essential for optimal growth and health of farmed fish and consumers [198, 214]. Besides the benefits for farmed fish, $n$-3 PUFA play a crucial role in human health as deficiencies can lead to cardiovascular and mental
disorders [153]. The World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO) and the European Food Safety Authority (EFSA) recommend a daily intake of 250–500 mg $n$-3 PUFA for human adults [53, 58, 227]. Therefore, the production of fish species that contribute to an adequate intake of $n$-3 PUFA is of significant value for human nutrition [12].

**Alternative ingredients for fishmeal and fish oil substitution**

Alternative feed ingredients for fishmeal and fish oil are selected on the basis of their nutritional composition and economical criteria. Currently used alternatives in the European Union (EU) include marine products other than fish (zooplankton, such as krill), terrestrial vegetable ingredients (proteins and lipids derived from beans, cereals and oilseeds), and microbial ingredients (single-cell protein and oil extracted from microorganisms). Other alternatives, but currently not used in the EU, include terrestrial animal by-products (poultry meal, blood meal and insect meal) and genetically modified plant products or algae rich in $n$-3 PUFA [86, 122, 141, 178, 198]. Vegetable-based ingredients are viable alternatives and already extensively applied in commercial salmon aquafeeds. In 2013, less than 30% of the Atlantic salmon diet was composed of marine ingredients while approximately 56% was composed of vegetable ingredients [65, 198, 232].

Replacing fish oils with vegetable oils alters the nutrient profile of aquafeeds. The use of vegetable oils increases dietary levels of monoene and $n$–6 fatty acids while levels of $n$–3 PUFA decrease [9, 10, 94, 137, 208-210]. Partial substitution up to 75% of fish oils by single or a mixture of vegetable oils, such as linseed, rapeseed, sunflower, soybean, palm or olive oil, did not negatively affect growth in different life stages of Atlantic salmon [12, 214]. Even complete replacement with a vegetable oil mixture of rapeseed oil, palm oil, and linseed oil did not significantly affect growth from juvenile fish up to slaughter size [211]. Partial replacement of fish oil by (mixtures of) vegetable oils did not significantly affect growth in rainbow trout (*Oncorhynchus mykiss*) [29, 46, 84, 166, 215]. In the carnivorous marine fishes such as European sea bass (*Dicentrarchus labrax*) and gilthead seabream, replacement of 60% of dietary fish oils by soybean oil, rapeseed oil and linseed oil or a mixture of these did not affect growth [93, 94, 165, 225]. Predictably, the use of vegetable oils in the diet of omnivorous and herbivorous fish such as tilapia (*Oreochromis niloticus*) and common carp (*Cyprinus carpio*), does not affect growth performances when requirements of essential fatty acid are met [12, 214]. Therefore, in general dietary fish oils can be replaced by substantial amounts of vegetable oils for many fish species without affecting growth or feed efficiency [12, 198, 214].
Vegetable-based aquafeeds are contaminated with PAHs

Unfortunately, the use of vegetable meal and oil to improve aquafeed marine sustainability comes with new challenges that were previously not associated with marine aquafeeds composed of traditional ingredients [12, 65, 143, 198]. Fish oils extracted from marine pelagic fish species contain relatively high levels of fat-soluble persistent organic pollutants (POPs), such as polychlorinated biphenyls (PCBs), dioxins [polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs)], polybrominated diphenyl ethers (PBDEs), and organochlorine pesticides that accumulate in the aquatic food chain [15, 88]. Farmed oily fish is an important source of POPs in the human diet and its consumption raised concerns for public health [88]. The (partial) replacement of fish oil by vegetable oil has substantially reduced POPs in farmed salmonids [11, 13, 46, 64]. However, technical processing of vegetable oils produces polycyclic aromatic hydrocarbons (PAHs). The inclusion of vegetable oils in aquafeeds introduces these potentially carcinogenic fat-soluble PAHs that were previously not associated with farming of salmon [14].

PAHs are ubiquitous environmental non-polar lipophilic contaminants mostly formed from incomplete combustion of organic matter from natural sources (e.g., oil seeps, forest fires, and volcanoes) and anthropogenic sources (e.g., combustion of fossil fuels, vehicle emissions, petroleum spills, and oil well blow-outs) [134, 157]. The carcinogenic and mutagenic properties of a broad spectrum of PAHs raise important public health concerns [52]. Apart from tobacco smoke, diet is the major source for PAH exposure in humans; meat products, oils, fats, cereals, fruits and vegetables are the main contributors [51].

PAHs can be categorized as ‘light’ PAHs that have 2 to 4 fused aromatic rings or ‘heavy’ PAHs with 4 to 6 fused aromatic rings. Vegetable oil obtained from oil-producing seeds such as soybeans, rapeseeds, olive seeds and sunflower seeds, can be contaminated indirectly from airborne PAH deposits on crops and/or directly by thermal feed processing like toasting and gas drying [51, 135, 202]. Of the 16 potentially genotoxic and carcinogenic PAH congeners listed by EFSA no less than 13\(^1\) were detected in vegetable-based aquafeeds [14, 16, 37, 202]. Besides these 13 PAH congeners, the non-genotoxic and/or non-carcinogenic PAHs fluoranthene (FA), naphthalene (NA), anthracene (AN) and phenanthrene (PHE) are also extensively present in vegetable oils [14, 37, 202].

In vegetable oil-based aquafeeds, elevated levels were detected of the two
PAH congeners benzo[a]pyrene (BaP) and especially PHE, compared to fish oil-based aquafeeds [14]. Based on the findings of Berntssen et al. (2010), BaP and PHE (Fig. 1.1) were chosen to use as relevant PAH indicators present in vegetable oil-based aquafeeds as the two PAHs differ in physical chemical properties such as lipophilicity (log octanol/water partition coefficient; K\text{ow}) as well as toxic action. The log K\text{ow} is defined as the ratio of the concentration of a compound in n-octanol and water at equilibrium at a specified temperature. Log K\text{ow} values can be used to predict bioaccumulation in aquatic organisms. Lipophilic compounds with high log K\text{ow} values (i.e., > 5) are of great concern because they cannot be efficiently cleared from the body to the water via gill ventilation, and thus have the potential to accumulate and bio-magnify in aquatic organisms [104]. BaP is highly lipophilic (log K\text{ow} = 6.31) and an agonist to the aryl hydrocarbon receptor that induces cytochrome P4501A (cyp1a) expression [112, 113]. PHE is less lipophilic than BaP (K\text{ow} = 4.5) and not an aryl hydrocarbon receptor agonist [19].

![Chemical structure of BaP \((C_{20}H_{12})\) and PHE \((C_{14}H_{10})\).](image)

**Lipophilic PAHs can interfere with lipid digestion and uptake**

The introduction of PAHs in aquafeeds with vegetable oils has increased PAH levels in fillets of Atlantic salmon reared on these diets [14].

The tissue concentration, distribution and bioavailability of lipophilic contaminants depend on luminal transport by micelles, intestinal uptake, metabolism and

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1 Benzo[a]anthracene (BaA), benzo[b]fluoranthene (BbFA), benzo[j]fluoranthene (BjFA), benzo[k] fluoranthene (BkFA), benzo[g,h,i]perylene (BghiP), benzo[a]pyrene (BaP), chrysene (CHR), cyclopenta[c,d]pyrene (CPP), dibenzo[a,h]anthracene (DBahA), dibenzo[a,e]pyrene (DBaeP), dibenzo[a,l]pyrene (DBalP), indeno[1,2,3-cd]pyrene (IP) and benzo[c]fluorine (BcFL).
excretion [33, 49, 95, 160, 231]. After oral ingestion, PAHs solubilize in the lipophilic core of emulsified oil droplets and micelles [97, 104, 160]. Vetter et al. (1985) observed that BaP remained strongly associated with lipids throughout lipid digestion, absorption and assimilation. The lipophilic nature of PAHs promotes solubilization in lipophilic domains where they can affect enzymatic digestion of lipids by digestive lipases, luminal transfer of free fatty acids by micelles, and their transmembrane and transepithelial uptake (Fig. 1.2).

Dietary fat is known to enhance intestinal uptake of a wide range of lipophilic compounds such as drugs, vitamins and contaminants. Indeed, in the seventies of the previous century, it was already postulated that lipid absorption could accompany intestinal BaP absorption [164]. Since dietary fat can have significant effects on the absorption of lipid derivatives, lipophilic drugs and fat-soluble vitamins [160, 222], it has been suggested that the intestinal transport and uptake of lipophilic organic contaminants, such as PAHs, are also influenced by lipids [47, 48, 77, 104, 222].

**Lipid structure and function**

Lipids comprise a heterogeneous group of non-polar compounds found in all living cells. Lipids, fats and oils include monoacylglycerides, diacylglycerides, triacylglycerides, FFA, phospholipids (PL), eicosanoids, resolvins, docosanoids, sterols, sterol esters, carotenoids, vitamins A and E, fatty alcohols, hydrocarbons and wax esters [58]. They are important forms of stored energy, structural components for cell membranes (e.g., phospholipids) and/or precursors to cell signaling molecules [1].

![Figure 1.2: Potential interactions between contaminating PAHs and dietary lipids on digestion, uptake across the intestinal mucosa, metabolism and further peripheral distribution of lipids and their digestion products. A) 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. B) The lipid composition of micelles is an important component in intestinal delivery and transfer of lipophilic PAHs. Therefore, the fatty acid composition of micelles could affect transepithelial PAH transport and intracellular PAH metabolism which in turn can alter PAH transfer to the systemic circulation. C) Micelles ferry lipophilic PAHs across the unstirred water layer to the apical brush border membrane of enterocytes. Once micelles arrive at the brush border membrane, PAHs are released from the micelles and can then be absorbed by enterocytes by passive diffusive diffusion and/or carrier-mediated transport. PAHs and/or their metabolites can also be excreted into the intestinal lumen by efflux transporters. Modified from Porter et al. (2007).](image-url)
General introduction
The most dominant lipids in the diet are triacylglycerides, contributing to 90-95% of the total energy derived from dietary lipids [92, 147]. Triacylglycerides are composed of three fatty acids esterified to a single glycerol and principally stored in adipose tissue (Fig. 1.3). Here, beta-oxidation of fatty acids generates acetyl-CoA that enters the citric acid cycle and is key in the generation of ATP [142]. Fatty acids are carboxylic acids with hydrocarbon chains ranging from short-chain (less than eight carbon long; \(< C_8\)), medium-chain (carbon chain length between \(C_8\) and \(C_{13}\)), long-chain (carbon chain length between \(C_{14}\) and \(C_{20}\)) to very long-chain fatty acids (more than 21 carbons) [58]. Unsaturated fatty acids contain one or more ethylenic (double) bonds that introduce a rigid bend in the hydrocarbon tail while saturated fatty acids contain no double bonds and have a relatively straight structure [205]. Lipases secreted by the exocrine pancreas into the intestinal lumen hydrolyze emulsified lipids into FFA, monoacylglycerides and, after complete hydrolysis, to glycerol [5, 147, 205]. In mammals, the exocrine pancreas secretes three major lipolytic enzymes - pancreatic lipase, phospholipases, and bile salt-activated lipase (BAL) [109, 223]. Digestive lipases hydrolyze ester bonds of triacylglycerides, phospholipids, cholesterylesters, wax and vitamin esters. Pancreatic lipase mainly hydrolyses the \(sn-1\) and \(sn-3\) positions of the triacylglycerides to release 2-monooacylglyceride and FFA [224]. 2-monooacylglyceride is the predominant form and is rapidly absorbed by the small intestine. Formation of 1-monooacylglyceride by isomerization promotes complete hydrolysis to glycerol and FFA [92]. Pancreatic lipase is the principal digestive lipase in mammals, is inhibited by bile salts and requires colipase for its hydrolytic activity [26, 124]. Phospholipase \(A_2\) is the key enzyme in the hydrolysis of phospholipids at the \(sn-2\) position into lysophospholipids and fatty acids [54, 170, 196]. In fish, several studies have concluded that BAL is the most important digestive lipase in species such as rainbow trout, Atlantic salmon, Atlantic cod (\textit{Gadus morhua}) and turbot (\textit{Scophthalmus maximus}) [23, 147, 170, 197, 205].

BAL has a broad substrate specificity and is more efficient than pancreatic lipase in hydrolyzing PUFA, which are abundant in the diet of marine and freshwater fish [35, 70, 223]. BAL is often referred to as carboxyl ester lipase, non-specific lipase, cholesterol esterase, or carboxyl-esterase, lysophospholipase. Contrary to pancreatic lipase, BAL is a non-specific lipase that requires activation by bile salts to hydrolyze mono-, di- and triacylglycerides, cholesteryl esters, phospholipids, lysophospholipids, ceramides and fat-soluble vitamins [90, 132, 147, 223].
Luminal transport of lipophilic compounds

When the critical micelle concentration is exceeded, biliary components in the intestinal lumen (e.g., bile salt and cholesterol) self-aggregate with FFA and, to lesser degrees, with mono-, di- and triacylglycerides, into water-soluble spherical aggregates called micelles [5, 156, 224, 231]. The hydrophilic ends of polar lipids face the intestinal lumen, creating a hydrophobic core favorable for the partitioning of lipophilic compounds. The solubilization of lipophilic compounds in micelles can increase their luminal concentration 100- to 1000-fold [156].

Micelles, and bile salts as well, facilitate the transport of lipophilic contaminants to the brush border membrane [226]. Micelles act as a vector ferrying monoacylglycerides, FFA, cholesterol, fat-soluble vitamins and even lipophilic contaminants across the unstirred water layer of the enterocyte’s apical brush border membrane [44, 48, 104, 160]. Indeed, the solubility of, for instance, the
PCBs Aroclor 1242 and 3,3′,4,4′-tetrachlorobiphenyl had increased 2.5-fold in micelles composed of unsaturated long-chain fatty acids compared to micelles composed of saturated medium-chain fatty acids [44, 110]. The solubility of the PAHs 3-methylcholanthrene and 7,12-dimethylbenz[a]anthracene had also increased in micelles composed of the mono-unsaturated fatty acid oleic acid (18:1\text{n-9}) compared to micelles composed of the medium-chain saturated octanoic acid (8:0) [110]. These results suggest that the composition, carbon-chain length and degree of saturation of fatty acids play an important role in the intestinal uptake of lipophilic compounds. Since fish oil-based diets contain more PUFAs with longer hydrocarbon chains and a higher degree of unsaturated bonds than vegetable oils, the intestinal bioavailability of lipophilic contaminants in alternative aquafeeds is likely to be affected by the dietary lipid composition.

**Transport across the apical membrane’s unstirred water layer**

The first step in intestinal transfer of lipophilic contaminants is the transfer of luminal micelles through the unstirred water layer towards the enterocyte’s apical membrane. The unstirred water layer is defined as the viscous mucus layer with high water content (ca. 95%) and glycoproteins, free proteins, lipids and mineral salts therein [231]. Micelles facilitate the movement of lipophilic compounds across the unstirred water layer towards the brush border membrane of enterocytes, and enhance intestinal absorption [44, 48, 104, 221, 222].

Transfer of PAHs from micelles can occur via a vesicle-mediated uptake process or via direct absorption following a collisional mechanism leading either to membrane fusion or to an exchange of membrane lipids [140, 160]. Alternatively, the dissociation of micelles is promoted by the acidic microclimate of the unstirred water layer when ionized fatty acids are converted to non-ionized fatty acids that reduce the solubility of FFA in micelles. The acidic microclimate in the unstirred water layer is generated by a H+/Na+-antiporter located in the brush border membrane of enterocytes. Once arrived at the brush border membrane, monoacylglycerides, FFA and lipophilic compounds released from micelles can then be absorbed across the membrane of enterocytes [145, 179].

**Lipid absorption across the brush border membrane**

In fish, lipids are absorbed throughout the entire intestine but mainly in the pyloric caeca (when present in a particular species) and proximal intestine [108, 205]. Besides the chemical composition, the lipid content of dietary lipids is an
important determinant of the intestinal bioavailability of lipophilic compounds. Dietary uptake of some highly lipophilic compounds ($\log K_{ow} > 6.3$) decreased with increased dietary lipid content, while the uptake of some moderately lipophilic compounds ($4.5 < \log K_{ow} < 6.3$) did not vary between diets differing in lipid content from $< 0.2\%$ to $13.5\%$ [55, 77, 103, 110, 177]. Furthermore, when the non-absorbable oil ‘Olestra’, a compound composed of sucrose bonded by ester links to short-chain ($C_6$ to $C_{8}$) fatty acids, was included in the diet, intestinal uptake decreased and subsequently fecal excretion of lipophilic compounds increased [67, 96, 136, 139].

Protein-independent passive diffusion and protein-dependent mechanisms have been implicated in the uptake and transport of FFA across a plasma membrane [102, 127]. It is believed that the uptake of long-chain fatty acids across the brush border membrane in enterocytes occurs by two concentration-dependent processes. At relatively low, micromolar luminal fatty acid concentrations, plasma membrane-associated protein transporters facilitate FFA uptake, whereas at high, millimolar concentrations the majority of fatty acids is taken up by the enterocytes passively [213].

The protein-independent diffusion model suggests that FFA are adsorbed to the membrane surface, move across the phospholipid bilayer by a ‘flip–flop’ conformational change and desorbed from the internal bilayer into the absorptive enterocyte. Flip-flop efficiency seems to be dependent on the FFA chain length as increasing chain lengths decrease uptake rates. FFA uptake rates are also dependent on the degree of unsaturation as FFA with one unsaturated bond showed increased uptake rates compared to saturated FFA of the same chain length [101].

A number of candidates are proposed to be involved in protein-dependent uptake of FFA, viz. the plasma membrane-associated fatty acid-binding protein (FABPpm), the fatty acid transport protein 4 (FATP4) and the fatty acid translocase CD36 [92, 127, 145]. Following transmembrane uptake, the absorbed lipid digestion products are resynthesized into triglycerides which are subsequently packaged with phospholipids and apolipoproteins to form chylomicrons [127]. In summary, there is strong evidence that passive diffusion and protein-mediated facilitated transport are important mechanisms for fatty acid uptake across the membrane.

**Transmembrane PAH transport pathways in enterocytes**

Following their arrival at the brush border membrane, PAHs are transported across
the intestinal epithelium [6, 91, 106, 172, 186, 189]. Although the exact mechanism has not been elucidated yet, it has been suggested that lipophilic contaminants such as PAHs have the capacity to diffuse through biological membranes because of their lipophilicity [44, 77, 104]. ‘Light’ PAHs, with a lower lipophilicity, appear to cross the intestinal barrier more rapidly, while ‘heavy’ lipophilic PAHs are more slowly transported [33]. The unstirred water layer creates a barrier to PAH absorption that increases with PAH lipophilicity [49]. Therefore, diffusion through the unstirred water layer seems to be a rate-limiting step for high-K_{ow} compounds, whereas micellar-mediated transport and diffusion through the phospholipid bilayer appears to be rate-limiting for low-K_{ow} compounds [104]. Besides passive uptake, energy-dependent transporters mediate the uptake of lipid derivatives, lipid-soluble vitamins and perhaps lipophilic contaminants as well. For instance, human intestinal brush border membrane vesicles showed ATP-dependent transmembrane BaP uptake [154].

The uptake of small lipophilic compounds such as lipophilic vitamins and cholesterol has long been assumed to be a process of simple passive diffusion. In the past decade, several studies have investigated the potential role of intestinal transport proteins for lipophilic vitamins and cholesterol, reviewed by Iqbal and Hussain (2009). Recently, it has been shown that the uptake of vitamin E is a rapid, saturable process. In contrast, the uptake of vitamin A at physiological concentrations is via saturable carrier-mediated processes but employs non-saturable diffusion-dependent processes at pharmacological concentrations [3, 92]. The absorption of lipid soluble vitamins involves molecular, biochemical, and cellular mechanisms closely related to overall lipid and lipoprotein homeostasis [92], and there might be parallel transport proteins for the intestinal uptake of lipophilic contaminants as well. For instance, scavenger receptor class B type I (SR-BI) plays a role in cholesterol as well as vitamin E absorption. Cholesterol is a steroid alcohol while vitamin E includes both tocopherols and tocotrienols (methylated phenols). Since SR-B1 seems to be involved in the intestinal uptake of the lipophilic compounds cholesterol and vitamin E, which have a chemically different structure, protein transporters that are involved in the uptake of diverse lipophilic compounds could, therefore, also be candidates for the uptake of lipophilic contaminants such as PAHs.

**PAH metabolism and excretion**

Net intestinal uptake of lipophilic PAHs is related to the transport properties of enterocytes and their capacity to metabolize PAHs [228]. Unlike POPs, PAHs typically
do not bioaccumulate in organisms as their whole-body concentrations can be significantly reduced through biotransformations where non-polar lipophilic contaminants are converted into more polar and water-soluble metabolites that can be excreted. The metabolic response to xenobiotics is often divided in three phases: oxidation, conjugation, and excretion [174]. The cytochrome P450 (Cyp) family plays a central role in phase I metabolism by catalyzing the oxidation of xenobiotics to less toxic metabolites.

Following uptake, the intestine is the first barrier for PAH uptake from the diet. Apart from the liver, the intestine plays an important role in PAH metabolism in metabolizing BaP [129]. Intracellularly, PAHs are endogenously activated to reactive metabolites by xenobiotic-metabolizing enzymes, forming diol-epoxides [16]. Cyp1a-mediated metabolism of BaP can result in toxic intermediates, such as benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE), that are very reactive and can bind to cellular proteins or form DNA adducts [130, 146, 219]. Of the currently 18 annotated Cyp families in fish, four families (Cyp1, Cyp2, Cyp3 and Cyp4) are induced by xenobiotics or involved in their biotransformation [216]. The Cyp1 family members (1a, 1b and 1c) are of particular interest for their ability to metabolize PAHs to reactive metabolites. In zebrafish (Danio rerio), all Cyp1s are highly inducible by the aryl hydrocarbon receptor, with the exception of Cyp1d1 [175, 216]. Many PAHs, but not all, have the ability to bind to the aryl hydrocarbon receptor, induce Cyp1a activity and enhance their own biotransformation [18, 19, 112, 180]. The aryl hydrocarbon receptor has a high affinity for ‘heavy’ PAH compounds while it has no or low affinity for ‘light’ PAHs [7, 8, 18].

In phase II reactions, oxidized phase I products are conjugated to form water-soluble substrates for enzymes such as UDP-glucuronosyltransferase, glutathione-S-transferase and sulfotransferase [174]. Both phase I and II reactions have been shown to be affected by nutrients and contaminants and play a central role in lipid metabolism, detoxification, reactive metabolite formation, cellular respiration and antioxidant activity [174]. The formation of reactive metabolites due to phase I activity is one of the best known cytotoxic mechanisms following PAH exposure [174].

When PAHs are oxidized by Cyp450s, enterocytes extrude the metabolites into the intestinal lumen [28, 33]. Excretion is most likely facilitated by ATP-binding cassette (ABC) proteins that excrete highly hydrophobic compounds into the gastrointestinal tract as native, non-metabolized xenobiotics or water-soluble conjugates from phase I and II. Indeed, exposure of tilapia (Oreochromis niloticus)
to waterborne BaP resulted in an up-regulation of \textit{abcc2} (multidrug resistance-associated protein 2) mRNA expression in the gills and an increase in \textit{abcg2} mRNA expression in the liver and proximal intestine [39]. The ABCG2 transporter is involved in the efflux of BaP conjugates in the human colon adenocarcinoma cell line Caco-2 [50] whereas ABCB1 (P-glycoprotein) and the multiple resistance associated proteins (MRP) ABCC1 (MRP1) and ABCC2 (MRP2) were ruled out as possible candidates involved in the efflux of BaP metabolites [27, 112].

PAHs that are not oxidized by Cyp450s remain associated with lipids during chylomicron formation and are secreted into the systemic circulation [121, 218]. Once PAHs have entered the systemic circulation they are primarily excreted via the bile and to a lesser extent via the urine [98, 176].

\textit{Aim and outline of this thesis}

The introduction of vegetable oils to substitute for fish oil in aquaculture feeds is a promising development towards more sustainable aquafeeds that are less dependent on ingredients obtained from wild fish. Unfortunately, the introduction, with vegetable oils, of contaminating PAHs in aquafeeds will to some extent counteract this positive development and seems to present a Catch-22 (paradoxical) situation. This presents a challenge in the development of alternative aquafeeds, and warrants further research.

The main aim of this thesis was to explore the interactions between contaminating PAHs and dietary lipid composition on uptake across the intestinal mucosa, metabolism and further peripheral distribution of lipids and their digestion products. The emphasis of this thesis is on generating fundamental knowledge on the combined effects of PAHs and lipids in model systems at several different levels of biological organization, e.g., at the level of enzymatic lipid hydrolysis, intestinal membrane delivery, intestinal membrane transfer and whole-body distribution.

Chapter 2 investigates the effects of dietary lipid composition on the intestinal uptake and peripheral distribution of BaP and PHE in Atlantic salmon fed either a ‘traditional’ fish oil-based diet or an alternative vegetable oil-based diet. In chapter 3, the effects of BaP and PHE on the enzymatic hydrolysis of rapeseed oil and fish oil in intestinal extracts is described. We hypothesized that the different lipid compositions of rapeseed oil and fish oil affect PAH solubility in micelles and, subsequently, lipase activity. Chapter 4 describes the effects of eicosapentaenoic
acid (EPA), a typical fish oil-derived fatty acid and oleic acid, a typical vegetable oil fatty acid on intestinal transmembrane uptake of BaP and PHE. The intestinal enterocyte is a typical polarized cell with distinct apical and basolateral membrane domains, differing in phospholipid and cholesterol make-up and each harboring specific integral membrane proteins, among which enzymes and transporters. A purified brush border membrane vesicle preparation was used to measure the first step of intestinal transmembrane PAH uptake. To further investigate the interaction between diet-contaminating PAHs and lipid hydrolysis products, BaP and PHE were solubilized in micelles composed of EPA or oleic acid and administrated to the mucosal side of intact intestinal epithelia mounted in Ussing chambers. The results of this study are described in chapter 5.

This thesis concludes with a general discussion, where the results of the studies described in the topical chapters, are discussed in the context of the main aim and put into a wider perspective of the production of novel alternative aquafeeds.
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Chapter 2

The effect of dietary lipid composition on the intestinal uptake and tissue distribution of benzo[a]pyrene and phenanthrene in Atlantic salmon (*Salmo salar*)
Intestinal uptake and tissue distribution of BaP and PHE

Abstract

Uptake of polycyclic aromatic hydrocarbons (PAHs) across the intestine is suggested to occur in association with dietary lipids. Partial replacement of fish ingredients by vegetable ingredients in aquafeeds has led to increased levels of PAHs in marine farmed fish. We therefore investigated, intestinal uptake, tissue distribution and PAH metabolism after a single dose of \(^{14}\)C-benzo[a]pyrene (BaP) or \(^{14}\)C-phenanthrene (PHE) given to Atlantic salmon (Salmo salar) acclimatized to a fish oil or vegetable oil-based diet. Both BaP and PHE were absorbed along the intestine. Fish oil-based feed increased BaP concentration in the pyloric caeca and that of PHE in the proximal intestine. In contrast, vegetable oil increased BaP concentrations in the distal intestine. Extraction of whole body autoradiograms removed PHE-associated radiolabeling almost completely from the intestinal mucosa, but not BaP-associated radiolabeling, indicating presence of BaP metabolites bound to cellular macromolecules. This observation correlates with the increased cyp1a expression in the proximal intestine, distal intestine and liver in the BaP exposed group. Furthermore, BaP-induced cyp1a expression was higher in the distal intestine of salmon fed fish oil compared to the vegetable oil fed group. PHE had no significant effect on cyp1a expression in any of these tissues.

We conclude that dietary lipid composition affects intestinal PAH uptake. Fish oil-based feed increased intestinal PAH concentrations probably due to an enhanced solubility in micelles composed of fish oil fatty acids. Increased BaP accumulation in the distal intestine of vegetable oil fed fish seems to be associated with a reduced Cyp1a-mediated BaP metabolism.
Introduction

Aquaculture is a fast-growing global food-producing sector, with the production of approximately 1.9 million metric tons of Atlantic salmon (Salmo salar) in 2014 [59]. Traditionally, marine fish oils and fishmeal have been used as the main feed ingredients in high energy commercial feeds for carnivorous farmed fish species such as Atlantic salmon. However, the rapidly growing aquaculture cannot continue to rely on fisheries for the supply of fish oil and fishmeal [24, 59, 166]. Therefore, there is a need to develop sustainable alternative aquafeed ingredients, such as oil and meal from vegetable sources. Partial or complete substitution of fish oils by vegetable oils does not seem to negatively affect growth, survival and/or feed nutrient utilization in several aquaculture species [9, 29, 93, 119, 166, 206, 208, 209]. In 2013, less than 30% of the Atlantic salmon diet was composed of marine ingredients while approximately 56% was composed of vegetable ingredients [232]. The use of vegetable oils will increase dietary levels of monoene and n-6 fatty acids. Contrary, levels of n-3 long-chain C20 and C22 polyunsaturated fatty acids (PUFAs) will decrease [9, 212].

Thermal processing of oil producing seeds and grains have been shown to elevate polycyclic aromatic hydrocarbons (PAH) contents in vegetable-based fish feeds [14, 51]. As a consequence, the use of vegetable feed ingredients in aquafeeds also increase the concentration of contaminants that have not been associated before with Atlantic salmon farming, such as PAHs [13, 16]. PAHs are ubiquitous lipid-soluble non-persistent organic pollutants that are biotransformed by oxygenation by the cytochrome P4501A (Cyp1a) family [18, 85]. Atlantic salmon fed ‘alternative’ diets based on partial replacement of fish oil and fishmeal with vegetable oil and vegetable meal, were found to have elevated tissue levels of several PAH congeners, including benzo(a)pyrene (BaP) and phenanthrene (PHE) [14]. BaP and PHE differ in their physico-chemical properties as well as their potential toxic actions. BaP consists of five fused benzene rings, is highly lipophilic (octanol/water partition coefficient, log \( K_{ow} = 6.31 \)) and is an agonist to the aryl hydrocarbon receptor that induces Cyp1a-mediated metabolism [112]. PHE has three fused benzene rings, is less lipophilic than BaP (log \( K_{ow} = 4.5 \)) and is not an aryl hydrocarbon receptor agonist [18], with, consequently, a slower metabolism than BaP [32].

Tissue concentration, distribution and bioavailability of PAHs depend on transport, uptake, metabolism and excretion. Intestinal transport and uptake of dietary PAHs has been suggested to occur in association with dietary lipids [49, 104,
Vetter et al. (1985) observed that lipids and BaP were co-transported from the intestinal lumen and co-incorporated in chylomicrons within one hour after digestion. Because PAHs have a low solubility in the aqueous environment of the gut lumen, it has been proposed that dietary lipids influence the uptake of highly hydrophobic xenobiotics [49, 221, 222]. In practice, aquafeeds are composed of vegetable and fish oil blends and an alteration in feed composition could affect the uptake of PAHs. In fish, lipids are absorbed throughout the entire intestine but predominantly in the proximal region and the pyloric caeca [100, 108, 205]. When PAHs enter the gastrointestinal tract, transport from the lumen to the apical brush border membrane of enterocytes can be facilitated by micelles [44, 221, 222]. The solubility of hydrophobic xenobiotics is higher in micelles composed of unsaturated long-chain fatty acids compared to saturated short-chain fatty acids [44, 110]. Furthermore, lipid digestion rates increase with the degree of unsaturation, and decrease with increased chain length of the constituting fatty acids [181]. The lower digestibility of vegetable n-9 and n-6 fatty acids compared to fish n-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is mainly due to the lower degree of unsaturation in plant PUFAs [208].

For dietary exposures, the intestine is the first barrier for PAH uptake from the diet. The intestine plays an important role in PAH metabolism with near similar abilities as liver (on protein basis) in metabolizing BaP [129]. PAHs are metabolized to epoxides and hydroxylated derivatives during phase I metabolism. Excretion of phase I products is facilitated by conjugation to more water-soluble glucuronides and sulfates during phase II metabolism. Fatty acid levels and in particular PUFAs can induce BaP metabolism by increasing intestinal cytochrome P450 activity [230]. All in all, the increased inclusion of vegetable oils in aquafeeds increase PAH concentrations in farmed fish while the presence of vegetable and fish oils in the diet could alter the solubility of PAHs, their intestinal uptake, bioavailability, metabolism and, ultimately, the animal’s exposure to PAHs.

Differences in solubility of PAHs in micelles and different digestion and absorption rates of different fatty acids can affect the uptake of PAHs from the diet. The objective of the present study was to investigate the effects of dietary lipid composition on the uptake and distribution of BaP and PHE across the intestinal tract in Atlantic salmon fed either a “traditional” fish oil-based diet or an alternative vegetable oil-based diet.
Material and Methods

Animals and diet formulation

The study was conducted at the Industrial and Aquatic Laboratory (I-Lab), Bergen, Norway. Locally bred seawater-acclimated Atlantic salmon (Salmo salar) weighing approximately 200 g were kept in indoor 1-m³ fibre glass tanks (26 fish per tank) supplied with running seawater with a salinity of approximately 31‰. The study was performed at a temperature of 10.0 ± 0.2 °C (average ± SD).

Fish were fed for three weeks with either a diet based on fish oil or a diet based on vegetable (rapeseed) oil to acclimatize the intestinal tract to the diet. The fish were reared under 12:12 dark:light conditions and fed by automatic feeders twice a day for two hours according to standardized in-house growth tables for salmonids modified after Austreng et al. (1987), with a feed intake of approximately 1.2% of body weight per day. The three weeks acclimatization period was chosen to ensure approximately 15 intestinal passages of the diet based on an assumed mean transit times of 28–29 hours in 140–145 g rainbow trout at 10 ºC fed two meals a day with a pelleted feed [60].

Table 2.1 shows that the fish oil-based diet was enriched in long-chain polyunsaturated omega-3 fatty acids (PUFA-n-3), whereas the vegetable oil-based diet had a low PUFA-n-3 content. The diets were designed to meet the nutritional requirements of Atlantic salmon. The choice of alternative feed ingredients as well as the composition of vegetable protein and oils was based on earlier studies on the replacement of fishmeal and fish oil and [14, 212]. The two diets were selected for low background levels of BaP and PHE of 0.06 and 5.1, and 0.07 and 8.3 µg ∙ kg⁻¹ in the fish oil and vegetable oil-based diets, respectively.

Gavage preparation, dosing and sampling.

A single pulse-chase dose of ¹⁴C labeled BaP or PHE was administrated [91] to follow the uptake across the intestinal tract over time. For the purpose of liquid scintillation counting of tissue homogenates and whole body autoradiography, salmon were given either a fish oil or a vegetable oil-based feed enriched with [7-¹⁴C]BaP (26.6 mCi ∙ mmol⁻¹; chemical purity was 99%; American Radiolabeled Chemicals, St Louis, U.S.A.) or [9-¹⁴C]PHE (52 mCi ∙ mmol⁻¹; chemical purity was 99%; Larodan Fine Chemicals AB, Malmö, Sweden). A single dose of 2.5 kBq ∙ g⁻¹ body mass, equivalent to approximately 0.64 µg BaP ∙ g⁻¹ body mass or 0.23 µg PHE ∙ g⁻¹
Table 2.1: Proximate feed (g · kg⁻¹) and fatty acid compositions (area % of total fatty acids) in pellets (4 mm) of the fish oil and the vegetable oil-based feeds.

<table>
<thead>
<tr>
<th></th>
<th>Fish oil-based feed</th>
<th>Vegetable oil-based feed</th>
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</thead>
<tbody>
<tr>
<td><strong>Proximate composition</strong></td>
<td></td>
<td></td>
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<tr>
<td>Fish meal ingredients</td>
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</tr>
<tr>
<td>Plant meal ingredients</td>
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<td>569</td>
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<tr>
<td>Vitamin and mineral mixture</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td><strong>Fatty acid composition</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14:0</td>
<td>2.4</td>
<td>0.6</td>
</tr>
<tr>
<td>16:0</td>
<td>15.1</td>
<td>14.8</td>
</tr>
<tr>
<td>18:0</td>
<td>2.2</td>
<td>2.3</td>
</tr>
<tr>
<td>Sum saturates</td>
<td>20.4</td>
<td>18.3</td>
</tr>
<tr>
<td>16:1n-7/9</td>
<td>2.3</td>
<td>0.6</td>
</tr>
<tr>
<td>18:1n-9/7</td>
<td>35.6</td>
<td>42.8</td>
</tr>
<tr>
<td>20:1n-9</td>
<td>2.6</td>
<td>1.3</td>
</tr>
<tr>
<td>22:1n-11/9</td>
<td>2.6</td>
<td>0.8</td>
</tr>
<tr>
<td>Sum monoenes</td>
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<td>45.9</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>13.5</td>
<td>16.6</td>
</tr>
<tr>
<td>20:4n-6</td>
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<td>0.0</td>
</tr>
<tr>
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<td>16.7</td>
</tr>
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<td>10.1</td>
<td>13.2</td>
</tr>
<tr>
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<td>0.1</td>
</tr>
<tr>
<td>20:4n-3</td>
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<td>0.0</td>
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<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>22:6n-3</td>
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<td>0.7</td>
</tr>
<tr>
<td>Sum n-3</td>
<td>16.7</td>
<td>14.8</td>
</tr>
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</table>

body mass was administered to each fish. The dose of 2.5 kBq · g⁻¹ body mass was chosen to ensure an adequate radioactive signal for autoradiogram development [2]. The dose 2.5 kBq · g⁻¹ body mass exceeds the natural background levels of PAH residues in feed (500- and 50000-fold for PHE and BaP, respectively). However the present study aimed to assess dietary oil matrix-PAH interaction by a single ¹⁴C pulse-chase study, rather than employing a feeding trial with natural background PAH residue levels.

Feed pellets of either the fish oil or the vegetable oil-based diet were separately crushed and mixed with water (1:1.5 w/v) to form a paste. PHE or BaP were dissolved in methanol and toluene, respectively, and added to the designated feed pastes. The pastes were air-dried (ca. 1 hour) to evaporate methanol and toluene. Each paste was thoroughly mixed and loaded in a 1-mL syringe with cut-away tip and smooth, polished edges. Control feeds were prepared using methanol and toluene.
vehicles only, and were otherwise treated similarly as the experimental diets. All syringes were air and light protected before oral administration.

For the purpose of the analysis of PAH metabolites and gene expression studies, the feed pastes were made as described above but mixed with a non-radio-labeled BaP or PHE (Sigma-Aldrich Co, St Louis, U.S.A.) in similar concentrations as for the liquid scintillation and autoradiograms (i.e., 0.64 µg BaP ∙ g⁻¹ body mass or 0.23 µg PHE ∙ g⁻¹ body mass was administered to each fish).

After exposure to a single dose the fish were euthanized at the designated time points by a blow to their head and selected organs were sampled. This pulse-chase experiment was designed to study initial effects of dietary lipids on PAHs uptake within 12 hours after oral administration. The experimental design obeyed Norwegian legislation and was approved by the Norwegian Animal Research Authority.

**Administration of experimental feeds**

Feeds were administered according to Amlund et al. (2006). Briefly, prior to oral administration of the experimental feed, fish were starved for 1.5 days in order to ensure an empty stomach and gastrointestinal tract. Fish were anaesthetized using 0.1% (w/v) tricaine methanesulfonate (MS-222) in seawater and weighed. The experimental feed was orally administrated to the stomach in a single dose of 0.3% body weight (v/w), which was chosen as to fill the stomach content. The 1-mL syringe with the designated feed paste was carefully inserted through the mouth and positioned at the posterior end of the stomach. The syringe was then slowly retracted while the feed paste was administered. Fish were placed in a separate tank to recover, and then transferred to the experimental tank. This also allowed a visual check of potential regurgitation, which did not occur in any of the fed fish.

**Intestinal transit time**

Fish reared on the same conditions as the experimental fish (n = 2 for each time point) were anaesthetized in seawater using 0.1% (w/v) MS-222 and were fed vegetable oil-based feed pastes to which 2% chromium(III)oxide was added. Dissection following euthanasia showed that three hours post-feeding the feed was located in the pyloric caeca and in the proximal part of the proximal intestine. After 6 hours the feed was mainly located in the proximal intestine
and after 12 hours the feed was also present in the distal intestine. The proximal intestine was defined as the region between the most caudal pyloric caecum and the junction with the distal intestine, which was larger, darker in appearance and characterized by annular rings. From this pilot study we chose sampling times to be 3, 6 and 12 hours.

**Liquid scintillation counting**

Fish \((n = 4\) for each treatment and time point) were sampled at 3, 6 and 12 hour post-feeding the experimental feeds. Immediately after weighing, 0.4 mL blood was sampled from the caudal vein by puncture with a heparinized needle directly caudal to the anal fin. Bile was collected by puncture of the gall bladder. Liver, gastrointestinal tract, spleen, gills, kidney and head kidney were dissected and collected in glass vials. The gastrointestinal tract was cleaned of its visceral fat and separated in stomach, pyloric caeca, proximal intestine and distal intestine. The mucosa of the stomach, proximal intestine and distal intestine was scraped off its underlying muscle layers with the aid of glass microscope slides. The mucosa of the pyloric caeca could not be collected. Instead the whole organ was measured. Organs were weighed and homogenized in 3 volumes of saline solution (150 mM NaCl) in a glass Potter-Elvehjem device fitted with a motorized PFTE pestle operating at 800 rpm.

Whole bile fluid, duplicate samples of blood (0.4 mL; to which 1 mL 2-propanol was added) and triplicate samples of 37.5 mg homogenized tissue were transferred to polyethylene vials and dissolved overnight in 1 mL of tissue solubilizer (SOLVABLE™, Packard BioScience BV, Groningen, The Netherlands) at 37 °C. Ten mL of liquid scintillation cocktail (Ultima Gold™ LTT, Packard BioScience BV, Groningen, The Netherlands) was added to the vials. Radioactivity was measured in a liquid scintillation counter (Tri-Carb® 2100TR, Packard), where the counting efficiency was controlled by measurement of the spectral quench parameters (SQP). The dependence of SQP on counting efficiency was determined using a \(^{14}\)C standard (No. 1210-122, lot No. 103209, LBK Wallac Oy, Turku, Finland) to which dichloromethane as quencher was added in increasing amounts to establish an efficiency vs. SQP curve. The measured SQP-value for each sample was then used to convert the measured radioactivity count rate to becquerel (Bq). The organ concentrations (ng PAH · g\(^{-1}\) organ) were calculated according Equation [1]:

\[
ng \text{ PAH/g organ} = \frac{(H_{\theta} - \text{Blank})}{SpA} \frac{SpA}{H_{\phi, \text{ww}}}
\]  

[1]
where $H_\theta$ is radioactivity (Bq) in the homogenate; Blank is the radioactivity (Bq) in a counted blank sample (Control fish which only had been given the experimental feed) for each selected organ. $SpA$ (Bq · ng$^{-1}$ PAH) is the specific radioactivity of the radiotracer and $H_\theta$ ww is the wet-weight (g) of the homogenate. Radioactivity in the homogenate samples was corrected for background radioactivity. Thereafter, the amount of PAHs (ng) in each homogenate was calculated by dividing the measured radioactivity by the $SpA$ of the radiotracer. Organ concentrations (ng PAH · g$^{-1}$ organ) were calculated by dividing the total amount of PAHs (ng) per organ by the homogenate wet-weight (g). Additionally the sample was corrected for organ weight (g) to obtain the total amount of PAHs (ng) per organ.

**Whole body autoradiography**

Fish ($n = 2$ for each treatment and time point) were sampled 3, 6 and 12 hours post-feeding. Each fish was immediately frozen on dry ice and stored at -20 °C until further sample preparation. Frozen fish were individually embedded in a pre-cooled (0 - 4 °C) aqueous gel of 1% carboxymethylcellulose (w/w in tap water) on dry ice and sectioned with a cryomicrotome (PMV, 450 MP, Sweden). Sagittal sections (20 µm) containing the pyloric caeca, proximal and distal intestine, liver, bile and kidney were collected on acrylic adhesive tape No. 821 (3M Co, Minnesota, USA). Neighboring sections were collected on acrylic adhesive tape No. 356 (3M Co, Minnesota, USA) for extraction with polar and non-polar solvents. All sections were freeze-dried overnight at -20 °C. Extraction was performed with 5% trichloroacetic acid (1 min), 50% methanol (30 s), 100% methanol (30 s), heptane (15 s), 100% methanol (30 s) and 50% methanol (30 s). After washing for 10 min in running tap water, sections were dried overnight at room temperature. Further processing of native sections took place at -20 °C to avoid melting and diffusion of fat in the sections [91], whereas extracted sections were prepared at room temperature. The autoradiograms obtained from native sections showed the locations of $^{14}$C-BaP or $^{14}$C-PHE and their related metabolites. Autoradiograms obtained from solvent-extracted sections were interpreted to show $^{14}$C-BaP or $^{14}$C-PHE and associated metabolites bound to cellular macromolecules [91]. Both native and extracted sections were exposed to X-ray-film (Structurix D7 DW ETE, Agfa-Gevaert, Belgium) for one week. The films were developed (XR D-1.5 NDT, Dürr, Germany) and fixated (XR F-1.5 NDT, Dürr, Germany) for four and eight min respectively, followed by rinsing in running tap water for 30 min and air-dried. The native sections and autoradiograms from native and extracted sections were digitalized (HP ScanJet 6200c). Images of the native sections have been adjusted to increase the contrast and autoradiograms have been inverted to photo
positives and adjusted to increase the contrast (Adobe Photoshop Elements, 8.0).

**Analysis of gene expression**

Fish (n = 4 for each treatment) were euthanized 12 hours post-feeding and the pyloric caeca, proximal intestine, distal intestine and liver were immediately dissected. The intestinal samples were cleaned of luminal content by washing with saline solution after which the mucosa of the proximal and distal intestine was scraped off its underlying muscle layers with a glass microscope slide. Samples thus obtained were immediately frozen in liquid nitrogen and stored at -80 °C until RNA isolation.

**RNA isolation**

Tissues were homogenized using a MagNA Lyser Green beads homogenizer (Roche Applied Science). Total RNA was extracted with Trizol reagent (Invitrogen Ltd, Paisley, UK) using the Qiagen® BioRobot® EZ1® (Hilden, Germany). Samples were treated with DNase according to the manufacturer’s instructions and diluted in 50 µL RNase-free MilliQ water. The quality of the RNA was assessed with the Nano-Drop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The RNA 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, CA, USA) was used to evaluate the RNA integrity of 12 randomly selected samples. The RNA integrity number was at least 8.1 for the liver samples and at least 5.8 for analyzed pyloric caeca, proximal- and distal intestine samples.

**RT-PCR**

Reverse transcriptase (RT)-PCR was performed using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA). PCR efficiency was calculated from a standard curve composed of serial dilutions (1000 to 31 ng) of a RNA pool from randomly chosen samples. Total RNA input was 500 ng in each reaction for all genes. Control reactions contained no template (no template control, ntc) or no reverse transcriptase (nrc). Reverse transcription was performed at 25°C for 10 min, 48°C for 60 min, 95°C for 5 min followed by a decrease to 4°C, using oligo dT primers (2.5 µM) for all genes in 50-µL total volume. The other chemicals (final concentration given in parentheses) were: MgCl2 (5.5 mM), dNTPs (500 µM of each), 10 · TaqMan RT buffer (1x), RNase inhibitor (0.4 U · µL⁻¹) and Multiscribe reverse transcriptase (1.67 U · µL⁻¹).
RT-qPCR

Two µL of the two-fold diluted cDNA from each RT reaction was transferred to a 384-well reaction plate. Real-time quantitative PCR (qPCR) was performed in 10-µL reactions on a LightCycler 480 Real-Time PCR System (Roche Applied Sciences, Basel, Switzerland). Real-time qPCR was performed using SYBR Green PCR Master Mix (Roche Applied Sciences, Basel, Switzerland), forward and reverse gene specific primer oligonucleotides (50 µM; see Table 2.2 for oligonucleotide sequences and target gene names). Reaction conditions were: a 5 min activation and denaturing step at 95 °C, followed by 45 cycles with each cycle consisting a 10 s denaturing step at 95 °C, a 10 s annealing step at 60 °C and finally a 10 s synthesis step at 72 °C, followed by a melt curve analysis and cooling to 4 °C.

Mean normalized expression (MNE) for each target gene was determined using a normalization factor calculated by the geNorm software based on the selected reference genes. PCR primer sequences used for the quantification of the reference genes encoding β-actin, elongation factor 1 alpha (elf1α), acidic ribosomal protein (arp) and the target genes are given in table 2.2. As target genes, phase I (i.e., cyp1a1, cyp2m, cyp3a27), phase II (glutathione S-transferase P (gstp), UDP glucuronosyltransferases (ugt)) and phase III (ATP-binding cassette (abcb1, abcc2, abcg2)) detoxification genes were selected as well as genes involved in transport proteins for fatty acid transport (fatty-acid-binding proteins (fabp 1, 5)). PCR efficiencies are presented in the supplementary information (Table S2.1).

Table 2.2: Primer oligonucleotide sequences used for quantitative real-time PCR.

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<th>Gene</th>
<th>GenBank accession #</th>
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<th>3’-5’ reverse primer</th>
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</table>
Preparation of bile samples and HPLC/fluorescence analyses

Bile was collected by puncture of the gall bladder by a 1 mL syringe from euthanized fish \((n = 4, \text{for each treatment and time point})\) 3 and 12 hours post-feeding. Preparation of hydrolyzed bile samples was performed as described by Krahn et al. (1992). Briefly, bile (20 µL) was mixed with an internal standard (triphenylamine, Sigma-Aldrich Co, St Louis, U.S.A.), diluted with demineralized water (50 µL), and hydrolyzed with glucuronidase/arylsulfatase (20 µL, 1 hour at 37 °C) [107]. Methanol (200 µL) was added, the sample was centrifuged (13000 g, 10 min) and the supernatant analyzed. HPLC was performed after Grung et al. (2009). Briefly: a Waters 2695 separation module with a 2475 fluorescence detector attached was used. The column used was a Waters PAH C18 (4.6 × 250 mm) with 5-µm particles. Twenty-five µL of extract was injected for each analysis [82]. Each run lasted 30 min where the mobile phase consisted of a 40:60 acetonitrile:ammonium acetate (0.05 mM, pH 4.1) and gradually increased to a 100% acetonitrile gradient at a flow of 1 mL ∙ min\(^{-1}\), the column was heated to 35 °C. Fluorescence excitation/emission wavelengths were measured at the optimum for each analyte: OH-PHE at 256/380 nm and OH-BaP at 350/430 nm. Standards of 1-OH-PHE and 3-OH-BaP (Chiron AS, Trondheim, Norway) were included measured at similar wavelengths and used to determine metabolite concentrations (µg OH-PAH ∙ g\(^{-1}\) bile).

Statistics

Organ PAH accumulation over time were analyzed using multilevel linear models in the statistical programming language R (version 3.0.2) [162]. In brief, a basic fixed intercept model predicting the relative BaP or PHE organ load from the intercept was created (BaP/PHE ~1) using the generalized least squares (gls) function of the package nlme [159]. A basic fixed intercept model was used instead of random intercepts (i.e., no need to account for inter-individual differences over time as the fish were terminally sampled at each time point). In a subsequent step, different time trajectories (linear and second order polynomial) were added to the models (BaP/PHE ~Time/Time\(^2\)). The produced models were compared to each other using Log likelihood tests and the best fitting (ANOVA, \(p \leq 0.05\)) and most parsimonious model was chosen for further analyses in which the type of dietary oil was added as predictor (BaP/PHE ~Time/Time\(^2\) + type of oil). Statistical significance was accepted at \(p \leq 0.05\). A comprehensive overview of all models created and all parameters determined is presented in the supplementary information (Table S2.2 – S2.3). Data were checked for homogeneity of variance by D’Agostino-Pearson normality test. Bile metabolite data were heterogeneous.
and assessed with a Kruskal-Wallis test between the control group, 3 and 12 hours post-feeding per PAH per feed type. A Mann-Whitney U test was performed to analyze differences per PAH between oil-based diets after 12 hours. The gene expression data 12 hours after exposure was assessed with a factorial (2x2) ANOVA to analyze the effects of the two factors “type of oil” (fish oil-based feed and vegetable oil-based feed) and presence or absence of “BaP” or “PHE”. When the interaction of the two factors was significant, multiple comparison post-hoc testing was performed in order to elucidate the simple effects among treatments. Results were considered statistically significant when $p < 0.05$ (two-tailed).

**Results**

**BaP and PHE organ concentrations**

Table 2.3 shows the PAH organ concentrations of salmon fed a single dose of either BaP or PHE pre-fed a fish oil or vegetable oil-based diet. The concentrations of both PAHs gradually increased along the entire intestinal tract during 12 hours. After 12 hours, the intestine (pyloric caeca, proximal and distal intestine) contained $24\% \pm 5.7\%$ (mean $\pm$ SD) and $19\% \pm 2.2\%$ of the given BaP dose in Atlantic salmon acclimatized to a fish oil or vegetable oil-based feed, respectively. The amount of PHE in the intestine, 12 hours post-feeding, was $18\% \pm 1.5\%$ in salmon pre-fed a fish oil-based diet and $14\% \pm 1.8\%$ in salmon pre-fed a vegetable oil-based diet. The highest PHE concentration was detected in the bile after 12 hours.

Figure 2.1 shows BaP and PHE concentrations during the first 12 hours in Atlantic salmon acclimatized to a fish oil or vegetable oil-based feed. The accumulation of BaP in the pyloric caeca, distal intestine and spleen differed significantly over time between fish fed a fish oil or vegetable oil-based feed. For PHE, only the proximal intestine concentration was significantly influenced by type of oil. No significant differences in PAH concentrations of other peripheral organs (liver, head kidney, kidney and gills) between the fish oil and vegetable oil groups were found.

Fish oil-based feed significantly increased BaP concentrations ($p = 0.041$) in the pyloric caeca compared to salmon fed a vegetable oil-based diet (Fig. 2.1A). A second order polynomial model describes the BaP concentrations that increase from 3 to 12 hours post-feeding. As opposed to the pyloric caeca, fish oil-based feed significantly decreased ($p = 0.037$) BaP concentrations in the distal part of the intestine (Fig. 2.1B). The model confirms a linear increase in BaP concentration over time. Of the other peripheral organs only spleen had significantly ($p = 0.008$)
higher BaP organ concentrations after the fish were fed a fish oil-based diet (Fig. 2.1C). Modeling BaP presence in the spleen showed an initial decrease from 3 to 6 hours followed by an increase 12 hours post-feeding. A detailed list of all regression model parameters for BaP organ concentrations are presented in the supplementary information (Table S2.2).

PHE organ concentrations were significantly increased ($p = 0.047$) in the proximal intestine of fish fed a fish oil-based diet (Fig. 2.1D). A second order polynomial

### Table 2.3: Organ accumulation of benzo[a]pyrene (BaP) or phenanthrene (PHE) in Atlantic salmon per organ (ng PAH · g⁻¹ organ). Atlantic salmon were fed either a fish oil or vegetable oil-based diet spiked with either BaP or PHE. Mean values ± SD, $n = 4$; are shown. An asterisk indicates a significant accumulation of a PAH over time between fish fed fish oil or vegetable oil in the respective organ and are shown in figure 2.1. Statistical parameters are presented in the supplementary information (Table S2.2 & S2.3).

<table>
<thead>
<tr>
<th></th>
<th>Fish oil-based feed</th>
<th>Vegetable oil-based feed</th>
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<tbody>
<tr>
<td></td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Stomach</td>
<td>1685 ± 332</td>
<td>1468 ± 932</td>
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<tr>
<td>Pyloric caeca*</td>
<td>1202 ± 479</td>
<td>6252 ± 2482</td>
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<tr>
<td>Proximal intestine</td>
<td>1382 ± 719</td>
<td>6495 ± 3717</td>
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<tr>
<td>Distal intestine*</td>
<td>69 ± 22</td>
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<tr>
<td>Liver</td>
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<td>151 ± 20</td>
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<tr>
<td>Bile</td>
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<td>734 ± 315</td>
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<tr>
<td>Blood</td>
<td>0.070 ± 0.022</td>
<td>1.1 ± 0.60</td>
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<tr>
<td>Third gill arch</td>
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<td>490 ± 339</td>
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<tr>
<td>Head Kidney</td>
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<tr>
<td>Kidney</td>
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<tr>
<td>Spleen*</td>
<td>43 ± 12</td>
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<tr>
<td>Stomach</td>
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<tr>
<td>Pyloric caeca*</td>
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<td>Distal intestine</td>
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<tr>
<td>Liver</td>
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<tr>
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<td>621 ± 446</td>
</tr>
<tr>
<td>Blood</td>
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<td>1.0 ± 0.28</td>
</tr>
<tr>
<td>Third gill arch</td>
<td>42 ± 45</td>
<td>226 ± 209</td>
</tr>
<tr>
<td>Head Kidney</td>
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<tr>
<td>Kidney</td>
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<td>55 ± 11</td>
</tr>
<tr>
<td>Spleen*</td>
<td>13 ± 11</td>
<td>33 ± 11</td>
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model describes PHE levels that increase from 3 to 12 hours post-feeding. A detailed list of regression model parameters of all PHE organ concentrations is presented in the supplementary information (Table S2.3).

**Organ distribution of BaP and PHE**

Figure 2.2 and 2.3 show the disposition of $^{14}$C-BaP and $^{14}$C-PHE 3 and 12 hours post-feeding. Three hours after oral administration, most of the BaP-associated radioactivity was detected in the mucosa of the pyloric caeca and proximal intestine. Radioactivity increased over time and after 6 hours BaP-associated radioactivity was present in liver and bile. Furthermore, after 12 hours, radioactivity was also detected in the kidney (inserts in Fig. 2.2D).

Three hours after administration, most PHE-associated radioactivity was present in the stomach and a very weak signal was detected in the intestinal tract (Fig. 2.3C). After 6 hours PHE-associated activity was detected in bile, liver, kidney and urine

**Figure 2.1:** Time-course of accumulation of benzo[a]pyrene (BaP) or phenanthrene (PHE) in Atlantic salmon per organ (ng PAH · g⁻¹ organ) which had significant difference between type of feed. Solid black lines represent fish oil-based feed spiked with either BaP or PHE and dashed lines represent vegetable oil-based feed spiked with either BaP or PHE. Only organs are shown which had significant different PAH accumulation over time between both diets. BaP: pyloric caeca (A), distal intestine (B), spleen (C). PHE: proximal intestine (D). Points represent experimental data (mean values ± SD; $n = 4$).
Intestinal uptake and tissue distribution of BaP and PHE

Figure 2.2: Autoradiograms of adjoining sections from Atlantic salmon, after a single oral dose of $[^{14}C]$-benzo[a]pyrene (2.5 kBq · g$^{-1}$ body mass) corresponding to approximately 0.64 µg BaP · g$^{-1}$ body mass (w/w). Sections represent a native section (top), the corresponding autoradiogram of the native section (middle) and an adjacent, solvents-extracted section (bottom). Bar = 1 cm. White areas correspond to high concentrations of radiolabeled compound. Numbers on the autoradiograms represent the following organs; 1) pyloric caeca, 2) proximal intestine, 3) distal intestine, 4) liver, 5) gall bladder, 6) kidney, 7) urinary tract, 8) central nervous system. A) 3 hours post-feeding a fish oil-based feed. B) 12 hours post-feeding a fish oil-based feed. C) 3 hours post-feeding a vegetable oil-based feed. D) 12 hours post-feeding a vegetable oil-based feed. An additional autoradiogram was inserted in D to include the kidney as no section with both proximal intestine and kidney was obtained.
Intestinal uptake and tissue distribution of BaP and PHE

Figure 2.3: Autoradiograms of adjoining sections from Atlantic salmon, after a single oral dose of [14C]-phenanthrene (2.5 kBq ∙ g⁻¹ body mass) corresponding to approximately 0.23 µg PHE ∙ g⁻¹ body mass (w/w). Sections represent a native section (top), the corresponding autoradiogram of the native section (middle) and an adjacent, solvents-extracted section (bottom). Bar = 1 cm. White areas correspond to high concentrations of radiolabeled compound. Numbers represent the following organs; 1) pyloric caeca, 2) proximal intestine, 3) distal intestine, 4) liver, 5) gall bladder, 6) kidney, 7) urinary tract, 8) central nervous system.

A) 3 hours post-feeding a fish oil-based feed. B) 12 hours post-feeding a fish oil-based feed. C) 3 hours post-feeding a vegetable oil-based feed. D) 12 hours post-feeding a vegetable oil-based feed. The autoradiogram of the extracted section in panel A has some artifacts (tape stuck on the film) which appear white and should not be misinterpreted as radioactivity (illustrated with an asterisk).
while most of the radioactivity had disappeared after 12 hours with the exception of the bile.

Distinctive differences were observed between BaP and PHE in the extracted autoradiograms. After the extraction procedure, the distribution of BaP-associated activity in the intestinal tract had not changed, indicating the presence of non-extractable cellular bound radioactivity. In contrast, BaP-associated radiolabeling was almost completely extracted from the bile, except for some activity in the gall bladder walls. Only low levels of PHE-associated activity were detected in the gallbladder and liver, indicating an almost complete extraction. No apparent differences were observed between salmon fed a fish oil or vegetable oil-based diet in either the BaP or the PHE group.

Figure 2.4: mRNA expression 12 hours post-feeding of cyp1a in the pyloric caeca (A), proximal intestine (B), distal intestine (C) and the liver (D) of Atlantic salmon acclimatized to a fish oil (black symbols) or a vegetable oil-based diet (open symbols) following a single oral dose of benzo[a]pyrene (BaP) or phenanthrene (PHE). Significant interactions are indicated with a dashed line following post-hoc testing with a dotted line. Main effects are indicated with a solid line. Significant differences are indicated with; * (p < 0.05), ** (p < 0.01) and *** (p < 0.001).
Up-regulation of cyp1a expression after BaP administration

Figure 2.4 shows the gene expression level of cyp1a in the pyloric caeca, proximal intestine, distal intestine and liver. Of all genes tested (see table 2.2), only the expression of cyp1a was significantly up-regulated following exposure to BaP. In the distal intestine, the factor “fish oil/vegetable oil” significantly interacted ($p = 0.015$) with BaP-induced cyp1a expression (Fig. 2.4C). In the proximal intestine and liver, BaP exposure resulted in a significantly increased cyp1a expression compared to controls. No significant changes in gene expression were observed for salmon fed PHE.

Bile metabolites of BaP and PHE

The levels of PAH metabolites in bile ($\mu$g OH-PAH $\cdot$ g$^{-1}$ bile) after diet exposure are illustrated in figure 2.5. The concentration of OH-BaP was significantly increased 12 hours post-feeding in fish fed fish oil ($p = 0.007$) when compared to the control group. Vegetable oil had no significant effect after 3 or 12 hours compared with the control group but a significant difference was detected between the 3 and 12 hours post-feeding ($p = 0.045$). After 12 hours the bile had significantly increased OH-PHE concentrations in fish fed a fish oil-based diet ($p = 0.012$) or a vegetable oil-based diet ($p = 0.006$). Between salmon pre-fed a fish oil or vegetable oil-based diet no significant differences in OH-BaP ($p = 0.10$) or OH-PHE ($p = 0.06$) concentrations were observed 12 hours post-feeding.

![Figure 2.5: PAH metabolites in bile (µg OH-PAH · g$^{-1}$ bile). Metabolites were measured in Atlantic salmon acclimatized either to a fish oil-based diet (black symbols) or a vegetable oil-based diet (open symbols) and 3 and 12 hours after a single oral dose of either benzo[a]pyrene (A; BaP) or phenanthrene (B; PHE). Mean values ± SD are shown, $n = 3$ for BaP and $n = 4$ for PHE. Letters above groups indicate significant differences assessed by a Kruskal-Wallis test. Groups that do not share corresponding letters are significantly different from each other ($p < 0.05$).](image)
Discussion

Atlantic salmon showed prominent differences between the intestinal uptake and organ distribution after intragastric administration of a single pulse-chase dose of BaP and PHE. BaP increased the expression of cyp1a in liver and intestine. In addition, the type of dietary fat, viz. fish oil or vegetable oil, altered intestinal BaP and PHE uptake and distribution.

BaP and PHE differences in tissue distribution and metabolism

Twelve hours post-feeding, both BaP and PHE were transported across the intestinal tract as evidenced by the increased concentration of radiolabeled compounds in liver and bile. Both compounds were still present along the entire intestinal tract, although PHE-associated radioactivity concentrations had declined in the intestine and were mostly found in bile. The high concentration of PHE, and to a lesser extent BaP, in bile and urine are evidence for hepatic and renal clearance pathways. Besides excretion of PAHs by bile to the feces, urine is also an important excretory pathway for water soluble PAH metabolites in other teleost species [115, 189].

A similar distribution pattern was observed when BaP was administrated intragastrically to channel catfish (Ictalurus punctatus), polar cod (Boreogadus saida) or rainbow trout (Oncorhynchus mykiss), where most BaP-associated radioactivity was detected in the intestinal tract and bile [6, 91, 106, 172]. Furthermore, oral administration of PHE to flounder (Platichthys flesus), polar cod and rainbow trout showed comparable results where most of the PHE-associated radioactivity was detected in the gall bladder [6, 186, 188].

The higher levels of unextractable BaP-associated radioactivity in the intestinal mucosa, compared to the extractable PHE-associated radioactivity, indicate macromolecule-bound BaP-residues [91, 172]. Cyp1a-mediated metabolism of BaP can result in toxic intermediates, such as benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE). Epoxide intermediates are very reactive and can bind to cellular macromolecules such as proteins or DNA forming adducts that can lead to cellular damage [130, 146, 219]. BaP is an aryl hydrocarbon receptor agonist [18, 19] and so induces its own metabolism by Cyp1a. BaP caused significant up-regulation of cyp1a expression in the proximal intestine, distal intestine and liver. This BaP-induced cyp1a expression coincides with macromolecule-bound BaP-residues observed in the autoradiograms of intestinal mucosa and liver.
Unfortunately, too little sample material was available to assess Cyp1a protein levels or ethoxyresorufin O-deethylase (EROD) activity together with cyp1a mRNA expression. However, several studies have shown the use of cyp1a mRNA expression as marker for elevated Cyp1a protein [114] or EROD activity [116] during short-term BaP exposures in fish. The presence of unextractable BaP-associated radioactivity could also contribute to the higher BaP concentrations in the pyloric caeca, proximal intestine and distal intestine compared to PHE concentrations as well as lower BaP bile levels (Table 2.3). Solvent extraction of whole-body sections resulted in faint traces of PHE-associated radioactivity. In contrast to BaP, PHE does not induce the aryl hydrocarbon receptor and has no effect on cyp1a mRNA expression, which is corroborated by our observations and other studies [8, 112].

For dietary exposures, the intestine is the first barrier for foodborne PAH uptake and plays an important role in PAH metabolism [129]. The lower intestinal metabolism of PHE compared to BaP may explain the higher accumulation of PHE in the liver and bile, as BaP metabolites can bind to intestinal cellular macromolecules while PHE is less extensively metabolized and probably transported faster across the intestinal tract, towards the liver and bile. Earlier studies have also emphasized the role of intestinal metabolism on intestinal transport of different dietary PAH congeners. Accordingly, BaP was metabolized at a faster rate and crossed the intestinal barrier slower compared to naphthalene which was absorbed faster but less readily metabolized [21]. Furthermore, when pyrene and PHE were incubated with intestinal Caco-2 cells, they were less extensively metabolized than BaP but transported faster across Caco-2 epithelia [33].

In addition to the intestine, the liver is a main site for phase I and phase II biotransformation of dietary PAHs and subsequently excretes the metabolic products in the bile of the gall bladder [82, 115, 189]. Metabolites of PAHs in fish bile have been extensively used as biomarkers for PAH exposure and studies have shown that PAH metabolites in fish bile correlate well with PAH exposure [82, 107]. Although PAH metabolites in bile mainly consists as conjugates, treatment of the bile samples with deconjugation enzymes is stimulated as available conjugation standards are poorly available [17]. The total bile concentration of PHE was higher than that of BaP (Table 2.3), although the given dose of PHE via gavage was lower. The higher PHE compared to BaP bile concentrations could probably be explained by the lower fraction of PHE metabolites bound to cellular macromolecules in the intestine and liver causing a faster intestinal and hepatic transports of PHE. In contrast, OH-BaP metabolite concentrations in the bile were higher than OH-PHE metabolite concentrations (Fig. 2.5). In addition, of the total
Intestinal uptake and tissue distribution of BaP and PHE

BaP bile concentration, 26% and 14% were OH-BaP metabolites with a fish oil and vegetable oil-based feed while the percentage of OH-PHE metabolites was only 4.7% and 2.1%, respectively. The higher percentage of OH-BaP metabolites in the bile coincides with the increased cyp1a expression and the unextractable BaP-associated radioactivity in the liver. Although PHE does not stimulate its own biotransformation, metabolites detected in the bile illustrate that PHE is metabolized in marine animals [187].

Effects of dietary oil on intestinal uptake of BaP and PHE

In addition to differences in PAH congeners, dietary fat composition also affected the intestinal uptake of PAHs in Atlantic salmon. Earlier studies have shown that dietary lipids influence intestinal uptake of hydrophobic xenobiotics [48, 110, 221, 222]. In the intestinal lumen, fatty acids and hydrophobic contaminants disperse after lipid hydrolysis into mixed micelles [44, 110, 222], which provide a more suitable hydrophobic environment for lipophilic molecules than the aqueous lumen [48]. Long fatty acid chains and a high degree of unsaturated bonds increase the solubility of PAHs in micelles [44, 110], which can lead to an increased intestinal delivery and transfer [44]. Fish oil-based diets have more PUFA’s such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) that have longer fatty acid chains and a higher degree of unsaturated bonds than vegetable oil-based diets (Table 2.1). Therefore, this knowledge coincides well with the increased BaP and PHE concentrations with a fish oil-based diet in the pyloric caeca and proximal intestine, respectively, which are the main sites for fatty acid uptake [100, 108, 205]. It seems that fish oil-based feed not only contributes to increased intestinal concentrations of BaP but also to increased peripheral distribution as indicated by higher organ concentration in spleen.

In contrast to the increased BaP levels in the pyloric caeca, BaP levels in the distal intestine were lower when Atlantic salmon was fed a fish oil-based diet compared to a vegetable oil-based diet. Although the distal intestine is not the main site for fatty acid uptake, for BaP exposed fish, the type of dietary oil significantly interacted with the cyp1a expression, with higher cyp1a expression in fish oil fed fish. The increased Cyp1a-mediated metabolism in fish oil fed fish versus vegetable oil fed fish could explain the lower intestinal BaP concentrations in the distal intestine in fish fed a fish oil-based diet. Indeed, when cyp1a expression was not increased in the pyloric caeca after BaP exposure fish oil resulted in increased BaP concentrations. Previous studies have shown that BaP metabolism by intestinal cytochrome P450 activity can also be induced by fatty acids levels and in particular by PUFAs [230]. When rats were fed a
Intestinal uptake and tissue distribution of BaP and PHE

cod liver oil diet that is rich in PUFAs, BaP metabolism by the intestinal cytochrome P450 system [80] and epoxide hydrolase activity of BaP [79] were both increased compared to BaP metabolism in rats fed a reference corn oil diet rich in saturated and mono-unsaturated fatty acids. As no differences were seen in the control group of this experiment between fish fed a fish oil-based diet or a vegetable oil-based diet, this could indicate that PUFAs have an additional stimulating effect on cyp1a expression in the presence of BaP [81].

In addition to the effects of dietary fat, other contaminants than BaP could also have induced the intestinal cytochrome P450 system in the fish oil fed fish. Earlier studies have shown that cyp1a expression is increased when multiple cyp1a inducers were introduced in combination with BaP, such as polyhalogenated aromatic hydrocarbons (PHAHs), dioxin-like polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-p-dioxins and furans (PCDD/F) [185, 190]. Fish oil is well known to contain elevated levels of dioxins and dioxin-like PCBs with an aryl hydrocarbon receptor affinity that induce Cyp1a activity [15]. In the present trial, the sum of dioxin and dioxin-like PCBs was 2.7 pg WHO2005-Toxic Equivalence Quotient (TEQ) \( \cdot \) g\(^{-1}\) for the fish oil-based diets and 0.43 pg WHO2005-TEQ \( \cdot \) g\(^{-1}\) for the vegetable oil-based diets. However, long-term trials with Atlantic salmon fed diets containing similar levels of dioxins and dioxin-like PCBs (2.31 pg WHO2005-TEQ \( \cdot \) g\(^{-1}\)) or decontaminated feeds (0.54 pg WHO2005-TEQ \( \cdot \) g\(^{-1}\)) showed no significant differences in hepatic cyp1a expression [15]. The stimulating effects on BaP metabolism by the cytochrome P450 in combination with higher levels of PUFA’s and the background level of dioxin-like components in fish oil-based feed could explain the significantly higher cyp1a expression in the distal intestine in salmons fed a fish oil-based diet compared to salmon fed the same level of spiked BaP in a vegetable oil-based feed. Fish oil induced metabolism is likely to reduce distal BaP intestinal concentrations by increasing the transfer into the systemic circulation or/and excretion to the intestinal lumen via cellular efflux transporters [217].

**Conclusions**

Dynamics and distinctions between uptake and metabolism are complex integrated processes, which make it difficult to distinguish dynamics in the present in vivo experiment. Nonetheless, this experiment shows that fish oil increases BaP and PHE concentrations in the pyloric caeca and proximal intestine respectively, compared to vegetable oil-based feed. This is probably due to a lower solubility of PAHs 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. In addition, it has to be
taken into account that replacement of fish oil with vegetable oil is likely to increase feed PAH levels and so increase their relative intestinal accumulation. Conversely, vegetable oil increased BaP concentrations in the distal intestine compared to fish oil-based feed, which is most likely due to a lower degree of BaP metabolism in vegetable oil fed fish. In the intestinal part with no BaP induced metabolism, (i.e., pyloric caeca), BaP behaved as PHE with elevated levels when fish were fed fish oils compared to vegetable oils.

The partial replacement of fish oils by vegetable oils in aquafeeds increase PAH concentrations in feed to farmed salmon. This study showed that fish oil could lead to an increased intestinal delivery and transfer of PAHs. In addition, vegetable oils could increase the intestinal transfer time by decreasing Cyp1a-mediated metabolism causing a decreased PAH metabolism. Further long-term feeding trials have to investigate the effects of aquafeed oil blends on the intestinal PAHs uptake and metabolism.

**Acknowledgements**

The authors thank Dr. Pål A. Olsvik for primer design and validation, Jacob Wessels for practical assistance with the liquid scintillation counting and Susanne Håvardstun Eide and Jan Ove Wedaa for excellent fish husbandry. This study was funded by the Research Council of Norway (RCN, project ID: 200506/I30 “Challenges towards sustainable aquafeeds: plant nutrients and contaminants interactions – Nutritox”).
Intestinal uptake and tissue distribution of BaP and PHE
Chapter 3

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

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 [59, 158, 199]. 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 [13, 14, 16]. 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 [134]. In aquafeeds, PAHs are formed by thermal processing of oil-containing seeds and grains during toasting or gas drying [135, 157, 202]. The diet contributes substantially to PAH exposure with cereals, vegetable fats and oils being the principal culprits [157]. Concern about these contaminants has been due to the carcinogenic, mutagenic/genotoxic and other toxic effects induced by PAHs [52].

After oral ingestion, the lipophilic nature of PAHs promotes their solubilization in oil droplets and mixed micelles in the intestinal lumen [97, 104, 160] 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 [147, 205]. 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 [5]. Biliary components (e.g., bile salts and cholesterol) spontaneously form mixed micelles with free fatty acids (FFA) and, to a lesser extent, with mono-, di- and triacylglycerides [5, 156, 224, 231].

Two key lipolytic enzymes are secreted by the exocrine pancreas in mammals, viz. bile salt-activated lipase (BAL) and pancreatic lipase [223]. Lipases hydrolyze ester bonds in triacylglycerides, phospholipids, cholesterylsters 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 [22, 23, 71, 147, 170, 197, 205]. 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 [35, 70, 223].
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 [44, 110] whereas solubility in micelles composed of long-chain triacylglycerides is often low [160]. Fish oil is an important source of n-3 unsaturated long-chain fatty acids such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) [12, 182]. Rapeseed oil is mainly used as a substitute for fish oils and contains high levels of oleic acid (ca. 60%; 18:1n-9) and moderate levels of linoleic acid (ca. 20%; 18:2n-6) and saturated short-chain fatty acids (Table 3.1). 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.

**Materials and Methods**

**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 in an indoor recirculating system containing 575 L (input of 1 L fresh tap water per min) 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 hour at a ration of 2% of the estimated body weight per day.

Previous studies showed that six hours post-feeding feed was mainly located in the proximal intestine in Atlantic salmon weighing 200 g [41]. Therefore, to ensure access to intestinal luminal contents, rainbow trout were anaesthetized six hours 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).

**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 10000 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

Table 3.1: 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).

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

**Validation of the modified titrimetric assay**

Lipase activity was assessed with a modified titrimetric assay [78] 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 triacylglyceride substrate are titrated with NaOH. To prevent dilution of substrate and enzyme concentrations in the incubate by the addition of NaOH titrant, the initial decrease in pH of the incubate was measured 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 [83]. The assay medium was completed with substrate emulsion, assay medium and dH₂O (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 x United States Pharmacopeia (U.S.P) specifications; 8.0 units lipase ∙ mg pancreatin⁻¹), dissolved 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 dH₂O 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 [78]. The liberation of FFA by lipase activity was expressed as pmol FFA per min 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. 3.1A). 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. 3.1B). 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. 3.1C), 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. 3.1D). 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. 3.1E). The initial hydrolysis rate with olive oil was best described by a substrate inhibition model with a substrate inhibition constant (Kᵢ) of 5.7 mM olive oil (Fig. 3.1F). Pre-incubation of porcine pancreatin for one hour with 10 nM of the lipase inhibitor orlistat decreased olive oil hydrolysis by 40%.

**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 *Oncorhynchus mykiss* and 20 mM bile salt (taurocholate) [22]. 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 dH₂O 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 less than 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 (Kₘ) value of 0.5 mM and a Vₘₐₓ of 11.8 pmol ∙ mg protein⁻¹ ∙ min⁻¹.
BaP and PHE inhibit intestinal lipase activity
BaP and PHE inhibit intestinal lipase activity

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 Oncorhynchus 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 [42]; therefore glass vials were used 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 Km 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.

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 ddH2O were added and again vortexed thoroughly [20]. 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 [87]. 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 x 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 visualized 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).

**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) [162] 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:

\[ p = \text{Limit} \cdot (1 - e^{-kt}) \]  \[1\]

where \( p \) represents the liberated FFA (pmol FFA · mg protein⁻¹) by lipase, \( \text{Limit} \) (pmol · mg protein⁻¹) represents the uptake at time (t) approaching \( \infty \), 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:

\[-kt = \ln \left(1 - \frac{p}{\text{Limit}}\right)\]  \[2\]
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:

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

where $v_0$ is enzyme initial rate (pmol FFA $\cdot$ mg protein$^{-1} \cdot$ min$^{-1}$), $[S]$ is the substrate concentration (mM), $V_{max}$ is the maximum rate (pmol $\cdot$ mg 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 [38]. 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 *** ($p < 0.001$).

**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 3.2).

Table 3.2: The effects of bile salt (taurocholate) on the hydrolysis of olive oil (pmol FFA $\cdot$ mg protein$^{-1} \cdot$ min$^{-1}$) in crude and desalted BAL extracts from the proximal intestine of rainbow trout. 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; $p < 0.001$).

<table>
<thead>
<tr>
<th>BAL extract</th>
<th>Bile salt</th>
<th>FFA release (pmol FFA $\cdot$ mg protein$^{-1} \cdot$ min$^{-1}$)</th>
<th>Average SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20 mM</td>
<td>0.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>-</td>
<td>2.5 ± 0.5$^a$</td>
<td></td>
</tr>
<tr>
<td>Desalted</td>
<td>0 mM</td>
<td>3.7 ± 0.5$^a$</td>
<td></td>
</tr>
<tr>
<td>Desalted</td>
<td>20 mM</td>
<td>15.5 ± 1.5$^b$</td>
<td></td>
</tr>
</tbody>
</table>
The time course of rapeseed and fish oil hydrolysis by BAL extracts proceeded linearly up to 30 min (Fig. 3.2A). The hydrolysis of rapeseed and fish oil is well described by a single first-order exponential rate equation (Fig. 3.2B) 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 \(\cdot\) mg protein\(^{-1}\)). The initial hydrolysis rates calculated at \(t = 0\) min were 9.2 and 9.0 pmol FFA \(\cdot\) min\(^{-1}\) \(\cdot\) 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. 3.3). 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.

Both PAHs inhibited the hydrolysis of rapeseed oil where BaP was 10-fold more potent than PHE (Fig. 3.4). Rapeseed oil hydrolysis had significantly decreased by 40% in the presence of 0.001 \(\mu\)M BaP (\(p < 0.01\)) and by 55% with 0.01 \(\mu\)M PHE (\(p < 0.01\)) compared to the control incubation (0 \(\mu\)M 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 \(\mu\)M could not be measured.
BaP and PHE inhibit intestinal lipase activity

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 3.3). 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%.
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 µg · L⁻¹, and are comparable with detected BaP and PHE concentrations of 1.0 and 17 µg · kg⁻¹ in aquafeeds [14]. 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 [89, 123, 223]. 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 [22, 71, 117, 203]. 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 [90, 132, 223]. 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 [90, 109, 132]. Crude luminal extracts prepared 6 hours 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 [90, 223].

Table 3.3: 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 rainbow trout (n = 2, mean ± SD).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>1 nM BaP</th>
<th>10 nM PHE</th>
<th></th>
<th></th>
<th>1 nM BaP</th>
<th>10 nM PHE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rapeseed oil</strong></td>
<td>0 µM PAH</td>
<td>16 ± 5</td>
<td>83 ± 6</td>
<td>65 ± 6</td>
<td>0 µM PAH</td>
<td>8 ± 2</td>
<td>50 ± 5</td>
</tr>
<tr>
<td>Triacylglycerides (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFA (%)</td>
<td>85 ± 5</td>
<td>17 ± 6</td>
<td>35 ± 6</td>
<td>92 ± 2</td>
<td>50 ± 5</td>
<td>44 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

Discussion

The lipophilic PAHs, BaP and PHE inhibit intestinal lipase activity 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 µg · L⁻¹, and are comparable with detected BaP and PHE concentrations of 1.0 and 17 µg · kg⁻¹ in aquafeeds [14]. 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.
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 [41, 44, 49, 77, 110, 221, 222]. When PAHs enter the gastrointestinal tract, micelles can facilitate luminal transfer across the unstirred water layer towards the brush border membrane of enterocytes [44, 110]. The solubility of lipophilic contaminants is higher in micelles composed of unsaturated long-chain fatty acids compared to saturated short-chain fatty acids [44, 110]. Fish oil contains more PUFAs that have longer fatty acid chains and a higher degree of unsaturated bonds than rapeseed oil (Table 3.1). 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 intact triacylglycerides cannot cross the intestinal brush border membrane [68, 69]. Double knock-out 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 [69]. 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 compounds are absorbed by the intestinal epithelium,
probably because higher luminal lipid contents reduce their bioavailability [42, 103]. 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 [77, 103]. 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 compounds [67, 96, 136, 139]. Although both BaP and PHE are absorbed along the entire teleost intestinal tract into the systemic circulation following gavage [6, 41, 189], 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 [42]. 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 [41]. 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 [42, 69, 103]. 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 [42].

**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 decrease 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.
Acknowledgments

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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*)
Abstract

Partial replacement of fish ingredients with vegetable ingredients has elevated levels of polycyclic aromatic hydrocarbons (PAHs) in Atlantic salmon reared on these feeds. PAH uptake in the intestinal tract is postulated to occur in association with lipid absorption and could well be affected by fatty acid composition. We therefore investigated the effects of a fish oil and vegetable oil fatty acid, eicosapentaenoic acid (EPA; 20:5n-3) and oleic acid (18:1n-9) respectively, on the uptake of benzo[a]pyrene (BaP) and phenanthrene (PHE) across the intestinal brush border membrane in the salmonid species rainbow trout (Oncorhynchus mykiss).

BaP and PHE were solubilized in micelles composed of either EPA or oleic acid and administrated to isolated brush border membrane vesicles (BBMV) derived from the pyloric caeca, proximal intestine and distal intestine. In the absence of free fatty acids (FFA), transmembrane uptake of BaP and PHE was 2-7 times lower than the fraction associated to or in the membrane. In the presence of FFA, transmembrane BaP uptake had decreased by 80 and 40% at the highest EPA and oleic acid concentration, respectively, whereas PHE uptake was virtually unaffected. In the presence of BaP, but not PHE, transmembrane EPA uptake in BBMV had decreased.

This study obtained evidence for PAH-dependent interactions with FFA uptake. In conclusion, intestinal BaP uptake is reduced by luminal FFA contents whereas PHE uptake is not. A large fraction of the administrated BaP and PHE remains associated with the cellular membrane of enterocytes and may interfere with uptake of nutrients.
**Introduction**

The steadily growing aquaculture industry has resulted in an increased pressure on limited available aquafeed ingredients, *viz.* fish oils and fishmeal [24, 59, 199]. The requirement of sustainable aquafeeds in aquaculture has led to the partial replacement of fish ingredients by vegetable ingredients. As of 2013, the diet of cultured Atlantic salmon (*Salmo salar*) consists of less than 30% of fish ingredients while approximately 56% is composed of vegetable ingredients [232]. Rapeseed oil is mainly used as a substitute for fish oils, but small amounts of palm oil and soybean oil are also used [198]. Rapeseed oil contains ca. 60% oleic acid (18:1n-9) and ca. 20% linoleic acid (18:2n-6), whereas fish oil is rich in *n*-3 long-chain polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3).

With the introduction of vegetable oils into aquafeeds, elevated levels of lipophilic contaminants were detected that have not been associated in Atlantic salmon farming before [13, 16]. Vegetable oil obtained from oil producing seeds and grains contained polycyclic aromatic hydrocarbons (PAHs), which are mostly formed due to the incomplete combustion of organic matter by so-called toasting or gas drying [135, 157, 202]. As a consequence, the use of vegetable oil in aquafeeds introduces PAH congeners, including benzo[a]pyrene (BaP) and phenanthrene (PHE), in Atlantic salmon reared on these diets [14]. PAHs are ubiquitous lipophilic non-persistent organic compounds that are potentially toxic [16]. BaP consists of five fused benzene rings, is highly lipophilic (octanol/water partition coefficient, $\log K_{ow} = 6.3$) while PHE has three fused benzene rings and is less lipophilic ($\log K_{ow} = 4.5$).

The first barrier for dietary PAH uptake is the mucosa of the intestinal tract. Although the exact mechanisms have not been elucidated yet, intestinal transport and uptake of lipophilic organic compounds such as PAHs have been suggested to occur in association with fat absorption [47, 48, 77, 104, 221]. A lipid-dependent uptake mechanism is suggested in which lipophilic compounds are absorbed from the intestinal tract with lipids during digestion and membrane transport [222]. Emulsified lipids are hydrolyzed by digestive lipases to yield free fatty acids (FFA), monoacylglycerides and, after complete digestion, glycerol [5, 147, 205].

Biliary components (*e.g.*, bile salts and cholesterol) together with FFA, and, to lesser degrees, mono-, di- and triacylglycerides form micelles (hereafter referred to as
micelles) [5, 156, 224, 231]. These micelles can enhance absorption of lipophilic compounds in enterocytes by facilitating transport of e.g., monoacylglycerides, FFA, fat-soluble vitamins and lipophilic compounds across the unstirred water layer which is positioned directly adjacent to the brush border membrane of enterocytes [44, 48, 104, 221, 222]. Despite the low solubility of lipids and lipophilic compounds in an aqueous environment, micelles greatly increase their aqueous concentrations [156]. Solubility of lipophilic compounds depends on the lipid composition of micelles [44, 110]. Besides micelle-mediated transport of lipophilic compounds, bile salts also facilitate the transport of PAHs across the unstirred water layer to the brush border membrane in the absence of lipids [226]. The transfer of PAHs from micelles might occur via a vesicular-mediated uptake process or via direct absorption following a collisional mechanism. Once micelles arrive at the brush border membrane, monoacylglycerides, FFA and lipophilic xenobiotics are released and can then be absorbed across the apical membrane of enterocytes [160].

The interactions of lipids with the uptake of lipophilic compounds has led to the suggestion that increased lipid consumption affects the bioavailability of PAHs [77]. In previous in vivo studies, a fish oil-based diet increased intestinal delivery and transfer of PAHs to the systemic circulation [41]. The objective of the present study was to investigate whether the uptake of PAHs at the first step of intestinal uptake in rainbow trout, at the apical brush border membrane of the enterocyte, is affected by two different fatty acids, i.e., EPA and oleic acid, from fish and vegetable origin, respectively. To reliably measure the first step of intestinal uptake it is necessary to isolate the apical brush border membrane in a purified homogenous preparation.

**Materials and Methods**

**Animals**

Rainbow trout (*Oncorhynchus mykiss*) weighing 200 to 600 g were obtained from a commercial hatchery (Keijzersberg, Blitterswijck, the Netherlands) and kept in an indoor recirculating system with a tank volume of 575 L. Water was aerated, biofiltered and UV-treated with an input of 1 L fresh tap water per min. Water temperature was kept at 15.0 ± 0.5 °C (mean ± S.D.). Trout were fed a commercial fish feed (Optiline trout, 3.0 mm, Skretting, Utah, USA) with an automatic feeder at 9.30 and 16.30 h at a ration of 2% of the estimated body weight per day. Rainbow trout were sacrificed in the morning by a single blow to the head one hour pre-
feeding. Experimental design followed Dutch legislation and was approved by the local ethical review committee (RU-DEC 2012-315).

**Isolation of intestinal brush border membrane vesicles**

Collection and isolation of brush border membrane vesicles (BBMV) was based on protocols described by Pelletier et al. (1986), Klaren et al. (1993) and Glover et al. (2003). Following euthanasia, the peritoneal cavity of two rainbow trout was opened and the intestinal tract was removed and placed on a Petri dish on ice. All subsequent steps were performed at 0-4 °C. Visceral fat was removed and the intestinal tract was divided in three sections; the pyloric caeca, proximal intestine and distal intestine. The proximal intestine was defined as the section directly caudal from the pyloric caeca to the beginning of the distal intestine, which is recognized by its larger diameter, darker appearance and annulo-spiral septa. All intestinal parts were flushed with ice-cold saline containing 150 mM NaCl, 1 mM dithiothreitol (DTT), 0.5 mM ethylene glycol tetraacetic acid (EGTA) and 10 mM HEPES/Tris at pH 7.4. All chemicals were purchased from Sigma-Aldrich Co, St Louis, USA, unless stated otherwise. Thirty caeca of the pyloric caeca, the proximal intestine and distal intestine were cut open lengthwise and rinsed with the same saline and blotted on tissue paper to remove excess fluid and mucus. The mucosa (< 2.0 g wet weight) of each intestinal segment from both fish was scraped off its underlying muscle layers with the aid of two glass microscope slides and pooled into one preparation. Scrapings were disrupted in a glass Dounce homogenizer by 25 strokes of a loosely fitting pestle (type A) in 35 mL homogenization buffer. Scrapings of the pyloric caeca and proximal intestine were disrupted in a buffer containing 50 mM mannitol, 2 mM EGTA, 0.5 mM MgSO4, 0.1 mM phenylmethanesulfonyl-fluoride (PMSF), and 10 mM HEPES/Tris at pH 7.4. Scrapings of the distal intestine were disrupted in a buffer containing 320 mM sucrose, 0.5 mM MgSO4, 0.1 mM PMSF and 10 mM HEPES/Tris at pH 7.4. The homogenates were centrifuged (Sorvall® RC 26 Plus centrifuge, SS-34 rotor) for 15 min at 1400 x g (K factor = 25708). The resulting pellet consisted of a white ‘fluffy’ top layer and sometimes with a firm brownish bottom layer. The white ‘fluffy’ top layer was aspirated and suspended in 30 mL of a buffer, containing 320 mM sucrose and 10 mM HEPES/Tris at pH 7.4. This suspension was again homogenized in a glass Dounce homogenizer by 25 strokes of a tightly fitting pestle (type B) after which solid MgCl2 was added to a final concentration of 10 mM. The homogenate was mildly inverted once and slowly shaken for 15 min. The suspension was centrifuged for 10 min at 20200 x g (K factor = 1779), the supernatant was collected and again centrifuged for 20 min at 43500 x g (K factor = 826). The pellet containing the brush border membranes was resuspended by 30 passages
through a 23-G needle in 200-300 µL assay buffer (150 mM KCl and 10 mM HEPES/Tris at pH 7.4). Occasionally, the pellet of the proximal intestine sedimented as a thin layer along the length of the centrifugal tube. In this case the scattered pellet was resuspended and centrifuged for 20 min at 43500 x g to obtain a compact pellet. The total isolation procedure lasted ca. 3.5 hours and experiments started within one hour after isolation.

Characterization of brush border membrane vesicle preparations

Protein concentrations of the membrane preparations were measured by spectrophotometry with a Coomassie Brilliant Blue reagent kit (Bio-Rad, München, Germany) using bovine serum albumin as a standard. Marker enzymes used to characterize the membrane preparations were alkaline phosphatase (EC 3.1.3.1) [63] and maltase (EC 3.2.1.20) [40] for brush border membranes, Na⁺/K⁺-ATPase (EC 3.6.1.3) [63] for basolateral membranes, and succinate dehydrogenase (SDH, EC 1.3.99.1) [63] and β-D-glucuronidase (EC 3.2.1.31) [62] for mitochondrial and lysosomal contamination, respectively. Membrane preparations were pre-incubated with 0.4 mg saponin ∙ mg protein⁻¹ for 5 min at 37 °C to permeabilize BBMV and unmask latent enzyme activity.

Data on enrichment and recovery of marker enzymes of the three intestinal preparations are presented in Table 4.1. Our preparations are enriched in BBMV as evidenced by purification in maltase and enrichment factors of 11, 9 and 6 for alkaline phosphatase in BBMV derived from the pyloric caeca, proximal and distal intestine, respectively. Membranes were not contaminated with mitochondria and only slightly with lysosomes. Contamination of basolateral membranes could not be determined as Na⁺/K⁺-ATPase activity was not detectable in the initial homogenate and membrane preparation. The enrichment and recovery of the marker enzymes correspond to those of other studies using rainbow trout and tilapia [73, 105, 152].

To determine the membrane orientation of vesicles, alkaline phosphatase activity in untreated preparations was compared to activity in saponin-treated preparations. The proportion of correctly orientated vesicles (i.e., right-side-out) was calculated as the ratio of alkaline phosphatase activity in permeabilized to untreated brush border membrane preparations. This analysis revealed 95.1 ± 1.9%, 99.8 ± 0.3% and 86.6 ± 6.1% right-side-out orientated vesicles (n = 3) in the pyloric caeca, proximal and distal intestinal preparations, respectively. These high percentages of resealed right-side-out vesicles are consistent with observations in other intestinal BBMV isolated from other fish species [73, 105].
Fatty acids inhibit intestinal transmembrane BaP uptake

Measurement of intravesicular space

Intravesicular space was determined according to Flik et al. (1990). BBMV were diluted to 0.3 mg protein ∙ mL⁻¹ in mannitol buffer containing 150 mM NaCl, 100 µM D-mannitol labeled with 100 kBq ∙ mL⁻¹ D-[1-¹⁴C]-mannitol (1.85 MBq,
PerkinElmer Inc, Massachusetts, USA) and 10 mM HEPES/Tris at pH 7.4 and pre-incubated for 30 min at 16 °C. Samples were quenched in 1 mL ice-cold stop buffer (150 mM NaCl, 2 mM D-mannitol and 10 mM HEPES/Tris at pH 7.4), and then filtered over an 80 kPa vacuum filtration manifold (Merck Millipore Corporation, Darmstadt, Germany) using glass microfiber filters with a pore size of 0.7 µm (VWR International, Amsterdam, the Netherlands). The filters were rinsed three times with 2.5 mL ice-cold stop buffer (150 mM NaCl, 100 µM D-mannitol) transferred to 6-mL polyethylene vials (PerkinElmer Inc, Massachusetts, USA) and dissolved in 4 mL liquid scintillation cocktail (Optiphase Hisafe 3™, PerkinElmer Inc, Massachusetts, USA). For every assay three 15-µL aliquots were withdrawn to determine the specific activity (dpm · mol⁻¹) of the radiotracer. Radioactivity (dpm) was measured in ³H and ¹⁴C channels in a liquid scintillation counter (Tri-Carb® 2900TR, PerkinElmer Inc, Massachusetts, USA). Radioactivity count rates were divided by the specific activity of the radiotracer and normalized for the protein content of the incubate; uptake was expressed as mol substrate per mg membrane protein. All samples were measured in duplicate and corrected by subtracting blank values (no BBMV present in the incubate).

The intravesicular space, calculated from the equilibrium mannitol content after 120 min (16 °C), was calculated to be 1.0 ± 0.3 µL · mg protein⁻¹ (mean ± SEM, n = 4) in the pyloric caecal membrane preparations, 3.3 ± 0.8 µL · mg protein⁻¹ in the proximal intestinal and 1.9 ± 0.9 µL · mg protein⁻¹ in the distal intestine membrane preparations, respectively.

### Uptake and membrane association of PAHs with fatty acids

Stock solutions of 2 mM EPA or oleic acid were prepared fresh daily in assay buffer (10 mM sodium taurocholate, 150 mM KCl and 10 mM HEPES/Tris at pH 7.4). Micelles were formed by sonication in a water bath for 20 min [151]. BBMV were diluted to 0.3 mg protein · mL⁻¹ in assay buffer, to which 1 µM [7-¹⁴C]BaP (26.6 mCi · mmol⁻¹; American Radiolabeled Chemicals, St Louis, USA) or 1 µM [9-¹⁴C]PHE (52 mCi · mmol⁻¹; Larodan Fine Chemicals AB, Malmö, Sweden) and 0, 10, 100 or 1000 µM unlabeled fatty acid (EPA or oleic acid) was added. Similar radioactivity concentrations of [³H]EPA (100 mCi · mmol⁻¹; Campro scientific, Berlin, Germany) or [9,10-³H]oleic acid (60 Ci · mmol⁻¹; Campro scientific, Berlin, Germany) were added to the designated experiments as BaP or PHE. PAH uptake could not be measured without the addition of bile salts as PAHs have a very low solubility in an aqueous environment without a detergent. Incubations were carried out at 16 °C, as this resembles the ambient temperature of our laboratory.
Fatty acids inhibit intestinal transmembrane BaP uptake

stock and that of feral rainbow trout (11-18 °C) [74, 75]. All assay buffers were pre-cooled 30 min before incubation.

Pilot experiments showed that PAHs and FFA associated very quickly, within seconds, with the BBMV in the incubate. Incubating at 0 °C did not resolve this. We therefore chose to measure transmembrane substrate uptake as the amount of substrate released from BBMV following perturbation of the vesicular space by detergent treatment. Transmembrane uptake in BBMV was assessed by disruption of the vesicular membrane with saponin, releasing PAH and FFA from the intravesicular space. BBMV were loaded with PAHs and FFA for 30 min and sampled, after which saponin was added. Five min later the substrate content of the BBMV was again sampled. The difference in radioactivity is the amount of substrate released from an intravesicular space and is interpreted to reflect transmembrane uptake. Samples (100 µL) were measured in duplicate at 0, 30 and 35 min. Blank incubations were carried out with water as a substitution for BBMV. Samples (75 µL) were filtered, rinsed three times with 2.5 mL ice-cold stop buffer (150 mM KCl and 10 mM HEPES/Tris at pH 7.4), dissolved in 4 mL liquid scintillation cocktail (Ultima Gold FTM, PerkinElmer Inc, Massachusetts, USA) and counted as described above.

**Disturbance of the osmotic space and temperature dependence**

To detect the most suitable membrane disrupting agent, pyloric caeca BBMV were incubated with assay buffer (10 mM sodium taurocholate, 150 mM KCl, 100 µM D-mannitol labeled with 100 kBq · mL⁻¹ D-[1-¹⁴C]-mannitol and 10 mM HEPES/Tris at pH 7.4) for 125 min at 0, 16 and 37 °C. After 120 min, the samples were incubated with either 0.4 mg saponin · mg protein⁻¹ or 0.1% Triton X-100 and allowed to incubate for 5 min to permeabilize the BBMV. Saponin induced the release of mannitol from the intravesicular space (Fig. S4.1), whereas Triton X-100 permeabilized the BBMV to such an extent that no membrane protein was retained by the glass microfibers filters as assessed by a Coomassie Brilliant Blue protein staining (data not shown). Therefore, saponin was chosen as the membrane-disrupting agent in our experiment. In the intravesicular space of BBMV, 150 pmol mannitol · mg protein⁻¹ was absorbed at 0 and 16 °C after 120 min, whereas no uptake was detected at 37 °C (Fig. 4.1). A similar uptake of mannitol was observed at 0 and 16 °C in studies with BBMV of rainbow trout [73]. Therefore, 16 °C was used for all experiments.
Passive adsorption and manipulation of PAHs

Due to their non-polar, lipophilic nature, PAHs adsorb strongly to labware surfaces. In a preliminary test the adsorption of PAHs to polystyrene, polypropylene, glass vials and glass vials etched with hydrofluoric acid or silanized with dichloromethylsilane were assessed. One µM [7-14C]BaP or 1 µM [9-14C]PHE was dissolved in assay buffer, incubated for 60 min at 16 °C and sampled at 0, 30 and 60 min. Samples (100 µL) were added to 6-mL polyethylene vials containing 4 mL liquid scintillation cocktail (Ultima Gold F™, PerkinElmer Inc, Massachusetts, USA). All vials and pipette tips used were washed 3 times with 100 µL methanol to rinse off PAHs. Radioactivity count rates were divided by the specific activity of the radiotracer to calculate total radionuclide adsorption (mol) to the vials and pipette tips. Our measurements showed that untreated glass vials had the highest recovery and lowest adsorption of PAHs (data not shown), therefore glass vials were used for all experiments.

Statistics

The effects of different concentrations of EPA and oleic acid on transmembrane uptake and membrane partitioning of PAHs in BBMV from different intestinal segments were analyzed using multilevel linear models in the statistical programming language R (version 3.2.3) [162]. In brief, fixed and random intercept models predicting the relative PAH or FFA concentration from the intercept were created for each section of the intestine. The models produced were compared to each other using log likelihood tests and the best fitting (ANOVA, \( p < 0.05 \)) and most parsimonious model was chosen for further analyses in which the concentration and type of fatty acid were added as predictors. Statistical significance was accepted at \( p < 0.05 \). A comprehensive overview of all models created and all

Figure 4.1: Mannitol uptake (pmol ∙ mg protein\(^{-1}\)) on the left y-axis and intravesicular space (µL ∙ mg protein\(^{-1}\)) on the right y-axis in brush border membrane vesicles (BBMV) of the pyloric caeca. BBMV were incubated at 0, 16, and 37 °C for 120 min and an additional 5 min incubation with saponin to disrupt the vesicular membrane and release of mannitol. Bars represent experimental data (mean values ± SEM; \( n = 6 \) for 0, 16 °C and \( n = 4 \) for 37 °C).
parameters determined is presented in the supplementary information (Table S4.1 – S4.6). Data were checked for homogeneity of variance by D’Agostino–Pearson normality test. A Student’s t-test was performed to analyze differences in the total PAH content of BBMV without FFA. Duplicate measurements were found to be stable as they differed by 16.4 ± 0.9% (mean ± SEM). Due to inter-sample heterogeneity (138.7 ± 33.9%) data were not pooled.

**Results**

Table 4.2 shows that the total BaP content of BBMV was 3–5 times higher ($p < 0.001$) in every intestinal segment compared to the total PHE content. In the absence of FFA, 12–42% of the total BaP and PHE content was released from the intravesicular space after perturbation of the intestinal brush border membrane with saponin, indicating uptake of BaP and PHE across the membrane. A large fraction remained associated to or in the saponin-treated membrane and was 2-7 times greater than the fraction released from the intravesicular space.

BaP as well as PHE, solubilized in micelles, were absorbed across the intestinal brush border membrane into the intravesicular space (Fig. 4.2). Transmembrane BaP uptake had significantly decreased ($p \leq 0.015$) by 80 and 40% at the highest EPA and oleic acid concentration, respectively, compared to BaP uptake in the absence of FFA (Fig. 4.2A-C). In the distal intestinal preparations, EPA was 3.5 times more potent than oleic acid in inhibiting transmembrane BaP uptake ($p < 0.040$). This pattern was observed in all three intestinal preparations, although not

<table>
<thead>
<tr>
<th>BaP</th>
<th>Pyloric caeca</th>
<th>Proximal intestine</th>
<th>Distal intestine</th>
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</thead>
<tbody>
<tr>
<td>Total BaP (nmol · mg protein$^{-1}$)</td>
<td>0.57 ± 0.05</td>
<td>1.00 ± 0.05</td>
<td>0.54 ± 0.06</td>
</tr>
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<td>Intravesicular space (%)</td>
<td>32 ± 4</td>
<td>18 ± 3</td>
<td>42 ± 5</td>
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<tr>
<td>Membrane-associated (%)</td>
<td>68 ± 4</td>
<td>82 ± 3</td>
<td>58 ± 5</td>
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<th>Pyloric caeca</th>
<th>Proximal intestine</th>
<th>Distal intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total PHE (nmol · mg protein$^{-1}$)</td>
<td>0.14 ± 0.03</td>
<td>0.29 ± 0.03</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td>Intravesicular space (%)</td>
<td>12 ± 4</td>
<td>14 ± 5</td>
<td>28 ± 7</td>
</tr>
<tr>
<td>Membrane-associated (%)</td>
<td>88 ± 4</td>
<td>86 ± 5</td>
<td>72 ± 7</td>
</tr>
</tbody>
</table>
statistically significant in the pyloric caeca ($p = 0.23$) and proximal intestine ($p = 0.28$). In contrast to BaP uptake, transmembrane PHE uptake was unaffected by oleic acid (Fig. 4.2D-F) and PHE uptake solubilized in EPA micelles was virtually undetectable. All regression model parameters for transmembrane BaP/PHE uptake and effects of FFA are presented in the supplementary information (Table S4.1 – S4.6).

Similarly to the PAHs, EPA and oleic acid were absorbed across the intestinal border membrane into the intravesicular space of BBMV (Fig. 4.3). In the presence of BaP, transmembrane EPA uptake was significantly lower when 1000 µM EPA was administrated to the BBMV compared to 1000 µM oleic acid in all intestinal segments (Fig. 4.3A-C; $p \leq 0.011$). The decreased EPA uptake coincides with the decreased transmembrane BaP uptake. Interestingly, in the presence of PHE, transmembrane uptake of EPA and oleic acid increased concentration-dependently in the pyloric caeca and proximal intestine, with an overall higher uptake of oleic acid (Fig. 4.3D-E; $p < 0.001$). In contrast to transmembrane uptake of oleic acid, EPA uptake did not increase dose-dependently in the distal intestine (Fig. 4.3F). The increased transmembrane uptake of EPA and oleic acid had no effect on transmembrane PHE uptake.
Fatty acids inhibit intestinal transmembrane BaP uptake

Figure 4.3: Transmembrane uptake of eicosapentaenoic acid (EPA) and oleic acid (nmol ∙ mg protein⁻¹) as measured by detergent treatment of brush border membrane vesicles (BBMV), isolated from the pyloric caeca (A, D), proximal intestine (B, E) and distal intestine (C, F) in the presence of 1 µM benzo[a]pyrene (BaP) and phenanthrene (PHE). EPA and oleic acid were administered in the form of micelles composed of 10 mM bile salts and 10, 100 or 1000 µM EPA (closed symbols, solid lines) or oleic acid (open symbols, dashed lines). The top panels (A-C) represent free fatty acid uptake in the presence of BaP, the bottom panels (D-F) in the presence of PHE (mean values ± SEM; n = 8).

Figure 4.4: Partitioning of 1 µM benzo[a]pyrene (BaP) and phenanthrene (PHE) (nmol ∙ mg protein⁻¹) to or in the brush border membrane after detergent treatment of brush border membrane vesicles, isolated from the pyloric caeca (A, D), proximal intestine (B, E) and distal intestine (C, F). BaP and PHE were administered solubilized in micelles composed of 10 mM bile salts and 10, 100 or 1000 µM eicosapentaenoic acid (EPA; closed symbols, solid lines) or oleic acid (open symbols, dashed lines), as indicated on the x-axes. The top panels (A-C) represent BaP partitioning, the bottom panels (D-F) PHE partitioning (mean values ± SEM; n = 8).
As opposed to transmembrane BaP uptake, the partitioning of BaP in or to the membrane was practically unaffected by FFA (Fig. 4.4A-C). Only oleic acid significantly decreased the membrane-associated BaP by 40% ($p = 0.001$) when 1000 µM oleic acid was administrated to BBMV derived from the proximal intestine. The partitioning of PHE in or to the membrane was unaffected by FFA (Fig. 4.4D-F), as it was for transmembrane PHE uptake.

**Discussion**

Prominent differences were seen between transmembrane uptake of BaP and PHE across the intestinal brush border membrane of rainbow trout. In the presence of FFA, transmembrane BaP uptake decreased but not that of PHE. Bile salts aid in the uptake of PAHs along the intestinal tract. Similar results were seen for channel catfish (*Ictalurus punctatus*) intestine where BaP uptake had decreased when solubilized in micelles composed of triacylglycerides and FFA compared to when only bile salts were present [226]. Unfortunately, the uptake of PAHs without the addition of bile salts could not be measured as PAHs have a very low solubility in an aqueous environment without a detergent.

The transmembrane PHE uptake was 4-fold lower compared to BaP. As PHE is less lipophilic ($\log K_{ow} 4.5$ vs. $6.31$) and has a smaller molecular size than BaP, differences can be expected in PAH uptake across the intestinal brush border membrane into the intravesicular space of BBMV. In a number of different vertebrate species, including fish, it has been reported that dietary uptake is less efficient for extreme lipophilic compounds [47, 76, 104]. Diffusion through the unstirred water layer would be a rate-limiting step for high-$K_{ow}$ compounds whereas diffusion through the phospholipid bilayer is rate-limiting for low-$K_{ow}$ compounds [104]. In our brush border membrane preparation, however, the unstirred water layer is most likely lost during the isolation procedure [61]. This would remove the rate-limiting step for BaP transport and could have led to the higher transmembrane uptake of BaP compared to PHE. In contrast to BaP, the less lipophilic PHE does not seem to leave the apical phospholipid bilayer of BBMV after associating to or into it. Phospholipids that form the bilayer structure of cell membranes are favorable sites for partitioning of lipophilic organic compounds such as PAHs [120, 161] and could affect the membrane fluidity and ultimately lead to abnormal enterocyte and gut architecture [233]. Besides partitioning to the brush border membrane, multiple *in vivo* studies have shown that following intragastric administration, BaP as well as PHE are absorbed along the entire teleost intestinal tract into the systemic circulation [6, 41, 189]. In contrast to the present BBMV model system,
other studies with intact CaCo-2 enterocytes showed a higher transcellular uptake of PHE compared to BaP [32, 33] which emphasizes the role of intestinal drug metabolism by the cytochrome P450 family (Cyps) on intestinal transport of different dietary PAH congeners [41]. In the present BMMV model, drug metabolism could only have a minor effect on PAH uptake, as Cyps are mainly located on the membrane of the endoplasmic reticulum and the inner mitochondrial membrane of cells [45, 174] that are removed during the isolation of BMMV.

EPA and oleic acid, administered as micelles, decreased transmembrane BaP uptake in BMMV in all sections of the intestinal tract. In analogy, a high-fat diet decreased intestinal uptake of orally administrated lipophilic compounds (PCBs and chlorobenzenes) in goldfish (Carassius auratus) and PCB 136 in adipose tissue of female C57BL/6 mice [77, 103]. Subsequently, fecal excretion of PCB 136 had increased in female mice in the highest fat diet [103]. 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 compounds [67, 96, 136, 139]. In a lipid-rich diet, less lipophilic compounds are absorbed via the intestinal tract, probably because a higher luminal fat content reduces bioavailability of lipophilic compounds. The reduced bioavailability of lipophilic compounds with increasing fat content coincides with the more potent inhibition of BaP uptake when administrated in micelles composed of EPA compared to oleic acid. Because transmembrane uptake of EPA was considerably lower than that of oleic acid in BMMV, a higher ambient EPA concentration and thus higher lipid content can reduce BaP bioavailability and uptake [103].

In contrast to this study, in primary hepatocytes of Atlantic salmon EPA uptake was significantly higher than oleic acid uptake [235]. However, when primary hepatocytes were pre-incubated with phloretin, EPA uptake was significantly more inhibited than oleic acid uptake (80 vs. 30% inhibition compared to control uptake). Phloretin inhibits the Na⁺/glucose co-transporter and has shown to partially inhibit fatty acid uptake in different model systems [125, 167, 235]. This suggests that FFA uptake is driven by passive diffusion as well as via a transmembrane transporter dependent on a Na⁺ gradient and that EPA uptake is more dependent on membrane-bound protein mediated uptake than oleic acid [235]. In this brush border membrane model FFA are translocated across the membrane alike PAH and the observed interactions likely occur at the level of transmembrane uptake. Transmembrane uptake of FFA and PAHs in the present study is most likely driven by passive diffusion, as no external chemical energy source (i.e., ATP) or electrochemical or osmotic gradient was provided. Therefore,
the significantly lower transmembrane EPA uptake could be due to the higher protein-mediated uptake of EPA compared to oleic acid.

Micelle-mediated uptake is thought to enhance the absorption of lipophilic compounds in enterocytes by facilitating movement across the unstirred water layer towards the brush border membrane of enterocytes [44, 48, 104, 221, 222]. Once lipophilic compounds arrive at the brush border membrane, possible mechanisms for transmembrane uptake encompass passive diffusion, carrier mediated (facilitated) passive transport and/or (primary or secondary) active transport. Gobas et al. (1993) postulated that passive diffusion is the principal transmembrane uptake route when dietary uptake rates and absorption efficiencies of lipophilic compounds decrease with increasing lipid content in consumed food. Conversely, if an increased lipid content results in a higher uptake and absorption efficiencies of lipophilic contaminants then transmembrane uptake would be mainly facilitated by micelles [77, 104]. The fugacity theory, which states that lipophilic compounds flow occurs from a high fugacity compartment to a low fugacity compartment, was used to explain transmembrane uptake of lipophilic compounds [77, 104]. These authors hypothesized that the decreased uptake of lipophilic compounds by dietary fat is driven by passive diffusion because lipid absorption concentrates lipophilic compounds in the intestinal lumen and creates a lipid-generated fugacity gradient from the gut lumen towards the gastro-intestinal tract for lipophilic compounds [77]. The present study showed that the presence of micelles did not facilitate transmembrane uptake of PAHs. However, the absence of micelle-mediated uptake across brush border membranes can also be explained by the absence of the unstirred water layer, in isolated membrane preparations, as discussed above. The notion of intestinal uptake of PAHs in association with fat uptake and aided by a lipid-generated fugacity gradient was supported by a Caco-2 in vitro digestion study where BaP bound to skimmed milk resulted in a two-fold higher BaP uptake than in a similar in vitro study with equivalent amounts of BaP bound to soil [220, 221]. Vasiluk et al. (2008) hypothesized that BaP was absorbed via passive diffusion, as the uptake of BaP and its transfer across Caco-2 monolayers were similar regardless whether BaP was added to the apical or basolateral side.

Uptake of PAHs across the brush border membrane of enterocytes is one of the side effects of the inclusion of vegetable oils in aquafeeds. After ingestion, fatty acids and PAHs disperse after lipid hydrolysis in the intestinal lumen into micelles. Micelles composed of fish oil result in an increased intestinal delivery and transfer of PAHs to the systemic circulation in vivo compared to vegetable oil-composed
micelles [41]. The present study shows that, in vitro, a high-lipid diet decreased PAH uptake across the intestinal brush border membrane. Other in vitro studies with Caco-2 cells showed substantially different results than our brush border membrane model. For instance, the higher transcellular uptake of PHE compared to BaP was not observed in the present study. This difference is attributed to the important role of intestinal metabolism with a faster metabolism of BaP compared to PHE. The absence of the unstirred water layer removed the rate-limiting step for BaP transport and illustrated that transmembrane uptake is less efficient for smaller ‘less’ lipophilic contaminants like PHE. This brush border membrane model emphasizes PAH-dependent interactions with FFA that take place at the first step of (passive) intestinal uptake.

**Conclusion**

We conclude that FFA and lipophilic PAHs interact in transmembrane uptake across the apical membrane of the enterocyte. Moreover, of the two FFA investigated, the fish oil derived fatty acid EPA is more potent than the vegetable-based fatty acid oleic acid in inhibiting BaP uptake. However, fish oil should not be supplemented to vegetable-based aquafeeds to reduce uptake of contaminants, as this would not enhance the development of sustainable aquafeeds. The fatty acid and lipid composition of an alternative aquafeed is an important determinant of PAH bioavailability and should be a considered in the development of novel sustainable aquafeeds. Furthermore, this study has also shown that PAHs not only translocate across, but for the largest part passively partition in the membrane. The physiological aspects of this membrane-associated fraction of lipophilic PAHs should be considered, as it is likely to disturb plasma membrane function and that of integral membrane proteins.

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Comparative Biochemistry and Physiology, Part C

In press
Chapter 5

Transepithelial transfer of phenanthrene, but not of benzo[a]pyrene, is inhibited by fatty acids in the proximal intestine of rainbow trout (*Oncorhynchus mykiss*)
Abstract

The inclusion of vegetable oils in aquafeeds introduces contaminating polycyclic aromatic hydrocarbons (PAHs) in Atlantic salmon (Salmo salar). Since lipophilic PAHs solubilize in micelles composed of lipids, bile salts and fatty acids, dietary lipid composition could alter intestinal transepithelial PAH transfer. To study the uptake and transepithelial transfer of PAHs and the effects of fatty acids, two labeled PAHs, viz. \(^{14}\text{C}-\text{benzo[a]}\text{pyrene (BaP)}\) or \(^{14}\text{C}-\text{phenanthrene (PHE)}\), were solubilized in micelles composed of tritiated fatty acids derived from fish oil or vegetable oil: \(^3\text{H-eicosapentaenoic acid (EPA; } 20:5n-3)\) or \(^3\text{H-oleic acid (18:1n-9)}\), respectively. PAHs, solubilized in micelles, were administrated to the mucosal side of intestinal segments of rainbow trout (Oncorhynchus mykiss) mounted in Ussing chambers for measurement of transepithelial transfer.

In the absence of EPA and oleic acid, PHE accumulation was two times higher than BaP in the mucosal and serosal layers of both proximal and distal intestine. Administration of PHE in micelles composed of oleic acid resulted in a 50% lower accumulation of PHE in the mucosal layers of the proximal intestine compared to EPA-composed micelles. Accumulation of EPA and oleic acid in the proximal intestinal mucosa correlated negatively with the transepithelial transfer of these fatty acids across the proximal intestinal epithelium. Transepithelial PHE transfer across the proximal intestine was reduced when co-exposed with micelles, with a lower reduction in co-exposure with EPA micelles compared to oleic acid micelles (30% and 80% lower transepithelial transfer, respectively), whereas BaP was not transferred across the intestine.

We conclude that the lipid composition of a vegetable oil-based aquafeed is an important determinant of PAH bioavailability. Therefore, lipid composition should be an important consideration in choosing vegetable oils as alternatives for fish oil in aquafeeds.
Introduction

Fishmeal and fish oil in aquafeeds for marine carnivorous fish are partially substituted by ingredients such as vegetable meal and vegetable oil and already extensively used in aquaculture [65, 198, 199, 214, 232]. The use of vegetable oils, however, alters the lipid profile of aquafeeds and introduces lipophilic contaminants such as polycyclic aromatic hydrocarbons (PAHs). The polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA; 20:5\(\text{n-3}\)) and docosahexaenoic acid (DHA; 22:6\(\text{n-3}\)) are important sources of dietary lipids in fish oils. Rapeseed oil is mainly used as a substitute for fish oils [198] and is rich in oleic acid (18:1\(\text{n-9}\)) and linoleic acid (18:2\(\text{n-6}\)) which are fatty acids with shorter hydrocarbon tails and fewer carbon-carbon double bonds than EPA and DHA.

PAHs are lipophilic organic contaminants that are introduced in vegetable-based aquafeeds by replacement of marine ingredients with plant feed ingredients, in particular vegetable oils [14]. PAHs are mostly formed by incomplete combustion of organic matter during toasting and/or gas drying of oil-producing seeds and grains [43, 135, 202]. Atlantic salmon (Salmo salar) reared on vegetable-based aquafeeds had substantially increased levels of the PAHs benzo[a]pyrene (BaP) and phenanthrene (PHE) in the fillet [14]. BaP and PHE differ in their potential toxicological and physico-chemical properties. BaP is highly lipophilic (octanol/water partition coefficient, log \(K_{\text{ow}} = 6.31\)) and an agonist of the aryl hydrocarbon receptor that induces cytochrome \(P4501A\) (cyp1a) mRNA expression and metabolism [112, 113]. PHE is less lipophilic than BaP (log \(K_{\text{ow}} = 4.5\)) and is not an aryl hydrocarbon receptor agonist [19] and is consequently less extensively metabolized than BaP [32]. The introduction of PAHs in aquafeeds raises concerns as to their potential toxic effects for fish and consumers [52].

After ingestion, the mucosa of the intestinal tract is the first barrier for uptake of dietary PAH. Intestinal uptake of lipophilic contaminants such as PAHs has been suggested to follow lipid absorption [47, 48, 77, 104]. Throughout lipid digestion, absorption and assimilation, Vetter et al. (1985) observed that BaP remained strongly associated with lipids and their hydrolysis products and was eventually detected in enterocytic lipid droplets.

Emulsified lipids are hydrolyzed by digestive lipases in the intestinal lumen to yield free fatty acids (FFA). Together with bile salts, FFA form self-aggregating spheres called mixed micelles (hereafter referred to as micelles) [5, 156, 224, 231]. Micelles can enhance intestinal absorption of lipophilic compounds by facilitating luminal transport of, e.g., monoacylglyceride, FFA, fat-soluble vitamins and lipophilic compounds across the unstirred water layer towards the apical brush border membrane of enterocytes [44, 48, 104, 160].
The lipid composition of micelles is an important component in intestinal delivery and transfer of lipophilic contaminants. Fish oil-based diets contain more PUFAs such as EPA and DHA that have longer fatty acid chains and a higher degree of unsaturated bonds which increase PAH solubility in micelles compared to vegetable-based diets [44, 110]. Indeed, in previous studies it was observed that a fish oil-based diet increased the intestinal uptake of BaP and PHE compared to a vegetable oil-based diet in Atlantic salmon [41]. Micelles act as a vector ferrying monoacylglycerides, FFA, cholesterol, fat-soluble vitamins and even lipophilic contaminants across the unstirred water layer towards enterocytes. The acidic microclimate of the unstirred water layer promotes micelle dissociation. Following dissociation, the micellar content is released, including lipophilic compounds such as PAHs, which can then be absorbed by enterocytes [145, 179]. Alternatively, micelles are absorbed by enterocytes via a vesicle-mediated uptake process or are directly absorbed following a collisional mechanism [160]. Overall, the chemical composition of lipids and their constituting fatty acids greatly determine the solubility of PAHs in micelles and their interaction with intestinal membranes.

Dietary lipids per se constitute a sink for lipophilic contaminants in the intestinal lumen, and their bioavailability is suggested to decrease with increasing lipid consumption [77]. Indeed, decreased trans-membrane BaP uptake with increased presence of EPA and oleic acid FFA was observed in intestinal brush border membrane vesicle preparations of rainbow trout (Oncorhynchus mykiss) [42].

This is a first study to detect possible interactions between PAHs and FFA during transepithelial transfer in intact intestinal epithelia. To this end, we measured the transfer of $^{14}$C-labelled BaP and PHE solubilized in micelles composed of two different tritiated fatty acids from fish (EPA) and vegetable (oleic acid) origin, across intact, metabolically active intestinal segments of rainbow trout mounted in Ussing chambers.

**Materials and Methods**

**Animals**

Rainbow trout (Oncorhynchus mykiss) with an average body weight of 540 ± 225 g (mean±SD) were obtained from a commercial hatchery 'Keijzersberg’in Blitterswijck, the Netherlands. Fish were kept at 15.0 ± 0.5 °C in an indoor recirculating system with a total volume of 575 L. Water was aerated, biofiltered and UV-treated with an input of 1 L fresh tapwater per min. 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.
Rainbow trout were sacrificed in the morning by a single blow to the head one hour post-feeding. To ensure that animals were in a postprandial state, animals in which no chymus was detected in the intestinal lumen were excluded from experimentation. Experimental design obeyed Dutch legislation and was approved by the local ethical review committee (RU-DEC 2012-315).

**Preparation of intestinal segments for Ussing chamber experiments**

Following euthanasia, the peritoneal cavity was opened and the intestinal tract, from directly caudal of the most posterior pyloric caeca to the anus, was dissected and placed on a Petri dish on ice. For each experiment either the proximal or distal intestine from one fish was chosen, using a random number generator, to mount in one of the four Ussing chambers in our laboratory. The proximal intestine was defined as the section caudal from the pyloric caeca to the beginning of the distal intestine, which is recognized by its larger diameter, darker appearance and annulo-spiral septa. The preparation and mount of intestinal segments for Ussing chamber measurements were performed according to Sundell et al. (2003). In brief, the intestine was dissected, opened lengthwise along a mesenteric border and rinsed in ice-cold Ringer solution (140 mM NaCl, 2.5 mM KCl, 1.5 mM CaCl2, 15 mM NaHCO3, 1 mM KH2PO4, 0.8 mM MgSO4, 10 mM glucose, 10 mM glutamate and 5 mM HEPES buffer at pH 7.4), saturated for 10 min with 99.7% air and 0.3% CO2. Remaining fat was removed from the serosal muscle layer while immersed in ice-cold Ringer solution using forceps and a dissecting microscope. From the anterior and posterior side of an intestinal segment, 0.5 cm was removed to be certain that there was no interference from adjacent intestinal regions. Each intestinal segment was randomly divided in five sections. Four of the five sections were placed in tissue holders with an aperture of 0.5 cm2 (type P2305; Physiologic Instruments, San Diego, CA, U.S.A.). The fifth section was not exposed to labeled compounds and was used to correct for background radioactivity. The tissue holders were randomly placed in modified Ussing chambers (type P2300; Physiologic Instruments), to avoid bias of electrogenic parameters. The mucosal and serosal half-cells were filled with 2.5 mL pre-gassed Ringer solution. The four Ussing chambers were mounted in water-jacketed chamber holders (type EM-CSYS-4; Physiologic Instruments, San Diego, CA, U.S.A.) connected to a thermostatic water bath maintained at 16 °C. Mixing and oxygenation was done by gassing the half-chambers with a 99.7/0.3% air:CO2 mixture.

Intestinal preparations were exposed to different lipophilic solutions as described by Oxley et al. (2007). Briefly, after an initial 45 min stabilization period, the Ringer solution of the mucosal half-chamber was withdrawn simultaneously with that of the serosal half-chamber to avoid hydrostatic pressure differences across the preparation. The mucosal half-chamber was refreshed with fresh mannitol Ringer
solution, devoid of glucose and Mg$^{2+}$ and Ca$^{2+}$ ions to avoid FFA precipitation [100, 234]. 1.5 mM CaCl$_2$ and 0.8 mM MgSO$_4$ were replaced isosmotically by 2.3 mM NaCl, 10 mM glucose was replaced by 10 mM mannitol to maintain osmolarity of the original Ringer solution. Ringer solution of the serosal half-chamber was refreshed with fresh Ringer solution containing glucose. After this, the tissues were allowed to equilibrate for 15 to 30 min.

**Electrophysiology**

Intestinal viability was assessed by continuous measurement of transepithelial potential (TEP), transepithelial resistance (TER), and short circuit current (SCC) of the intestinal epithelium. In short, TER mainly reflects the paracellular permeability to ions. SCC equals the sum of active transports that are electrogenic (i.e., carrying charged substrates) in the apical and basolateral membranes of the enterocytes. The TEP reflects the electrochemical gradient across the epithelium [192].

A pair of current (Ag-wire coated with Cl) and voltage (Ag/AgCl pellet) electrodes, connected via 3 M KCl agar bridges to an amplifier (model VCC MC6, Physiologic Instruments), recorded differences under voltage-clamp conditions at 0 mV. At 20-s intervals, two 5-mV pulses in opposite direction were passed across the intestinal segment. The currents were measured and the TER was calculated using Ohm's law.

The measurements were logged on a personal computer using a data acquisition system (DataQ DI-400, Physiological Instruments) and Acquire & Analyze software (version 2.3, Physiological Instruments). Electrical parameters were measured every 20 s. Potential differences between Ag/AgCl electrodes and the resistance originating from the electrode/agar bridge and the Ringer solution were corrected by determining these parameters in the chambers without mounted intestinal epithelium. All tissues were allowed to equilibrate for 45 min before the start of the experiment. Electrophysiological measurements were averaged per 20-s window, amounting to 15 data points per 5-min period. Tissue viability was checked by continuous monitoring of SCC and TER.

**Experimental treatments**

Stock solutions of 2 mM oleic acid or EPA were prepared freshly daily in mannitol-Ringer buffer containing 20 mM sodium taurocholate. Micelles were formed by sonication in a water bath for 20 min [151]. Experimental Ringer solutions were prepared by diluting the stock solution containing EPA or oleic acid with mannitol-Ringer solution to concentrations of 0, 20, 200 and 2000 µM unlabeled fatty acid. Radiolabeled BaP or PHE were added to concentrations of 2 µM
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[7-14C]BaP (American Radiolabeled Chemicals, St Louis, USA) or 2 µM [9-14C]PHE (Larodan Fine Chemicals AB, Malmö, Sweden). Tritiated fatty acids served as radiotracers for EPA and oleic acid (Campro Scientific GmbH, Berlin, Germany). The radioactive concentrations in the experiments were 10 and 20 kBq ∙ mL⁻¹ for BaP and PHE, respectively, with similar radioactive concentrations of tritiated EPA and oleic acid.

After the second equilibration period, 2.5 mL of the Ringer solution of the mucosal and serosal half-chamber was removed. The experiment started at \( t = 60 \) min by adding 2.5 mL of the experimental Ringer solution to the mucosal half-chamber to obtain a final concentration in the Ussing chambers of 1 µM [9-14C]PHE or [7-14C]BaP and 0, 10, 100 or 1000 µM fatty acid (EPA or oleic acid).

The serosal chamber received 2.5 mL Ringer solution (containing glucose) only. Duplicate 250-µl samples were taken at \( t = 60 \) min from the mucosal and serosal half-chamber every 30 min for a total period of 180 min. Sample volumes were replaced by the same volume of experimental Ringer solution and serosal Ringer, respectively. Ringer samples were taken from each half-chamber before the experiment started to correct for background radioactivity.

Control incubations (without PAHs and FFA) were carried out as described above, with the exception that at the start of the experiment 2.5 mL mannitol Ringer solution was added.

Samples taken from the half-chambers were directly transferred to 6-mL polyethylene vials (PerkinElmer Inc., Massachusetts, USA) and dissolved in 4 mL liquid scintillation cocktail (Optiphase Hisafe 3™, PerkinElmer Inc., Massachusetts, USA). For every assay three 15-µL aliquots from every experimental concentration were withdrawn to determine the specific activity (dpm ∙ mol⁻¹) of the radiotracer. Radioactivity (dpm) was measured in \(^{3}H\) and \(^{14}C\) channels in a liquid scintillation counter (Tri-Carb® 2900TR, PerkinElmer Inc., Massachusetts, USA). Radioactivity count rate was divided by the specific activity of the radiotracer and normalized for the volume of the sample and aperture of the intestinal segment. The flux of the tracee in a chamber was presented as mol per cm². All half-cell samples were measured in duplicate and corrected by subtracting blank values.

Apparent permeability of a substrate, expressed in cm ∙ min⁻¹, under steady-state conditions substrate was calculated using the equation:

\[
P_{app} = \frac{dQ}{dT} \cdot \frac{1}{AC_0}
\]

where \( dQ/dT \) is the appearance rate (mol ∙ min⁻¹) in the serosal half-cell of the
Ussing chamber, $A$ is the area of intestinal surface (cm$^2$) exposed in the chamber and $C_0$ (mol · cm$^{-3}$) is the initial concentration in the mucosal half-cell (with the assumptions that the concentration in the mucosal half-chamber remains constant, and that the concentration in the serosal half-chamber is virtually zero as compared to the mucosal half-chamber).

**Tissue accumulation of PAHs and fatty acids**

After termination of the experiment, the intestinal segment was removed from the Ussing chamber and the mucosal layers were separated from the serosal layers (tunica muscularis and tunica serosa) with the aid of glass microscope slides. The mucosal and serosal layers were weighed separately, transferred to 6-mL polyethylene vials (PerkinElmer Inc, Massachusetts, U.S.A.) and dissolved overnight in 1 mL solubilizer (Solvable, PerkinElmer Inc, Massachusetts, U.S.A.) at 37°C. Samples of 250-µL of the dissolved tissue were placed in 6-mL polyethylene vials, 4 mL liquid scintillation cocktail (Ultima Gold, PerkinElmer Inc, Massachusetts, U.S.A.) was added and radioactivity was determined as described above. All tissue samples were measured in duplicate and corrected by subtracting blank values (unexposed tissue of the same intestinal segment). Tissue level was expressed as pmol substrate per g tissue (wet weight).

**Statistical analysis**

Statistical analyses were performed using the statistical programming language R (version 3.2.3) [162]. Data were checked for homogeneity of variance by a D'Agostino–Pearson normality test. The data sets that did not show homogeneity of variance were log-transformed. The log-transformed data were then subjected to appropriate analysis of variance. PAH concentrations in the mucosal and serosal layers in the absence of FFA were assessed with two-way ANOVA to analyze the effects of the two factors “intestinal layers” (consisting of two levels: mucosal layer and serosal layer) and “PAH” (levels: BaP and PHE). PHE fluxes in the proximal intestine were assessed with a two-way ANOVA to analyze the effects of the two factors “FFA” (EPA and oleic acid) and “FFA concentration” (0, 10, 100 and 1000 µM). Results were considered statistically significant when $p < 0.05$ (two-tailed).

The effects of EPA and oleic acid on PAH accumulation in the mucosal and serosal layers of the proximal and distal intestine and electrophysiology were analyzed using multilevel linear models. In brief, fixed and random intercept models predicting the relative PAH or FFA concentration from the intercept were created for each intestine. Calculated model parameters were compared using Akaike’s information criterion (AIC), and the best fitting (ANOVA, $p < 0.05$) and the most parsimonious model was chosen for further analyses in which time, concentration
or type of fatty acid were added as predictors. Statistical significance was accepted at \( p < 0.05 \). A comprehensive overview of all models created and of all parameters determined is presented in the supplementary information (Tables S5.1 – S5.12).

**Results**

**Intestinal PAH accumulation**

In the absence of FFA, PHE concentrations in the mucosal and serosal layers of the proximal intestine were two times higher \( (p < 0.001) \) than those of BaP (Fig. 5.1A). The concentrations of BaP and PHE were 10 times higher \( (p < 0.001) \) in the mucosal layers of the proximal intestine compared to the serosal layers. In the mucosal and serosal layers of the distal intestine, PHE had a 1.7 fold higher total accumulation \( (p = 0.016) \) than BaP (Fig. 5.1B). The BaP and PHE concentrations were two and four times higher, respectively \( (p < 0.001) \) in the mucosal layers of the distal intestine than in the serosal layers (Fig. 5.1B).

BaP and PHE, solubilized in micelles, accumulated in the mucosal and serosal layers of the proximal (Fig. 5.2) and distal intestine (Fig. 5.3). The accumulation of BaP in the mucosal and serosal layers of both intestinal segments was unaffected by EPA and oleic acid. In contrast to BaP, PHE accumulation was 50% lower in the mucosal layers of the proximal intestine when administrated via 1000 µM oleic acid-micelles \( (p < 0.001) \) compared to EPA-composed micelles (Fig. 5.2C). The accumulation of PHE in the serosal layers of the proximal intestine was unaffected by EPA (Fig. 5.2D) but virtually undetectable when PHE was administrated in micelles composed of...
oleic acid ($p < 0.001$). PHE accumulation in the mucosal and serosal layers of the distal intestine was unaffected by increasing FFA concentrations (Fig. 5.3A–B). All regression model parameters for BaP/PHE accumulation and effects of FFA are presented in the supplementary information (Tables S5.1 – S5.2).

**Tissue accumulation and transepithelial transfer of fatty acids**

In the mucosal and serosal layers of the proximal and distal intestine, a concentration-dependent increase of EPA and oleic acid accumulation was observed ($p < 0.001$; Fig. 5.4). Oleic acid accumulated 7.5 and 4.5 times higher in the mucosal layers of the proximal and distal intestine, respectively, than EPA ($p \leq 0.002$). The lower accumulation of EPA compared to oleic acid correlated significantly ($R^2 > 0.74$; $p < 0.001$) with the 2–4 times higher transfer of EPA across both intestinal segments compared to oleic acid in the presence of BaP and PHE, respectively (Fig. 5.4). The apparent permeability of EPA under steady state conditions was 2 and 1.6 times higher than that of oleic acid ($p < 0.05$) in the proximal and distal intestine, respectively, and did not differ between BaP or PHE presence (Table 5.1).
Fatty acids inhibit transepithelial PHE transfer

Figure 5.3: Accumulation of 1 µM benzo[a]pyrene (BaP) and phenanthrene (PHE) in the mucosal and serosal layers of the distal intestine. BaP and PHE were administered to the mucosal half-chamber solubilized in micelles composed of 10 mM bile salts without and with 10, 100 and 1000 µM eicosapentaenoic acid (EPA; closed symbols, solid lines) or oleic acid (open symbols, dashed lines), as indicated on the x-axes. The top panels (A–B) represent BaP accumulation, the bottom panels (C-D) PHE accumulation (mean values ± SD; n = 8).

Figure 5.4: Correlation between the accumulation and transepithelial transfer at t = 180 min of eicosapentaenoic acid (EPA) and oleic acid in the presence of 1 µM benzo[a]pyrene (BaP; A) and phenanthrene (PHE; B) in the mucosal layers of the proximal intestine (n = 8). EPA and oleic acid were administered to the mucosal half-chamber solubilized in micelles composed of 10 mM bile salts with 10, 100 and 1000 µM EPA (closed symbols) or oleic acid (open symbols). The solid line is the best linear fit and dotted lines represent the 95% confidence intervals.
Table 5.1. Apparent permeability of EPA and oleic acid (10^-6 cm · min⁻¹) in the proximal and distal intestine in the presence of 1 µM BaP or PHE (mean ± SD; n = 8). Lower case letters indicate significant differences of apparent permeability between EPA and oleic acid per intestinal segment (p < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>EPA (10^-6 cm · min⁻¹)</th>
<th>Oleic acid (10^-6 cm · min⁻¹)</th>
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<tr>
<td></td>
<td>BaP</td>
<td>PHE</td>
</tr>
<tr>
<td>Proximal intestine</td>
<td>0.44 ± 0.01</td>
<td>0.46 ± 0.03</td>
</tr>
<tr>
<td>Distal intestine</td>
<td>0.24 ± 0.05</td>
<td>0.22 ± 0.06</td>
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Transepithelial PAH transfer

PHE was transferred across the proximal intestine whereas BaP was not. EPA as well as oleic acid decreased unidirectional PHE fluxes across the proximal intestine (Fig. 5.5; p = 0.003). The PHE flux decreased by 40% when co-exposed with the highest EPA concentration. When PHE was administered in oleic acid micelles, the PHE fluxes were decreased by 45-65% compared to PHE uptake in the absence of FFA. In the distal intestine, BaP and PHE were not transferred across the intestinal segment (data not shown). The presence of FFA had no effect on the transepithelial transfer on any of the PAHs in the distal intestine.

Electrophysiology

The effect of BaP and PHE on the electrical properties of the intestinal epithelium was assessed over the 240 min incubation period by concomitant measurement of TEP, SCC and TER in the proximal and distal intestine (Fig. S5.1 – S5.4). The intestinal segments of the proximal and distal intestine displayed stable TEP, SCC and TER values after mucosal administration of BaP and PHE, indicating good viability. Linear regression modeling revealed increased, i.e., less negative, TEP and SCC values in the proximal intestine when PHE was administrated in micelles.
Fatty acids inhibit transepithelial PHE transfer composed of 1000 µM EPA or oleic acid. FFA concentrations had no effect on the stability of TEP, TER and SCC after PAH exposure in the distal intestine.

**Discussion**

Of the two PAHs investigated PHE was transferred across the proximal intestine whereas BaP was not. PHE and BaP both accumulated in mucosal as well as serosal layers of the proximal and distal intestine, with an overall higher accumulation of PHE than BaP. In the presence of FFA, transepithelial PHE transfer decreased in the proximal intestine while BaP was not transferred across the intestine. The transepithelial transfer of PHE but not of BaP might be explained by its less extensive metabolism and subsequently faster transfer across the proximal intestine while BaP is metabolized and its metabolites bind to intestinal cellular macromolecules [41]. Although both PAHs accumulated in the distal intestine, BaP and PHE were not transferred across these sections of the intestine.

**Intestinal PAH accumulation and transepithelial transfer**

Accumulation of PHE was 2-fold higher in the proximal and distal intestine compared to BaP. Dietary uptake is less efficient for extreme lipophilic compounds (log $K_{ow} > 6.3$) in a number of different vertebrate species, including fish [47, 76, 104]. Diffusion of high-$K_{ow}$ compounds through the unstirred water layer is believed to be a rate-limiting step while diffusion through the phospholipid bilayer is considered to be rate-limiting for low-$K_{ow}$ compounds [104]. Indeed, the unstirred water layer, adjacent to the brush border membrane of enterocytes, creates an absorption barrier that increases with molecule lipophilicity [49]. The transmembrane uptake of BaP was higher than PHE *in vitro* in intestinal brush border membrane vesicles of rainbow trout that lacked an unstirred water layer [42]. In contrast, PHE had a higher absorption than BaP in intact intestinal epithelia that contained an unstirred water layer (present study). The higher lipophilicity (log $K_{ow}$) of BaP compared to PHE (log $K_{ow}$ 6.31 vs. 4.5) can well explain the lower accumulation of BaP compared to PHE in the intestinal mucosa. In support, PHE concentrations were higher compared to that of BaP in intact intestinal Caco-2 cells [33, 34]. CaCo-2 cells are cellular monolayers from colonic origin that exhibit a typical enterocytic differentiation characterized by a polarization of the cell layer with the presence of tight junctions, apical brush border membranes with their associated enzymes and epithelial electrical properties [111, 169] that display transmembrane uptake of PAHs. Therefore, the *in vitro* PAH accumulation in intestinal segments of rainbow trout and intestinal Caco-2 cells most likely resembles PAH uptake *in vivo*. Additionally, transmembrane uptake in isolated brush border membrane vesicles of rainbow trout illustrate membrane effects that already take place at the first step of intestinal transmembrane uptake.
The accumulation of PAH measured in the mucosal and serosal layers is the sum of the intracellular and plasma membrane-associated fractions. The largest PAH fraction is most likely passively partitioned in the membrane [42]. Accumulation of BaP and PHE in the serosal layers of the proximal and distal intestine is evidence that both PAHs are transferred across the tunica mucosa (consisting of the mucosal epithelium and lamina propria) towards the circular and longitudinal muscle layers of the tunica muscularis [229]. The continuous blood circulation of the lamina propria, which transports absorbed intestinal nutrients to peripheral organs, is absence in Ussing chamber experiments. In Atlantic salmon, BaP and PHE were both transported across the intestinal epithelium into the systemic circulation and distributed to peripheral organs [41]. Therefore, the in vitro accumulation of BaP and PHE in the serosal layers of the proximal and distal intestine would in vivo most likely have entered the systemic circulation.

In contrast to PHE, BaP was not transferred across the epithelium in any of the intestinal segments during the 180 min incubation. Since BaP is an aryl hydrocarbon receptor agonist it induces its own metabolism by Cyp1a [18, 19]. Intestinal CaCo-2 cells have been shown to express CYP1A and CYP3A that can metabolize xenobiotics [111]. When BaP was incubated with intestinal Caco-2 cells, it was more extensively metabolized than pyrene and PHE and transferred to a lesser extent across CaCo-2 cell monolayers (Cavret and Feidt, 2005). Therefore, the transepithelial transfer of PHE but not of BaP might be explained by the less extensive PHE metabolism and subsequently higher transfer of the native compound across the proximal intestine while, in contrast, BaP is metabolized and binds to intestinal cellular macromolecules [41]. Although PHE does not stimulate its own biotransformation, PHE is metabolized in marine fish such as European flounder (Platichthys flesus), Atlantic salmon and rainbow trout [41, 187].

When PAHs are metabolized by Cyp450s, intestinal enterocytes extrude the metabolites into the intestinal lumen [28, 33]. Excretion is most likely facilitated by ATP-binding cassette (ABC) proteins that excrete highly hydrophobic compounds into the gastrointestinal tract as native, non-metabolized xenobiotics or water-soluble conjugates. Waterborne exposure of BaP resulted in an up-regulation of abcc2 (multidrug resistance-associated protein 2) mRNA expression in the gills and an increase in abcg2 mRNA expression in the liver and proximal intestine of tilapia (Oreochromis niloticus) [39]. The ABCG2 transporter is indeed involved in the efflux of BaP conjugates in Caco-2 cells [50]. Therefore, the absence of transepithelial BaP transfer, in contrast to PHE, could also have been caused by Cyp1a-mediated BaP metabolism following efflux of BaP metabolites to the mucosal half-cell (i.e., intestinal lumen).
**FFA effects on intestinal PAH uptake and transepithelial transfer**

PHE accumulation in the mucosal layers of the proximal intestine was lower when PHE was administrated to the mucosal side of the intestine solubilized in micelles composed of 1000 µM oleic acid compared to micelles composed of the same concentration EPA. In Atlantic salmon, a vegetable oil-based fish feed (i.e., rich in oleic acid) also decreased intestinal PHE concentrations in the proximal intestine compared to a fish oil-based fish feed [41]. The lipophilic core of micelles provides a more suitable hydrophobic environment for lipophilic compounds than the aqueous intestinal lumen [48]. However, the composition of micelles can affect intestinal delivery and transfer of lipophilic compounds [44]. Micelles composed of shorter fatty acid chain-lengths and a lower degree of unsaturated bonds have a decreased PAH solubility [44, 110]. In the present study, the lower hydrocarbon chain length and degree of saturation of oleic acid compared to EPA might explain why oleic acid but not EPA reduced intestinal PHE accumulation in the mucosal layer of the proximal intestine at high concentrations.

In fish, lipids are absorbed along the entire intestinal tract but predominantly in the proximal region and, when present, in the pyloric caeca [100, 108, 205]. The higher mucosal accumulation of oleic acid compared to EPA coincides with previous in vitro studies performed with isolated enterocytes of rainbow trout [150, 155]. It has been postulated that an increased intestinal lipid accumulation reduces lipid transport across the basolateral membrane and consequently reduces the absorption of lipids across the brush border membrane [5]. Indeed, the 2 - 4 times lower transepithelial transfer of oleic acid compared to EPA strongly correlates with the higher FFA accumulation in the proximal and distal intestine. In addition, the apparent permeability of oleic acid was also lower than EPA in the proximal as distal intestine (Fig. 5.4). The apparent digestibility of saturated and long-chain monounsaturated fatty acids in vivo by fish is generally lower compared to PUFAs [4, 66, 181, 208]. In this study, the total FFA absorption (i.e., FFA accumulation + transepithelial FFA transfer) was also higher for EPA than oleic acid that reflects in vivo digestibility studies. Therefore, the degree of lipid accumulation in enterocytes varies with the concentration and type of dietary oil composition and could reduce lipid absorption across the brush border membrane of enterocytes [5].

EPA and oleic acid, administrated as micelles, decreased transepithelial PHE transfer in the proximal intestine. If an increased intestinal lipid accumulation decreases lipid transport across the basolateral membrane, this could also affect the transepithelial transfer of lipophilic PAH across the intestine. Indeed, our study showed that an increased intestinal lipid accumulation reduced PHE transfer across the proximal intestine. In addition, lipid accumulation in enterocytes is not
only affected by lipid concentration but also by a lipid’s chemical properties, that differ between the sources from which lipids are obtained. In gilthead seabream (*Sparus aurata*) and Atlantic salmon, intracellular lipid accumulation increased dose-dependently in fish fed vegetable oils while this was observed to a much lesser degree in fish fed fish oils as sole dietary lipid source [30, 148, 149]. Likewise, the higher dose-dependent accumulation of oleic acid than that of EPA in the mucosal layers of the proximal intestine coincides with the increased inhibition of transepithelial PHE transfer of 60% by oleic acid whereas EPA only inhibited PHE transfer by the highest concentration by 40%.

A high-lipid diet decreased intestinal uptake of orally administrated lipophilic compounds (PCBs and chlorobenzenes) in goldfish (*Carassius auratus*) [77]. In female C57BL/6 mice, higher dietary lipids decreased PCB 136, which subsequently increased the fecal excretion of lipophilic compounds [103]. Furthermore, transmembrane uptake of BaP had decreased in intestinal brush border membrane vesicle preparations of rainbow trout in the presence of high EPA and oleic acid concentrations [42]. Lipophilic compounds are absorbed less by the intestinal epithelium in a high lipid diet probably because higher luminal lipid contents reduce their bioavailability [42, 103]. Gobas et al. (1993) postulated that passive diffusion is the principal uptake route when dietary uptake rates and absorption efficiencies of lipophilic compounds decrease with increasing dietary lipid content. Conversely, if an increased lipid content results in higher uptake and absorption efficiencies of lipophilic contaminants then transepithelial uptake would be mainly facilitated by micelles [77, 104]. In a previous study with brush border membrane vesicles, the absence of micelle-mediated uptake across brush border membranes was postulated to be caused by the absence of the unstirred water layer [42]. However, intestinal segments used in this study constitute intact epithelia that still have an unstirred water layer. Therefore, since transmembrane [42] as well as transepithelial PAH transfer decreased with increasing FFA concentrations passive diffusion and/or carrier mediated (facilitated) passive transport could be the main uptake mechanism for lipophilic PAHs. Another possibility is that PHE and FFA used in this study compete for the same specific binding site of a transport protein in the proximal intestine, as increasing FFA concentrations decreased PHE accumulation as well as transepithelial PHE transfer.

Interestingly, PHE was not transferred across the distal intestine but was transferred across the proximal intestine. In general, the distal intestine of salmonids has a higher TER and lower apparent permeability of paracellular marker molecules compared to the proximal intestine [100, 191, 193]. The inverse of the TER, the conductivity, is regarded to reflect paracellular permeability, i.e., conductance across tight junctions [192]. The absence of transepithelial PHE transfer across the distal intestine could indicate that the proximal intestine, but not the distal
intestine, expresses a membrane PHE transport protein. Alternatively, besides a transcellular route \[42\], PHE might be transferred via a second transport route (e.g., paracellular) across the intestine.

**FFA decrease TEP and SCC values in the presence of PHE**

Micelles composed of EPA or oleic acid decreased an active transport mechanism in the proximal intestine in the presence of PHE, but only at the highest FFA concentration (1000 µM), as evidenced by significant interaction effects on TEP (EPA and oleic acid) and SCC (oleic acid only) (Tables S7 - S8). The observed decrease in TEP was accompanied by a decrease in SCC. A lowered TEP reflects a decreased ability of the epithelium to maintain an electrochemical gradient. Factors increasing the total paracellular permeability across the intestinal epithelium, reflected in TER, are predicted to decrease TEP \[191, 194\]. However, since TER showed no changes over time after PHE incubation this suggests that the decrease in TEP is caused not by increased paracellular leakage of charged molecules but mainly by disturbance in ion-transporting mechanisms \[194\]. The decreased TEP and SCC coincide with the results of Oxley et al., (2007) where an experimental incubation of 1 mM unsaturated fatty acids decreased TEP and SCC values in saltwater-adapted Atlantic salmon. *In vitro* studies have shown that *n*-3 and *n*-6 unsaturated fatty acids of 18 and 20 carbon chain-lengths inhibit Na⁺/K⁺-ATPase activity \[128, 195\]. Therefore, the observed decrease in SCC and TEP could be due to an inhibition of the Na⁺/K⁺-ATPases activity by unsaturated fatty acids and a collapse of a transmembrane Na⁺-gradient that drives sodium-coupled cotransport and exchange. Under normal physiological conditions intracellular FFA bind to lipoproteins, therefore, it is unlikely that these lipids are physiological regulators of Na⁺/K⁺-ATPase \[126\] and why only the highest incubation concentration decreased intestinal electrogenic properties.

**Conclusion**

This study has shown that fatty acids decrease the PHE accumulation and transepithelial PHE fluxes. The inhibitory effects of FFA were on established BaP and PHE concentrations of 1.3 and 0.9 µg · L⁻¹, respectively, and are comparable with detected BaP and PHE concentrations of 1.0 and 17 µg · kg⁻¹ in aquafeeds \[14\]. Micelles composed of oleic acid decreased PHE accumulation in the proximal intestine compared to EPA micelles. This is probably due to a lower solubility of PAHs in micelles that consist of shorter vegetable oil derived fatty acid chain lengths that have a lower degree of unsaturated bonds compared to fish oil fatty acids. Partial replacement of fish oil with vegetable oil is likely to increase aquafeed PAH levels and so increase their intestinal exposure. In addition, the inclusion of vegetable oil fatty acids could lead to higher intestinal accumulation of fatty acids.
and possibly a decreased flux of vegetable oils to the systemic circulation that could, ultimately, negatively impact organismal energy metabolism. Since increasing concentrations of EPA and oleic acid decreased transepithelial PHE transfer in the proximal intestine the lipid composition and content of alternative aquafeeds are important determinants of PAH bioavailability and should be a consideration in the development of novel sustainable aquafeeds.

Acknowledgments

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Fatty acids inhibit transepithelial PHE transfer
Chapter 6

General discussion
Nutrient-contaminant interactions on intestinal uptake

The steadily and rapidly growing aquaculture increases pressure on the limited supply of marine aquafeed ingredients. The increased use of vegetable feed ingredients to replace fish oil and fishmeal that are traditionally used in aquafeeds introduces new challenges in the development of novel, alternative aquafeeds.

One particular challenge is that substitution of fishmeal and oil with vegetable ingredients leads to increased levels of contaminating polycyclic aromatic hydrocarbons (PAHs) in aquafeeds. Nutrients such as tocopherols, flavonoids and fatty acids are well known to interact with contaminants such as PAHs and pesticides [131, 138, 222]. The intestine is the site where nutrients and contaminants are absorbed from the aquafeed diet and is also the primary site where nutrient-toxicant interactions take place. These interactions constituted the main research theme of this thesis.

We wished to generate fundamental knowledge on nutrient-toxicant interactions, in particular those between lipids (and its digestion products) and two typical lipophilic PAH contaminants: benzo[a]-pyrene (BaP) and phenanthrene (PHE). We approached this by first performing an in vivo pulse-chase study to investigate the metabolic fate and peripheral distribution of BaP and PHE. We then employed several in vitro models to gain further insights in the interactions between dietary lipids, their hydrolysis products and BaP and PHE at the level of enzymatic lipid hydrolysis, intestinal membrane transport and intestinal transepithelial uptake.

The main findings of the research presented in this thesis are:
- BaP and PHE, administered via the diet, are transported from the intestinal lumen across the intestinal wall into the systemic circulation. Intestinal uptake of PAHs and the distribution to peripheral organs were stimulated by dietary fish oil compared to a vegetable oil (Chapter 2).
- Exposure to BaP induces intestinal and hepatic cyp1a expression. This can explain the detection of BaP metabolites that appear to be bound to cellular proteins and/or DNA. PHE did not induce cyp1a expression (Chapter 2).
- BaP and PHE inhibited lipase activity in vitro which effect was dependent on the type of substrate, i.e., rapeseed oil or fish oil (Chapter 3).
- BaP and PHE avidly associated with or in the enterocyte's apical membrane in a purified membrane preparation. The presence of free fatty acids (FFA) inhibited transmembrane BaP uptake across the intestinal apical
membrane whereas PHE uptake was not affected. (Chapter 4).

A study using Ussing chambers revealed that BaP and PHE accumulated in the mucosal and serosal layers of intact intestinal epithelium. FFA inhibited transepithelial PHE fluxes in vitro across the proximal intestine. BaP did not cross the intestinal epithelium at all (Chapter 5).

**PAH distribution is dependent on intestinal barrier functions**

Dietary BaP and PHE are both transported across the intestinal epithelium into the systemic circulation and are distributed to various peripheral organs in vivo in a number of different teleost species [6, 41, 91, 106, 172, 186]. Both PAHs were detected in the mucosa of the pyloric caeca and proximal intestine 6-12 hours post-feeding in Atlantic salmon (*Salmo salar*) (Chapter 2). The resolution of our whole-body autoradiographs was not sufficient to detect the exact location where BaP and PHE resided in the intestinal mucosa. However, in brush border membrane vesicles the largest fraction of BaP and PHE passively partitioned in the membrane’s lipid bilayer rather than translocate across the membrane into the vesicle’s lumen (Chapter 4). Indeed, phospholipids that form the bilayer structure of cell membranes are preferential sites for the partitioning of lipophilic contaminants such as PAHs [120, 161]. Lipophilic contaminants can affect membrane fluidity and ultimately disturb plasma membrane integrity and the function of integral membrane proteins [233]. We predict that the largest fraction of dietary PAHs shall initially partition in the apical brush border membrane of enterocytes where they may interfere with nutrient uptake.

Differences in the intestinal uptake of high and low-\(K_{ow}\) PAH congeners seem to be dependent on the presence of an unstirred water layer. The unstirred water layer, adjacent to the brush border membrane of enterocytes, creates an absorption barrier which permeability decreases with increasing molecule lipophilicity [49]. It is postulated that diffusion through the unstirred water layer is a rate-limiting step for high-\(K_{ow}\) compounds (e.g., BaP) whereas diffusion through the phospholipid bilayer is rate-limiting for low-\(K_{ow}\) compounds (e.g., PHE) [104]. This notion is supported by our observations of a higher absorption of BaP than PHE in intestinal brush border membrane vesicles that lack an unstirred water layer in vitro, and of a higher absorption of PHE than BaP in intact intestinal epithelia that do contain an unstirred water layer (Chapter 4 & 5).

The intestine is the first barrier for dietary PAH uptake and plays an important role in PAH metabolism [129]. Because BaP is an aryl hydrocarbon receptor agonist
that induces \textit{cyp1a} expression, an enzyme that is involved in phase I xenobiotic metabolism, BaP is more extensively metabolized compared to PHE [21, 33, 112, 113]. Cyp1a-mediated metabolism of BaP can result in toxic intermediates that are very reactive and can bind to cellular proteins and/or DNA leading to cellular damage [130, 146, 219]. Intestinal CaCo-2 cells have been shown to express CYP1A and CYP3A that can metabolize xenobiotics [111]. When intestinal Caco-2 cells were exposed to BaP and PHE, BaP was more extensively metabolized than PHE and transferred to a lesser extent across CaCo-2 cell monolayers [33]. Conversely, while PHE was transferred across an intact epithelium of the proximal and distal intestine of rainbow trout (\textit{Oncorhynchus mykiss}) mounted in Ussing chambers, BaP was not (Chapter 5). Similarly, BaP was not detected in peripheral organs three hours post-feeding in Atlantic salmon while in the same time period low PHE concentrations were already detected in the liver (Chapter 2). The more extensive peripheral distribution of PHE compared to BaP can thus be explained by the less extensive metabolism of PHE and subsequently faster transfer of the native compound across the proximal intestine. In contrast, BaP is metabolized by Cyp1a to derivatives that can bind to cellular proteins and/or DNA. Hence, less BaP is transferred into the systemic circulation [91, 172]. The activities of intracellular xenobiotic-metabolizing enzymes are an important determinant of the transfer of PAHs from the intestine and subsequent distribution to peripheral organs.

Aquafeeds contain PAH mixtures [14]. Therefore, dietary PAHs mixtures with compounds such as PHE, that do not induce \textit{cyp1a} mRNA levels, could be metabolized at an increased rate by the presence of BaP-induced Cyp1a levels. Pre-exposure of rat hepatocytes of rat and human with the Cyp1a inducer 2,3,7,8-tetrachlorodibenzo-\textit{p}-dioxin resulted in an increased BaP metabolism [190]. Although PHE does not stimulate its own biotransformation, metabolites were detected in the bile which illustrated that PHE is metabolized in Atlantic salmon (Chapter 2). The stimulating effect on PAH metabolism by Cyp1a inducers is likely to reduce intestinal PAH concentrations by increasing PAH transfer into the systemic circulation or and/or excretion to the intestinal lumen via cellular efflux transporters [217].

\textbf{Lipase inhibition by PAHs is dependent on the diet’s lipid composition}

Dietary lipids affect the intestinal uptake of lipophilic compounds [44, 48, 110, 222]. Indeed, in Atlantic salmon, a vegetable (rapeseed) oil-based diet decreased intestinal and peripheral distribution of BaP and PHE \textit{in vivo} compared to a fish oil-based diet (Chapter 2). When ingested, the lipophilic nature of PAHs promotes
their solubilization in oil droplets and micelles in the intestinal lumen \[97, 104, 160\]. The lipid composition of micelles is an important determinant of the intestinal delivery of lipophilic contaminants \[44, 110\]. The solubility of lipophilic compounds is higher in micelles composed of long-chain fatty acids with a higher degree of unsaturated bonds than in micelles composed of more saturated shorter-chain fatty acids \[44, 110\]. Fish oil-based diets contain more C\(_{20}\) and C\(_{22}\) polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA; 20:5\(\text{n-3}\)) and docosahexaenoic acid (DHA; 22:6\(\text{n-3}\)). Hence, the solubility of PAHs in fish oil-composed micelles can be predicted to be higher than in vegetable oil-composed micelles rich in monoene shorter-chain fatty acids such as oleic acid (18:1\(\text{n-9}\)). The dependence of PAH solubility on micelle composition can explain why PHE accumulation in the proximal intestine of rainbow trout \textit{in vitro} was lower when co-exposed with micelles composed of oleic acid compared to EPA (Chapter 5). The shorter hydrocarbon chain length and lower degree of saturation of oleic acid compared to EPA explains why a diet based on the vegetable-derived oleic acid reduced the accumulation of PHE in the proximal intestine \textit{in vivo} as well as \textit{in vitro} (Chapter 2 & 5). This suggests that by manipulation of the lipid content and composition of an aquafeed the intestinal uptake of PAHs can be modulated.

The lipid composition of aquafeeds not only provides the substrate and, hence, the catalytic activity of lipases per se, but also affects the potency of lipase inhibitors. Lipase activity had decreased in yellowtail kingfish (\textit{Seriola lalandi}) fed a rapeseed/canola oil diet while it was not affected in gilthead seabream (\textit{Sparus aurata}) and European sea bass (\textit{Dicentrarchus labrax}) fed a vegetable oil blend diet containing rapeseed, linseed and palm oil \[25, 31, 173\]. These vegetable oil blend diets had a higher lipid content of long-chain fatty acids with a higher degree of unsaturated bonds such as EPA, DHA and α-linolenic acid (18:3\(\text{n-3}\)) and less monoene shorter-chain fatty acids such as oleic acid compared to the rapeseed/canola oil diet \[25, 31, 173\]. These results illustrate that, in general, lipase activity is inhibited in aquafeeds rich in monoene short-chain fatty acids. Our studies, however, show...
that the reduced lipase activity of vegetable oil-based diets could also be caused by luminal presence of PAHs (i.e., BaP and PHE) that inhibit enzymatic rapeseed oil hydrolysis (Chapter 3). Therefore, the occurrence of PAHs in aquafeeds by partial replacement of fish ingredients by vegetable ingredients could possibly decrease lipid digestion \textit{in vivo} and negatively impact organismal energy metabolism.

To minimize the inhibitory effects of PAHs on lipase activity, aquafeed manufacturers could include vegetable oils rich in PUFA and with lower levels of monoene fatty acids. This would exclude rapeseed oil, currently one of the most used oils in aquafeeds, as sole oil source of dietary lipid because rapeseed oil contains high levels of monoene fatty acids such as oleic (40%). Linseed oil, for example, contains higher levels of the PUFA α-linolenic acid (40%) and has lower levels of oleic (15%) than rapeseed oil [10, 9, 66]. A 55:30:15-mixture of rapeseed, palm, and linseed oil, respectively, can be used as an adequate replacement to partially replace fish oil as it has no detrimental effects on fish growth, but will establish a dietary lipid profile of saturated, monoene and \textit{n}-3 PUFA that is similar to fish oil [168, 211]. If adjustments in vegetable oil composition of aquafeeds are beneficial for fish growth and they counteract the inhibitory effect of PAHs on lipase, it might be a potential solution to decrease intestinal PAH uptake.

**Lipids in the diet modulate intestinal PAH transfer**

In a lipid-rich diet, less lipophilic compounds are absorbed by the intestinal epithelium [77, 103]. Indeed, transmembrane uptake of BaP in intestinal brush border membrane vesicle preparations of rainbow trout decreased when co-exposed with increasing concentrations of micelles composed of EPA or oleic acid (Chapter 4). Increasing concentrations of EPA and oleic acid, administrated as micelles, also decreased the unidirectional PHE flux across the proximal intestine of rainbow trout \textit{in vitro}, which effect was more prominent with oleic acid than with EPA (Chapter 5). The higher intracellular accumulation of oleic acid compared to EPA in the proximal intestine coincides with a reduced transepithelial transfer of oleic acid compared to EPA (Chapter 5). Lipophilic compounds, such as tocopherol and astaxanthin, follow lipoprotein transport pathways [36, 118, 163]. Fat-soluble nutrients possibly follow lipid-associated transport pathways across the intestine as well. An increased lipid accumulation in enterocytes decreases transport of lipids and lipophilic nutrients across the basolateral membrane [5]. Therefore, we hypothesize that an increased intracellular lipid accumulation in enterocytes also reduces the transport of lipophilic compounds across the basolateral membrane. The reduced transepithelial PHE transfer across the proximal intestine seems to be
related to an increased intracellular lipid accumulation and was more prominent in combination with oleic acid that accumulated to a higher degree than EPA (Chapter 5). This suggests that the intestinal transfer of PAHs can be modulated by manipulation of the lipid content and composition of an aquafeed.

**Complete fish oil substitution by vegetable oil decreases PAH uptake**

Marine ingredients are substituted with plant ingredients and already extensively applied in commercial salmon feeds. However, in particular aquafeeds that are composed of fish oil combined with vegetable oil seem to have detrimental effects on PAH uptake. The inclusion of vegetable oils in aquafeeds introduces PAHs into the feeds, but specifically the presence of fish oils rich in long-chain unsaturated fatty acids increase luminal transfer, intestinal uptake and peripheral distribution of PAHs in salmonids. Therefore, the replacement of fish oil in these alternative aquafeeds seems warranted.

In aquafeeds, fish oil is mainly included because it provides the dietary requirements for $n$-3 PUFAs that are essential for optimal growth and health of farmed fish and consumers [198, 214]. The composition of marine carnivorous fish feeds has changed considerably during the last 30 years. In 1990, 90% of the global aquaculture feed ingredients was of marine origin while nowadays only 30% of the ingredients is composed of marine ingredients which contain approximately 8% EPA + DHA combined [184, 201, 232]. Recently, it has been shown that low concentrations (5% of the total fatty acid content) of the $n$-3 fatty acids EPA and DHA in aquafeeds did not seem to affect production performance of Atlantic salmon during a seawater production cycle [184].

All vertebrate species, including fish, have a minimal dietary requirement for both $n$-6 and $n$-3 PUFAs. The biologically active forms of essential fatty acids are generally the C$_{20}$ and C$_{22}$ metabolites of linoleic acid (18:2$n$-6) and α-linolenic acid [12, 72, 198, 214]. *In vivo*, de novo synthesis of fatty acids takes place by the addition or removal of 2-C acetyl units and the synthesis of an unsaturated bond in a fatty acid hydrocarbon chain [72]. However, vertebrates lack Δ-12 and Δ-15 desaturases to form de novo linoleic acid and α-linolenic acid, hence, these fatty acids are essential and must be provided by the diet [72, 205, 214]. Elongation and further desaturation of α-linolenic can successively result in the synthesis of EPA and DHA [72, 205]. Atlantic salmon have specific requirements of EPA + DHA combined (more than 2.7% of the total fatty acids content, provided in 1:1 ratio) for optimal long-term growth in seawater [168, 183, 184]. These aquafeeds also
contained 11-12% α-linolenic acid, which can be converted to EPA and DHA by Atlantic salmon [72, 168, 205]. When EPA + DHA concentrations in aquafeeds can be decreased from 8% to 2.7%, the low inclusion of fish oils necessary for optimal growth will likely have beneficial effects on PAH uptake as a reduction in fish oils could decrease PAH accumulation and subsequently PAH toxicity.

Rainbow trout and Atlantic salmon both showed no reduction in growth when fed aquafeeds where fish oil was completely substituted by vegetable oils [12, 171]. However, these diets contained fish meal as the main protein source. Fish meal also contains phospholipids rich in EPA and DHA, resulting in dietary levels of EPA + DHA at 3% and above [168]. Complete substitution of fish oils by linseed oils decreased n-3 PUFA levels in fish fillets but Atlantic salmon fed on linseed oil-based diets had increased desaturase mRNA levels in hepatocytes, implicating an increased capacity to convert α-linolenic acid into EPA and DHA [204]. Vegetable oils rich in α-linolenic such as linseed oil are best used in aquafeeds to decrease intestinal PAH uptake. However, since linseed oil contains high PUFA levels, intestinal transfer of PAHs by PUFA-composed micelles could still elevate the intestinal PAH uptake and hence mimic the detrimental effects of fish oils. It seems, therefore, that a vegetable oil blend with a lipid composition that contains sufficient n-3-PUFA levels, to reduce the inhibitory PAH effects on lipase activity, and shorter saturated, monoene fatty acids to decrease intestinal PAH uptake is most appropriate in aquafeeds.

**Production processes of vegetable oils can decrease PAH levels in aquafeeds**

Vegetable oils used in aquafeeds are mainly contaminated by environmental pollution of the vegetable raw material and by thermal feed processing like toasting and gas drying [134, 157]. Drying of oil-containing seeds with combustion gases (using oil, diesel or propane fuel) tremendously increases PAH levels in vegetable oils [43]. The drying technique had no effect on heavy PAH concentrations such as BaP, but Dennis et al. (1991) showed that air drying (electrically heated or unheated) of rapeseeds gave lower levels of the PAHs fluoranthene, pyrene and chrysene compared to drying with combustion gases. A transition to cleaner drying techniques could already improve the quality of aquafeed ingredients considerably.

The inclusion of crude vegetable oils in aquafeeds has increased the dietary concentration of 13 potentially genotoxic and carcinogenic PAH congeners 10-fold. In addition, concentrations of the non-genotoxic and/or non-carcinogenic PAHs...
fluoranthene (FA), naphthalene (NA), anthracene (AN) and PHE had increased 4-fold [14]. The European Union has set an upper limit for BaP of 2 µg ∙ kg⁻¹ in oils and fats intended for human consumption and 10 µg ∙ kg⁻¹ for the sum of BaP, benzo[a]anthracene, benzo[b]fluoranthene and chrysene [56, 57]. However, no upper limit currently exists for PAH concentrations in feed ingredients of animal feeds while BaP and PHE concentrations in vegetable-based aquafeeds are 1.0 µg and 17.0 µg ∙ kg⁻¹, respectively [14].

When Atlantic salmon was reared on a crude vegetable oil-based diet, fillets contained 0.3 µg ∙ kg⁻¹ BaP and 3.2 µg ∙ kg⁻¹ PHE. Although fillet concentrations of BaP were 7-fold lower than the EU upper limit for human consumption, prominent *in vitro* effects were detected, *e.g.*, lipase inhibition by low concentrations of BaP and PHE. Refining processes, such as treatment with active carbon and/or deodorization, have been known to drastically reduce PAH levels in vegetable oils [43, 133, 202]. Deodorization processes are most effective in the removal of the most volatile light PAHs, whereas bleaching by activated carbon is more efficient in removing heavy PAHs [37, 135, 202]. Refining of vegetable oils decreased the content of light and heavy PAHs by 70-80% and 30-80%, respectively [43, 202]. Therefore, the introduction of refining processes during aquafeed production could greatly reduce PAH levels, but, unfortunately, not completely remove PAHs from aquafeeds [16]. The most known toxic effect of BaP is the formation of tumors due to the carcinogenic or mutagenic/genotoxic properties. Therefore, refining processes should be focused more on removing BaP from aquafeeds than the non-carcinogenic PHE. Since vegetable oils used for human consumption are refined to reduce the concentrations of potentially hazardous lipophilic compounds (such as PAHs) it could be commercially feasible to process vegetable oils in such a way that they do not introduce toxic PAH levels in aquafeeds.

**Conclusion and future perspectives**

In this thesis we used several *in vitro* and *in vivo* models to investigate interactions between lipids, their hydrolysis products and PAHs at the level of enzymatic lipid hydrolysis, intestinal membrane transport, intestinal transepithelial uptake and peripheral distribution. With the partial inclusion of vegetable oils, aquafeed manufacturers will have to consider the effects of fish oil and rapeseed oil on the uptake of high as well as low-*K_{ow}* PAH congeners. We have clearly shown that interactions are dependent upon type of oil, lipid content, fatty acid composition and PAH congener. Prominent effects on lipid digestion and intestinal uptake by PAHs in concentrations that are below EU upper limits were detected *in vitro,*
and these could very well negatively impact organismal energy metabolism. To decrease PAH uptake in salmonids, the most realistic and commercially feasible solution for the aquaculture industry is refining of vegetable oils to decrease PAH levels in aquafeeds. Also, the lipid composition and content of an aquafeed is an important determinant for PAH bioavailability and should be considered in the development of novel aquafeeds.
References


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References


Torstensen, B.E., Frøyland, L. and 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. Aquaculture Nutrition. 10, 175–192.


Supplementary information
## Chapter 2

Table S2.1: PCR efficiencies for each gene per organ. Genes that were not determined for transcriptional levels are indicated with ND

<table>
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<th>Gene</th>
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<th>Distal intestine</th>
<th>Liver</th>
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<td>ND</td>
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Table S2.2: Regression models fitted to the predicted benzo[a]pyrene (BaP) concentration following the effect of vegetable oil-based feed on the organ concentrations. Fish oil-based feed is the reference level with value 0. MC is the p value for the model criteria (ANOVA, \( p < 0.05 \)), b is the estimate for the ‘Intercept’, slope for ‘Time’ and ‘Time’², and the coefficient for ‘vegetable feed’ of the regression models. Lower and Higher Cl: 95% confidence intervals, SE: associated standard errors; df: degrees of freedom, Within each model, \( p \)-values give significant differences among slopes. Linear model: \( y = \text{intercept} + (b_{\text{time}} \cdot \text{Time}) + b_{\text{vegetable feed}} \). Polynominal model: \( y = \text{intercept} + (b_{\text{time}} \cdot \text{Time}) + (b_{\text{time}^2} \cdot \text{Time}^2) + b_{\text{vegetable feed}} \), where the intercept is the initial BaP organ concentration (ng BaP ∙ g⁻¹ organ) at three hours.

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Table S2.2 continued

Kinetic change over time of BaP per organ (ng BaP - g⁻¹ organ)

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Table S2.3: Regression models fitted to the predicted phenanthrene (PHE) concentration following the effect of vegetable oil-based feed on the organ concentrations. Fish oil-based feed is the reference level with value 0. MC is the p value for the model criteria (ANOVA, p < 0.05), b is the estimate for the ‘Intercept’, slope for ‘Time’ and ‘Time2’, and the coefficient for ‘vegetable feed’ of the regression models. Lower and Higher CI: 95% confidence intervals, SE: associated standard errors; df: degrees of freedom. Within each model, p-values give significant differences among slopes. Linear model: y = intercept + (b_time ∙ Time) + bVegetable feed. Polynomial model: y = intercept + (bTime ∙ Time) + (bTime² ∙ Time²) + bVegetable feed, where the intercept is the initial BaP organ concentration (ng PHE ∙ g⁻¹ organ) at three hours.

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<tr>
<th>Organ</th>
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<th>Higher CI</th>
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Table S4.1: Regression models fitted to the predicted transmembrane benzo[a]pyrene (BaP) and phenanthrene (PHE) uptake in brush border membrane vesicles following the effect of increasing concentrations of eicosapentaenoic acid (EPA) and oleic acid. EPA is the reference level with value 0. Model criterion (MC) is the p-value for the model criteria and only used when significant (ANOVA; \( p < 0.05 \), b is the estimate for the ‘Intercept’, slope for ‘free fatty acid (FFA) concentration’ and ‘Interaction (FFA concentration * Oleic acid)’ and the coefficient for ‘Oleic acid’ of the regression models. Lower and Higher CI: 95% confidence intervals, SE: associated standard errors, df: degrees of freedom and t-value. Within each model, p-values give significant differences among slopes. Linear model: \( y = \text{Intercept} + (b_{\text{FFA concentration}} \cdot \text{FFA concentration}) + (b_{\text{interaction}} \cdot \text{FFA concentration}) + \text{Oleic acid} \), where the intercept is the BaP or PHE concentration (nmol · mg protein\(^{-1}\)) in the intravesicular space of BBMV at 0 µM FFA.

### Transmembrane uptake of BaP (nmol · mg protein\(^{-1}\)) with increasing concentrations of FFA

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### Transmembrane uptake of PHE (nmol · mg protein\(^{-1}\)) with increasing concentrations of FFA

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Table S4.2: Regression models fitted to the predicted change of transmembrane benzo[a]pyrene (BaP) and phenanthrene (PHE) uptake in brush border membrane vesicles (BBMV) following the effect of increasing concentrations of free fatty acids (FFA) i.e., oleic acid or eicosapentaenoic acid (EPA). Model criterion (MC) is the p-value for the model criteria and only used when significant (ANOVA; p < 0.05), b is the estimate for the Intercept and the slope for ‘FFA concentration’ and ‘FFA concentration^2’ of the regression models. Lower and Higher Cl: 95% confidence intervals, SE: associated standard errors, df: degrees of freedom and t-value. Within each model, p-values give significant differences among slopes. Linear model: y = Intercept + (b FFA concentration ∙ FFA concentration), polynomial model: y = Intercept + (b FFA concentration ∙ FFA concentration) + (b FFA concentration^2 ∙ FFA concentration^2) where the intercept is the BaP or PHE concentration (nmol · mg protein⁻¹) in the intravesicular space of BBMV at 0 µM FFAs.

### Transmembrane BaP (nmol · mg protein⁻¹) uptake with increasing concentrations of EPA or oleic acid

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### Transmembrane PHE (nmol · mg protein⁻¹) uptake with increasing concentrations of EPA or oleic acid

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<td>0.021</td>
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<td>3.4·10⁻⁶</td>
<td>32</td>
<td>2.34</td>
<td>0.026</td>
</tr>
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<td>Proximal intestine</td>
<td>Oleic</td>
<td>Intercept</td>
<td>0.110</td>
<td>0.056</td>
<td>0.165</td>
<td>0.027</td>
<td>28</td>
<td>4.15</td>
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</tr>
<tr>
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<td>Intercept</td>
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<td>Intercept</td>
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</tr>
<tr>
<td></td>
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<td>0.002</td>
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Supplementary information
Table S4.3: Regression models fitted to the predicted transmembrane free fatty acids (FFA) i.e., oleic acid or eicosapentaenoic acid (EPA); Ln(nmol · mg protein \(^{-1}\) + 1)) uptake in brush border membrane vesicles (BBMV) in the presence of 1 µM benzo[a]pyrene (BaP) and phenanthrene (PHE). EPA is the reference level with value 0. Model criterion (MC) is the \(p\)-value for the model criteria (ANOVA; \(p\) < 0.05), \(b\) is the estimate for the ‘Intercept’, slope for ‘FFA concentration’ and ‘Interaction (FFA concentration * Oleic acid)’ and the coefficient for ‘Oleic acid’ of the regression models. Lower and Higher Cl: 95% confidence intervals, SE: associated standard errors, df: degrees of freedom and \(t\)-value. Within each model, \(p\)-values give significant differences among slopes. Linear model: \(y = \text{Intercept} + (b_{\text{FFA concentration}} \cdot \text{FFA concentration}) + (b_{\text{interaction}} \cdot \text{FFA concentration}) + \text{Oleic acid},\) where the intercept is the FFA uptake (Ln(nmol · mg protein \(^{-1}\) +1)) in the osmotic space of BBMV at 10 µM FFAs.

### Transmembrane uptake of FFA (Ln(nmol · mg protein \(^{-1}\) + 1)) in the presence of BaP

<table>
<thead>
<tr>
<th>Organ</th>
<th>Estimator</th>
<th>MC</th>
<th>(b)</th>
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<th>Higher Cl</th>
<th>SE</th>
<th>df</th>
<th>(t)-value</th>
<th>(p)-value</th>
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<tbody>
<tr>
<td><strong>Pyloric caeca</strong></td>
<td>Intercept</td>
<td></td>
<td>0.349</td>
<td>0.081</td>
<td>0.618</td>
<td>0.133</td>
<td>45</td>
<td>2.63</td>
<td>0.012</td>
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<tr>
<td></td>
<td>FFA concentration</td>
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<td>-4.4(\times)10(^{-4})</td>
<td>4.8(\times)10(^{-4})</td>
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<td>0.196</td>
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<tr>
<td></td>
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<td>0.011</td>
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<td>6.6(\times)10(^{-4})</td>
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<tr>
<td><strong>Distal intestine</strong></td>
<td>Intercept</td>
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<td>0.146</td>
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<td>2.1(\times)10(^{-4})</td>
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<td>Oleic acid</td>
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<td>0.152</td>
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<td>Interaction</td>
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<td>8.2(\times)10(^{-4})</td>
<td>2.4(\times)10(^{-4})</td>
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<td>2.88</td>
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### Transmembrane uptake of FFA (Ln(nmol · mg protein \(^{-1}\) + 1)) in the presence of PHE

<table>
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<tr>
<th>Organ</th>
<th>Estimator</th>
<th>MC</th>
<th>(b)</th>
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<th>Higher Cl</th>
<th>SE</th>
<th>df</th>
<th>(t)-value</th>
<th>(p)-value</th>
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<tbody>
<tr>
<td><strong>Pyloric caeca</strong></td>
<td>Intercept</td>
<td></td>
<td>0.125</td>
<td>-0.117</td>
<td>0.367</td>
<td>0.120</td>
<td>45</td>
<td>1.04</td>
<td>0.305</td>
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<tr>
<td></td>
<td>FFA concentration</td>
<td>&lt;0.001</td>
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<td>7.4(\times)10(^{-4})</td>
<td>1.4(\times)10(^{-3})</td>
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<td>0.150</td>
<td>45</td>
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<td>Intercept</td>
<td></td>
<td>0.090</td>
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<td>0.371</td>
<td>0.139</td>
<td>45</td>
<td>0.65</td>
<td>0.522</td>
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<td>4.2(\times)10(^{-4})</td>
<td>1.2(\times)10(^{-3})</td>
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<td>-0.096</td>
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<td>2.84</td>
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Table S4.4: Regression models fitted to the predicted change of free fatty acids (FFA), i.e., oleic acid or eicosapentaenoic acid (EPA); Ln(nmol · mg protein⁻¹ + 1), uptake in brush border membrane vesicles (BBMV) in the presence of 1 µM benzo[a]pyrene (BaP) and phenanthrene (PHE). EPA is the reference level with value 0. Model criterion (MC) is the p-value for the model criteria (ANOVA; p < 0.05), b is the estimate for the Intercept and the slope for ‘FFA concentration’ of the regression models. Lower and Higher CI: 95% confidence intervals, SE: associated standard errors, df: degrees of freedom and t-value. Within each model, p-values give significant differences among slopes. Linear model: y = Intercept + (b_{FFA concentration} · FFA concentration) where the intercept is the FFA uptake (Ln(nmol · mg protein⁻¹ + 1)) in the osmotic space of BBMV at 10 µM FFAs.

<table>
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<tr>
<th>Organ</th>
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<th>Estimator</th>
<th>MC</th>
<th>b</th>
<th>Lower CI</th>
<th>Higher CI</th>
<th>SE</th>
<th>df</th>
<th>t-value</th>
<th>p-value</th>
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<tr>
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<td>Intercept</td>
<td>&lt;0.001</td>
<td>0.094</td>
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<td>21</td>
<td>0.68</td>
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<tr>
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<td>FFA concentration</td>
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<td>9.2·10⁻⁴</td>
<td>4.2·10⁻⁴</td>
<td>1.4·10⁻³</td>
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<td>1.2·10⁻³</td>
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<tr>
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<th>b</th>
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<th>SE</th>
<th>df</th>
<th>t-value</th>
<th>p-value</th>
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<td>Pyloric caeca</td>
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<tr>
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<td>5.3·10⁻⁴</td>
<td>1.5·10⁻³</td>
<td>2.4·10⁻⁴</td>
<td>21</td>
<td>4.35</td>
<td>&lt;0.001</td>
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<tr>
<td></td>
<td>EPA</td>
<td>Intercept</td>
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<td>0.210</td>
<td>0.042</td>
<td>0.379</td>
<td>0.081</td>
<td>24</td>
<td>2.58</td>
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</table>
Table S4.5: Regression models fitted to the predicted benzo(a)pyrene (BaP) and phenanthrene (PHE) membrane partitioning following the effect of increasing concentrations of the free fatty acids (FFA), eicosapentaenoic acid (EPA) and oleic acid. EPA is the reference level with value 0. Model criterion (MC) is the p-value for the model criteria (ANOVA; $p < 0.05$), b is the estimate for the ‘Intercept’, slope for ‘FFA concentration’ and ‘Interaction (FFA concentration * Oleic acid)’ and the coefficient for ‘Oleic acid’ of the regression models. Lower and Higher CI: 95% confidence intervals, SE: associated standard errors, df: degrees of freedom and t-value. Within each model, p-values give significant differences among slopes. Linear model: $y = \text{Intercept} + (b_{\text{FFA concentration}} \cdot \text{FFA concentration}) + (b_{\text{Interaction}} \cdot \text{FFA concentration}) + \text{Oleic acid}$ where the intercept is the PAH concentration (nmol \cdot mg protein$^{-1}$) associated with the brush border membrane at 0 µM FFAs.

### Membrane-associated BaP (nmol \cdot mg protein$^{-1}$) with increasing concentrations of FFA

<table>
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<th>Estimator</th>
<th>MC</th>
<th>b</th>
<th>Lower CI</th>
<th>Higher CI</th>
<th>SE b</th>
<th>df</th>
<th>t-value</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Pyloric caeca</td>
<td>Intercept</td>
<td>0.375</td>
<td>0.321</td>
<td>0.429</td>
<td>0.027</td>
<td>59</td>
<td>13.85</td>
<td>&lt; 0.001</td>
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<td>Intercept</td>
<td>0.880</td>
<td>0.745</td>
<td>1.016</td>
<td>0.069</td>
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<td>2.4 \cdot 10^{-4}</td>
<td>7.1 \cdot 10^{-5}</td>
<td>60</td>
<td>1.39</td>
<td>0.173</td>
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</tr>
<tr>
<td>Oleic acid</td>
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<td>0.021</td>
<td>-0.191</td>
<td>0.233</td>
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<tr>
<td>Interaction</td>
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<td>-3.94</td>
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<tr>
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<td>Intercept</td>
<td>0.316</td>
<td>0.223</td>
<td>0.409</td>
<td>0.047</td>
<td>58</td>
<td>6.77</td>
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</tr>
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</table>

### Membrane-associated PHE (nmol \cdot mg protein$^{-1}$) with increasing concentrations of FFA

<table>
<thead>
<tr>
<th>Organ</th>
<th>Estimator</th>
<th>MC</th>
<th>b</th>
<th>Lower CI</th>
<th>Higher CI</th>
<th>SE b</th>
<th>df</th>
<th>t-value</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Pyloric caeca</td>
<td>Intercept</td>
<td>0.080</td>
<td>0.025</td>
<td>0.134</td>
<td>0.028</td>
<td>60</td>
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<tr>
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<td>-1.04</td>
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<tr>
<td>Oleic acid</td>
<td>0.011</td>
<td>0.110</td>
<td>0.027</td>
<td>0.194</td>
<td>0.040</td>
<td>60</td>
<td>2.78</td>
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<tr>
<td>Proximal intestine</td>
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<td>0.148</td>
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<tr>
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<td>1.7 \cdot 10^{-4}</td>
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<td>0.121</td>
<td>0.012</td>
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<td>0.052</td>
<td>60</td>
<td>2.34</td>
<td>0.036</td>
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Table S4.6: Regression models fitted to the predicted change of benzo[a]pyrene (BaP) and phenanthrene (PHE) membrane partitioning following the effect of increasing concentrations of the free fatty acids (FFA) eicosapentaenoic acid (EPA) and oleic acid. The designated FFA is the reference level with value 0. Model criterion (MC) is the $p$-value for the model criteria (ANOVA; $p < 0.05$), b is the estimate for the ‘Intercept’ and slope for ‘FFA concentration’ of the regression models. Lower and Higher Cl: 95% confidence intervals, SE: associated standard errors, df: degrees of freedom and t-value. Within each model, $p$-values give significant differences among slopes. Linear model: $y = \text{Intercept} + (b \cdot \text{FFA concentration})$ where the intercept is the PAH concentration (nmol · mg protein$^{-1}$) associated with the brush border membrane at 0 µM FFA.

<table>
<thead>
<tr>
<th>Organ</th>
<th>FFA</th>
<th>Estimator</th>
<th>MC</th>
<th>b</th>
<th>Lower Cl</th>
<th>Higher Cl</th>
<th>SE b</th>
<th>df</th>
<th>t-value</th>
<th>$p$-value</th>
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<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intercept</td>
<td></td>
<td>0.328</td>
<td>0.261</td>
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<tr>
<td></td>
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<td>0.416</td>
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<td>0.490</td>
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<td>11.39</td>
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<td>$&lt;0.001$</td>
<td>-3.1·10$^{-4}$</td>
<td>-4.8·10$^{-4}$</td>
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<td>8.5·10$^{-5}$</td>
<td>28</td>
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<td>0.001</td>
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<tr>
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<td>EPA</td>
<td>Intercept</td>
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<tr>
<td>Proximal intestine</td>
<td>Oleic acid</td>
<td>Intercept</td>
<td>$&lt;0.001$</td>
<td>-4.0·10$^{-5}$</td>
<td>-3.7·10$^{-6}$</td>
<td>9.0·10$^{-6}$</td>
<td>32</td>
<td>-4.71</td>
<td>&lt; 0.001</td>
<td></td>
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<tr>
<td></td>
<td>EPA</td>
<td>Intercept</td>
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<td>0.253</td>
<td>0.175</td>
<td>0.331</td>
<td>0.038</td>
<td>27</td>
<td>6.63</td>
<td>&lt; 0.001</td>
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<td>0.296</td>
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<td>0.058</td>
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<td>Distal intestine</td>
<td>Oleic acid</td>
<td>Intercept</td>
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<td>0.268</td>
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<td>Intercept</td>
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<td>32</td>
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| Membrane-associated BaP (nmol · mg protein$^{-1}$) with increasing concentrations of EPA of oleic acid

<table>
<thead>
<tr>
<th>Organ</th>
<th>FFA</th>
<th>Estimator</th>
<th>MC</th>
<th>b</th>
<th>Lower Cl</th>
<th>Higher Cl</th>
<th>SE b</th>
<th>df</th>
<th>t-value</th>
<th>$p$-value</th>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>Intercept</td>
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<td>32</td>
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<td>&lt; 0.001</td>
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</tbody>
</table>
| Membrane-associated PHE (nmol · mg protein$^{-1}$) with increasing concentrations of EPA of oleic acid

<table>
<thead>
<tr>
<th>Organ</th>
<th>FFA</th>
<th>Estimator</th>
<th>MC</th>
<th>b</th>
<th>Lower Cl</th>
<th>Higher Cl</th>
<th>SE b</th>
<th>df</th>
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<th>$p$-value</th>
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<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Intercept</td>
<td>$&lt;0.001$</td>
<td>-1.6·10$^{-5}$</td>
<td>-2.3·10$^{-5}$</td>
<td>-9.5·10$^{-6}$</td>
<td>3.5·10$^{-6}$</td>
<td>32</td>
<td>-4.71</td>
<td>&lt; 0.001</td>
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</tbody>
</table>
Figure S5.1: Effect of benzo[a]pyrene (BaP) solubilized in micelles composed of 10 mM bile salts without or with 10, 100 and 1000 µM eicosapentaenoic acid (EPA; A-C-E) or oleic acid (B-D-F) on transepithelial potential (TEP; A-B), short-circuit current (SCC; C-D) and transepithelial resistance (TER; E-F) of the proximal intestine. At t = 45 min, Ringer of the mucosal half-cell was replaced with mannitol Ringer (1st dotted vertical line). At t = 60 min, mannitol Ringer of the mucosal half-cell was replaced with experimental treatments (2nd dotted vertical line). Control incubations are without the addition of BaP and FFA to the incubation medium. Points represent experimental data (mean ± 95% CI; n = 8).
Figure S5.2: Effect of phenanthrene (PHE) solubilized in micelles composed of 10 mM bile salts without or with 10, 100 and 1000 µM eicosapentaenoic acid (EPA; A-C-E) or oleic acid (B-D-F) on transepithelial potential (TEP; A-B), short-circuit current (SCC; C-D) and transepithelial resistance (TER; E-F) of the proximal intestine. At t = 45 min, Ringer of the mucosal half-cell was replaced with mannitol Ringer (1st dotted vertical line). At t = 60 min, mannitol Ringer of the mucosal half-cell was replaced with experimental treatments (2nd dotted vertical line). Control incubations are without the addition of PHE and FFA to the incubation medium. Points represent experimental data (mean ± 95% CI; n = 8).
Figure S5.3: Effect of benzo[a]pyrene (BaP) solubilized in micelles composed of 10 mM bile salts without or with 10, 100 and 1000 µM eicosapentaenoic acid (EPA; A-C-E) or oleic acid (B-D-F) on transepithelial potential (TEP; A-B), short-circuit current (SCC; C-D) and transepithelial resistance (TER; E-F) of the distal intestine. At t = 45 min, Ringer of the mucosal half-cell was replaced with mannitol Ringer (1st dotted vertical line). At t = 60 min, mannitol Ringer of the mucosal half-cell was replaced with experimental treatments (2nd dotted vertical line). Control incubations are without the addition of BaP and FFA to the incubation medium. Points represent experimental data (mean ± 95% CI; n = 8).
Figure S5.4: Effect of phenanthrene (PHE) solubilized in micelles composed of 10 mM bile salts without or with 10, 100 and 1000 µM eicosapentaenoic acid (EPA; A-C-E) or oleic acid (B-D-F) on transepithelial potential (TEP; A-B), short-circuit current (SCC; C-D) and transepithelial resistance (TER; E-F) of the distal intestine. At t = 45 min, Ringer of the mucosal half-cell was replaced with mannitol Ringer (1st dotted vertical line). At t = 60 min, mannitol Ringer of the mucosal half-cell was replaced with experimental treatments (2nd dotted vertical line). Control incubations are without the addition of PHE and FFA to the incubation medium. Points represent experimental data (mean ± 95% CI; n = 8).
Table S5.1: Regression models fitted to the predicted accumulation of benzo[a]pyrene (BaP) and phenanthrene (PHE) in the mucosal and serosal layers of the proximal and distal intestine following the effect of increasing concentrations of eicosapentaenoic acid (EPA) and oleic acid. EPA is the reference level with value 0. Model criterion (MC) is the $p$-value for the model criteria and only used when significant (ANOVA; $p < 0.05$), $b$ is the estimate for the ‘Intercept’, slope for ‘free fatty acid (FFA) concentration’ and ‘Interaction (FFA concentration * Oleic acid)’ and the coefficient for ‘Oleic acid’ of the regression models. Lower and Higher CI: 95% confidence intervals, SE: associated standard errors, df: degrees of freedom and t-value. Within each model, p-values give significant differences among slopes. Linear model: $y = \text{Intercept} + (b_{\text{FFA concentration}} \cdot \text{FFA concentration}) + (b_{\text{Interaction}} \cdot \text{FFA concentration}) + \text{Oleic acid}$, where the intercept is the BaP or PHE accumulation (pmol · g tissue$^{-1}$) in the mucosal or serosal layer at 0 µM FFA.

### PAH accumulation (pmol · g tissue$^{-1}$) in intestinal layers of the proximal intestine with increasing concentrations of FFAs

<table>
<thead>
<tr>
<th>PAH</th>
<th>Intestinal layers</th>
<th>Estimator</th>
<th>MC</th>
<th>$b$</th>
<th>Lower CI</th>
<th>Higher CI</th>
<th>SE</th>
<th>df</th>
<th>t-value</th>
<th>$p$-value</th>
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<tbody>
<tr>
<td>BaP</td>
<td>Mucosa</td>
<td>Intercept</td>
<td>&lt;0.001</td>
<td>152.26</td>
<td>42.56</td>
<td>102.46</td>
<td>18.64</td>
<td>47</td>
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<tr>
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<td>Serosa</td>
<td>Intercept</td>
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<td>10.04</td>
<td>2.55</td>
<td>7.70</td>
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<tr>
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<td>-0.02</td>
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<td>1.19</td>
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<td>-0.21</td>
<td>-0.06</td>
<td>0.04</td>
<td>49</td>
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<td>Intercept</td>
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<td>-0.01</td>
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<td>15</td>
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<td>&lt;0.001</td>
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### PAH accumulation (pmol · g tissue$^{-1}$) in intestinal layers of the distal intestine with increasing concentrations of FFAs

<table>
<thead>
<tr>
<th>PAH</th>
<th>Intestinal layers</th>
<th>Estimator</th>
<th>MC</th>
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<th>Lower CI</th>
<th>Higher CI</th>
<th>SE</th>
<th>df</th>
<th>t-value</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BaP</td>
<td>Mucosa</td>
<td>Intercept</td>
<td>66.40</td>
<td>56.63</td>
<td>76.17</td>
<td>4.89</td>
<td>63</td>
<td>13.59</td>
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<td>Intercept</td>
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<td>4.12</td>
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<td>6.23</td>
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Table S5.2: Regression models fitted to the predicted accumulation of free fatty acids (FFA) i.e., oleic acid or eicosapentaenoic acid (EPA), in the mucosal and serosal layers of the proximal and distal intestine in the presence of 1 µM benzo[a]pyrene (BaP) or phenanthrene (PHE). EPA is the reference level with value 0. Model criterion (MC) is the p-value for the model criteria (ANOVA; \( p < 0.05 \)), b is the estimate for the ‘Intercept’, slope for ‘free fatty acid (FFA) concentration’ and the coefficient for ‘Oleic acid’ of the regression models. Lower and Higher Cl: 95% confidence intervals, SE: associated standard errors, df: degrees of freedom and t-value. Within each model, p-values give significant differences among slopes. Linear model: \( y = \text{Intercept} + (b_{\text{FFA concentration}} \cdot \text{FFA concentration}) + \text{Oleic acid} \), where the intercept is the FFA accumulation Ln(nmol \cdot g tissue\(^{-1}\)) in the mucosal or serosal layer at 10 µM FFA.

### FFA accumulation (Ln(nmol \cdot g tissue\(^{-1}\))) in intestinal layers of the proximal intestine in the presence of 1 µM PAH

<table>
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<tr>
<th>PAH</th>
<th>Intestinal layers</th>
<th>Estimator</th>
<th>MC</th>
<th>b</th>
<th>Lower Cl</th>
<th>Higher Cl</th>
<th>SE</th>
<th>df</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BaP</td>
<td>Mucosal</td>
<td>Intercept</td>
<td></td>
<td>0.33</td>
<td>0.12</td>
<td>0.54</td>
<td>0.10</td>
<td>48</td>
<td>3.14</td>
<td>0.003</td>
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<tr>
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<td></td>
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<td>10.91</td>
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<tr>
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<td>Intercept</td>
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<td>-0.18</td>
<td>-0.38</td>
<td>0.02</td>
<td>0.10</td>
<td>48</td>
<td>-1.77</td>
<td>0.083</td>
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<tr>
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<td>1.2 10(^{-3})</td>
<td>1.9 10(^{-3})</td>
<td>1.7 10(^{-4})</td>
<td>48</td>
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</tr>
<tr>
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<td>Mucosal</td>
<td>Intercept</td>
<td></td>
<td>0.24</td>
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<tr>
<td></td>
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<td>1.7 10(^{-3})</td>
<td>1.4 10(^{-4})</td>
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</tr>
<tr>
<td></td>
<td>Oleic acid</td>
<td></td>
<td></td>
<td>0.77</td>
<td>0.52</td>
<td>1.02</td>
<td>0.13</td>
<td>51</td>
<td>6.08</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Serosal</td>
<td>Intercept</td>
<td></td>
<td>-0.29</td>
<td>-0.54</td>
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<td>2.0 10(^{-3})</td>
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<td>51</td>
<td>9.93</td>
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<tr>
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<td></td>
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</table>

### FFA accumulation (Ln(nmol \cdot g tissue\(^{-1}\))) in intestinal layers of the distal intestine in the presence of 1 µM PAH

<table>
<thead>
<tr>
<th>PAH</th>
<th>Intestinal layers</th>
<th>Estimator</th>
<th>MC</th>
<th>b</th>
<th>Lower Cl</th>
<th>Higher Cl</th>
<th>SE</th>
<th>df</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BaP</td>
<td>Mucosal</td>
<td>Intercept</td>
<td></td>
<td>-0.06</td>
<td>-0.33</td>
<td>0.21</td>
<td>0.14</td>
<td>48</td>
<td>-0.45</td>
<td>0.655</td>
</tr>
<tr>
<td></td>
<td>FFA concentration</td>
<td></td>
<td></td>
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<td>0.22</td>
<td>0.92</td>
<td>0.17</td>
<td>48</td>
<td>3.27</td>
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<tr>
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<td></td>
<td>-0.23</td>
<td>-0.46</td>
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<td>Intercept</td>
<td></td>
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<td>-0.43</td>
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<tr>
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<td>Mucosal</td>
<td>Intercept</td>
<td></td>
<td>-0.57</td>
<td>-0.74</td>
<td>-0.39</td>
<td>0.09</td>
<td>56</td>
<td>-6.33</td>
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</tr>
<tr>
<td></td>
<td>FFA concentration</td>
<td></td>
<td></td>
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<td>1.5 10(^{-3})</td>
<td>2.1 10(^{-3})</td>
<td>1.5 10(^{-4})</td>
<td>56</td>
<td>11.75</td>
<td>&lt;0.001</td>
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Supplementary information
Table S5.3: Regression models fitted to the predicted transepithelial transfer of free fatty acids (FFA) *i.e.*, oleic acid or eicosapentaenoic acid (EPA); \( \text{Ln}(\mu\text{mol} \cdot \text{cm}^2 + 1) \) across the proximal intestine in the presence of 1 µM benzo[a]pyrene (BaP) and phenanthrene (PHE). Transepithelial transfer of EPA or oleic acid with the concentration of 10 µM is the reference level with value 0. Model criterion (MC) is the \( p \)-value for the model criteria (ANOVA; \( p < 0.05 \)), \( b \) is the estimate for the ‘Intercept’, slope for ‘Time’ and the coefficient for ‘FFA conc’ of the regression models. Lower and Higher CI: 95% confidence intervals, SE: associated standard errors, df: degrees of freedom and t-value. Within each model, \( p \)-values give significant differences among slopes. Linear model: \( y = \text{Intercept} + (b_{\text{time}} \cdot \text{Time}) + \text{FFA conc} \), where the intercept is the transepithelial FFA transfer across the proximal intestine at 60 min.

<table>
<thead>
<tr>
<th>FFA</th>
<th>Estimator</th>
<th>MC</th>
<th>b</th>
<th>Lower Cl</th>
<th>Higher Cl</th>
<th>SE b</th>
<th>df</th>
<th>t-value</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EPA</strong></td>
<td>Intercept</td>
<td>-0.71</td>
<td>-0.87</td>
<td>-0.55</td>
<td>0.08</td>
<td>196</td>
<td>-8.57</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>&lt; 0.001</td>
<td>5.5 \times 10^{-3}</td>
<td>4.7 \times 10^{-3}</td>
<td>6.3 \times 10^{-3}</td>
<td>4.1 \times 10^{-4}</td>
<td>196</td>
<td>13.32</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>FFA conc (100 µM)</td>
<td>0.28</td>
<td>0.16</td>
<td>0.40</td>
<td>0.06</td>
<td>196</td>
<td>4.61</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FFA conc (1000 µM)</td>
<td>0.99</td>
<td>0.88</td>
<td>1.11</td>
<td>0.06</td>
<td>196</td>
<td>16.35</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td><strong>Oleic acid</strong></td>
<td>Intercept</td>
<td>-0.47</td>
<td>-0.63</td>
<td>-0.31</td>
<td>0.08</td>
<td>128</td>
<td>-5.69</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>&lt; 0.001</td>
<td>3.5 \times 10^{-3}</td>
<td>2.7 \times 10^{-3}</td>
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<td>4.2 \times 10^{-4}</td>
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<td>8.30</td>
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<tr>
<td></td>
<td>FFA conc (100 µM)</td>
<td>0.22</td>
<td>0.10</td>
<td>0.34</td>
<td>0.06</td>
<td>128</td>
<td>3.53</td>
<td>&lt; 0.001</td>
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</tr>
<tr>
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<td>FFA conc (1000 µM)</td>
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<td>0.06</td>
<td>128</td>
<td>12.73</td>
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<table>
<thead>
<tr>
<th>FFA</th>
<th>Estimator</th>
<th>MC</th>
<th>b</th>
<th>Lower Cl</th>
<th>Higher Cl</th>
<th>SE b</th>
<th>df</th>
<th>t-value</th>
<th>( p )-value</th>
</tr>
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<tbody>
<tr>
<td><strong>EPA</strong></td>
<td>Intercept</td>
<td>-0.74</td>
<td>-0.88</td>
<td>-0.61</td>
<td>0.07</td>
<td>145</td>
<td>-10.92</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>&lt; 0.001</td>
<td>5.5 \times 10^{-3}</td>
<td>4.8 \times 10^{-3}</td>
<td>6.2 \times 10^{-3}</td>
<td>3.5 \times 10^{-4}</td>
<td>145</td>
<td>15.52</td>
<td>&lt; 0.001</td>
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<tr>
<td></td>
<td>FFA conc (100 µM)</td>
<td>0.48</td>
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<td>0.58</td>
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<td>145</td>
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<tr>
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<td>FFA conc (1000 µM)</td>
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<td>1.11</td>
<td>1.31</td>
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<td>145</td>
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<tr>
<td><strong>Oleic acid</strong></td>
<td>Intercept</td>
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<td>-0.54</td>
<td>-0.32</td>
<td>0.06</td>
<td>172</td>
<td>-7.64</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>&lt; 0.001</td>
<td>3.1 \times 10^{-3}</td>
<td>2.5 \times 10^{-3}</td>
<td>3.7 \times 10^{-3}</td>
<td>3.0 \times 10^{-4}</td>
<td>172</td>
<td>10.16</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>FFA conc (100 µM)</td>
<td>0.21</td>
<td>0.12</td>
<td>0.29</td>
<td>0.04</td>
<td>172</td>
<td>4.77</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FFA conc (1000 µM)</td>
<td>0.66</td>
<td>0.58</td>
<td>0.75</td>
<td>0.04</td>
<td>172</td>
<td>15.00</td>
<td>&lt; 0.001</td>
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Table S5.4: Regression models fitted to the predicted transepithelial transfer of free fatty acids (FFA) i.e., oleic acid or eicosapentaenoic acid (EPA); Ln(µmol ∙ cm⁻² + 1) across the proximal intestine in the presence of 1 µM benzo[α]pyrene (BaP) and phenanthrene (PHE). Transepithelial transfer of EPA or oleic acid with the concentration of 10 µM is the reference level with value 0. Model criterion (MC) is the p-value for the model criteria (ANOVA; p < 0.05), b is the estimate for the ‘Intercept’, slope for ‘Time’ and ‘Time²’ and thre coefficient for ‘FFA conc’ of the regression models. Lower and Higher Cl: 95% confidence intervals, SE: associated standard errors, df: degrees of freedom and t-value. Within each model, p-values give significant differences among slopes. Linear model: \( y = \text{Intercept} + (b_{\text{Time}} \cdot \text{Time}) + \text{FFA conc} \); polynominal model: \( y = \text{intercept} + (b_{\text{Time}} \cdot \text{Time}) + (b_{\text{Time}^2} \cdot \text{Time}^2) + b_{\text{FFA conc}} \) where the intercept is the transepithelial FFA transfer across the distal intestine at 60 min.

### Transepithelial transfer of FFA (Ln(µmol ∙ cm⁻²+1)) in the presence of 1 µM BaP

<table>
<thead>
<tr>
<th>FFA</th>
<th>Estimator</th>
<th>MC</th>
<th>b</th>
<th>Lower Cl</th>
<th>Higher Cl</th>
<th>SE b</th>
<th>df</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPA</td>
<td>Intercept</td>
<td>&lt;0.001</td>
<td>-0.59</td>
<td>-0.88</td>
<td>-0.30</td>
<td>0.15</td>
<td>171</td>
<td>-4.01</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td></td>
<td>3.910⁻³</td>
<td>-2.410⁻⁴</td>
<td>8.110⁻³</td>
<td>2.110⁻³</td>
<td>171</td>
<td>1.84</td>
<td>0.068</td>
</tr>
<tr>
<td></td>
<td>Time²</td>
<td></td>
<td>1.010⁻⁶</td>
<td>-1.310⁻⁵</td>
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<td>7.110⁻⁶</td>
<td>171</td>
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</tr>
<tr>
<td></td>
<td>FFA conc (100 µM)</td>
<td></td>
<td>0.22</td>
<td>0.12</td>
<td>0.33</td>
<td>0.05</td>
<td>171</td>
<td>4.16</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>FFA conc (1000 µM)</td>
<td></td>
<td>0.74</td>
<td>0.64</td>
<td>0.85</td>
<td>0.05</td>
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<td>&lt; 0.001</td>
</tr>
<tr>
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<td>Intercept</td>
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<td>0.08</td>
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<tr>
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<td>Time</td>
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<td>1.210⁻³</td>
<td>5.110⁻³</td>
<td>1.810⁻³</td>
<td>3.310⁻⁴</td>
<td>190</td>
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<tr>
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<td>FFA conc (100 µM)</td>
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<td>-0.07</td>
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<td>0.05</td>
<td>190</td>
<td>0.43</td>
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<tr>
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<td>FFA conc (1000 µM)</td>
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<td>0.38</td>
<td>0.29</td>
<td>0.48</td>
<td>0.05</td>
<td>190</td>
<td>7.74</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

### Transepithelial transfer of FFA (Ln(µmol ∙ cm⁻²+1)) in the presence of 1 µM PHE

<table>
<thead>
<tr>
<th>FFA</th>
<th>Estimator</th>
<th>MC</th>
<th>b</th>
<th>Lower Cl</th>
<th>Higher Cl</th>
<th>SE b</th>
<th>df</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
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<tr>
<td>EPA</td>
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<td>-0.51</td>
<td>-0.66</td>
<td>-0.37</td>
<td>0.07</td>
<td>196</td>
<td>-6.86</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td></td>
<td>3.710⁻³</td>
<td>3.110⁻³</td>
<td>4.410⁻³</td>
<td>3.310⁻⁴</td>
<td>196</td>
<td>11.11</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>FFA conc (100 µM)</td>
<td></td>
<td>0.17</td>
<td>0.07</td>
<td>0.27</td>
<td>0.05</td>
<td>196</td>
<td>3.47</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>FFA conc (1000 µM)</td>
<td></td>
<td>0.76</td>
<td>0.66</td>
<td>0.86</td>
<td>0.05</td>
<td>196</td>
<td>15.48</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>Intercept</td>
<td>&lt;0.001</td>
<td>-0.33</td>
<td>-0.46</td>
<td>-0.19</td>
<td>0.07</td>
<td>173</td>
<td>-4.70</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td></td>
<td>2.610⁻³</td>
<td>1.910⁻³</td>
<td>3.210⁻³</td>
<td>3.310⁻⁴</td>
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</tr>
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<td>FFA conc (10 µM)</td>
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<td>0.08</td>
<td>0.27</td>
<td>0.05</td>
<td>173</td>
<td>3.53</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>FFA conc (100 µM)</td>
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<td>0.43</td>
<td>0.62</td>
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<td>10.88</td>
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</tr>
<tr>
<td></td>
<td>FFA conc (1000 µM)</td>
<td></td>
<td>0.38</td>
<td>0.29</td>
<td>0.48</td>
<td>0.05</td>
<td>190</td>
<td>7.74</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
Table S5.5: Regression models fitted to the predicted transepithelial potential (TEP; mV), short circuit current (SCC; µA·cm⁻²) and transepithelial resistance (TER; Ω·cm²) over time in the presence of 1 µM benzo[a]pyrene (BaP) of the proximal intestine following the effect of increasing concentrations of eicosapentaenoic acid (EPA). Control incubation (without BaP and EPA) is the reference level with value 0. Electrophysiology parameters (E.P), model criterion (MC) is the p-value for the model criteria and only used when significant (ANOVA; p < 0.05), b is the estimate for the 'Intercept', the slope estimate for 'Time', and 'Interaction (Time * Treatment)' and coefficient for 'Treatment' of the regression models. Lower and Higher Cl: 95% confidence intervals, SE: associated standard errors, df: degrees of freedom and t-value. Within each model, p-values give significant differences among slopes. Linear model: y = intercept + (bTime ∙ Time) + btreatment + (bInteraction ∙ Time); polynomial model: y = intercept + (bTime ∙ Time) + (bTime² ∙ Time²) + btreatment + (bInteraction ∙ Time) + (bInteraction² ∙ Time²) where the intercept is the electrophysiology parameter at 100 min.

<table>
<thead>
<tr>
<th>E.P.</th>
<th>Estimator</th>
<th>MC</th>
<th>b</th>
<th>Lower CI</th>
<th>Higher CI</th>
<th>SE b</th>
<th>df</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
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<td>Intercept</td>
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<td>-1.00</td>
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<td>1337</td>
<td>-9.77</td>
<td>&lt; 0.001</td>
</tr>
<tr>
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<td>1337</td>
<td>6.37</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Treatment BaP</td>
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<td>-0.23</td>
<td>0.42</td>
<td>0.17</td>
<td>1337</td>
<td>0.57</td>
<td>0.568</td>
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<td>0.61</td>
<td>0.17</td>
<td>1337</td>
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*Effects of BaP on electrophysiology with increasing concentrations EPA*

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Table S5.6: Regression models fitted to the predicted transepithelial potential (TEP; mV), short circuit current (SCC; μA · cm⁻²) and transepithelial resistance (TER; Ω · cm²) over time in the presence of 1 μM benzo[a]pyrene (BaP) of the proximal intestine following the effect of increasing concentrations of oleic acid (OA). Control incubation (without PHE and EPA) is the reference level. Electrophysiology parameters (E.P), model criterion (MC) is the p-value for the model criteria and only used when significant (ANOVA; p < 0.05), b is the estimate for the 'Intercept', the slope estimate for 'Time', and 'Interaction (Time * Treatment)' and coefficient for 'Treatment' of the regression models. Lower and Higher CI: 95% confidence intervals, SE: associated standard errors, df: degrees of freedom and t-value. Within each model, p-values give significant differences among slopes. Linear model: y = intercept + (b_{Time} · Time) + b_{Treatment} + (b_{Interaction} · Time) where the intercept is the electrophysiology parameter at 100 min.

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| SCC           | Intercept       | < 0.001 | -16.48 | -19.92  | -13.03    | 1.77  | 992  | -9.33   | < 0.001 |
|               | Time            | < 0.001 | 0.03   | 0.02    | 0.05      | 0.01  | 992  | 4.61    | < 0.001 |
|               | Treatment BaP   |        | -0.81  | -5.68   | 4.07      | 2.50  | 992  | -0.32   | 0.747   |
|               | Treatment BaP + 10 µM EPA |       | -2.46  | -7.33   | 2.42      | 2.50  | 992  | -0.98   | 0.325   |
|               | Treatment BaP + 100 µM EPA |       | -1.85  | -6.73   | 3.02      | 2.50  | 992  | -0.74   | 0.458   |
|               | Treatment BaP + 1000 µM EPA |      | -5.98  | -10.85  | -1.10     | 2.50  | 992  | -2.39   | 0.017   |
|               | Interaction BaP | 2.4 · 10⁻³ | -1.8 · 10⁻² | 2.3 · 10⁻² | 1.0 · 10⁻² | 992  | 0.23  | 0.815   |
Table S5.6 continued

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Table S5.7: Regression models fitted to the predicted transepithelial potential (TEP; mV), short circuit current (SCC; µA · cm⁻²) and transepithelial resistance (TER; Ω · cm²) over time in the presence of phenanthrene (PHE) of the proximal intestine following the effect of increasing concentrations of eicosapentaenoic acid (EPA). Control incubation (without PHE and EPA) is the reference level. Electrophysiology parameters (E.P), model criterion (MC) is the p-value for the model criteria and only used when significant (ANOVA; p < 0.05), b is the estimate for the 'Intercept', the slope estimate for 'Time', and 'Interaction (Time * Treatment)' and coefficient for 'Treatment' of the regression models. Lower and Higher Cl: 95% confidence intervals, SE: associated standard errors, df: degrees of freedom and t-value. Within each model, p-values give significant differences among slopes. Linear model: y = intercept + (bTime ∙ Time) + btreatment + (bInteraction ∙ Time); polynominal model: y = intercept + (bTime ∙ Time) + (bTime² ∙ Time²) + btreatment + (bInteraction ∙ Time) + (bInteraction² ∙ Time²) where the intercept is the electrophysiology parameter at 100 min.

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Table S5.8: Regression models fitted to the predicted transepithelial potential (TEP; mV), short circuit current (SCC; µA ∙ cm⁻²) and transepithelial resistance (TER; Ω ∙ cm²) over time in the presence of phenanthrene (PHE) of the proximal intestine following the effect of increasing concentrations of oleic acid (OA). Control incubation (without PHE and OA) is the reference level. Electrophysiology parameters (E.P), model criterion (MC) is the p-value for the model criteria and only used when significant (ANOVA; p < 0.05), b is the estimate for the 'Intercept', the slope estimate for 'Time,' and 'Interaction (Time * Treatment)' and coefficient for 'Treatment' of the regression models. Lower and Higher CI: 95% confidence intervals, SE: associated standard errors, df: degrees of freedom and t-value. Within each model, p-values give significant differences among slopes. Linear model: y = intercept + (b_{Time} ∙ Time) + b_{Treatment} + (b_{Interaction} ∙ Time) where the intercept is the electrophysiology parameter at 100 min.

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Table S5.8 continued

**Effects of PHE on electrophysiology with increasing concentrations oleic acid**
Table S5.9: Regression models fitted to the predicted transepithelial potential (TEP; mV), short circuit current (SCC; µA · cm⁻²) and transepithelial resistance (TER; Ω · cm²) over time in the presence of 1 µM benzo[a]pyrene (BaP) of the distal intestine following the effect of increasing concentrations of eicosapentaenoic acid (EPA). Control incubation (without BaP and EPA) is the reference level. Electrophysiology parameters (E.P), model criterion (MC) is the p-value for the model criteria and only used when significant (ANOVA; p < 0.05), b is the estimate for the 'Intercept', the slope estimate for 'Time', and 'Interaction (Time * Treatment)' and coefficient for 'Treatment' of the regression models. Lower and Higher Cl: 95% confidence intervals, SE: associated standard errors, df: degrees of freedom and t-value. Within each model, p-values give significant differences among slopes. Linear model: y = intercept + (b₁ Time) + b₂ Treatment + (b₃ Interaction) where the intercept is the electrophysiology parameter at 100 min.

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Table S5.10: Regression models fitted to the predicted transepithelial potential (TEP; mV), short circuit current (SCC; µA · cm⁻²) and transepithelial resistance (TER; Ω · cm²) over time in the presence of 1 µM benzo[a]pyrene (BaP) of the distal intestine following the effect of increasing concentrations of oleic acid (OA). Control incubation (without PHE and EPA) is the reference level. Electrophysiology parameters (EP), model criterion (MC) is the p-value for the model criteria and only used when significant (ANOVA; p < 0.05), b is the estimate for the 'Intercept', the slope estimate for 'Time', and 'Interaction (Time * Treatment)' and coefficient for 'Treatment' of the regression models. Lower and Higher CI: 95% confidence intervals, SE: associated standard errors, df: degrees of freedom and t-value. Within each model, p-values give significant differences among slopes. Linear model: \( y = \text{intercept} + (b_{\text{Time}} \cdot \text{Time}) + b_{\text{Treatment}} + (b_{\text{Interaction}} \cdot \text{Time}) \) where the intercept is the electrophysiology parameter at 100 min.

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#### Effects of BaP on electrophysiology with increasing concentrations oleic acid in the distal intestine

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Table S5.11: Regression models fitted to the predicted transepithelial potential (TEP; mV), short circuit current (SCC; µA ∙ cm⁻²) and transepithelial resistance (TER; Ω ∙ cm⁻²) over time in the presence of phenanthrene (PHE) of the distal intestine following the effect of increasing concentrations of eicosapentaenoic acid (EPA). Control incubation (without PHE and EPA) is the reference level. Electrophysiology parameters (E.P), model criterion (MC) is the p-value for the model criteria and only used when significant (ANOVA; p < 0.05), b is the estimate for the 'Intercept', the slope estimate for 'Time', and 'Interaction (Time * Treatment)' and coefficient for 'Treatment' of the regression models. Lower and Higher Cl: 95% confidence intervals, SE: associated standard errors, df: degrees of freedom and t-value. Within each model, p-values give significant differences among slopes. Linear model: y = intercept + (bTime ∙ Time) + bTreatment + (bInteraction ∙ Time) where the intercept is the electrophysiology parameter at 100 min.

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Table S5.12: Regression models fitted to the predicted transepithelial potential (TEP; mV), short circuit current (SCC; µA · cm⁻²) and transepithelial resistance (TER; Ω · cm²) over time in the presence of phenanthrene (PHE) of the distal intestine following the effect of increasing concentrations of oleic acid (OA).

Control incubation (without PHE and OA) is the reference level. Electrophysiology parameters (E.P), model criterion (MC) is the p-value for the model criteria and only used when significant (ANOVA; p < 0.05), b is the estimate for the 'Intercept', the slope estimate for 'Time', and 'Interaction (Time * Treatment)' and coefficient for 'Treatment' of the regression models. Lower and Higher Cl: 95% confidence intervals, SE: associated standard errors, df: degrees of freedom and t-value. Within each model, p-values give significant differences among slopes. Linear model: y = intercept + (b₉Time ∙ Time) + btreatment + (binteraction ∙ Time); polynomial model: y = intercept + (b₉Time ∙ Time) + (b₉Time² ∙ Time²) + btreatment + (binteraction ∙ Time) + (binteraction² ∙ Time²) where the intercept is the electrophysiology parameter at 100 min.

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### Table S5.12 continued

**Effects of PHE on electrophysiology with increasing concentrations OA in the distal intestine**

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Summary

The rapidly growing aquaculture has led to an increased pressure on the limited supply of marine aquafeed ingredients. Traditionally, marine fish oils and fishmeal have been used as the main feed ingredients in commercial feeds for carnivorous farmed fish species such as Atlantic salmon (Salmo salar). However, the rapidly growing aquaculture cannot continue to rely on fisheries for the supply of fish oil and fishmeal. There is therefore a need to develop alternative aquafeed ingredients, such as oil and meal from vegetable sources.

Replacing fish oils with vegetable oils alters the nutrient profile of aquafeeds. The use of vegetable oils increases dietary levels of monoene and omega-6 fatty acids. Contrary, levels of omega-3 long-chain polyunsaturated fatty acids (PUFAs) decrease. As a result of thermal processing of oil-containing seeds and grains, the inclusion of these oils in aquafeeds introduces contaminating polycyclic aromatic hydrocarbons (PAHs).

The introduction of vegetable oils in aquafeeds has increased levels of PAHs, such as benzo[a]pyrene (BaP) and phenanthrene (PHE), in fillets of Atlantic salmon reared on these diets. BaP and PHE are lipophilic compounds that differ in their physico-chemical properties as well as their potential toxic actions.

The intestine is the site where nutrients and contaminants are absorbed from the aquafeed diet and is also the primary site where nutrient-toxicant interactions take place. One of our first experiments showed that BaP and PHE entered intestinal enterocytes 6-12 h post-feeding in Atlantic salmon. Thereafter, they were both transported across the intestinal epithelium into the systemic circulation and distributed to various peripheral organs (e.g., liver). However, in an apical membrane vesicle preparation isolated from enterocytes, the largest fraction of BaP and PHE partitioned within the membrane rather than transferred across. The presence of PAHs in the plasma membrane of enterocytes could affect fluidity of the lipid bilayer, disturb membrane integrity and the function of integral membrane proteins. These effects are likely to interfere with nutrient uptake.

The intestine is the first barrier for dietary PAH uptake and plays an important role in the metabolic breakdown of PAHs. BaP is an aryl hydrocarbon receptor agonist that induces cyp1a expression, an enzyme involved in the detoxification of xenobiotics. Cyp1a metabolizes BaP to derivatives that can bind to cellular proteins and/or DNA. Hence, BaP is less transferred from enterocytes into the systemic circulation compared to PHE. The activities of intracellular enzymes of the Cyp-family that are involved in the metabolic breakdown are an important determinant of the whole-body distribution of PAHs.
Dietary lipids can affect the intestinal uptake of PAHs. In Atlantic salmon a vegetable oil-based diet decreased intestinal and peripheral PAH distribution compared with a fish oil-based diet. When ingested, the lipophilic nature of PAHs promotes their solubilization in oil droplets and micelles in the intestinal lumen. The solubility of PAHs is higher in micelles composed of long-chain fatty acids with a higher degree of unsaturated bonds (i.e., fish oil) than in micelles composed of more saturated shorter-chain fatty acids (i.e., vegetable oil). Oleic acid, a fatty acid derived from vegetable oil has a shorter chain length and less unsaturated bonds than the fish oil-derived fatty acid eicosapentaenoic acid (EPA). The dependence of PAH solubility on micelle composition can explain why the accumulation of PHE in the proximal intestine of rainbow trout (Oncorhynchus mykiss) in vitro was lower when co-exposed with micelles composed of oleic acid than EPA. This explains why a diet based on the vegetable oil-derived oleic acid reduces the accumulation of PHE in the proximal intestine in vivo as well as in vitro.

BaP and PHE potently inhibited the hydrolysis of rapeseed oil by rainbow trout intestinal lipase, whereas fish oil hydrolysis was largely insensitive to inhibition. Since micelles composed of rapeseed oil contain lower concentrations of PAHs than 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. Therefore, the occurrence of PAHs in aquafeeds could decrease lipid digestion in vivo and negatively impact organismal energy metabolism.

In a lipid-rich diet, less lipophilic compounds are absorbed by the intestinal epithelium. Indeed, transmembrane uptake of BaP in intestinal apical membrane vesicles had decreased more when co-exposed with increasing concentrations of micelles composed of EPA than with oleic acid micelles. Increasing concentrations of EPA and oleic acid, administrated as micelles, also decreased the unidirectional PHE flux across intact epithelium of the proximal intestine of rainbow trout in vitro.

In this thesis several in vitro and in vivo models were used to investigate interactions between lipids, their hydrolysis products and PAHs at the level of enzymatic lipid hydrolysis, intestinal membrane transport, intestinal transepithelial uptake and peripheral distribution. In conclusion, lipid-PAH interactions depend upon the type of oil, lipid content, fatty acid composition and PAH congener. Therefore, the lipid composition and content of an aquafeed is an important determinant for PAH bioavailability.
Samenvatting

Traditioneel werden visolie en vismeel gebruikt als belangrijkste ingrediënten in commercieel visvoer voor carnivore vissen zoals de Atlantische zalm (Salmo salar). De aquacultuur blijft groeien en daarmee ook de vraag naar de beperkte hoeveelheid mariene visvoer ingrediënten. Daarom is het noodzakelijk om alternatieve ingrediënten te gaan gebruiken in visvoer, zoals bijvoorbeeld olie en eiwitten uit plantardige bronnen. Het vervangen van visolie met plantaardige oliën verandert het nutriëntenprofiel van visvoer. Het gebruik van plantaardige oliën verhoogt de concentratie van enkelvoudige onverzadigde vetzuurketens terwijl de concentratie van omega-3 meervoudig onverzadigde lange vetzuurketens afneemt. Naast het gewijzigde nutriëntenprofiel leidt de toevoeging van plantaardige oliën tot de introductie van de giftige polycliscyclische aromatische koolwaterstoffen (PAKs) die ontstaan tijdens het productieproces van visvoer.

De introductie van PAKs in plantaardig visvoer heeft geleid tot verhoogde niveaus van PAKs als benzo[a]pyreen (BaP) en fenantreen (PHE) in filets van Atlantische zalm. BaP en PHE zijn lipofiele stoffen die verschillen in hun fysische-chemische eigenschappen, evenals in hun potentiële toxische acties.

In de darm worden voedingsstoffen en toxische stoffen opgenomen uit het dieet. Daarnaast is het ook de primaire plaats waar interacties tussen voedingsstoffen en toxische stoffen kunnen plaatsvinden. In één van de eerste experimenten is geobserveerd dat BaP en PHE beide werden gedetecteerd in darmcellen (enterocyten) van Atlantische zalm, 6 tot 12 uur nadat ze waren gevoed. Daarna werden beide PAKs getransporteerd naar de bloedsomloop en verder gedistribueerd naar verschillende organen (zoals de lever). In membraanblaasjes, opgezuiverd uit de apicale celmembranen van darmcellen, zat de grootste fractie van BaP en PHE in het membraan, in plaats van over het membraan getransporteerd te worden. De PAKs die in het celmembran van darmcellen zitten kunnen de bewegelijkheid van fosfolipiden in het membraan beïnvloeden en de membraanintegriteit en de functie van membraanewitten verstoren. Uiteindelijk kan dit leiden tot verstoring van de opname van voedingsstoffen.

De darm is de eerste barrière voor de opname van PAKs en speelt een belangrijke rol in de stofwisseling ervan. BaP stimuleert de genexpressie van cyp1a, een enzym dat betrokken is bij de afbraak van toxische stoffen. Wanneer BaP wordt afgebroken door Cyp1A kunnen er tussenvormen ontstaan die zeer reactief zijn en kunnen binden aan cellulaire eiwitten en/of DNA. BaP wordt meer gemetaboliseerd dan PHE wat als gevolg heeft dat BaP ook minder getransporteerd wordt door het darmepithel dan PHE. De enzymatische activiteiten van eiwitten die betrokken zijn bij de metabole afbraak van PAK zijn daarom belangrijke factoren die
meespelen tijdens de opname van PAKs uit de darm en daaropvolgend naar de perifere organen.

Vetten die in het dieet aanwezig zijn kunnen de opname van PAKs in de darm beïnvloeden. Een dieet op basis van plantaardige oliën zorgde ervoor dat in Atlantische zalm zowel de concentraties van PAKs in de darm als in andere organen lager was dan wanneer de vissen werden gevoerd met een dieet op basis van visolie. De lipofiele aard van PAKs zorgt ervoor dat ze goed oplossgen in oliedruppels en micellen in het lumen van de darm. De oplosbaarheid van PAKs is hoger in micellen die bestaan uit lange vetzuurketens en een hogere mate van onverzadigde verbindingen (zoals visolie) dan in micellen die voornamelijk bestaan uit verzadigde kortere vetzuurketens (zoals plantaardige olie). Oliezuur (oleïne), een vetzuur afgeleid uit plantaardige oliën, heeft een kortere vetzuurketen en minder onverzadigde verbindingen dan de visolie afgeleide eicosapentaeenzuur (EPA). Waarschijnlijk is dat de reden waarom de PHE accumulatie in de proximale darm van de regenboogforel (Oncorhynchus mykiss) lager was wanneer PAKs waren opgelost in micellen die bestonden uit oliezuur dan EPA.

BaP en PHE verminderden de hydrolyse van koolzaadolie door darmlipase in regenboogforellen. De hydrolyse van visolie daaren-tegen was grotendeels ongevoelig voor beide PAKs. Aangezien PAKs minder goed oplossen in micellen die bestaan uit koolzaadolie dan wanneer ze bestaan uit visolie, zijn er meer PAKs vrij beschikbaar om lipase activiteit te remmen. Dit betekent dat de aanwezige PAKs in plantaardig visvoer de vetvertering kunnen verlagen. Omdat vetten belangrijke energiebronnen zijn kan dit uiteindelijk negatieve gevolgen hebben voor het energiemetabolisme.

In een vetrijk dieet worden lipofiele stoffen minder goed geabsorbeerd door het darmepitheel. De opname van BaP over het membraan in membraanblaasjes was verlaagd bij toenemende concentraties EPA en oliezuur, toegediend in de vorm van micellen. Daarnaast verlaagden de toenemende micel-concentraties EPA en oliezuur ook de PHE-flux over het epitheel van de proximale darm van de regenboogforel in vitro.

In dit proefschrift zijn verschillende in vitro en in vivo modellen gebruikt om de interacties tussen vetten, hun afbraakproducten en PAKs te bekijken op het niveau van enzymatische hydrolyse, transport over het darmmembraan, over het gehele darmepitheel en de verdeling over perifere orgaan. Geconcludeerd kan worden dat interacties tussen PAKs en vetten afhankelijk zijn van het type vet (olie), de vetconcentratie, de vetzuursamenstelling en type PAK. Daarom is de vetzuursamenstelling en vetconcentratie van visvoer een belangrijke factor om de biologische beschikbaarheid van PAKs te beoordelen.
List of publications


Curriculum vitae
