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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*)



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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; 20:5*n*–3) and oleic acid (18:1*n*–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 mixed micelles composed of either EPA or oleic acid and administered 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) trans-membrane uptake of BaP and PHE was 2–7 times lower than the fraction associated to or in the membrane. In the presence of FFA, trans-membrane 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, trans-membrane EPA uptake in BBMV had decreased.

This study obtained evidence for PAH-dependent interactions with FFA uptake. We conclude that intestinal BaP uptake is reduced by luminal FFA contents whereas PHE uptake is not. A large fraction of the administered BaP and PHE remains associated with the cellular membrane of enterocytes and may interfere with uptake of nutrients.

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1. Introduction

The steadily growing aquaculture industry has resulted in an increased pressure on limited available aquafeed ingredients, viz. fish oils and fishmeal (Bostock et al., 2010; FAO, 2014; Tacon and Metian, 2008). 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 <30% of fish ingredients while approximately 56% is composed of vegetable ingredients (Ytrestøyl et al., 2015). Rapeseed oil is mainly used as a substitute for fish oils, but small amounts of palm oil and soybean oil are also used (Sørensen et al., 2011). Rapeseed oil contains ca. 60% oleic acid (18:1*n*–9) and ca. 20% linoleic acid (18:2*n*–6), whereas

fish oil is rich in *n*–3 long-chain polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA; 20:5*n*–3) and docosahexaenoic acid (DHA; 22:6*n*–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 (Berntssen et al., 2005, 2015). 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 roasting or gas drying (Moret et al., 2005; Phillips, 1999; Teixeira et al., 2007). 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 (Berntssen et al., 2010). PAHs are ubiquitous lipophilic non-persistent organic chemicals that are potentially toxic (Berntssen et al., 2015). 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).

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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 chemicals such as PAHs have been suggested to occur in association with fat absorption (Drouillard and Norstrom, 2000; Dulfer et al., 1998; Gobas et al., 1993; Kelly et al., 2004; Vasiluk et al., 2008). A lipid-dependent uptake mechanism is suggested in which lipophilic chemicals are absorbed from the intestinal tract with lipids during digestion and membrane transport (Vetter et al., 1985). Emulsified lipids are hydrolyzed by digestive lipases to yield free fatty acids (FFA), monoacylglycerols and, after complete digestion, glycerol (Bakke et al., 2010; Olsen and Ringø, 1997; Tocher, 2003).

Biliary components (e.g., bile salts and cholesterol) together with FFA, and, to lesser degrees, mono-, di- and triacylglycerols form mixed micelles (Bakke et al., 2010; Phan and Tso, 2001; Wang et al., 2013; Yeap et al., 2013). These mixed micelles can enhance absorption of lipophilic compounds in enterocytes by facilitating transport of e.g. monoacylglycerols, FFA, fat-soluble vitamins and lipophilic chemicals across the unstirred water layer which is positioned directly adjacent to the brush border membrane of enterocytes (Doi et al., 2000; Dulfer et al., 1996; Kelly et al., 2004; Vasiluk et al., 2008; Vetter et al., 1985). Despite the low solubility of lipids and lipophilic chemicals in an aqueous environment, mixed micelles greatly increase their aqueous concentrations (Phan and Tso, 2001). Solubility of lipophilic chemicals depends on the lipid composition of mixed micelles (Doi et al., 2000; Laher and Barrowman, 1983). Besides micelle-mediated transport of lipophilic chemicals, bile salts also facilitate the transport of PAHs across the unstirred water layer to the brush border membrane in the absence of lipids (Weber and Lanno, 2001). The transfer of PAHs from micelles might occur via a vesicular-mediated uptake process or via direct absorption following a collisional mechanism. Once mixed micelles arrive at the brush border membrane, monoacylglycerols, FFA and lipophilic xenobiotics are released and can then be absorbed across the apical membrane of enterocytes (Porter et al., 2007).

The interactions of lipids with the uptake of lipophilic chemicals has led to the suggestion that increased lipid consumption affects the bio-availability of PAHs (Gobas et al., 1993). In previous *in vivo* studies, a fish oil based diet increased intestinal delivery and transfer of PAHs to the systemic circulation (de Gelder et al., 2016). 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.

2. Materials and methods

2.1. Animals

Rainbow trout (*Oncorhynchus mykiss*, weighing 200 to 600 g) were obtained from a commercial hatchery (Keijzersberg, Blitterswijk, 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 minute. Water temperature was kept at 15.0 ± 0.5 °C (mean \pm 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 1 h pre-feeding. Experimental design followed Dutch legislation and was approved by the local ethical review committee (RU-DEC 2012-315).

2.2. 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, viz. 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 Mg₂SO₄, 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 Mg₂SO₄, 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 ×g (K factor = 25.708). 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 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 20,200 ×g (K factor = 1779), the supernatant was collected and again centrifuged for 20 min at 43,500 ×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 43,500 ×g to obtain a compact pellet. The total isolation procedure lasted ca. 3.5 h and experiments started within 1 h after isolation.

2.3. 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) (Flik et al., 1983) and maltase (EC 3.2.1.20) (Dahlqvist, 1964) for brush border membranes, Na⁺/K⁺-ATPase (EC 3.6.1.3) (Flik et al., 1983) for basolateral membranes, and succinate dehydrogenase (SDH, EC 1.3.99.1) (Flik et al., 1983) and β-D-glucuronidase (EC 3.2.1.31) (Fishman and Bernfeld, 1955) 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 S1 of the supplementary data. Our preparations were enriched in BBMVs as evidenced by purification in maltase and enrichment factors of 11, 9 and 6 for alkaline phosphatase in BBMVs 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 (Glover et al., 2003; Klaren et al., 1993; Pelletier et al., 1986).

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 $95.1 \pm 1.9\%$, $99.8 \pm 0.3\%$ and $86.6 \pm 6.1\%$ 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 BBMVs isolated from other fish species (Glover et al., 2003; Klaren et al., 1993).

2.4. Measurement of intravesicular space

Intravesicular space was determined according to Flik et al. (1990). BBMVs were diluted to $0.3 \text{ mg protein} \cdot \text{mL}^{-1}$ in mannitol buffer containing 150 mM NaCl, 100 μM D-mannitol labeled with $100 \text{ kBq} \cdot \text{mL}^{-1}$ D-[1- ^{14}C]-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 ($\text{cpm} \cdot \text{mol}^{-1}$) of the radiotracer. Radioactivity was measured 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 BBMVs present in the incubate).

The intravesicular space, calculated from the equilibrium mannitol content after 120 min (16 °C), was calculated to be $1.0 \pm 0.3 \mu\text{L} \cdot \text{mg protein}^{-1}$ (mean \pm SEM, $n = 4$) in the pyloric caecal membrane preparations, $3.3 \pm 0.8 \mu\text{L} \cdot \text{mg protein}^{-1}$ in the proximal intestinal and $1.9 \pm 0.9 \mu\text{L} \cdot \text{mg protein}^{-1}$ in the distal intestine membrane preparations, respectively.

2.5. Uptake and membrane association of PAHs in combination 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). Mixed micelles were formed by sonication in a water bath for 20 min (Oxley et al., 2007). BBMVs were diluted to $0.3 \text{ mg protein} \cdot \text{mL}^{-1}$ in assay buffer, to which 1 μM [7- ^{14}C]BaP (26.6 mCi $\cdot \text{mmol}^{-1}$; American Radiolabeled Chemicals, St Louis, USA) or 1 μM [9- ^{14}C]PHE (52 mCi $\cdot \text{mmol}^{-1}$; 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 $\cdot \text{mmol}^{-1}$; Campro Scientific, Berlin, Germany) or [9,10- ^3H]oleic acid (60 Ci $\cdot \text{mmol}^{-1}$; 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 stock and that of feral rainbow trout (11–18 °C) (Glover and Wood, 2008a,b). All assay buffers were pre-cooled 30 min before incubation.

Pilot experiments showed that PAHs and FFA associated very quickly, within seconds, with the BBMVs in the incubate. Incubating at 0 °C did not resolve this. We therefore chose to measure trans-membrane substrate uptake as the amount of substrate released from BBMVs following perturbation of the vesicular space by detergent treatment. Trans-membrane uptake in BBMVs was assessed by disruption of the vesicular membrane with saponin, releasing PAHs and FFA from the intravesicular space. BBMVs were loaded with PAHs and FFA for 30 min and sampled, after which saponin was added. Five minutes later the substrate content of the BBMVs was again sampled. The difference in radioactivity is the amount of substrate released from an intravesicular space and is interpreted to reflect trans-membrane 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 BBMVs. 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 F™, PerkinElmer Inc., Massachusetts, USA) and counted as described above.

2.6. Disturbance of the osmotic space and temperature dependence

To detect the most suitable membrane disrupting agent, BBMVs, isolated from the pyloric caeca, were incubated with assay buffer (10 mM sodium taurocholate, 150 mM KCl, 100 μM D-mannitol labeled with $100 \text{ kBq} \cdot \text{mL}^{-1}$ D-[1- ^{14}C]-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 $\cdot \text{mg protein}^{-1}$ or 0.1% Triton X-100 and allowed to incubate for 5 min to permeabilize the BBMVs. Saponin induced the release of mannitol from the intravesicular space (Supplementary Fig. 1), whereas Triton X-100 permeabilized the BBMVs to such an extent that no membrane protein was retained by the glass microfiber 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 BBMVs, 150 μmol mannitol $\cdot \text{mg protein}^{-1}$ was absorbed at 0 and 16 °C after 120 min, whereas no uptake was detected at 37 °C (Supplementary Fig. S1). A similar uptake of mannitol was observed at 0 and 16 °C in studies with BBMVs of rainbow trout (Glover et al., 2003). Therefore, 16 °C was used for all experiments.

2.7. 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 was assessed. One μM [7- ^{14}C]BaP or 1 μM [9- ^{14}C]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

Table 1

Total benzo[a]pyrene (BaP) and phenanthrene (PHE) content in brush border membrane vesicles derived from the pyloric caeca, proximal intestine and distal intestine (mean \pm SEM; $n = 16$). Vesicles were incubated with 1 μM BaP or PHE in the absence of eicosapentaenoic acid (EPA) and oleic acid. The fractions of BaP and PHE that were released from the intravesicular space upon membrane perturbation with saponin, and the fractions that remained associated to, or in, the brush border membrane after detergent treatment are given in percentages.

	Pyloric caeca	Proximal intestine	Distal intestine
BaP			
Total BaP (nmol·mg protein ⁻¹)	0.57 \pm 0.05	1.00 \pm 0.05	0.54 \pm 0.06
Intravesicular space (%)	32 \pm 4	18 \pm 3	42 \pm 5
Membrane-associated (%)	68 \pm 4	82 \pm 3	58 \pm 5
PHE			
Total PHE (nmol·mg protein ⁻¹)	0.14 \pm 0.03	0.29 \pm 0.03	0.09 \pm 0.03
Intravesicular space (%)	12 \pm 4	14 \pm 5	28 \pm 7
Membrane-associated (%)	88 \pm 4	86 \pm 5	72 \pm 7

that untreated glass vials had the highest recovery and lowest adsorption of PAHs (data not shown), therefore glass vials were used for all experiments.

2.8. Statistics

The effects of different concentrations of EPA and oleic acid on trans-membrane 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) (R Development Core Team, 2013). 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 parameters determined is presented in the supplementary material (Supplementary Tables S2–S7). 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 \pm 0.9% (mean \pm SEM). Due to inter-sample heterogeneity (138.7 \pm 33.9%) data of 0 μM FFA were not pooled.

3. Results

Table 1 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 mixed micelles, were absorbed across the intestinal brush border membrane into the intravesicular space (Fig. 1). Trans-membrane 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. 1A–C). In the distal intestinal preparations, EPA was 3.5 times more potent than oleic acid in inhibiting trans-membrane BaP uptake ($p < 0.040$). This pattern was observed in all three intestinal preparations, although not statistically significant in the pyloric caeca ($p = 0.23$) and proximal intestine ($p = 0.28$). In contrast to BaP uptake, trans-membrane PHE uptake was unaffected by oleic acid (Fig. 1D–F) and PHE uptake solubilized in EPA micelles was virtually undetectable. All regression model parameters for trans-membrane BaP/PHE uptake and effects of FFA are presented in the supplementary material (Tables S2–S7).

Similarly to the PAHs, EPA and oleic acid were absorbed across the intestinal border membrane into the intravesicular space of BBMV (Fig. 2). In the presence of BaP, trans-membrane EPA uptake was significantly lower when 1000 μM EPA was administered to the BBMV compared to 1000 μM oleic acid in all intestinal segments (Fig. 2A–C; $p \leq 0.011$). The decreased EPA uptake coincides with the decreased trans-membrane BaP uptake. Interestingly, in the presence of PHE, trans-membrane 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. 2D–E; $p < 0.001$). In contrast to trans-membrane uptake of oleic acid, EPA uptake did not increase dose-dependently in the distal intestine (Fig. 2F). The increased trans-

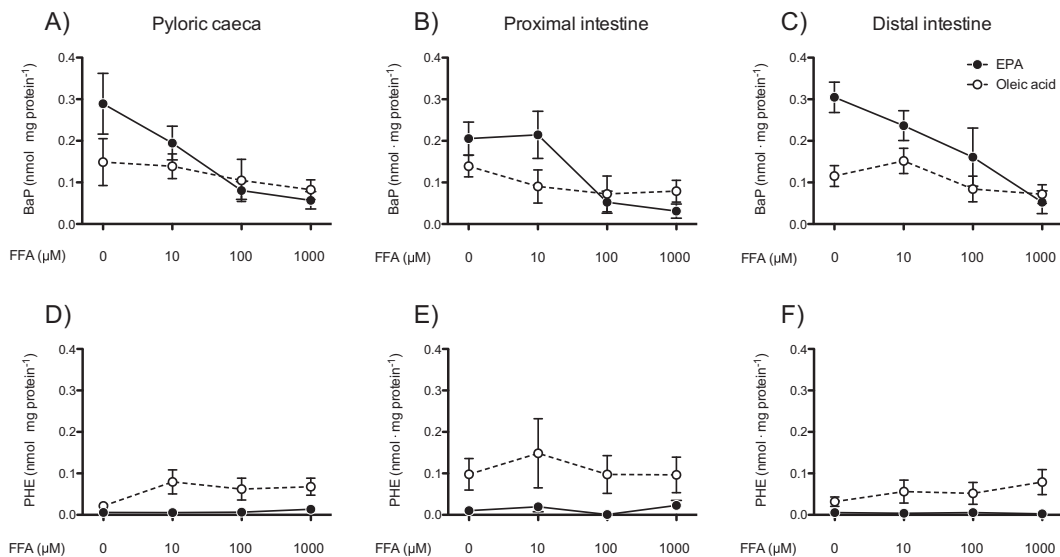


Fig. 1. Trans-membrane uptake of 1 μM benzo[a]pyrene (BaP) and phenanthrene (PHE) (nmol·mg protein⁻¹) as measured by detergent treatment of brush border membrane vesicles, isolated from the pyloric caeca (A, D), proximal intestine (B, E) and distal intestine (C, F). BaP and PHE were administered solubilized in mixed micelles composed of 10 mM bile salts + 0, 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–C) represent BaP uptake, the bottom panels (D–F) PHE uptake (mean values \pm SEM; $n = 8$).

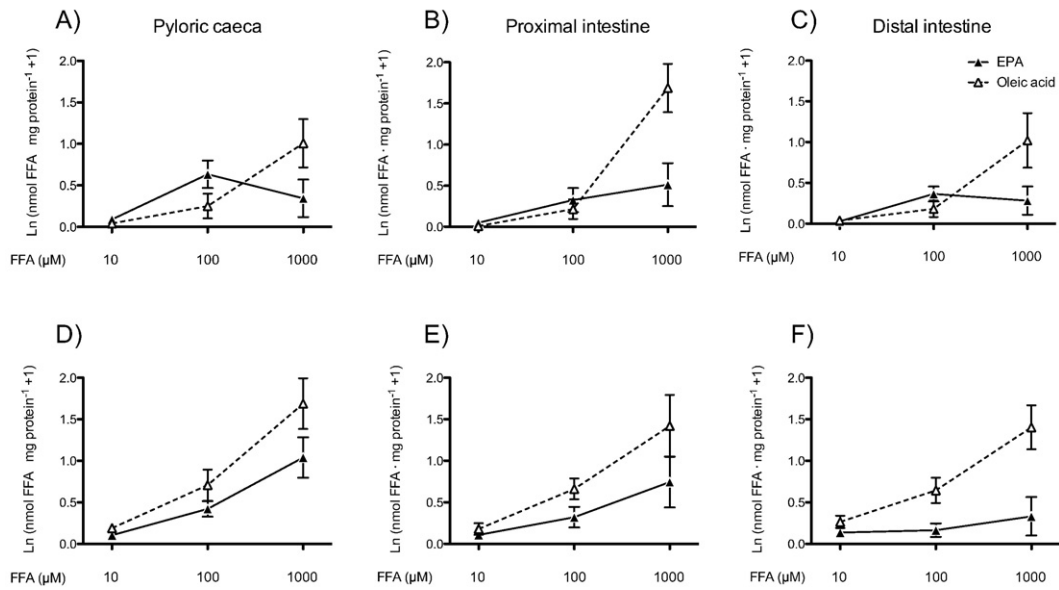


Fig. 2. Trans-membrane uptake of eicosapentaenoic acid (EPA) and oleic acid ($\text{nmol} \cdot \text{mg protein}^{-1}$) as measured by detergent treatment of brush border membrane vesicles (BBMV), isolated from the pyloric caeca (A, D), proximal intestine (B, E) and distal intestine (C, F) in the presence of $1 \mu\text{M}$ benzo[a]pyrene (BaP) and phenanthrene (PHE). EPA and oleic acid were administered in the form of mixed micelles composed of 10 mM bile salts and $10, 100$ or $1000 \mu\text{M}$ 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 \pm SEM; $n = 8$).

membrane uptake of EPA and oleic acid had no effect on trans-membrane PHE uptake.

As opposed to trans-membrane BaP uptake, the partitioning of BaP in or to the membrane was practically unaffected by FFA (Fig. 3A–C). Only oleic acid significantly decreased the membrane-associated BaP by 40% ($p = 0.001$) when $1000 \mu\text{M}$ oleic acid was administered to BBMV derived from the proximal intestine. The partitioning of PHE in or to the membrane was unaffected by FFA (Fig. 3D–F), as it was for trans-membrane PHE uptake.

4. Discussion

Prominent differences were seen between trans-membrane uptake of BaP and PHE across the intestinal brush border membrane of rainbow

trout. In the presence of FFA, trans-membrane 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 mixed micelles composed of triacylglycerides and FFA compared to when only bile salts were present (Weber and Lanno, 2001). 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 trans-membrane 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

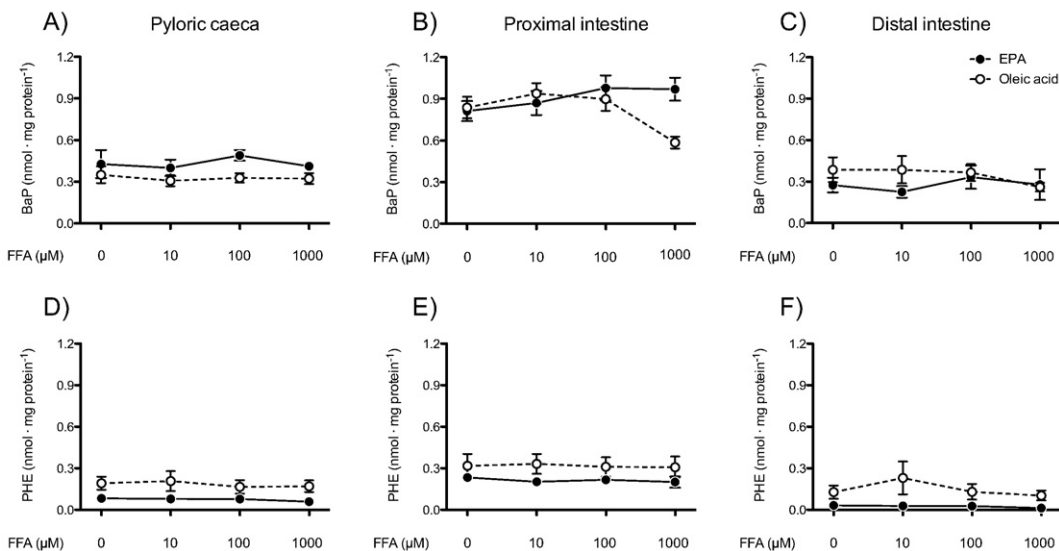


Fig. 3. Partitioning of $1 \mu\text{M}$ benzo[a]pyrene (BaP) and phenanthrene (PHE) ($\text{nmol} \cdot \text{mg protein}^{-1}$) to or in the brush border membrane after detergent treatment of brush border membrane vesicles, isolated from the pyloric caeca (A, D), proximal intestine (B, E) and distal intestine (C, F). BaP and PHE were administered solubilized in mixed micelles composed of 10 mM bile salts + $0, 10, 100$ or $1000 \mu\text{M}$ 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 \pm SEM; $n = 8$).

reported that dietary uptake is less efficient for extreme lipophilic compounds (Drouillard and Norstrom, 2000; Gobas et al., 1988; Kelly et al., 2004). Diffusion through the unstirred water layer would be a rate-limiting step for high- K_{ow} chemicals whereas diffusion through the phospholipid bilayer is rate-limiting for low- K_{ow} chemicals (Kelly et al., 2004). In our brush border membrane preparation, however, the unstirred water layer is most likely lost during the isolation procedure (Fingerote et al., 1994). This would remove the rate-limiting step for BaP transport and could have led to the higher trans-membrane 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 chemicals such as PAHs (Liland et al., 2014; Qu et al., 2007) and could affect the membrane fluidity and ultimately lead to abnormal enterocyte and gut architecture (Yuen et al., 2007). 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 (Bakke et al., 2015; de Gelder et al., 2016; Solbakken et al., 1984). In contrast to the present BBMV model system, other studies with intact Caco-2 enterocytes showed a higher trans-cellular uptake of PHE compared to BaP (Cavret et al., 2004; Cavret and Feidt, 2005) which emphasizes the role of intestinal drug metabolism by the cytochrome P450 family (CYPs) on intestinal transport of different dietary PAH congeners (de Gelder et al., 2016). In the present BBMV 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 (Dressman and Thelen, 2009; Schlenk et al., 2008) that are removed during the isolation of BBMV.

EPA and oleic acid, administered as mixed micelles, decreased trans-membrane BaP uptake in BBMV in all sections of the intestinal tract. In analogy, a high-fat diet decreased intestinal uptake of orally administered lipophilic chemicals (PCBs and chlorobenzenes) in goldfish (*Carassius auratus*) and PCB 136 in adipose tissue of female C57BL/6 mice (Gobas et al., 1993; Kania-Korwel et al., 2008). Subsequently, fecal excretion of PCB 136 had increased in female mice in the highest fat diet (Kania-Korwel et al., 2008). Increasing the lipid content of a diet with a nonabsorbable mixture of long-chain fatty acids bonded by ester links to sucrose (Olestra) also increased fecal excretion of lipophilic chemicals (Geusau et al., 1999; Jandacek, 2005; Moser and McLachlan, 1999; Mutter et al., 1988). In a lipid-rich diet, less lipophilic chemicals are absorbed via the intestinal tract, probably because a higher luminal fat content reduces bioavailability of lipophilic chemicals. The reduced bioavailability of lipophilic chemicals with increasing fat content coincides with the more potent inhibition of BaP uptake when administered in mixed micelles composed of EPA compared to oleic acid. Because trans-membrane uptake of EPA was considerably lower than that of oleic acid in BBMV, a higher ambient EPA concentration and thus higher lipid content can reduce BaP bioavailability and uptake (Kania-Korwel et al., 2008).

In contrast to this study, in primary hepatocytes of Atlantic salmon EPA uptake was significantly higher than oleic acid uptake (Zhou et al., 2010). 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 (Luiken et al., 1999; Richards, 2004; Zhou et al., 2010). This suggests that FFA uptake is driven by passive diffusion as well as via a trans-membrane transporter dependent on a Na^+ gradient and that EPA uptake is more dependent on membrane-bound protein mediated uptake than oleic acid (Zhou et al., 2010). In this brush border membrane model FFA are translocated across the membrane alike PAH and the observed interactions likely occur at the level of trans-membrane uptake. Trans-membrane 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 significant lower trans-membrane 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 chemicals in enterocytes by facilitating movement across the unstirred water layer towards the brush border membrane of enterocytes (Doi et al., 2000; Dulfer et al., 1996; Kelly et al., 2004; Vasiluk et al., 2008; Vetter et al., 1985). Once lipophilic chemicals arrive at the brush border membrane, possible mechanisms for trans-membrane 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 trans-membrane uptake route when dietary uptake rates and absorption efficiencies of lipophilic chemicals 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 trans-membrane uptake would be mainly facilitated by mixed micelles (Gobas et al., 1993; Kelly et al., 2004). The fugacity theory, which states that lipophilic chemical flow occurs from a high fugacity compartment to a low fugacity compartment, was used to explain trans-membrane uptake of lipophilic chemicals (Gobas et al., 1993; Kelly et al., 2004). These authors hypothesized that the decreased uptake of lipophilic chemicals by dietary fat is driven by passive diffusion because lipid absorption concentrates lipophilic chemicals in the intestinal lumen and creates a lipid-generated fugacity gradient from the gut lumen towards the gastro-intestinal tract for lipophilic chemicals (Gobas et al., 1993). The present study showed that the presence of micelles did not facilitate trans-membrane 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 (Vasiluk et al., 2007, 2008). 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 mixed 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 (de Gelder et al., 2016). The present study shows that, in vitro, a high-lipid diet decreased PAH uptake across the intestinal brush border. Other in vitro studies with Caco-2 cells showed substantially different results than our brush border membrane model. For instance, the higher trans-cellular 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 trans-membrane 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.

5. Conclusion

We conclude that FFA and lipophilic PAHs interact in trans-membrane 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 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.cbpc.2017.02.003>.

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