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

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ABSTRACT

The inclusion of vegetable oils in aquafeeds introduces contaminating polycyclic aromatic hydrocarbons (PAHs) in salmonids. Since lipophilic PAHs solubilize in micelles composed of lipids, bile salts and fatty acids, dietary lipid composition can alter intestinal transepithelial PAH transfer. We studied the uptake of two PAHs, *viz.* benzo[*a*]pyrene (BaP) and phenanthrene (PHE), in rainbow trout (*Oncorhynchus mykiss*) intestine. We also investigated the effects of two fatty acids, *viz.* fish oil-derived eicosapentaenoic acid (EPA, 20:5*n*-3) and vegetable oil-derived oleic acid (18:1*n*-9) on intestinal uptake. Radiolabeled PAHs were solubilized in micelles composed of tritiated EPA and oleic acid, respectively, and administrated to intestinal segments mounted in Ussing chambers.

In the absence of micelles, PHE accumulation was two times higher than BaP in the mucosal and serosal layers of 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 by 30% in co-exposure with EPA-composed micelles compared to 80% with oleic acid micelles. BaP was not transferred across the intestine.

We conclude that the lipid composition of an 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.

1. Introduction

Fishmeal and fish oil in aquafeeds to marine carnivorous species are partially substituted by ingredients such as vegetable meal and vegetable oil and already extensively used in aquaculture (Gatlin et al., 2007; Sørensen et al., 2011; Tacon and Metian, 2008; Turchini et al., 2009; Ytrestøyl et al., 2015). 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*n*-3) and docosahexaenoic acid (DHA; 22:6*n*-3) are important sources of dietary lipids in fish oils. Rapeseed oil is mainly used as a substitute for fish oils (Sørensen et al., 2011) and is rich in oleic acid (18:1*n*-9) and linoleic acid (18:2*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 (Berntssen et al., 2010). PAHs are mostly formed by incomplete combustion of organic matter during toasting and/or gas drying of oil-producing seeds and grains (Dennis et al., 1991; Moret et al., 2005; Teixeira et al., 2007). 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 (Berntssen et al., 2010). BaP and PHE differ in their potential toxicological and physico-chemical properties. BaP is highly lipophilic (octanol/water partition coefficient, log

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K_{ow} = 6.31) and an agonist of the aryl hydrocarbon receptor that induces the expression of the *cytochrome P450, family 1, subfamily A* (*cyp1a*) gene and its metabolism by Cyp1a (Lampen et al., 2004; Landers and Bunce, 1991). PHE is less lipophilic than BaP (log K_{ow} = 4.5) and is not an aryl hydrocarbon receptor agonist (Billiard et al., 2004) and is consequently less extensively metabolized than BaP (Cavret et al., 2004). The introduction of PAHs in aquafeeds raises concerns as to their potential toxic effects for fish and consumers (EFSA, 2008).

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 (Drouillard and Norstrom, 2000; Dulfer et al., 1998; Gobas et al., 1993; Kelly et al., 2004). Vetter et al. (1985) observed that throughout lipid digestion, absorption and assimilation, 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) (Bakke et al., 2010; Phan and Tso, 2001; Wang et al., 2013; Yeap et al., 2013). 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 (Doi et al., 2000; Dulfer et al., 1996; Kelly et al., 2004; Porter et al., 2007).

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 (Doi et al., 2000; Laher and Barrowman, 1983). 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 (de Gelder et al., 2016). 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 (Niot et al., 2009; Shiau, 1990). Alternatively, micelles are absorbed by enterocytes *via* a vesicle-mediated uptake process or are directly absorbed following a collisional mechanism (Porter et al., 2007). 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 (Gobas et al., 1993). Indeed, decreased transmembrane 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*) (de Gelder et al., 2017). Net transepithelial uptake of lipophilic toxicants is the net result of the concentration and chemical composition of lipids.

We here report on 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–labeled 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.

2. Materials and methods

2.1. 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 tap water per minute. 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 1 h 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).

2.2. 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 the 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 $CaCl_2$, 15 mM $NaHCO_3$, 1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 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% CO_2. 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 cm^2 (type P2305; Physiologic Instruments, San Diego, CA, USA). 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, USA) 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/CO_2 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 (Jutfelt et al., 2007; Zakelj et al., 2004). 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.

2.3. 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 (Sundell and Sundh, 2012).

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 zero mV. At 20-s intervals, two 5-mV pulses in opposite direction were passed across the intestinal segment and SCCs were measured. TER was calculated using Ohm's law.

Measurements were logged on a personal computer using a data acquisition system (DataQ DI-400, Physiological Instruments) and Acquire & Analyze software (v 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.

2.4. 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 (Oxley et al., 2007). 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 [7-^{14}C]BaP (American Radiolabeled Chemicals, St Louis, USA) or 2 μM [9-^{14}C]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-^{14}C]PHE or [7-^{14}C]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 3 h. 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·cm^{-2}. All half-cell samples were measured in duplicate and corrected by subtracting blank values.

Apparent permeability of a substrate, P_{app} (expressed in cm·min^{-1}), under steady-state conditions substrate was calculated using the equation:

$$P_{app} = \frac{dQ}{dt} \cdot \frac{1}{A \bullet C_0}$$

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_o (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 compared to the mucosal half-chamber).

2.5. 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 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 levels were expressed as pmol substrate per g tissue (wet weight).

2.6. Statistical analysis

Statistical analyses were performed using the statistical programming language R (version 3.2.3) (R Development Core Team, 2013). 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 prior to analysis of variance (ANOVA). 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

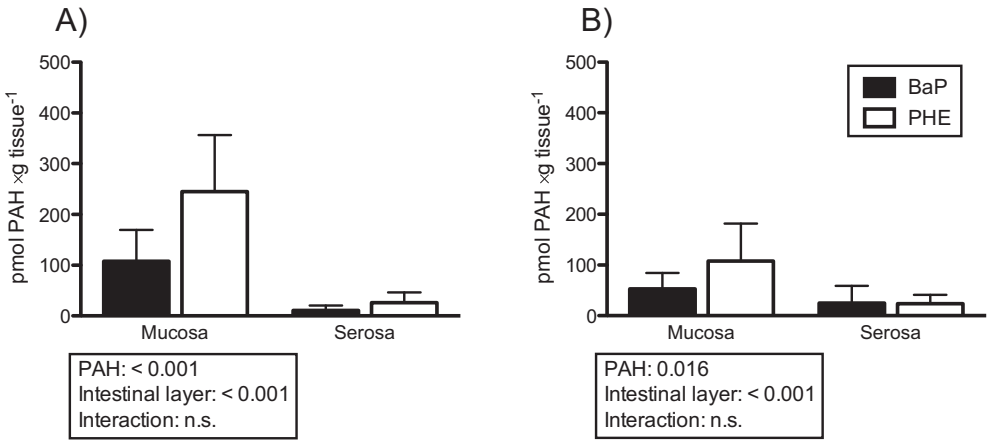

Fig. 1. Benzo[*a*]pyrene (BaP) and phenanthrene (PHE) accumulation in the mucosal and serosal layers of the proximal (A) and distal intestine (B). Intestinal segments were incubated with 1 μM BaP or PHE in the absence of eicosapentaenoic acid (EPA) and oleic acid. The *p*-values are for the main effects of the factors "PAH" (BaP, PHE) and "intestinal layer" (mucosa, serosa) and their interaction, as obtained from the two-way ANOVA; n.s., not significant. Bars represent mean values ± sd ($n = 16$).

overview of all models created and of all parameters determined is presented in the supplementary material (Tables S1–S12).

3. Results

3.1. 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. 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. 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. 1B).

BaP and PHE, solubilized in micelles, accumulated in the mucosal and serosal layers of the proximal (Fig. 2) and distal intestine (Fig. 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. 2C). The accumulation of PHE in the serosal layers of the proximal intestine was unaffected by EPA (Fig. 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. 3A–B). All regression model parameters for BaP/PHE accumulation and effects of FFA are presented in the supplementary material (Tables S1–S12).

3.2. 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. 4). Oleic acid accumulated 7.5 and 4.5 times more than EPA in the mucosal layers of the proximal and distal intestine, respectively ($p \leq 0.002$). The lower accumulation of

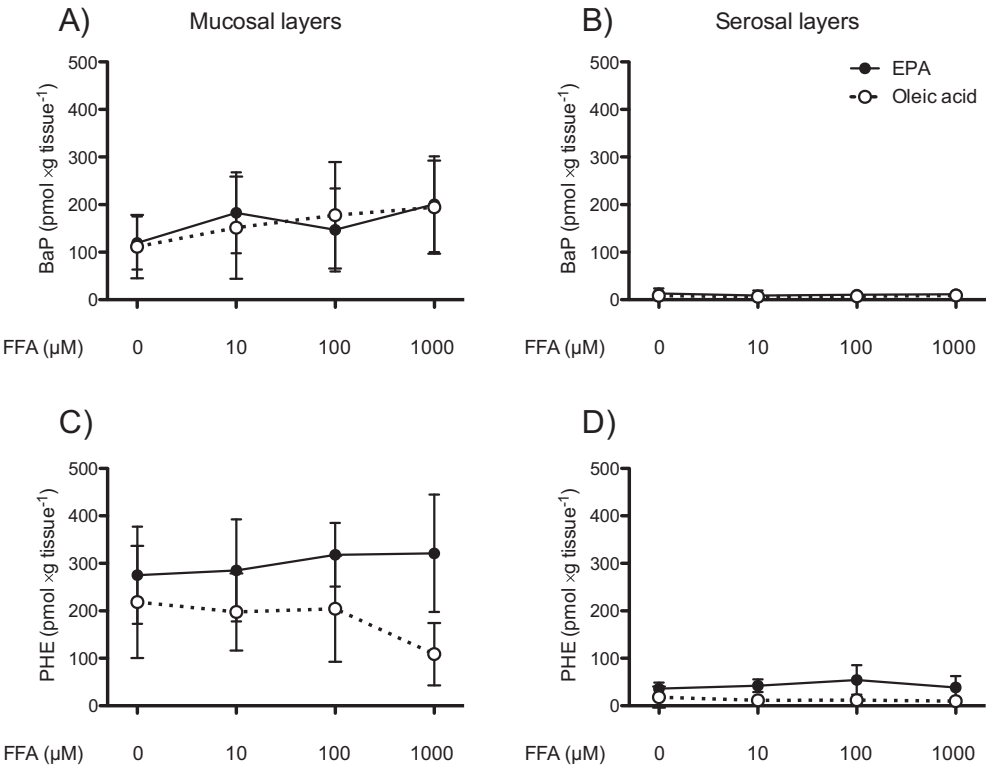

Fig. 2. Accumulation of 1 μM benzo[*a*]pyrene (BaP) and phenanthrene (PHE) in the mucosal and serosal layers of the proximal intestine. BaP and PHE were administered to the mucosal half-chamber solubilized in micelles composed of 10 mM bile salts without and with 10, 100 and 1000 μM eicosapentaenoic 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; $n = 8$).

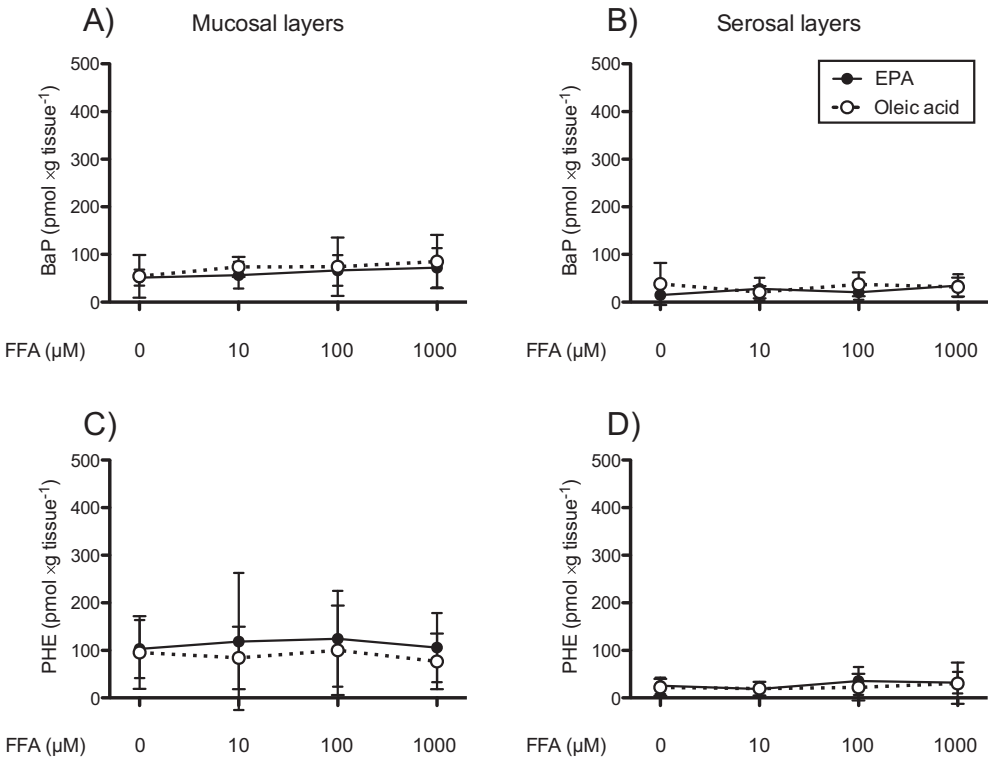

Fig. 3. Accumulation of 1 μM benzo[*a*]pyrene (BaP) and phenanthrene (PHE) in the mucosal and serosal layers of the distal intestine. BaP and PHE were administered to the mucosal half-chamber solubilized in micelles composed of 10 mM bile salts without and with 10, 100 and 1000 μM eicosapentaenoic acid (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; $n = 8$).

EPA compared to oleic acid correlated significantly ($R^2 > 0.74$; $p < 0.001$) with its 2–4 times higher transfer across both intestinal segments compared to oleic acid in the presence of BaP and PHE, respectively (Fig. 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 1).

3.3. 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 ($p = 0.003$) (Fig. 5). The PHE flux decreased by 40% when co-exposed with the highest EPA concentration. When PHE was administrated 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. The presence of FFA had no effect on the transepithelial transfer on any of the PAHs in the distal intestine.

Table 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 ($p < 0.05$).

Intestine	EPA (10^{-6} cm · min^{-1})		Oleic acid (10^{-6} cm · min^{-1})	
	BaP	PHE	BaP	PHE
Proximal	0.44 [a] ± 0.01	0.46 [a] ± 0.03	0.24 [b] ± 0.02	0.19 [b] ± 0.02
Distal	0.24 [a] ± 0.05	0.22 [a] ± 0.06	0.13 [b] ± 0.02	0.16 [b] ± 0.03

3.4. 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. S1–S4). 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 significantly increased, *i.e.,* less

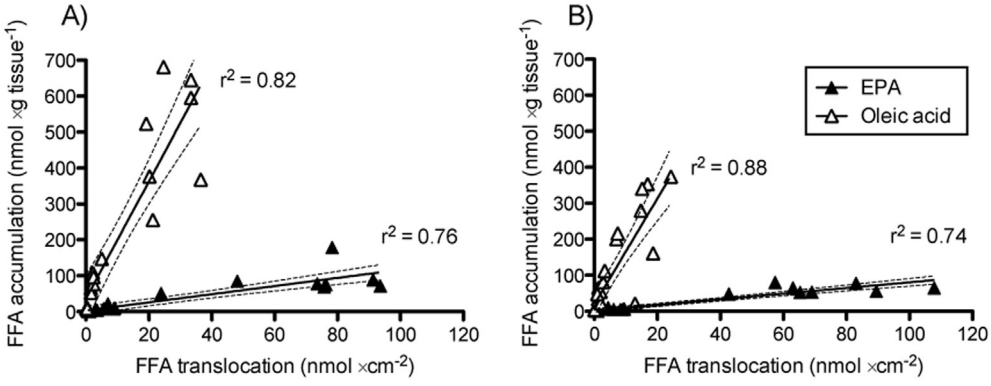

Fig. 4. Correlation between the accumulation and transepithelial transfer at $t = 180$ min of eicosapentaenoic acid (EPA) and oleic acid in the presence of 1 μM benzo[*a*]pyrene (BaP; A) and phenanthrene (PHE; B) in the mucosal layers of the proximal intestine ($n = 8$). EPA and oleic acid were administered 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.

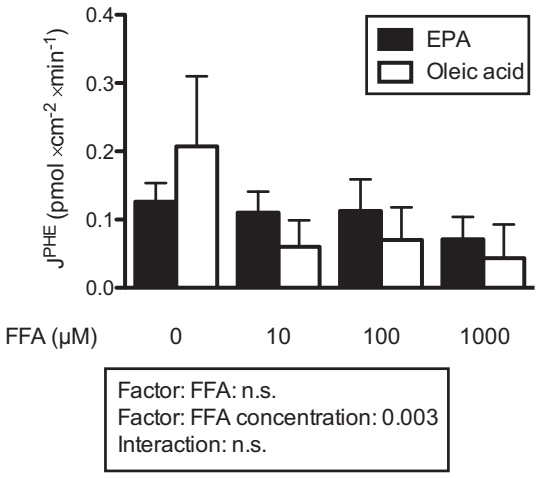

Fig. 5. Transepithelial transfer of phenanthrene (PHE) in the proximal intestinal after 180 min exposure. PHE was administered to the mucosal half-chamber solubilized in micelles composed of 10 mM bile salts without and with 10, 100 and 1000 μM eicosapentaenoic acid (EPA) or oleic acid. The *p*-values are for the main effects of the factors "FFA" (EPA, oleic acid) and "FFA concentration" (0, 10, 100, 1000 μM) and their interaction, as obtained from the two-way ANOVA; n.s., not significant. Bars represent mean values ± sd (n = 8).

negative, TEP and SCC values in the proximal intestine when PHE was administrated in micelles 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.

4. Discussion

The present study investigated the effect of FFA on transepithelial transfer of PAHs. 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 (de Gelder et al., 2016). Although both PAHs accumulated in the distal intestine, BaP and PHE were not transferred across this section of the intestine.

4.1. 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 (Drouillard and Norstrom, 2000; Gobas et al., 1988; Kelly et al., 2004). Diffusion of high-K_{ow} chemicals 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} chemicals (Kelly et al., 2004). Indeed, the presence of an unstirred water layer, adjacent to the brush border membrane of enterocytes, creates an absorption barrier that increases with molecule lipophilicity (Dulfer et al., 1998). This notion is supported by observations of the present study as well as previous studies (de Gelder et al., 2017). The transmembrane uptake of BaP was higher than PHE *in vitro* in intestinal brush border membrane vesicles that lack an unstirred water layer (de Gelder et al., 2017). In contrast, PHE had a higher absorption than BaP in intact intestinal epithelia that do contain 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 of rainbow trout. In support, PHE concentrations were higher compared to that of BaP in intact intestinal Caco-2 cells (Cavret and Feidt, 2005; Cavret et al., 2005). 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 associated enzymes and epithelial electrical properties (Lampen et al., 1998; Rousset et al., 1985) 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 (de Gelder et al., 2017). 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 (Wilson and Castro, 2010). The continuous blood perfusion of the lamina propria, which transports absorbed intestinal nutrients to peripheral organs *in vivo*, is abolished in *in vitro* 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 (de Gelder et al., 2016). 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 (Billiard et al., 2002, 2004). Intestinal Caco-2 cells have been shown to produce CYP1A and CYP3A that can metabolize xenobiotics (Lampen et al., 1998). When BaP was incubated with intestinal Caco-2 cells, it was more extensively metabolized than pyrene and PHE, and was 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 (de Gelder et al., 2016).

When PAHs are metabolized by Cyp450 enzymes, intestinal enterocytes excrete the metabolites into the intestinal lumen (Buesen et al., 2003; Cavret and Feidt, 2005). 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*) (Costa et al., 2012). The ABCG2 transporter is indeed involved in the efflux of BaP conjugates in Caco-2 cells (Ebert et al., 2005). Therefore, the absence of transepithelial BaP transfer, in contrast to PHE, could also have been caused by Cyp1a-mediated BaP metabolism followed by efflux of BaP metabolites to the mucosal half-cell (*i.e.,* intestinal lumen).

4.2. Effects of EPA and oleic acid 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 (de Gelder et al., 2016). The lipophilic core of micelles provides a more suitable hydrophobic environment for lipophilic compounds than the aqueous intestinal lumen (Dulfer et al., 1996). However, the composition of micelles can affect intestinal delivery and transfer of lipophilic compounds (Doi et al., 2000). Micelles composed of shorter fatty acid chain-lengths and a lower degree of unsaturated bonds have a decreased PAH solubility (Doi et al., 2000; Laher and Barrowman, 1983). 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 (Jutfelt et al., 2007; Krogdahl et al., 1999; Tocher, 2003). The higher mucosal accumulation of oleic acid compared to EPA coincides with previous *in vitro* studies performed with isolated enterocytes of rainbow trout (Oxley et al., 2005; Pérez et al., 1999). 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 (Bakke et al., 2010). Indeed, the 2 to 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 and distal intestine (Fig. 4). The apparent digestibility of saturated and long-chain monounsaturated fatty acids *in vivo* by fish is generally lower compared to PUFAs (Austreng et al., 1987; Geurden et al., 2009; Sigurgisladottir et al., 1992; Torstensen et al., 2000). 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 (Bakke et al., 2010).

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 sea bream (*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 (Caballero et al., 2003; Olsen et al., 1999, 2003). 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%.

The interactions between lipids and fatty acids, and lipophilic xenobiotics measured *in vitro* are observed *in vivo* as well. Indeed, a high-lipid diet decreased intestinal uptake of orally administrated lipophilic compounds (PCBs and chlorobenzenes) in goldfish (*Carassius auratus*) (Gobas et al., 1993). In female C57BL/6 mice, higher dietary lipids decreased PCB 136, which subsequently increased the fecal excretion of lipophilic compounds (Kania-Korwel et al., 2008). Furthermore, transmembrane uptake of BaP decreased in intestinal brush border membrane vesicle preparations of rainbow trout in the presence of high EPA and oleic acid concentrations (de Gelder et al., 2017). Lipophilic compounds are absorbed less by the intestinal epithelium in a high lipid diet probably because higher luminal lipid contents reduce their bioavailability (de Gelder et al., 2017; Kania-Korwel et al., 2008). 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 (Gobas et al., 1993; Kelly et al., 2004). 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 (de Gelder et al., 2017). However, intestinal segments used in this study constitute intact epithelia that still have an unstirred water layer. Therefore, since transmembrane (de Gelder et al., 2017) 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 (Jutfelt et al., 2006; Sundell et al., 2003; Sundh et al., 2010). The inverse of the TER, the conductivity, is regarded to reflect paracellular permeability, *i.e.,* conductance across tight junctions (Sundell and Sundh, 2012). 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 (de Gelder et al., 2017), PHE might be transferred *via* a second transport route (*e.g.,* paracellular) across the intestine.

4.3. 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 (1000 μM) FFA concentration, as evidenced by significant interaction effects on TEP (EPA and oleic acid) and SCC (oleic acid only) (supplementary materials Fig. S2, Tables S7 and 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 (Sundell et al., 2003; Sundh et al., 2011). 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 (Sundh et al., 2011). 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. Further, *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 (Mayol et al., 1999; Swarts et al., 1990). Therefore, the observed decrease in SCC and TEP could be due to an inhibition of the Na^+/K^+-ATPases activity by unsaturated fatty acids resulting in a reduced 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 (MacGregor and Walker, 1993) and why only the highest incubation concentration decreased intestinal electrogenic properties.

5. 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 $\mu g \cdot l^{-1}$, respectively, and are comparable with detected BaP and PHE concentrations of 1.0 and 17 $\mu g \cdot kg^{-1}$ in aquafeeds (Berntssen et al., 2010). 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-derived fatty acids could lead to an increased intestinal accumulation of fatty acids and possibly a decreased flux of vegetable oils to the systemic circulation. Ultimately, this could negatively affect 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2017.11.006.

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