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PCB-126 spiked to polyethylene microplastic ingested by juvenile Atlantic cod (*Gadus morhua*) accumulates in liver and muscle tissues

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ABSTRACT

In the present study, polyethylene (PE) microplastics (150–300 μm) were added to Atlantic cod (*Gadus morhua*) feeds at 1 %, either in their present form (Virgin PE) or spiked with PCB-126 (Spiked PE). The feeds were given to juvenile cod for a 4-week period. The fish grew from 11 to 23 g with no significant difference between dietary treatments. Cod fed spiked PE showed a significantly higher concentration of PCB-126 in liver and muscle samples compared to control and fish ingesting virgin PE. In accordance with the accumulation of PCB-126 in the liver, the expression of hepatic *cyp1a* was higher in cod fed spiked PE. Notably, we observed that spiked PE, as well as virgin PE, have an effect on skin. Overall changes indicated a reduced skin barrier in fish fed a diet containing PE. Indicating that PE itself through interaction with gut tissue may influence skin health in fish.

1. Introduction

Pollution of plastics and related contaminants is an emerging concern in the marine environment (Andrady, 2011; Cole et al., 2011). In contrast, the environmental presence of polychlorinated biphenyls (PCB) and their detrimental effects on aquatic life have been a concern for decades and caused political actions to ban the production and use of these chemicals in the 1970s (Markowitz and Rosner, 2018). Plastic pollution with its visible harm through entanglement and gastrointestinal blockage of birds, marine mammals and fish have in similar ways increased the societal awareness and political pressure to make humans less dependent on plastic products and find new solutions for reuse or alternatives (Rochman et al., 2013a). In addition to the visible plastic litter, there is also a great concern towards the presence and putative adverse effects of microplastics (particles <5 mm), either industrial produced (primary) or degraded plastics (secondary) (Wright et al., 2013). It has been demonstrated that microplastics can cause gastrointestinal blockage in smaller animals (Cole et al., 2015), affect nutrient utilization, or be absorbed into tissues (reviewed by Bhagat et al. (2020)).

Microplastics have a large volume to surface area that can facilitate adsorption of chemical pollutants, such as PCB (Koelmans, 2015). Moreover, it has been suggested that this interaction can be used as an indicator for environmental contamination and to calculate their concentrations in the water (Ogata et al., 2009). Adsorption of chemical pollutants may also increase the detrimental effect of microplastics (Capó et al., 2021; Rochman et al., 2013b), e.g., glycogen depletion, fat vacuolation, and single cell necrosis were observed at increased levels in liver of medaka fed marine-polluted polyethylene (PE) microplastics in comparison to medaka fed virgin PE. These observations were shown alongside an increased body burden of contaminants.

PCB-126 is a dioxin-like congener that is ubiquitously present in the environment. PCB 126 is hardly metabolized and known to bind to the aryl hydrocarbon receptor (Ahr) with high potency (Foekema et al., 2008). Ahr is ligand-activated transcription factor and member of the basic helix-loop-helix PER-ARNT-SIM superfamily and has been widely studied due to its role in mediating cellular responses to environmental pollutants. Ahr can act as a xenosensor and modulate the transcription of genes that encode proteins involved in the biotransformation of xenobiotic compounds, such as cytochrome P450 1A (Cyp1a) (Stegeman, 1989). Expression of cyp1α mRNA has been shown to increase in dose-dependent manner with exposure of PCB-126 to liver slices of Atlantic salmon (*Salmo salar*) and Atlantic cod (*Gadus morhua*) (Aranguren-Abadía et al., 2020; Lemaire et al., 2011). The ingestion, biotransformation, and accumulation of PCB 126 in fish is associated with toxicity, including changes in the thyroid function, oxidative stress in the liver

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etc. (Brown et al., 2004; van der Oost et al., 2003).

Atlantic cod is a commercially important marine fish species for fisheries in the north Atlantic Ocean. Atlantic cod has also commonly been used as an indicator species in marine pollution monitoring programs, such as the Protection of the Marine Environment of the North-East Atlantic (OSPAR) convention, and for water column monitoring of offshore petroleum activities in Norway. PCB pollution in Atlantic cod have been examined by Norwegian Food authorities for decades, while recent interest in microplastic pollution has financed some surveys on the presence of plastics in this species (although detection method to quantify different sizes and polymers of plastics are still in development). These studies have shown low abundance of plastic in Atlantic cod along the coast of Norway (9 of 302 individuals had plastic items in the stomach, Bråte et al. (2016)) and Newfoundland cod (5 of 205 gastrointestinal tracts of cod had plastic items, Liboiron et al. (2016)). Both studies detected mainly mesoplastics (5–25 mm), and both studies observed organic food in the cod that had ingested plastics and suggested that similar rate of gut clearance for plastic as for ingested food. Discussions related to particle sizes that can cross tissue barriers in animals are ongoing (Ogonowski et al., 2018) and suggested to be limited by the pore sizes of the barriers (e.g. gills and gut). However, in the review by Hussain et al. (2001), translocation across mammalian gut are observed of microplastics at sizes ranging from 0.2 to 150 μm (humans), 3–100 μm (dogs) 0.1–10 μm (rabbits) and 30–40 μm (rodents), and even larger microplastic sizes are observed in the liver of fish (Avio et al., 2015; Collard et al., 2017). However, microplastics >100 μm have not shown to effect barrier functions or cause intestinal inflammation (studied in rainbow trout (*Onchorynchus mykiss*) by Ašmonaitė et al. (2018)). But contaminants adsorbed to these could be transferred through the intestinal cells and microplastics have shown to affect skin, gills, and gut of the fish (Huang et al., 2022).

In the present study, we spiked PCB-126 to 150–300 μm PE microplastics. These were added at a concentration of 1 %, mixed with other feed ingredients and extruded to feed pellets that were given to juvenile Atlantic cod. Performance, PCB accumulation, effect on liver gene expression, and skin barrier properties were examined.

2. Material and methods

2.1. Microplastic source, spiking and feed production

Industrial PE powder (Plastinvent, Radøy, Norway) was sieved to a size distribution between 150 and 300 μm. A fraction of the sieved PE was spiked with PCB-126 (Chiron, Trondheim, Norway). The concentration of PCB-126 was based on previous studies showing induction of cyp1a in fish exposed to 10–18 ng PCB-126/g fish (Gagnon, 2002; Soffientino et al., 2010; Roy et al., 2011; Janz and Metcalfe, 1991; Newsted et al., 1995). The assimilation efficiency (AE) of PCB-126 from food with plastic has been shown to be from 13 % to 55 % (Grigorakis and Drouillard, 2018; Granby et al., 2018). To achieve a similar effect level in cod PCB-126 was spiked to about 5 μg/g plastic assuming an AE of 20 %, and 1 % plastic in the food. PCB-126 were therefore dissolved in acetone to a concentration of 0.16 mg/mL. 20 mL of the PCB solution was then mixed with 300 g of plastic powder and 500 mL methanol (Sigma-Aldrich, Stuttgart/Germany) in a Duran glass bottle with a plastic lid. The plastic suspension was mixed for one hour while shaking (125 rpm), before 670 mL milliQ water added sequentially every hour in a period of 4 h (total 2680 mL milliQ water). After the last addition of water, the suspension was mixed overnight. After a total of 24 h of mixing, the suspension was filtrated with vacuum equipment to remove methanol and water. The plastic was then rinsed with clean milliQ water three times, filtered, and let to airdry in aluminum foil trays for five days until it was completely dry.

Analysis on GC–MS/MS (ISO-17025) at SINTEF Industry (Trondheim, Norway) verified the content of PCB-126 in spiked PE microplastics (4.1 μg/g) compared to its content in virgin PE microplastics that was below detection limits for the method (<0.03 μg/g).

Three feeds were produced at Nofima Feed Technology Centre (Fyllingsdalen, Norway): control feed, and two feeds where 1 % of the wheat flower in the control was replaced by virgin PE or spiked PE in the feed mix prior to extrusion (Table 1). The concentration of PE in the feeds were at a level that have minimum effect on the nutritional composition of the feeds, assuming any effects on the fish of the two test feeds to be caused by the inclusion of virgin or spiked PE. The feeds were produced using a combined preconditioner and co-rotating twin-screw extruder system (TX-52, Wenger Manufacturing Inc., Sabetha, KS, United States), and dried in a dual layer carousel dryer (Model 200.2; Paul Klockner GmbH, Nistertal, Germany).

2.2. Fish experiment

All animal procedures and handling described in this study were carried out according to Norwegian Animal welfare laws and were approved by the Norwegian Animal Research Authority (Forsøksdyrutvalget; FOTS ID 24381). Atlantic cod juveniles hatched spring 2020 were transported from The Centre for Marine Aquaculture (Nofima, Kvaløya, Norway) to University of Bergen, Bergen High Technology Centre (Norway) September 2020. In total 450 juvenile Atlantic cod (average BW 10.3 g) were distributed randomly in 9 tanks and fed 1.5 mm AgloNorse® (Trofi, Tromsø, Norway) *ab libitum*. After three weeks acclimatization the juveniles were re-distributed in the tanks after weighted in bulk and counted equally into nine $1m^3$ tank (n = 48–49 fish), and thereafter fed the respective diets to triplicate tanks for 34 days at the following conditions: 24 h light, flow-through sea water, 34 ‰ salinity, 9.3 ± 0.2 °C, and oxygen saturation at 87.4 ± 1.6 %. The diets were fed continuously based on Nofima feeding tables.

At termination of the trial, October 2020, 5 fish per tank were euthanized with a lethal dose of Finquel (100 mg/L). Length and body weight of each fish were measured. Samples of muscle and liver for PCB-126 analysis were pooled per tank and frozen on dry ice and stored at −80 °C. Individual liver tissue for *cyp1α* analysis by qPCR were placed in RNAlater (Themo Fisher Scientific, Waltham, MA, USA), while individual skin samples for histological examination were stored in formaldehyde. Length and body weight were measured of the remaining fish in the tanks. The specific growth rate was calculated as ((ln(final weight)-ln(start weight))/days in trial) × 100, and the Fulton's condition factor was calculated as body weight divided by $length^3$ × 100.

2.3. PCB analysis

PCB-126 in PE-powders was quantified by ISO-17025 on a GC–MS/MS after extraction of PCB from the PE using hexane and ultrasound at

Table 1
Composition experimental diets.

Ingredient composition (%)	Control	Virgin PE	Spiked PE
Fish meal	25.00	25.00	25.00
Soy protein concentrate	20.00	20.00	20.00
Wheat gluten meal	15.00	15.00	15.00
Corn gluten meal	5.00	5.00	5.00
Wheat flower	13.80	12.80	12.80
Møllers cod liver oil	6.00	6.00	6.00
Rapeseed oil	6.00	6.00	6.00
Soya lecithin	1.50	1.50	1.50
Cholineklorid	0.50	0.50	0.50
Virgin PE powder		1.00	
Spiked PE powder			1.00
Monosodiumphosphate	2.00	2.00	2.00
krillhydrolysat	2.00	2.00	2.00
Vitamin mix	2.00	2.00	2.00
Mineral mix	0.59	0.59	0.59
Stay C	0.10	0.10	0.10
Taurine	0.50	0.50	0.50
Yttrium oxide	0.01	0.01	0.01

SINTEF Industries (Trondheim, Norway). PCBs in fish meal, fish oil and feeds were analyzed by Conform EC 2017/644 (food) and EC 2017/771 (feed), while liver and muscle analysis of PCBs were performed with an internal method at Eurofins (Moss, Norway) using an GC–MS/MS to quantify PCB congeners.

2.4. Gene expression analysis

RNA was extracted from homogenized liver samples using the TRI Reagent User Guide (Thermo Fisher Scientific). The concentrations of the extracted RNA were determined with a NanoDrop^{Tm} One Micro-Volume UV–Vis Spectrophotometer (Thermo Fisher Scientific). The 260/280 nm absorbance ratios were >1.85 for all samples and the integrity of the RNA was confirmed using agarose gel electrophoresis. cDNA was generated from 1 μg total RNA following the instructions from the iScript cDNA Synthesis kit (Bio-Rad Laboratories Hercules, CA, USA). qPCR analyses of *cyp1a* and *actb* gene expression were conducted as described previously (Yadetie et al., 2018) The *actb* gene was used as a reference for normalization. Expression levels were compared between controls and treated samples using the ΔΔCq method (Schmittgen and Livak, 2008) and further statistical analysis was performed using the log2-transformed fold-changes in expression.

2.5. Histology analysis

Skin samples were carefully dissected, orientated and placed in tissue embedding cassette (Simport, Quebec, Canada). Samples were decalcified in EDTA (Merck KGaA, Darmstadt, Germany) solution, pH 7 for 2 days. Tissue processing was performed using an automated tissue processor (TP1020, Leica Biosystems, Nussloch GmbH, Germany) where the samples were dehydrated through 100 % alcohol and then a clearent Xylene bath before infiltration in melted 60 °C paraffin (Merck KGaA, Darmstadt, Germany). Paraffin-embedded tissue samples were cut in 5 μm sections using a Microtome (Leica RM 2165), mounted on polysin-coated slides (VWR, Avantor, Pennsylvania, USA) and dried overnight at 37 °C. The sections were deparaffinized and rehydrated, and staining was performed using an automated special stainer (Autostainer XL Leica Biosystems, Nussloch GmbH, Germany). Paraffin sections were stained with Alcian Blue Periodic Acid Schiff (AB/PAS, pH 2.5, Alcian Blue 8GX, Sigma Aldrich, Darmstadt, Germany). All slides were examined by light microscope slide scanner (Leica Microsystems, Wetzlar, Germany) and manually evaluated in Aperio Image Scope (Leica). Measurements for skin were done in a region of ca. 1000 μm per section. In each region, epidermal thickness and thickness of the club cell region were measured in 5 different locations of the selected regions in the dorsal and the ventral area, and mucous cells and club cells counted.

2.6. Statistics

Statistical analysis was performed using Statistica 14.0 (TIBCO Software Inc., Palo Alto, USA) for Windows. All results were subjected to analysis of variance (ANOVA) for the mean effect of the dietary treatment, followed by Tukey post hoc test for differences between diets. Significant differences were observed at $P < 0.05$.

3. Results

There were no differences between the dietary groups in growth, condition factor or mortality of Atlantic cod in the present study (Table 2). The juveniles grew from a body weight of 10–11 g at start to 23 g at termination of the experiment, with a specific growth rate of 2.3–2.5 % per day. Low mortality, < 5 %, were observed in the experiment.

The spiked PE-powder were analyzed to contain 4.07 ± 0.72 μg PCB-126 per g plastic powder, while virgin-PE content of PCB-126 was below detection limits for the method (< 0.033 ± 0.006 μg/g). Spiked-PE included at 1 % in the feed resulted in a PCB-126 content of 112,000 pg/g feed, while the control feed had a PCB-126 content of 85 pg/g, and 49 pg/g in feed included 1 % virgin PE (Table 3). The two latter feeds contained PCB-126 that was mainly derived from fish meal (19.8 pg/g), while the cod liver oil contributed with a low content (0.9 pg/g). Lower content of PCB-126 in feeds with virgin-PE could be due to that some of the PCB have sorbed to the plastic and was not extracted during analysis. The content of PCB-126 in spiked PE feed were reflected in the content of liver and muscle, and with a significantly higher content of PCB-126 in the tissues compared to control and virgin PE feed groups (ANOVA, $P < 0.01$). The content of PCB-126 were in juveniles fed Spiked PE magnitudes higher in liver (301.667 pg/g) compared to the muscle (2157 pg/g).

There was no statistically significant difference in the *cyp1a* expression in the liver of juvenile cod fed with 1 % virgin PE and the control group (1.3 fold-change). However, the hepatic *cyp1a* expression in juveniles fed PCB-126 spiked PE was significantly higher in comparison to the control, demonstrating a 1.8 fold higher expression of this gene (ANOVA, $P < 0.001$) (Fig. 1).

Histology on skin revealed that both virgin PE and spiked-PE affected skin in a similar manner (Fig. 2). In both groups, the thickness of the epidermis, the thickness of the club cell layer and the number of mucous cells and club cells per 100 μm were reduced compared to fish fed control feed, both at the dorsal and the ventral side of the lateral line. Dietary differences were also observed in the morphology of the keratocytes, in fish fed virgin PE and PE-spiked feed the outermost layer of keratocytes in the epidermis had breaches and appeared either with looser structure or with more condensed cells compared to control.

4. Discussion

Microplastic pollution is an increasing concern in the marine environment, and its association with persistent chemicals can potentially increase any detrimental effects of microplastics in aquatic animals (Ašmonaitė et al., 2018; Teuten et al., 2009). In the present study we showed that sorbed PCB-126 to PE microplastics are transferred to tissues in Atlantic cod and increase detoxification enzyme activity in the liver. The microplastics had a size range of 150–300 μm, which are assumed not to be absorbed through the gut tissue of the juvenile fish (Bhagat et al., 2020), demonstrating that larger microplastics not absorbed into tissues may still harm the animals through transfer of hazardous environmental chemicals.

Transport pathways for microplastics across barriers are scarcely studied but are in mammals believed to occur through endocytosis or

Table 2
Fish performance.

	Control			Virgin PE			Spiked PE			ANOVA (*P*-value)
Body weight (g) start	11.00	±	1.00	10.67	±	0.33	10.00	±	0.58	0.609
Body weight (g) end	23.45	±	0.39	23.13	±	1.05	23.07	±	0.28	0.914
Length (cm) end	14.19	±	0.05	14.01	±	0.18	14.05	±	0.08	0.539
K-factor end	0.82	±	0.01	0.84	±	0.02	0.83	±	0.01	0.509
Specific growth rate	2.25	±	0.25	2.27	±	0.08	2.47	±	0.18	0.676
Mortality (%)	4.76	±	1.80	2.72	±	2.72	2.05	±	0.01	0.702

Mean ± S.E., n = 3. Statistics by one-way ANOVA ($P < 0.05$).

Table 3
PCB-126 content (pg/g) in feed, liver and muscle at termination of the experiment.

	Control			Virgin PE			Spiked PE			ANOVA
Feed	85			49			112			
Liver	1901	±	1463[a]	260	±	98[a]	301,667	±	9387[b]	<0.001
Muscle	64	±	32[a]	564	±	393[a]	2157	±	162[b]	0.002

Mean ± S.E., n = 3. Statistics by one-way ANOVA, followed by Tukey post hoc test ($P < 0.05$).

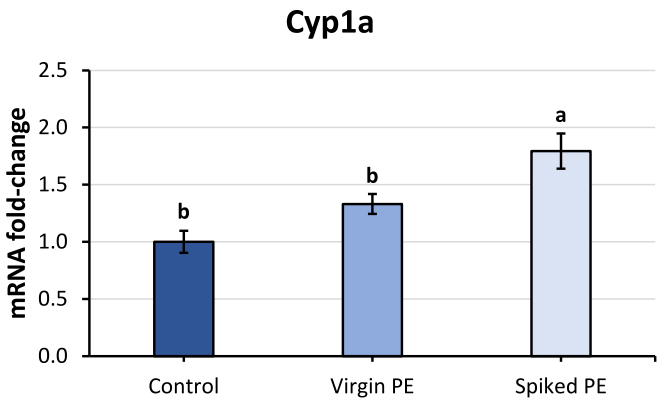

Fig. 1. qPCR analyses of liver *cyp1α* mRNA expression in juvenile cod exposed to virgin PE, Spiked PE, and control feed (mean ± S.E., n = 15). Statistics by one-way ANOVA mean effect of diet ($P < 0.001$), followed by Tukey post hoc test showing significant differences ($P < 0.05$) with superscript letters.

phagocytosis by macrophages and transported into tissues (Wright and Kelly, 2017; Yoo et al., 2011). Environmental conditions and health can affect the barriers' ability to resist absorption of unwanted substances. Uptake of microplastics into or via barriers may increase due to increased permeability of tissue barriers caused by e.g. diseases (Schmidt et al., 2013), inflammation (reviewed in Ahmad et al. (2017)) and temperature (Dokladny et al., 2006), but this has not been studied in fish. Furthermore, even though microplastics are not absorbed, they could cause reduced nutrient digestibility/absorption due to interactions with enzymes, reduced appetite due to gut filling of unabsorbed content, and gastrointestinal blockage at extreme high concentration (Wright et al., 2013). Unabsorbed microplastics can also cause intestinal inflammation due to contact with mucosal surfaces, and can even be incorporated in the intestinal tissue (Pedà et al., 2016). This could increase gut retention time of the microplastic, and as such to a larger extent desorb attached contaminants that subsequently could be easily absorbed by the animals.

However, contaminants attached to the microplastics can also be desorbed to the feed matrix during feed production, storage and during digestion. These will then be absorbed associated with nutrients, particularly those with lipophilic properties (Ašmonaitė et al., 2020). PCB sorbed to microplastics and tube fed to fasted cod larvae with no dietary input, was not desorbed from the microplastic during passage through the gastrointestinal tract (Norland et al., 2021). In the present study, we cannot say with certainty the mechanisms for desorption of PCB-126 from microplastic and how it was absorbed into the fish. However, spiked PCB-126 to PE microplastics were transferred to liver and muscle in juvenile Atlantic cod showing significantly higher tissue content of PCB-126 compared to fish given control feed or feed with 1 % virgin microplastics. The concentration of PCB-126 in the liver was twice as high (301,667 pg/g) as in the feeds (112,000 pg/g), reflecting the accumulation of contaminants in the liver due to its high fat content in cod. While the lean muscle composition of this species is reflected with much lower content of PCB-126 (2157 pg/g). Although, the other groups had significantly lower content of PCB-126 in the tissues were there observed higher level than in the feed and large variation between tanks. Liver composition of PCB-126 in the control group were 7-folds higher compared to fish fed virgin PE, while in muscle samples were the concentration 9-folds lower in the control compared to virgin PE. Fat content in the tissue and extraction efficiency of fat by the method could affect the calculated content of PCB-126. Furthermore, individual variation may have affected the composition in the pooled tissue samples per tank.

Expression of *cyp1a* mRNA of 15 individual samples of liver per dietary group showed quite consistent results, with a significantly higher gene expression in liver sampled from cod fed PCB-126 spiked PE compared to the other groups. Furthermore, juveniles fed virgin PE appeared to show higher expression compared to the control group, although this was not statistically significant. The expression of *cyp1a* mRNA indicates increased activity of detoxification enzymes, which may also be stimulated by other chemical compounds, such as plastic additives. We have no information of such chemicals in the PE used in the present study, but it cannot be excluded as a possibility.

The sizes of microplastics used in the presents study (150–300 μm) are not assumed to be absorbed through the gut. However, we found changes in the skin that has previously been associated with reduced robustness of the skin in several species, including Atlantic cod, polar cod (Ytteborg et al., 2020) and Atlantic salmon (Karlsen et al., 2021). The observed changes did not indicate severe pathology, but reduced thickness of the epidermis, number of club cells and mucus cells, in addition to looser cell structures and more breaches, may indicate a weakened skin barrier. A weakened skin barrier may result in an overall less robust fish, weaker wound healing properties and make the fish more prone to infections, as a healthy epidermis is vital (Sveen et al., 2019). As we have previously observed PCB polluted PE to affect skin histology in cod larvae (unpublished), the observation that virgin PE also influences skin is interesting. This may further indicate that microplastics itself through interaction with gut tissue influence skin health in fish. These changes are in line with the findings that PCB-126 accumulated in liver and muscle and effected expression of *cyp1a* in both PE fed fish groups. In skin, most changes were found in the spiked PE, but the changes seen in Virgin PE too are alarming and should be further studied to evaluate the biological significance of these changes and the impact they may have on cod health and overall robustness.

5. Conclusion

Atlantic cod fed diets included 150–300 μm PE microplastics with and without PCB-126 were in the present study not shown to effect growth or mortality. PCB-126 spiked to PE were accumulated in liver and muscle and produced an increased expression of *cyp1a* in liver. However, minor differences in the *cyp1*a expression in liver and skin histology changes in dietary group fed 1 % virgin PE compared to the control, may indicate that PE itself have a negative impact on cod health. Further studies are therefore needed to understand intestinal interaction of microplastics on cod health.

CRediT authorship contribution statement

André S. Bogevik: Conceptualization, Investigation, Resources, Supervision, Visualization, Writing – original draft. **Elisabeth Ytteborg:** Formal analysis, Resources, Visualization, Writing – review & editing. **Alexander Klevedal Madsen:** Formal analysis, Writing – review & editing. **Ann-Elise Olderbakk Jordal:** Investigation, Supervision,

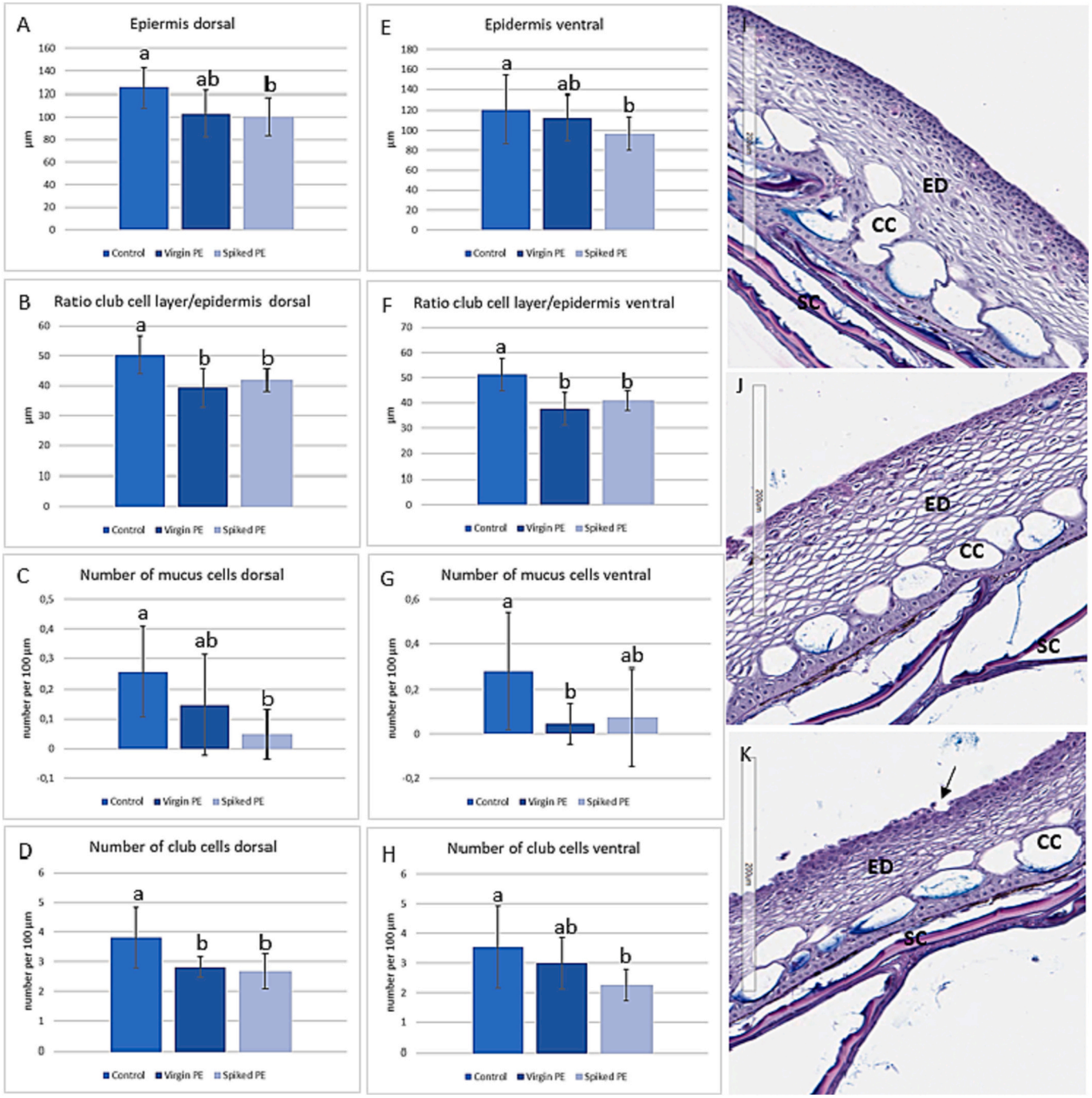

Fig. 2. Morphological measures of epidermal thickness, ratio club cell layer/thickness of epidermis, number of mucus cells and number of club cells in fish from the control, Virgin-PE and spiked-PE groups, measured in the A–D) Dorsal and the E–H) Ventral side of the skin (mean ± S.E., n = 15). Statistics by one-way ANOVA, followed by Tukey post hoc test showing significant differences ($P < 0.05$) with superscript letters. I–J) Representative images of the epidermis from I) control showing a dense keratocyte layer in the epidermis. Virgin-PE and spiked-PE had different morphological alterations in this layer, including J) looser structure in the epidermis and K) breaches in the epidermis (arrow). ED: epidermis, CC: club cell, SC: scale. Scale bar as indicated.

Writing – review & editing. **Odd André Karlsen:** Conceptualization, Formal analysis, Resources, Writing – review & editing. **Ivar Rønnestad:** Conceptualization, Investigation, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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