# Untangling mechanisms of crude oil toxicity: linking gene expression, morphology and PAHs at two developmental stages in a cold-water fish

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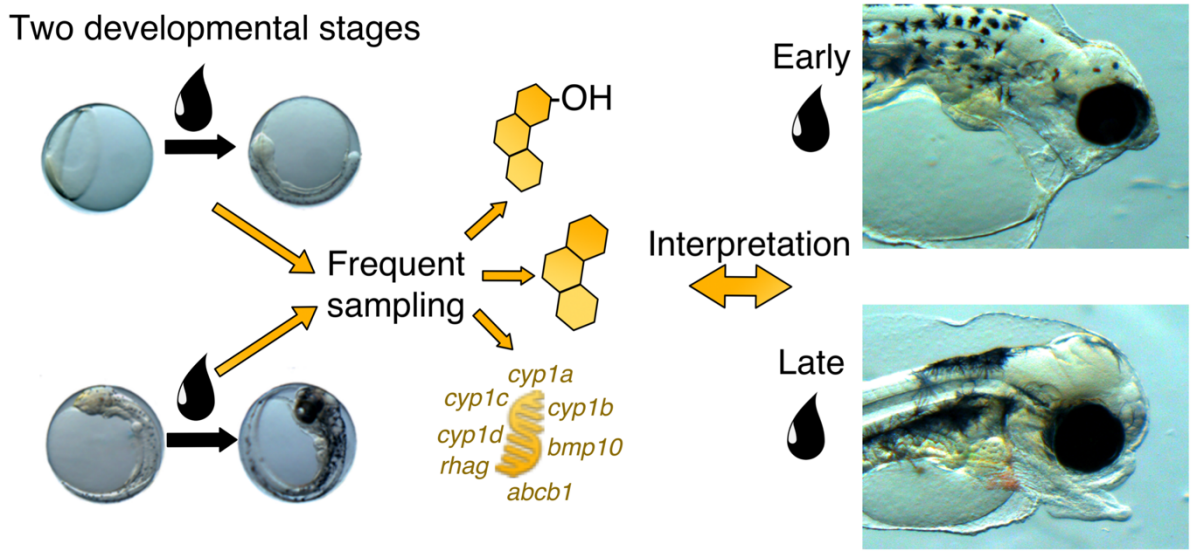
## Abstract

Early life stages of fish are highly sensitive to crude oil exposure and thus, short term exposures during critical developmental periods could have detrimental consequences for juvenile survival. Here we administered crude oil to Atlantic haddock (*Melanogrammus aeglefinus*) in short term (3-day) exposures at two developmental time periods: before first heartbeat, from gastrulation to cardiac cone stage (*early*), and from first heartbeat to one day before hatching (*late*). A frequent sampling regime enabled us to determine immediate PAH uptake, metabolite formation and gene expression changes. In general, the embryotoxic consequences of an oil exposure were more severe in the *early* exposure animals. Oil droplet fouling in the highest doses resulted in severe cardiac and craniofacial abnormalities. Gene expression changes of Cytochrome 1 a,b,c and d (*cyp1a,b,c,d*), Bone morphogenetic protein 10 (*bmp10*), ABC transporter b1 (*abcb1*) and Rh-associated G-protein (*rhag*) were linked to PAH uptake, occurrence of metabolites of phenanthrene and developmental and functional abnormalities. We detected circulation-independent, oil-induced gene expression changes and separated phenotypes linked to proliferation, growth and disruption of formation events at early and late developmental stages. Our study gives an increased knowledge about developmentally dependent effects of crude oil toxicity. Thus, providing more knowledge and detail to new and several existing adverse outcome pathways of crude oil toxicity.

## Graphical abstract




## Highlights

- Oil droplet fouling occurred in the whole water column and increased the oil toxicity.
- Early exposure resulted in higher PAH uptake due to lower metabolism resulting in more severe abnormalities.
- A rapid and circulation-indepenent regulation of *bmp10* suggested a direct oil-induced effect on calcium homeostasis.
- Expression of *rhag* indicated a direct oil-induced effect on osmoregulatory cells and osmoregulation.
- Severe eye abnormalities especially in the late exposure was linked to inappropriate overexpression of *cyp1b* in the eyes .


## Key words

Crude oil exposure, PAH uptake and metabolism, gene expression, cyp1a, cyp1b, cyp1c, cyp1d, bmp10, abcb1, rhag, development, Atlantic haddock, cold-water marine fish, phenotypes, cardiotoxicity, craniofacial, phenanthrene metabolites


## 1. Introduction

Globally, fish habitats are frequently impacted by accidental oil spills. For fish, consequences of crude oil toxicity are stage dependent, and early life stages (eggs and larvae) are thought to be highly vulnerable for several reasons. Early life stages drift and have no opportunity to avoid contaminated areas compared to juvenile and mature fish (Carroll et al., 2018; Olsen et al., 2010). Relative content of lipids and surface-area-to-volume ratio are higher

in early life stages compared to juveniles/adults, making them more susceptible to accumulation of lipophilic environmental toxicants (Petersen and Kristensen, 1998). Finally, critical developmental processes like patterning and organogenesis can be disrupted by crude oil exposure (Incardona, 2017; Pasparakis et al., 2019; Sørhus et al., 2017). However, there are large interspecies sensitivity differences, e.g. the Atlantic haddock (*Melanogrammus aeglefinus*) embryo is approximately 10 times more sensitive to crude oil than its close relative Atlantic cod (Gadus morhua) due to oil droplet fouling on the haddock chorion (Sørensen et al., 2017).

Crude oil is a complex mixture of thousands of different components, however, the content of polycyclic aromatic hydrocarbons (PAHs) appears to correlate to the toxicity in developing fish (Adams et al., 2014; Carls and Meador, 2009). Deactivation and excretion of the larger PAHs (4-6 rings) is initiated by activation of the aryl hydrocarbon receptor (AhR), which in turn induces PAH-degrading enzymes, such as cytochrome P4501A (Cyp1a). Cyp1a induction has for a long time been used as a sensitive biomarker for crude oil toxicity (Goksøyr, 1995). The detoxification process is divided into three phases, with activation of substrate in phase I by *e.g.* cyp1a, followed by conjugation in phase II, and finally transport and excretion in phase III (Wells et al., 2009). Although PAHs are themselves toxic, reactive intermediates (reactive oxygen species, ROS) created during the detoxification process can cause toxicity such as oxidative damage to cellular macromolecules and disruption of signal transduction (Wells et al., 1997).

Cardiac morphology abnormalities can be a result of AhR-dependent mechanisms (Wells et al., 1997), circulation abnormalities (Andres-Delgado and Mercader, 2016) and direct disruption of signaling pathways, i.e calcium (Ebert et al., 2005; Rottbauer et al., 2001; Sørhus et al., 2017). In addition, tricyclic PAHs are shown to cause cardiotoxicity through an AhR-independent manner (Incardona et al., 2004; Marris et al., 2019). Tricyclic PAHs are poor inducers of AhR, and instead act by blocking the repolarizing potassium efflux and a reduction in intracellular calcium cycling (Brette et al., 2014; Brette et al., 2017). The disruption of ionic signaling leads to defects in cardiac rhythm and contractility (Incardona, 2014; Incardona et al., 2009; Sørhus et al., 2016b) as well as calcium regulated cardiomyocyte proliferation (Ebert et al., 2005; Huang et al., 2012; Rottbauer et al., 2001; Sørhus et al., 2017). Beyond these circulatory defects that affect cardiac development (Andres-Delgado and Mercader, 2016), other extra-cardiac abnormalities can be secondary to circulatory defects, including craniofacial and eye development, and osmoregulatory and lipid metabolism deficiencies (Incardona et al., 2004; Incardona and Scholz, 2016; Laurel et al., 2019; Sørhus et al., 2017; Sun et al., 2019)

Numerous other pathways/endpoints have also been reported beyond the effects downstream of circulatory defects described above. PAH-related disruption of retinoid signaling interrupts eye development (Lie et al., 2019). Craniofacial development and growth can be disrupted by inappropriate signaling in formation processes like midline signaling (Abramyan, 2019; Delling et al., 2013; Kimmel et al., 2001) or secondary to impact on craniofacial muscle apparatus (Shwartz et al., 2012). Severe oil-induced aberrations lead to early larval mortality, yet even the smaller developmental impacts can affect survival later in life (Heintz et al., 2000; Hicken et al., 2011). Recently, a study linked oil-induced changes in lipid composition and dynamics to reduced growth and survival in polar cod (*Boreogadus saida*) (Laurel et al., 2019). Consistently, impacts on lipid metabolism pathways have been reported in several RNAseq studies (Sørhus et al., 2017; Xu et al., 2017). Several gene expression studies have started to unravel initiating events and key events of adverse outcome pathways of crude oil (Laurel et al., 2019; Lie et al., 2019; McGruer et al., 2019; Sørhus et al., 2017), but there are still knowledge gaps in understanding the underlying mechanisms of crude oil toxicity.

In this study we aim to improve our understanding of cause and effect relationships by linking immediate transcriptional changes to morphological phenotypes, PAH uptake and metabolism. A frequent and thorough sampling regime during and after exposure enabled us to detect immediate changes. We focused specifically on genes found interesting in a previous study (Sørhus et al., 2017) that are involved in various parts of development and function: cardiac development and calcium homeostasis, osmoregulation and ammonium waste excretion, transport of xenobiotic products, and enzymes possibly involved in phase I xenobiotic metabolism.

This study was designed to understand key events of adverse outcome pathways. First, exposure at the early embryonic stage prior to first heartbeat (2.5-5.5 days post-fertilization (dpf)) allowed us to detect circulation-independent, oil-induced gene expression changes. Second, exposure both during and after completion of organogenesis (7.5-10.5 dpf) enabled us to separate phenotypes linked to proliferation, growth and disruption of formation events at early and late developmental stages. Finally, increased knowledge about uptake, induction and rate of PAH metabolism helps to pinpoint sources of crude oil toxicity. A greater understanding of crude oil toxicity in the framework of adverse outcome pathways is essential for predicting more accurately both acute and delayed consequences of accidental and operational oil spills.

## 2. Materials and methods

### 2.1. Animal collection, maintenance and exposure set up

Fertilized Atlantic haddock (*Melanogrammus aeglefinus*) eggs were collected from tanks with wild-caught broodstock haddock maintained at the Institute of Marine Research, Austevoll Research station. The fertilized eggs were kept in indoor incubators (8±1°C), until transfer of ≈5000 eggs to 50 L green poly~~t~~ethylene plastic experimental tanks. Flow through in the tanks was set to 32 L/hr, the water temperature 8.0°C, and light regime was 12 hours light, 12 hours dark provided by broad spectrum 2x36W Osram Biolux 965 (Osram GmbH, Munich, Germany).

The crude oil used was a weathered blend from the Heidrun oil field of the Norwegian sea. The crude oil is representative of the oil found in Lofoten island areas, an area of both haddock spawning and potential oil industry development. The exposure regime was identical to previous studies (Sørhus et al., 2016b).

Two short (72 hours) exposure experiments were performed, hereafter referred to as *early* and *late* exposure (Fig. 1A). The *early* exposure started at 2.5 dpf and ended at 5.5 dpf (10% epiboly to 10-20 somite/cardiac cone stage), while the *late* exposure started at 7.5 dpf and ended at 10.5 dpf (30-40 somite stage to hatching gland stage). The experimental setup consisted of a control and three exposure doses in a total of 18 tanks (Fig. 1B): control (*C*) 0.0 µg oil/L; low (*L*) 30 µg oil/L; medium (*M*) 100 µg oil/L; and high 300 µg oil/L. In our previous oil exposure experiments with haddock embryos, eggs were allowed to float freely (Sørensen et al., 2017; Sørhus et al., 2015; Sørhus et al., 2016b). Haddock eggs have a natural high buoyancy which allocates them close to the surface, and are potentially exposed to any slick formation. Therefore, eggs were added to plankton mesh chambers (Fig. 1B) in four replicate tanks of each treatment and submerged into the water column (*sub*). To identify an effect of oil droplet binding at the surface, the high dose was administered in three ways: like the other doses, where the eggs were held submerged under the water surface (*H sub*); where the eggs were allowed to float at the surface (*H surf*); and where the eggs were allowed to float and the exposure water was filtered to remove oil microdroplets (water soluble fraction; *H WSF*).

After exposure, ≈1000 eggs were transferred to recovery flow through tanks with clean water where they were kept until termination of experiment at 3 days post-hatching (dph). Throughout this work, multiple terms are used to define timepoints, based on days post-fertilization (dpf), hours post-exposure start (hpes), and days post-hatching (dph) (Fig 1A).

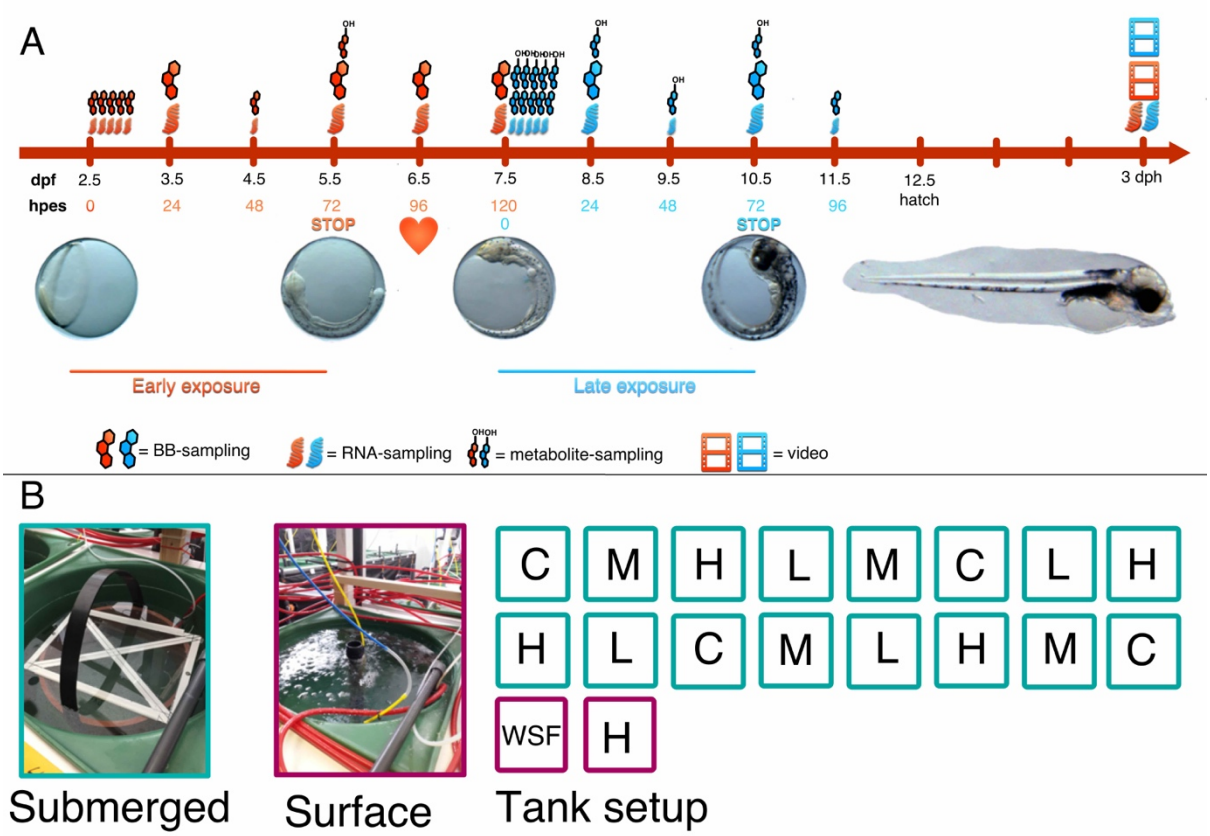



*Figure 1:* ***Experimental design and overview.*** *A) Timeline for experiments in relation to development. Atlantic haddock (Melanogrammus aeglefinus) embryos were exposed from 2.5 days post fertilization (dpf) to 5.5 dpf (early exposure) and from 7.5 dpf to 10.5 dpf (late exposure). Heart symbol indicates time point for observation of first heartbeat (6.5 dpf). Samplings were performed from 1-120 hours post exposure start (hpes) for the early exposure, and from 1-96 hpes for the late exposure. Videos were conducted 3 days post hatching (dph) for both experiments. Red and blue labeling of timepoints and sampling symbols represent early and late exposure, respectively. Small and large sampling symbols indicate sampling of high doses and control and sampling of all groups, respectively. B) Tank setup. C = control, L = Low dose, M = Medium dose, H = High dose, WSF = Filtered oil dispersion (water soluble fraction). The turquoise tanks were submerged.*

## 2.2. Sampling regime

167 Duplicate sample collection from all 18 tanks for total RNA extraction, body burden
168 and metabolites occurred at 24, 72 hpes and 3 dph for both experiments, as well as 96 and 120
169 hpes for the *early* exposure. A more thorough time series with triplicate samples was collected
170 from a tank of each of four selected treatments (*C*, *H sub*, *H surf* and *H WSF*) following the
171 sampling regime described in Fig. 1A, with sampling points at 1, 2, 4, 8, 12, 24, 48, 72, 96 and
172 120 hpes. Fewer replicates were taken for *H surf* and *H WSF* at some of the latest time points
173 due to high mortality. The precise number of eggs in each sample pool was counted from
174 microscope pictures.
175
176 **2.3. Analytical chemistry**
177 *2.3.1. PAH analysis in exposure water and body burden samples*
178 Water samples (1 L) were taken from each exposure tank at the beginning and the end
179 of each experiment, preserved by acidification (HCl, pH < 2) and then stored at 4 °C in the dark
180 until further handling. Details are given in Sørensen et al. (2017). In short, the water samples
181 were extracted twice by partitioning to dichloromethane (30 mL) in a separatory funnel (2 min).
182 Deuterated internal standards (naphthalene-d8, biphenyl-d8, acenaphtylene-d8, anthracene-
183 d10, pyrene-d10, perylene-d12 and indeno[1,2,3-c,d]perylene-d12) were added prior to
184 extraction to account for analyte~~s~~ loss during extraction. The combined extracts were
185 concentrated by solvent evaporation prior to analysis by GC-MS. The following PAH
186 compounds were measured: C0-, C1-, C2-, C3-, C4-naphthalenes (NAP), anthracene (ANT),
187 acenaphthylene (AC), acenaphtene (AE), C0-, C1-, C2-, C3-fluorene (FLU), C0-, C1-, C2-, C3,
188 C4-phenanthrene/anthracenes (PHE), C0-, C1-, C2-, C3-, C4-dibenzothiophenes (DBT),
189 fluoranthene (FL), C0-, C1-, C2-, C3-pyrene (PYR), benzo[a]anthracene (BAA), C0-, C1-, C2-
190 , C3, C4-chrysene (CHR), benzo[b+j+k]fluoranthene (BKF), benzo[e]pyrene (BEP),
191 benzo[a]pyrene (BAP), perylene (PER), benzo[g,h,i]perylene (BP), indeno[1,2,3-c,d]pyrene
192 (IND) and dibenzo[a,h]anthracene (DBA).
193 Samples for PAH body burden were collected frequently (Fig. 1A) to detect immediate
194 changes in PAH uptake. The 48 hpes time point for the *late* exposure was compromised during
195 analysis and data are not available. Each sample collected consisted of 50-100 pooled animals
196 preserved by flash-freezing in liquid nitrogen and stored at −80 °C. Tissue samples were
197 extracted by solid-liquid extraction and cleaned-up by solid phase extraction (SPE) prior to
198 analysis. The method is described in detail in Sørensen et al. (2017). Briefly, after addition of
199 internal standards the samples were homogenized in 2 mL dichloromethane-hexane (1:1, v/v),

200 followed by addition of sodium sulphate (150 mg) and two rounds of vortex extraction (30 sec)
201 and centrifugation (2000 rpm, 2 min). The combined organic extract was concentrated to ~1 mL
202 prior to clean-up by SPE (Agilent Bond Elut SI, 500 mg). The SPE columns were conditioned
203 with hexane and the extract eluted with 6 mL 10% dichloromethane in hexane. The purified
204 extract was concentrated to 100 µL under a gentle stream of $N_2$.
205 Water and body burden samples were analyzed on an Agilent 7890 gas chromatograph
206 coupled to an Agilent 7010c triple quadrupole mass detector (GC-MS/MS) as described
207 previously in Sørensen et al. (2017). For the body burden samples, a matrix-matched calibration
208 was used, made from the blank extraction of haddock and cod eggs.

209 *2.3.2. Phenatharene metabolite analysis in haddock eggs*

210 There are today no analytical methods that are able to detect the whole metabolome of
211 PAH in fish (Beyer et al., 2010). In the present study we have included detection of phenathrene
212 metabolites as representatives of complex PAH metabolism. We analyzed the main products of
213 phenathrene metabolism in fish which are dominated by dihydroxylated-phenanthrene together
214 with minor levels of monohydroxylkated-phenanthrenes (Goksoyr et al., 1987; Pampanin et al.,
215 2016; Pangrekar et al., 2003; Sette et al., 2013).

216 The following standards of target compounds were purchased from Chiron AS
217 (Trondheim, Norway): 1-hydroxyphenanthrene (1-OH-PHE), 2-hydroxyphenanthrene (2-OH-
218 PHE), 3-hydroxyphenanthrene (3-OH-PHE), 4-hydroxyphenanthrene (4-OH-PHE), 9-
219 hydroxyphenanthrene (9-OH-PHE) , *trans*-9,10-dihydroxy-9,10-dihydrophenanthrene (9,10-
220 diol-PHE) and surrogate standard 2-hydroxynaphthalene-d7 (2-OH-NPH-d7). The standard
221 *trans*-1,2-dihydroxy-1,2-dihydrophenanthrene (1,2-diol-PHE) was purchased from MRI
222 Global Carcinogen Repository (Kansas City, MO, USA) . Samples for metabolite analysis were
223 collected at 72 hpes in *early* exposure and 1-72 hpes in *late* exposure (Fig. 1A), flash frozen
224 and kept at -80 °C until analysis. Between 50-100 eggs pooled from the same treatment were
225 homogenized and a 50 µL aliquot of the homogenized mixture was diluted with water to 200
226 µL and spiked with surrogate standard. The homogenate was then added to phospholipid
227 removal cartridges (Phenomenex Phree cartridges) contaning methanol and eluted under
228 vacuum to remove phospholipids and proteins, followed by enzymatic hydrolysis by adding a
229 pH-5 buffer containing 2000 U of β-glucuronidase/sulfatase (Sigma-Aldrich) and incubating
230 for 1 hour at 40 °C. The hydrolysis reaction was then suspended with the addition of glacial
231 acetic acid. We used SPE with Strata-X 200 mg/6 mL cartridges (Phenomenex Inc.) pre-

232 conditioned with methanol and distilled water. Sample was added and washed with water and
233 63:37 methanol:water (v/v) before drying 30 minutes under vacuum. Target metabolites were
234 finally eluted using 65:35 methanol:diethyl ether (v/v). Final volume was reduced to 3 mL
235 before addition of internal standards and an aliquot was transferred for final analysis of the
236 PAH metabolites.

237 Instrumental analysis was performed with a Waters Acquity high performance liquid
238 chromatograph (LC) coupled with an AB Sciex QTRAP 5500 tandem mass spectrometer (MS)
239 operating in negative electrospray ionization mode with ion spray voltage of 4500 V and
240 multiple reaction mode (MRM) with the source temperature at 650 °C. The LC was equipped
241 with a 0.2-µm pre-filter followed by a 2.1 x 5.0 mm (1.7-µm particle size) reversed phase guard
242 column (BEH Shield RP18, Waters Co) and a 2.1 x 150 mm (1.7 µm particle size) reversed-
243 phase column (BEH Shield RP18, Waters Co). Water (solvent A) and methanol (solvent B)
244 were used as a mobile-phase with a 29-minute linear gradient as follows (solvent A/solvent B):
245 initial gradient was 50/50 at 0.2 mL/min; 15 min to 20/80 at 0.2 mL/min; 0.1 min to 100%
246 solvent B and 0.1 min to 0.3mL/min; held at 100% B for 6.8 min at 0.3 mL/min; 0.5 min to
247 return to initial gradient 50/50 at 0.3 mL/min; held at 50/50 for 6.0 min at 0.3 mL/min; 0.1 min
248 to 0.2 mL/min and kept for 0.4 min. The column temperature was maintained at 45ºC.

249 Sum OHPHEs (ΣOHPHEs) is the sum concentrations of 1-OH-PHE, 2-OH-PHE + 3-
250 OH-PHE (estimate concentration, as these are chromatographically co-eluting isomers), 4-OH-
251 PHE and 9-OH-PHE. Sum PHE-diols (ΣOHPHEs) is the sum concentration~~s~~ of 1,2-diol-PHE
252 and 9,10-diol-PHE. Sum PHE metabolites (Σ PHE metabolites) is the sum concentration~~s~~ of all
253 metabolites measured. The quantitative results were converted from ng metabolites/embryo to
254 ng/g by using average mass of one haddock egg (0.00218 g).

255

256 **2.4. Detection of Cyp1a activity**

257 Ethoxyresorufin-O-deethylase (EROD) assay was applied to detect Cyp1a activity.
258 Activity was measured at 72 hpes (5.5 dpf) in the *early* exposure. The embryos were at the end
259 of Organogenesis 1, at the 10-30 somite/cardiac cone developmental stage (Fridgeirsson, 1978;
260 Hall et al., 2004; Sørhus et al., 2016a). The procedure was performed as described in Gonzalez-
261 Doncel et al. (2011). Briefly, 7-ethoxyresorufin (7ER) was added to live embryos and the
262 Cyp1a-catalyzed fluorescing product, resorufin, was detected with fluorescence microscopy. A
263 stock of 7ER (48 mg 7ER/L) (Sigma-Aldrich, Germany) was made in 100% DMSO (Thermo
264 Fisher Scientific). Control, DMSO and crude oil exposed embryos were transferred to 24-well

265 plates where they were incubated in the dark for 1 hour in 20 µg 7ER/L or DMSO in sterile sea
266 water. After exposure the solution was drained off and exchanged with sterile sea water. Images
267 of 12-20 embryos per treatment were captured with a digital camera (SPOT Insight 5 Mpx)
268 coupled to a Nikon AZ100 fluorescence microscope. For each set of embryos, two fluorescence
269 images were captured using the rhodamine filter (Ex 545/25 nm and Em 605/70 nm) to detect
270 EROD activity and DAPI filter (Ex 350/50 nm and Em 460/50 nm) to detect the internal
271 fluorescence of PAH compounds. In addition, a corresponding brightfield image was captured
272 for each set of fluorescence. Exposure time and gain for all fluorescence images were set at 300
273 ms and 4 g. Semi-quantitative measurements of EROD fluorescence in the embryos was
274 estimated using the adjusted light intensity in the whole egg in the various treatments. Adjusted
275 light intensity was measuered using ImageJ software (Schneider et al., 2012;
276 http://imagej.nih.gov/ij/, 1997–2016) and was calculated using the following formula: Adjusted
277 light intensity = (light intensity in Treatment with 7ER)/(light intensity in Treatment with
278 DMSO).
279
280 **2.5. Imaging of live embryos/larvae and measurements of cardiac function**
281 Images were taken at all sampling points to define developmental stage, morphological
282 abnormalities and degree of oil droplet accumulation. At 3 dph, all groups were imaged to
283 assess morphological and cardiac functional endpoints. Images were collected for all exposure
284 tanks in both *early* and *late* exposures. Digital still micro-images and 20-second videos of live
285 3 dph larvae were obtained using an Olympus SZX-10 Stereo microscope equipped with a 1.2
286 megapixel resolution video camera (Unibrain Fire-I 785c) controlled by BTV Pro 5.4.1
287 software (www.bensoftware.com). Image calculations were calibrated with a stage micrometer.
288 Animals were immobilized in a glass petri dish filled with 3 % Methylcellulose (Sigma-
289 Aldrich, Germany) in seawater and kept at 8°C using a temperature-controlled microscope stage
290 (Brook Industries Inc. Lake Villa, IL. USA). Length of ethmoid plate, mandible, area of edema
291 and length, systolic and diastolic diameter of ventricle and atrium were measured using ImageJ
292 (Schneider et al., 2012; http://imagej.nih.gov/ij/, 1997–2016) with the ObjectJ plugin
293 (https://sils.fnwi.uva.nl/bcb/objectj/index.html) from images as described previously (Sørhus et
294 al., 2016b). Additionally, eye diameter was quantified, and eye shape and deformities were
295 described according to categories: normal shape (no), bend shape (be), irregular shape (irr),
296 protruding lens (pl). Six distinct craniofacial phenotypes were identified. Degree of looping of
297 heart was also described (normal looping (n), poor looping (p) and severly poor looping (sp)).
298 The area occupied by edema fluid was quantified as the difference between the total area of the

299 yolk sac and the area of the yolk mass, expressed as a percentage of total yolk sac area.
300 Similarly, ventricular and atrial diastolic (D) and systolic diameter (S) were used to estimate
301 the fractional shortening (FS = (D − S)/D). Measurements from both images and videos were
302 performed blind.
303
304 **2.6. Oil induced gene expression changes**
305 All animals collected for RNA extraction were frozen in liquid nitrogen and stored at
306 −80 °C after being imaged (as described in the previous section). Total RNA was extracted
307 from frozen pools with 10 eggs/larvae with Maxwell HT simplyRNA (Promega corporation)
308 according to the manufacturer's instructions using Biomek 4000 Automated liquid handler
309 (Beckman coulter). Quantity and quality of the extracted total RNA was checked using
310 Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and
311 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) respectively. cDNA was
312 subsequently generated using SuperScript VILO cDNA Synthesis Kit (Life Technologies
313 Corporation), according to the manufacturer's instructions, and was normalized to obtain a
314 concentration of 50 ng/µL. The described frequent sampling regime was followed to detect
315 immediate gene expression changes (Fig. 1A and see the previous section *"Sampling regime"*).
316 Specific primers and probes for real-time qPCR analysis of Atlantic haddock were
317 designed with Primer Express software (Applied Biosystems, Carlsbad, California, USA)
318 (*cyp1a* and the technical reference *ef1a)* or Integrated DNA Technology (IDT) probe and
319 primer design software (IDT Inc., Iowa, USA) (*cyp1b, cyp1c, cyp1d, abcb1, rhag* and technical
320 reference *rxrba*), according to the manufacturer's guidelines. Primer and probe sequences are
321 given in Supplementary table S1. Real-time qPCR assays were performed in duplicate, using
322 384-well optical plates on a QuantStudio 5 Real Time PCR System (Thermofisher Scientific)
323 with settings as follows: 95 °C for 20 s, followed by 40 cycles of 95°C for 1 s and 60°C for 20
324 s. Duplicates with standard deviation$^2$ ($SD^2 \geq 0.05$ were either rerun or eliminated from the
325 dataset. No-template control, reverse transcriptase enzyme control and genomic DNA controls
326 were included. For each 5 µl PCR reaction, a 1.5 µl cDNA 1:40 dilution (1.9 ng) was mixed
327 with 200 nM fluorogenic probe, 900 nM sense primer, 900 nM antisense primer in 1xTaqMan
328 Fast Advanced Master Mix (Applied Biosystems, Carlsbad, California, USA). Gene expression
329 data were calculated relative to control in the first sampling point using the ΔΔΔCt method
330 generating reference residuals from *ef1a* and *rxrba*, as described in detail in Edmunds et al.
331 (2014).
332

### 2.7. Localization of *rhag* gene expression

Location of *rhag* gene expression was examined using a method derived from previous protocols (Hall et al., 2003; Thisse and Thisse, 2008; Valen et al., 2016). We performed whole mount *in situ* hybridization on unexposed 10 dpf embryos to determine location of *rhag* in early life stages of fish. Detailed protocol can be found in Supplementary information.

### 2.8. Statistics

Statistical differences in Cyp1a activity, morphology, cardiac function, PAH water concentration, PAH body burden, and OHPAH metabolites were tested using one-way ANOVA with Dunnet´s multiple comparison in R (The R Foundation for Statistical Computing Platform). In the gene expression data, statistically significant differences were tested using both One-way and Two-way ANOVA with Dunnet´s multiple comparison in R. ~~for.~~ PAH water concentration, PAH body burden, OHPAH metabolites and gene expression data were log-transformed due to large differences in levels and thus standard deviation. In craniofacial and eye phenotypes and appearance of silent ventricle, statistical differences between treatments and control were evaluated using chi-square test. Statistical difference was denoted $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***). Error bars in all figures represent the standard deviation of the mean.

### 2.9. Ethics statement

The animals were monitored daily, and any dead larvae were removed. All sampled embryos and yolk sac larvae were euthanized immediately in liquid nitrogen. The Austevoll Aquaculture Research station has the following permission for catch and maintenance of adult Atlantic haddock: H-AV 77, H-AV 78 and H-AV 79. These are permits given by the Norwegian Directorate of Fisheries. Furthermore, the Austevoll Aquaculture Research station has a permit to run as a Research Animal facility using fish (all developmental stages), with code 93 from the national IACUC; NARA, although no approval is necessary to perform studies with fish embryos and yolk sac larvae.

## 3. Results

Overall, both *early* and *late* exposure resulted in functional and morphological abnormalities, but they were more severe in the *early* exposure. Tissue uptake of PAHs was higher in the *early* exposure, followed by a stronger, but delayed *cyp1a* induction. The *H WSF*

367 group showed very few oil induced abnormalities in both exposures. Gene expression patterns
368 were unique between *early* vs *late* exposures.
369
370 **3.1. Oil droplet fouling in surface and submerged embryos**
371 Oil droplet fouling was observed on embryos in both submerged (*L, M, H sub*) and
372 surface (*H surf*) exposures regardless of timing, and was evident after only 1 hour of exposure
373 (Fig 2A and B, top panels). The oil droplet fouling effect was higher in the surface (*H surf*)
374 exposure due to the accumulation of oil microdroplets on the surface (Fig. 2). While oil droplets
375 in the *early* exposure were distributed all over the surface, the *late* exposure embryos mainly
376 accumulated oil droplets at one area on the chorion (Fig 2B, filled black arrows). In the filtered
377 high dose group (*H WSF*), no oil droplets were detected on the chorion (Fig 2A and B, right
378 panel). After transfer to clean water, some of the droplets were washed off, however, in addition
379 to a reduced number of apparent oil droplets (brown), translucent droplets were now visible on
380 the surface (white arrow head Supplementary Fig. S1). This phenomenon was not observed in
381 the *late* exposure due to hatching that occurred shortly after end of exposure (11 dpf).

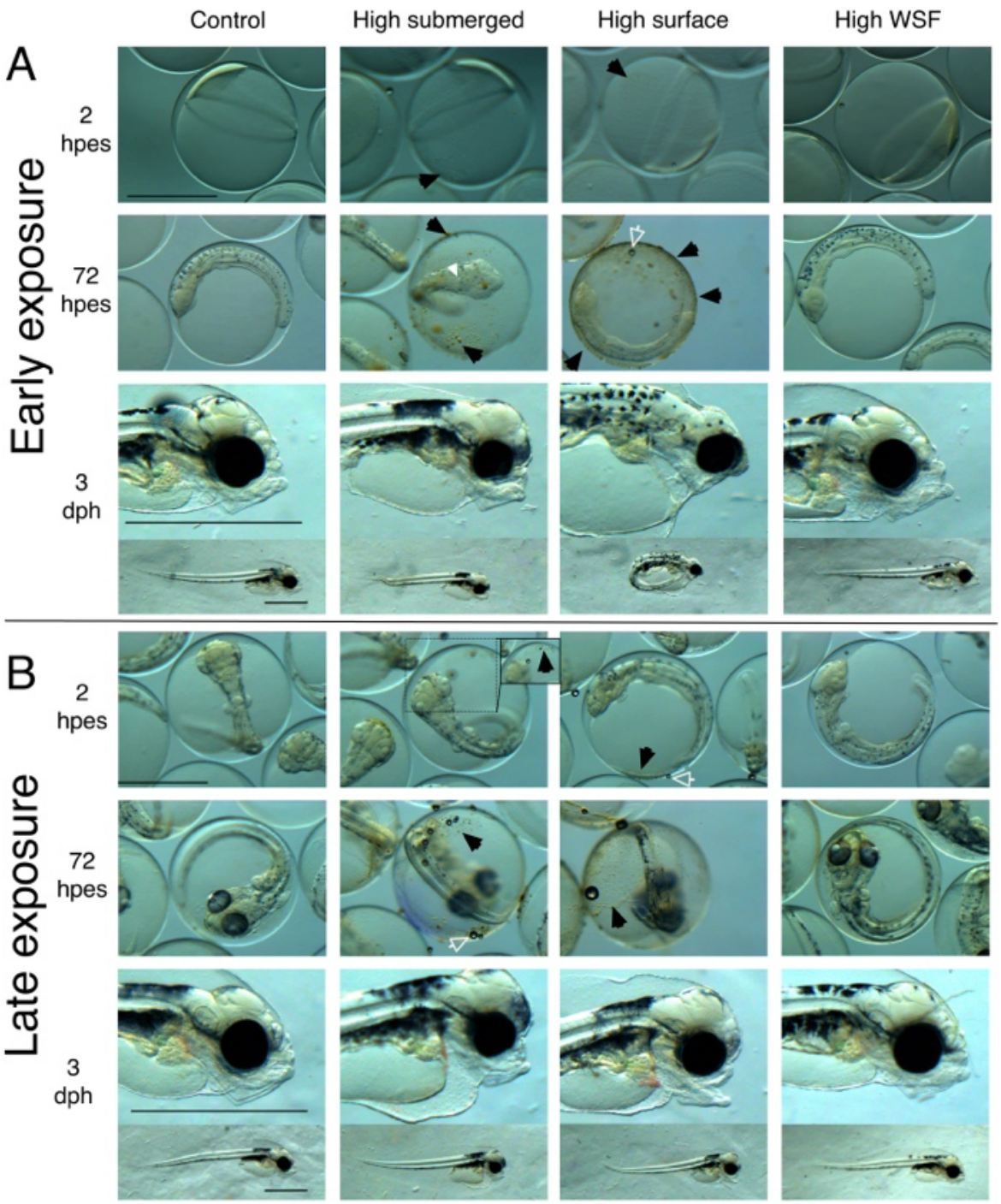



*Figure 2:* ***Oil droplet fouling and morphology.*** *A) early and B) late embryonic exposure of Atlantic haddock. Top panels show 2 hours post exposure start (hpes), middle panels 72 hpes (exposure stop) and lower panels display representative phenotypes at 3 days post hatching (dph). Black arrows : oil droplets. White arrow head: indicates cardiac cone. White open arrow: air bubble trapped on the chorion. WSF: water soluble fraction.*

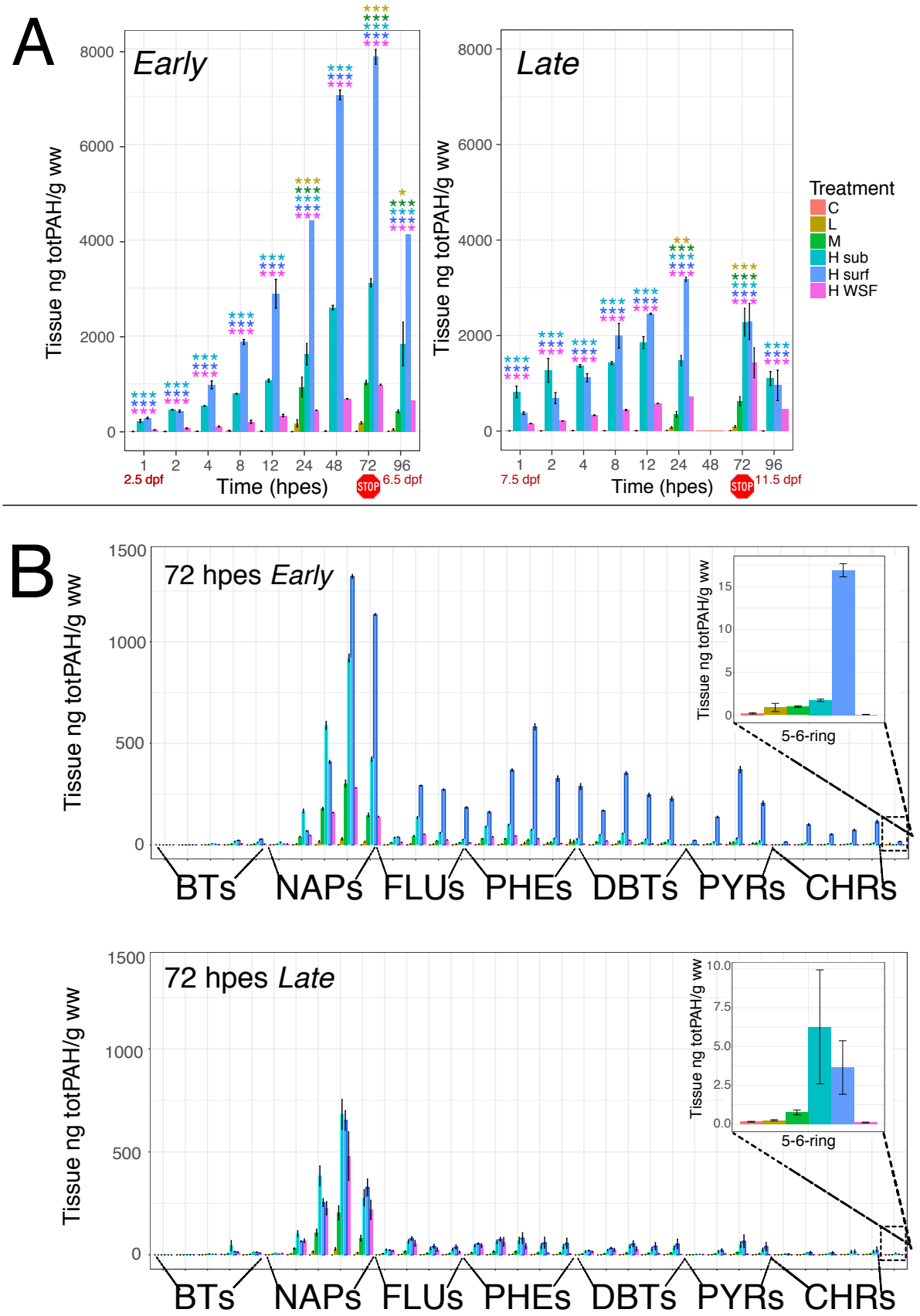
*Scale bar: 1000 µm.*

### 3.2. Oil exposure and PAH uptake

### *3.2.1. PAH water and tissue concentration*

We successfully delivered doses of dispersed crude oil to the exposure tanks during the experiments. Total PAHs (dissolved and droplet associated) were monitored at the beginning and end of each exposure experiment (Fig. S2) . The water concentration of PAH was lower in the *H WSF* than the other high dose groups, and reflected the PAH contribution from the oil droplets. There were some differences in the total PAH concentration between the two experiments, but there were ~~a~~ clear gradients among the different treatment in both studies: total PAH concentration (µg PAH/L): Average (start and stop) concentrations after *Early* exposure *C* = 0.04 +/- 0.01, *L* = 0.47 +/- 0.61, *M* = 2.3 +/- 2.4, *H sub* = 5.2 +/- 1.7, *H surf* = 6.4 +/- 0.8 , *H WSF* = 3.7 +/- 1.0. *Late* exposure C = 0.04+/-0.01, L = 0.5+/-0.2, M = 2.2+/-0.8, *H sub* = 9.7 +/- 2.7 *H surf* = 8.3 +/- 2.9, *H WSF* = 6.3 +/-0.9

An immediate and prominent uptake of PAHs into the embryo was detected (Fig. 3). Body burden measurements of all doses at all time-points in both experiments had statistically significant elevated levels of PAH compared to control. Average total PAH tissue concentrations in the exposed groups for *early* exposure ranged from 38 (1 hpes, *H WSF*) to as high as 7800 ng/g ww (72 hpes, *H surf*). Values in the *late* exposure were between 75 (24 hpes, *L)* and 3000 ng/g ww (72 hpes, *H surf*). Notably, despite higher water concentrations in the *late* exposure, the highest tissue concentrations were found in the *early* exposure. Moreover, the tissue concentration in the *early* exposure peaked at a later time point than in the *late* exposure (72 hpes vs 24 hpes). The levels in the *H WSF* groups (in which the water was filtered) were more similar to the medium group, despite sharing the same oil content of the high dose (300 µg oil/L) (Fig. 3A). Figure 3B shows the profile of PAHs at 72 hpes, where, notably, the high molecular weight (HMW) PAH group (inset in Fig. 3B) found in medium, *H sub* and *H surf* groups, was lacking in the *H WSF* group. These HMW PAHs, *e.g.* PYRs and CHRs, accumulated substantially more in the *H surf* than in other groups. In addition, in the *H surf*, the levels of these HMW PAHs were up to four times higher in *early* vs *late* exposure (Fig. S3). Detailed profiles for individual PAHs at each time point are documented in Supplementary Dataset S1.


A
Early
Late
Tissue ng totPAH/g ww
Time (hpes)
2.5 dpf
6.5 dpf
7.5 dpf
11.5 dpf
STOP
Treatment
C
L
M
H sub
H surf
H WSF
B
72 hpes Early
72 hpes Late
5-6-ring
BTs
NAPs
FLUs
PHEs
DBTs
PYRs
CHRs

419

*Figure 3:* ***Tissue uptake of polycyclic aromatic hydrocarbons (PAHs).*** *A) Total body burden of PAHs from 1-96 hours post exposure start (hpes) in early (Left panel) and late (right panel) exposure. Exposure stop was after 72 hours. Statistical difference from control was tested at each time point with One-way ANOVA and significant differences are indicated by **=p<0.01 or ***: p<0.001. Note, at 96 hpes only one replicate from H surf and H WSF (early exposure) and H WSF (late exposure ) were analyzed. B) PAH profile at exposure stop (72 hpes) in early (left panel) and late (right panel) exposure. Inset: magnification of 5-6 ring distribution in the treatment groups. Dpf: days post exposure, C: control, L: low dose, M: medium dose, H sub: high dose submerged, H surf: high dose surface, H WSF: high dose water soluble fraction, BTs: Benzothiophenes , NAPs: Naphtalenes, FLUs: Fluorenes, PHEs: Phenanthrenes, DBTs: Dibenzothiophenes, PYRs: Pyrenes, CHRs: Chrysenes.*

### 3.2.2. *Hydroxylated phenanthrene metabolites*

Hydroxylated phenanthrene metabolites were analyzed in the control and three high dose groups (*H sub*, *H surf* and *H WSF*) at *early* (only at exposure stop, 72 hpes) and *late* exposure (1-72 hpes). Metabolites assessed are presented as sum of monohydroxyphenanthrenes (ΣOHPHE) and sum of dihydrodiol phenanthrenes (ΣPHE-diols) (Fig. 4). Within these groups 1,2-diol-PHE was the dominating metabolite and accounted for approximately 90% of total detected metabolites (average of all treatments). Of the ΣOHPHE, 1-OH-PHE was the most abundant and contributed with 9% of the total metabolites (Supplementary Dataset S2).

Metabolite levels at exposure stop (72 hpes) for *early* exposure were approximately 3 times lower than *late* exposure (Fig 4A). In the *late* exposure, where we analysed for a metabolite profile over time, we observed significant levels of phenanthrene metabolites in all high dose groups after 24 hpes, and these levels continued to increase until exposure stop (Fig. 4B). The maximum body burden of PHE metabolites was found in the *H surf* group (72 hpes) in a concentration of 45 ~~56~~ ng/g ww, which is in the similar concentration ranges as the body burden of the parent PHE (95 ng/g ww found after 24 hpes). The metabolites in the *H WSF* were approximately 1/3 that of *H sub*, although they followed a similar trend over time. (Fig. 4B). The correlation between phenanthrene metabolites and body burden (Fig. 4C) demonstrates that the rate of biotransformation exceeds the rate of PAH uptake. By 12 hpes (*H sub*) and 24 hpes (*H surf*), the curve turns back on itself indicating that the formation of metabolites is increasing while the PAH body burden has begun to decrease (even under continuous exposure).

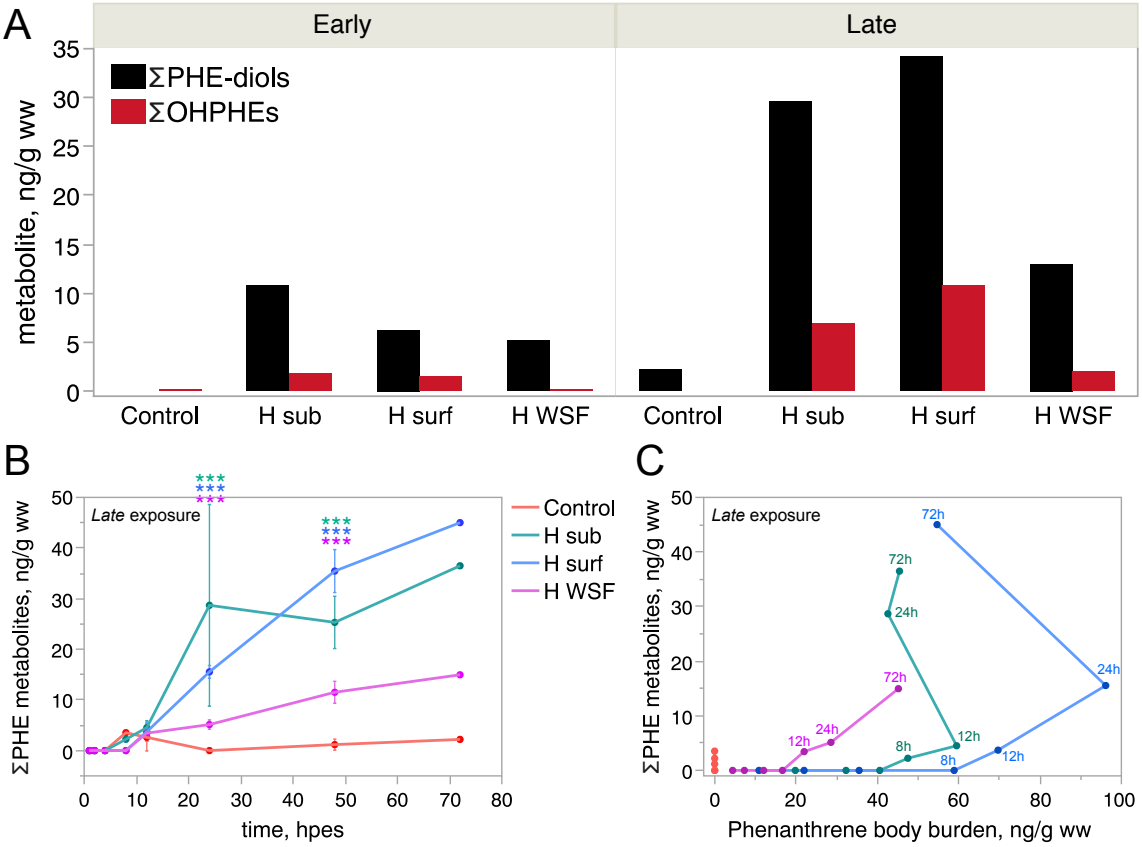



*Figure 4:* ***Tissue concentration of phenanthrene metabolites.*** *A) Concentrations of PHE-diols and OHPHEs at 72 hpes in early and late exposures (one sample per treatment). B) Concentrations of ΣPHE metabolites over time in late exposure. Statistical difference from control was tested at 12 hpes, 24, hpes and 48 hpes with one-way ANOVA with Dunnet's multiple comparison, and significant differences are indicated by ***: p<0.001. C) Sum of phenanthrene metabolites versus mean body burden of phenanthrene in late exposure. The backwards curve suggests that metabolism is faster than uptake towards the end of the exposure. H sub: high dose submerged, H surf: high dose surface, H WSF: high dose WSF.*

### 3.3. Temporal expression of *cyp1a, cyp1b, cyp1c* and *cyp1d* and EROD activity

#### *3.3.1. Temporal expression of Cyp1s*

Expression of *cyp1a* was immediate in both experiments. It was increasing throughout the *early* exposure (Fig. 5A),and the expression stayed steady from 12-72 hpes at the late exposure (Fig 5B). In the *early* exposure a 5- and 6- fold significant change of *cyp1a* was detected after 8 hours of exposure in *H sub* and *H surf*, respectively (Fig. 5A). All treatments at investigated time-points were up-regulated from 12 hpes (Fig. 5A). As with PAH body burden, the *cyp1a*

469 expression in the *H WSF* group fell between the levels detected in low and medium dose in all
470 time points except at 120 hpes, which reflects the toxicity contribution of oil droplets in the
471 exposure that is reduced when the exposure water is filtered to yield the WSF exposure. In the
472 *late* exposure, the induction of *cyp1a* was much more rapid with 2 fold up-regulation after only
473 2 hours of exposure in the *H surf* group (Fig. 5B). From 8 hpes and onward significant up-
474 regulation of *cyp1a* was seen in all treatment groups including low and medium at 24 and 72
475 hpes (Fig. 5B). Similar to the *early* exposure, the *H WSF* group *cyp1a* expression fell between
476 levels detected in the medium and low dose groups (Fig. 5B).

477 Induction of *cyp1a* correlated with tissue uptake of PAH (Fig. S4). However, one of the
478 low dose tanks, L4, showed low PAH uptake and *cyp1a* induction in both *early* and *late*
479 exposures. It was therefore considered an outlier and eliminated for further analysis in both
480 experiments. Embryos from the L4 tank clustered to the control tanks rather than the low dose
481 tanks at both 24 hpes and 72 hpes (Fig. S5, 72 hpes).

482 The expression of the other *cyp1* paralogs, *cyp1b* and *cyp1c*, was delayed in the *early*
483 exposure and genereally followed the same pattern as *cyp1a* expression in the *late* exposure
484 (Fig. 5). The expression profile over time for the control group show a highly dynamic temporal
485 expression of *cyp1b, cyp1c* and *cyp1d* while *cyp1a* showed a stable expression throughout
486 embryonic development (Fig. S6A-D). In the early exposure, *cyp1c* was not appreciably
487 expressed regardless of treatment until 48 hpes (Fig. 5C) and at 72 hpes a developmentally
488 related 200 fold up-regulation in the control group was observed (Fig. S6). Similarly, a general
489 up-regulation of *cyp1b* was seen from 24 hpes, with a significant upregulation in surface dose
490 at the two latest time points (Fig. 5C). In comparison, at peak at 96 hpes *H surf* showed a 221
491 fold up-regulation of *cyp1a* while *cyp1b* and *cyp1c* showed a 2.5 fold and 82 fold up-regulation,
492 respectively, compared to control at 96 hpes. For *cyp1d*, almost no transcripts were detected at
493 any of the time points in either dose/control in the *early* exposure. In the *late* exposure *cyp1d*
494 was generally up-regulated at 72 hpes, with a significant up-regulation in *M* and *H sub* groups
495 (Fig. S7). After 10 days of recovery (*early* exposure) at 3 dph one of the groups *H surf*, still had
496 significant up-regulation of one of the *cyp1s*, namely *cyp1b* (Fig. S8A-D, left panel). While at
497 3 dph after 5 days of recovery (*late* exposure), significant up-regulation in *H surf* group was
498 observed for all *cyp1*s, and in *H sub* group for *cyp1a, b* and *c* (Fig. S8A-D, right panels).

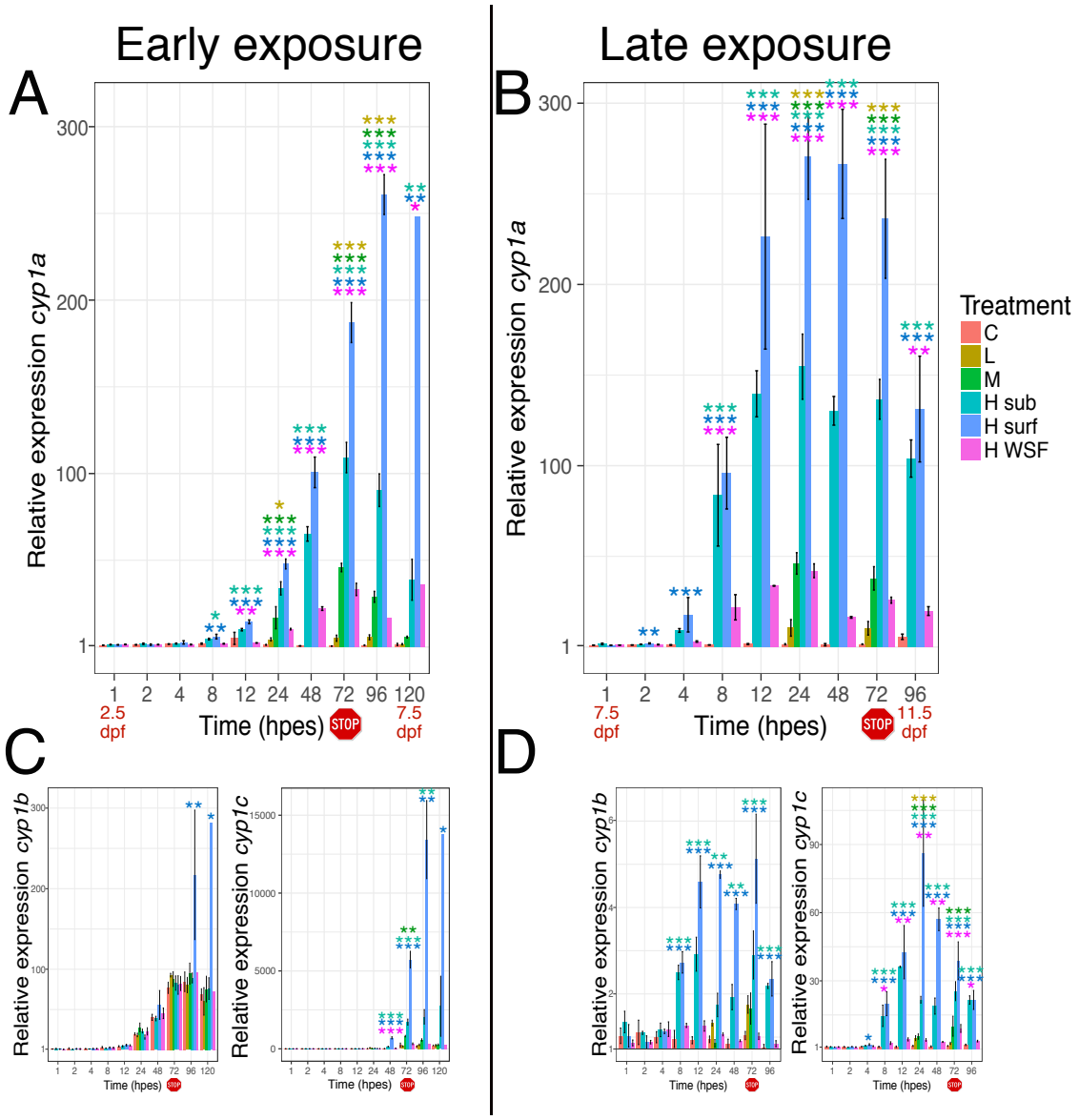



*Figure 5:* ***Relative expression of cyp1s.*** *Relative expression of cyp1a after early (A) and late (B) exposure. C) shows relative expression of cyp1b (left panel) and cyp1c (right panel) after early exposure, while D) displays cyp1b (Left panel) and cyp1c (right panel) after Late exposure. Expression was related to control group at exposure start to detect both developmental and treatment related expression. Exposure stop is indicated with a stop icon. Statistical difference from control at each time point was tested with One-way ANOVA with Dunnet´s multiple comparsions and significant differences are indicated by *=p<0.05, **=p<0.01 or ***: p<0.001. At 120 hpes in the early exposure, only one sample was collected for H surf and H WSF. Hpes: hours post exposure start, Dpf: days post exposure, C: control, L: low dose, M: medium dose, H sub: high dose submerged, H surf: high dose surface, H WSF: high dose WSF.*

### *3.3.2. Cyp1a activity*

512 Ethoxyresorufin-O-deethylase (EROD) assay was applied to detect Cyp1a activity and
513 was measured at 72 hpes (5.5 dpf) in the *early* exposure. Cyp1a activity was detected in the
514 skin, mainly in the trunk, and in the otoliths in the *H sub* and *H surf* exposures. Low and
515 medium doses showed EROD activity in the trunk, while very little activity was detected in *H*
516 *WSF* (Fig. S9). No specific fluorescence was detected in the embryos incubated in DMSO
517 (images not shown). The internal fluorescence activity of the PAHs in the oil droplets are
518 shown in Figure S9C.
519
520 **3.4. Crude oil induced malformations**
521 Craniofacial, eye and growth abnormalities were seen in both exposures (Table 1). We
522 defined six distinct dose-dependent craniofacial phenotypes, given here in increasing severity:
523 mild Bulldog (MBD) and Bulldog (BD) with reduced upper jaw, Upper Jawbreaker (UJB) with
524 a posteriorly twisted upper jaw, Total Jawbreaker (TJB) with posteriorly twisted jaws in
525 general, Darth Vader (DV) with an underdeveloped upper jaw and hanging lower jaw and
526 Hunchback (HB) with severe reduction of all jaw structures with reduced brain structures,
527 abnormal finfold, spinal curvature and poor dorsal rotation of the head (Fig. 6).
528 In the *early* exposure, most severe craniofacial malformations were observed in the *H*
529 *surf* group (Fig. 6A, lower panel Table 1), where all animals were characterized as DVs or HBs
530 (Fig. 6A). The *H sub* group possessed fewer malformations (50%) and the group comprised of
531 BD/MBD, UJB and TJB animals and a few DVs (Fig. 6A, Table 1). In the *H WSF* dose, no
532 malformed phenotypes were observed while low and medium dose both had occasions of the
533 milder phenotypes (MBD/BD and UJB, 28%) (Fig. 6A, Table 1). The abnormal jaw phenotypes
534 were linked to the shorter ethmoid plate. In accordance with the phenotypic observations, both
535 *H sub* and *H surf* groups with the most severe jaw effects also showed a significantly reduced
536 ethmoid plate (14% and 72% shorter, respectively). While the *H WSF*, low dose and medium
537 dose showed no significant reduction in ethmoid plate compared to control (Table 1). Similarly,
538 the total length of the larvae were only significantly reduced in the *H sub* and *H surf* groups
539 (Table 1).
540 In the *late* exposure, the craniofacial malformations were generally milder, and the
541 difference between the *H sub* and *H surf* groups were less evident (Fig. 6). The same phenotypes
542 were present (MBD/BD, TJB and DV) but with an increased proportion in the surface group
543 where all had craniofacial malformations (Fig. 6, Table 1). The *H WSF* group had
544 approximately the same occurrence of the mildest phenotype (MBD/BD, 15 vs 19%) as the low
545 dose (Table 1). In comparison, the medium dose had slightly lower occurrence of the mildest

phenotypes (MBD/BD, 17%) , but additionally had incidents of the more severe phenotypes (TJB and DV, 6%) (Table 1). Along with craniofacial phenotype, the ethmoid plate was significantly reduced in the *H sub* and *H surf* groups (Table 1). Total length of the larvae was reduced in all exposed groups (Table 1).

Eye shape and diameter were affected by oil exposure at both time points. Four eye phenotypes were observed: no phenotype, normal round shape (no), bend shape (be), irregular shape (irr) and protruding lens (pl) (Table 1). The frequency of any eye phenotype was higher in the *early* exposed animals, and the most severe phenotype, protruding lens, was only found in *H sub* (8%) and *H surf* (12%) groups. No effect on eye shape was seen in *H WSF* regardless of timing of exposure (Table 1). Severe reduction in eye diameter was found in the *early* exposed *H surf* and *H sub* animals (Table 1). Less effect on eye size was observed in the *late* exposure, only medium showed significantly reduced eye diameter.

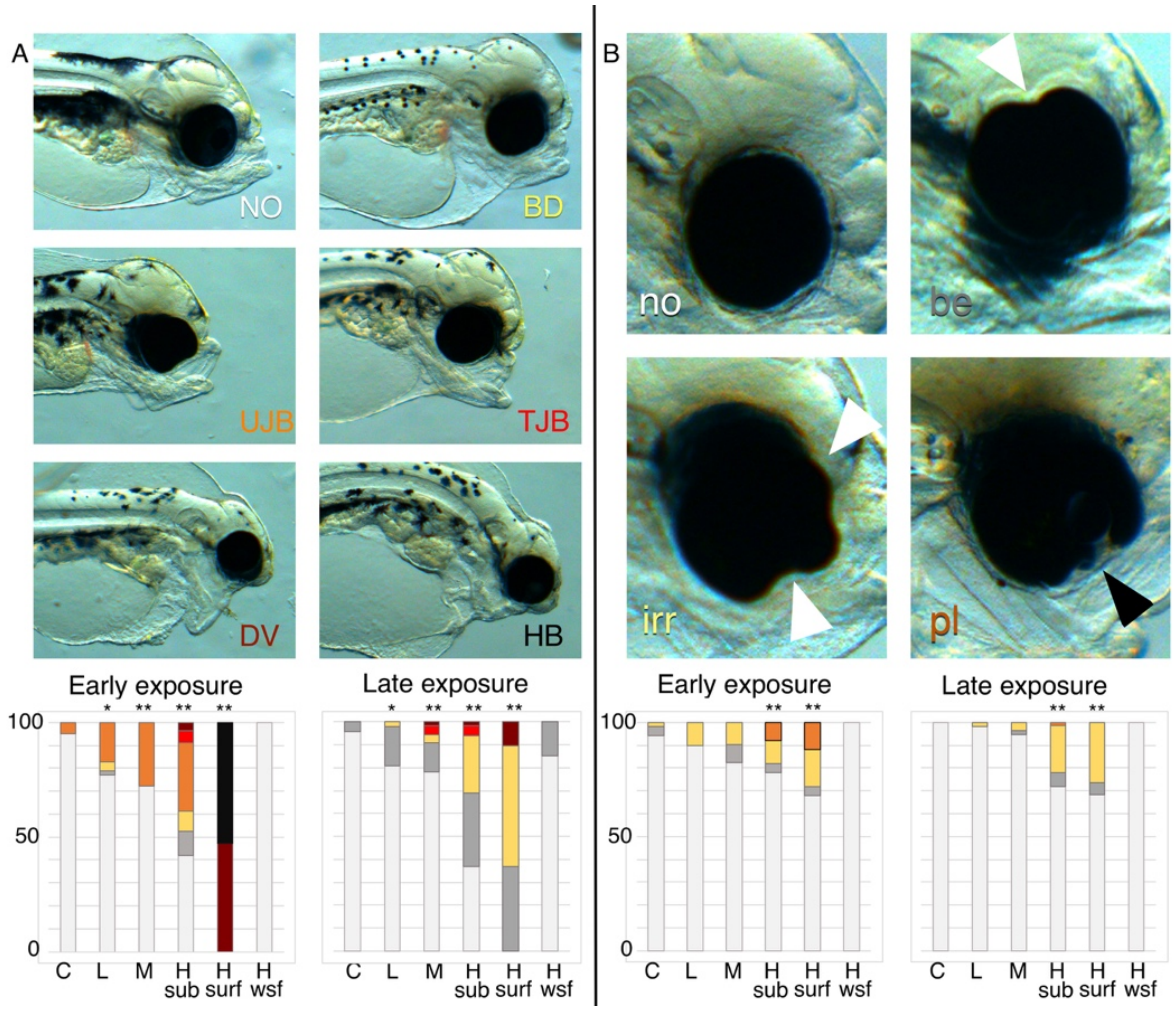



*Figure 6:* ***Oil induced craniofacial and eye phenotypes.*** *A) Oil induced craniofacial phenotypes, NO: normal, BD: Bulldog, UJB: Upper jawbreaker, TJB: Total jawbreaker, DV: Darth Vader, HB: Hunchback. The bottom panel shows the distribution of the craniofacial phenotypes after early and late exposure at 3 days post hatching. Coloration code: white: NO, grey: mild BD, yellow: BD, orange:*

*UJB, red: TJB, dark red: DV, black: HB. B) Oil induced eye phenotypes, no: normal eye shape (white), be: bend shape (grey), irr: irregular shape (yellow), pl: protruding lens (orange). Bottom panel shows the distribution of eye phenotypes in early and late exposures. White arrow heads indicate irregularities in the eye shape, and black arrow head indicate protruding lens.*

*Statistical difference from control was tested with Chi-square, and p<0.01 is indicated by **. C: control, L: low dose, M: medium dose, H sub: high dose submerged, H surf: high dose surface, H WSF: high dose water soluble fraction.*

| TABLE 1 | | Total length | | Ethmoid plate | | Eye diameter | | Craniofacial phenotype | | | | | | | Eye phenotype | | | | Cardiac looping | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Average +/- SD | N | Average | N | Average +/- SD | N | No | MBD | BD | UJB | TJB | DV | HB | no | be | irr | pl | nlo | plo | splo | p value |
| Early exposure 3 dph | Control | 4836 +/- 325 | 59 | 170.3 +/- 28,1 | 51 | 174,9 +/- 17,4 | 52 | 95 | 0 | 0 | 5 | 0 | 0 | 0 | 94 | 4 | 2 | 0 | 93 | 7 | 0 | |
| | Low dose | 4777 +/- 394 | 47 | 161,8 +/- 24,1 | 47 | 184,3 +/- 12,7 | 47 | 77 | 2 | 4 | 17 | 0 | 0 | 0 | 90 | 0 | 9 | 0 | 76 | 24 | 0 | 0.0054 ** |
| | Medium dose | 4732 +/- 270 | 61 | 174,1 +/- 19,4 | 61 | 177,2 +/- 12,3 | 59 | 72 | 0 | 0 | 28 | 0 | 0 | 0 | 82 | 8 | 10 | 0 | 60 | 40 | 0 | <0.00001 *** |
| | High submerged | 4567 +/- 399*** | 60 | 146,6 +/- 19,4*** | 61 | 167,4 +/- 15,6*** | 60 | 42 | 11 | 9 | 30 | 5 | 4 | 0 | 78 | 4 | 10 | 8 | 31 | 57 | 11 | <0.00001 *** |
| | High surface | 3547 +/- 371*** | 18 | 46,6 +/- 37,0*** | 19 | 130,9 +/- 8,8*** | 20 | 0 | 0 | 0 | 0 | 0 | 47 | 53 | 68 | 4 | 16 | 12 | 0 | 0 | 100 | <0.00001 *** |
| | High WSF | 4769 +/- 269 | 20 | 163,7 +/- 24,1 | 20 | 186,8 +/- 12,5 | 20 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 95 | 5 | 0 | 0.86 |
| Late exposure 3 dph | Control | 4704 +/- 172 | 75 | 155,9 +/- 21,2 | 79 | 177,2 +/- 9,4 | 78 | 96 | 4 | 0 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 88 | 12 | 0 | |
| | Low dose | 4589 +/- 193* | 59 | 148,2 +/- 17,0 | 59 | 178,6 +/- 8,7 | 59 | 81 | 17 | 2 | 0 | 0 | 0 | 0 | 98 | 0 | 2 | 0 | 95 | 5 | 0 | 0.24 |
| | Medium dose | 4559 +/- 153*** | 58 | 154,0 +/- 26,3 | 59 | 172,8 +/- 9,9* | 58 | 78 | 13 | 4 | 0 | 4 | 2 | 0 | 95 | 2 | 4 | 0 | 74 | 26 | 0 | 0.047 * |
| | High submerged | 4487 +/- 293*** | 97 | 123,8 +/- 26,9*** | 74 | 173,4 +/- 10,8 | 78 | 37 | 32 | 25 | 0 | 4 | 1 | 0 | 72 | 6 | 21 | 1 | 42 | 58 | 0 | <0.00001 *** |
| | High surface | 4320 +/- 175*** | 20 | 116,4 +/- 18,6*** | 20 | 172,9 +/- 8,9 | 19 | 0 | 37 | 53 | 0 | 0 | 11 | 0 | 68 | 5 | 26 | 0 | 20 | 80 | 0 | <0.00001 *** |
| | High WSF | 4532 +/- 179** | 20 | 149,3 +/- 30,2 | 20 | 182,6 +/- 7,1 | 19 | 85 | 15 | 0 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 90 | 10 | 0 | 0.91 |

*Table 1: **Morphological abnormalities after early and late embryonic exposure**. WSF; water soluble fraction, SD; standard deviation, N; total number of animals analyzed, No; normal phenotype, MBD; mild bulldog phenotype, BD; bulldog phenotype, UJB; upper jawbreaker phenotype, TJB; total jawbreaker phenotype, DV; Darth Vader phenotype, HB; hunchback phenotype, no; normal eye shape be; bend eye shape, irr; irregular eye shape, pl; protruding lens, nlo; normal looping, plo; poor looping, splo; severely poor looping. Statistical significant difference from control is indicated by asterisks; p<0.05 *, p<0.001 **. P<0.0001 ***.*

### 3.5. Cardiac malformations

#### *3.5.1. Morphological abnormalities.*

The size of the heart was reduced in both exposures, but only *early* exposure resulted in severly malformed hearts with poor looping (Table 2). After *early* exposure, an effect on atrial dimensions was only detected in the *H surf* and *H sub* group (Table 2). The ventricular diastolic diameter was reduced in all exposure groups (9-43%) except for *H WSF*, and the *H surf* group also showed reduced ventricular systolic diameter (38%) (Table 2). There was no effect on atrial dimensions after *late* exposure in any of the doses. However, ventricular systolic and diastolic diameters were reduced in both *H sub* (12% and 19%, respectively) and *H surf* groups (19% and 31%, respectively) in the *late* exposure (Table 2). The hearts were not as malformed as in the *early* exposure, and no incidences of severly poor looping was observed (Table 2).

### *3.5.2. Functional abnormalities*

In both exposures ventricular contractility (VFS) was reduced (Table 2). Notably, reduced contractility was also evident in the lower doses in the *early* exposure. In addition, several animals suffered from a non-contracting or constantly contracting ventricle, defined as silent ventricle (SiV). In the *early* exposure, we observed a dose-dependent prevalence of SiV in all groups with oil droplets, with the most occurrences (73%) in the *H surf* group with the severely abnormally shaped hearts. The only group in *early* exposure with no incidence of SiV was *H WSF* . In the *late* exposure, there were fewer SiVs in all doses, however, we still observed SiV in 60 % of the *H surf* animals, even though their hearts´ morphology looked normal compared to the *early* exposed animals. Reduced heart rate (bradycardia) was only observed in the high surface at the *early* exposure, while increased heart rate (tachycardia) was seen in *H WSF* in the *late* exposure (Table 2).

Edema formation, a consequence of circulation abnormalities, was seen among more treatments in the *late* exposure (Table 2). In the *early* exposure, both *H WSF* (26%) and *H surf* (30%) group had increased frequency of edema compared to the control group (15%). While in the *late* exposure, medium (17%) and all high doses (*H WSF* (23%), *H sub* (23%) and *H surf* (28%)) all showed increased frequency of edema compared to control (12%) (Table 2). Yolk sac size is dependent on hatching time and rate of yolk consumption. We observed an increased average yolk sac size, indicating a decreased yolk utilization in the *H surf* group after *early* exposure. The opposite was observed in the *late* exposure, a decreased yolk sac, thus an increased yolk utilization rate in the *M* and *H WSF* groups (Table 2).

| TABLE 2 | | AS | | AD | | VS | | VD | | AFS % | | VFS % | | Edema | | Yolk sac | | BPM | | SiV % | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Average +/- SD | N | Average +/- SD | N | Average +/- SD | N | Average +/- SD | N | Average +/- SD | N | Average +/- SD | N | Average +/- SD | N | Average +/- SD | N | Average +/- SD | N | % | N |
| Early exposure 3 dph | Control | 101.4 +/- 14.3 | 42 | 121.7 +/- 18.4 | 42 | 89.9 +/- 13.8 | 54 | 105.8 +/- 19.7 | 54 | 17.8 +/-6.9 | 42 | 14.4 +/- 6.9 | 54 | 15 +/- 8.3 | 61 | 21.3 +/- 8.1 | 61 | 93.1 +/- 8.7 | 61 | 2 | 54 |
| | Low dose | 94.0 +/- 15.3 | 20 | 115.4 +/- 18.4 | 20 | 85.9 +/- 9.1 | 34 | 96.0 +/- 13.3* | 34 | 18.1 +/- 8.2 | 20 | 9.8 +/- 6.9* | 32 | 12.3 +/- 6.1 | 48 | 24.3 +/- 9.8 | 48 | 90.4 +/- 7.4 | 38 | 13** | 48 |
| | Medium dose | 93,5 +/- 13.9 | 22 | 113.9 +/- 17.1 | 22 | 85.4 +/- 13.5 | 50 | 94.1 +/- 15.2** | 50 | 20.2 +/- 8.8 | 22 | 8.8 +/- 7.8** | 50 | 13.3 +/- 8.9 | 61 | 23.7 +/- 10.7 | 61 | 89.2 +/- 9.5 | 53 | 24*** | 51 |
| | High submerged | 91.5 +/- 18.2* | 42 | 114.6 +/- 20.2*** | 44 | 84.1 +/- 15.6 | 57 | 91.7 +/- 19.5*** | 57 | 19.8 +/- 8.9 | 42 | 7.8 +/- 9.1*** | 57 | 17.6 +/- 10.9 | 61 | 24.1 +/- 11.2 | 61 | 90.5 +/- 10.3 | 64 | 20*** | 40 |
| | High surface | 86.2 +/- 14.0** | 17 | 101.0 +/- 18.6 | 17 | 56.1 +/- 20.1*** | 15 | 59.8 +/- 20.1*** | 15 | 13.9 +/- 9.0 | 17 | 5.5 +/-12.4*** | 15 | 30.0 +/- 13.1*** | 18 | 42.5 +/- 11.9*** | 18 | 74.9 +/- 15.2*** | 21 | 73*** | 15 |
| | High wsf | 99.6 +/- 11.4 | 15 | 119.9 +/- 11.7 | 15 | 86.1 +/- 8.5 | 19 | 105.6 +/- 9.0 | 19 | 16.8 +/- 7.0 | 15 | 18.4 +/-4.8 | 19 | 25.9 +/- 7.1*** | 20 | 16.3 +/- 6.6 | 20 | 91.7 +/- 5.6 | 20 | 0 | 20 |
| Late exposure 3 dph | Control | 98.0 +/- 7.8 | 50 | 122.6 +/- 10.8 | 50 | 85.7 +/- 16.3 | 61 | 102.9 +/- 19.2 | 61 | 19.7 +/- 6.3 | 50 | 16.2 +/- 6.6 | 61 | 12.2 +/- 8.2 | 79 | 25.8 +/- 6.8 | 79 | 83.2 +/- 7.7 | 59 | 2 | 61 |
| | Low dose | 99.3 +/- 10.3 | 37 | 122 +/- 13.1 | 37 | 83.1 +/- 10.1 | 49 | 102.2 +/- 12.9 | 49 | 17.8 +/- 8.2 | 47 | 17.0 +/- 5.4 | 47 | 12.0 +/- 6.8 | 59 | 25.1 +/- 5.8 | 59 | 82.9 +/- 4.7 | 77 | 0 | 65 |
| | Medium dose | 97.6 +/- 12.3 | 40 | 120.9 +/- 14.0 | 40 | 83.0 +/- 16.0 | 50 | 100.2 +/- 19.3 | 50 | 18.6 +/- 7.5 | 40 | 16.7 +/- 7.9 | 50 | 17.2 +/- 9.4** | 58 | 22.1 +/- 8.3** | 58 | 83.4 +/- 5.0 | 60 | 4 | 50 |
| | High submerged | 97.9 +/- 9.5 | 57 | 121.8 +/- 18.4 | 57 | 75.4 +/- 12.3*** | 69 | 83.1 +/- 15.3*** | 69 | 18.8 +/- 8.0 | 57 | 9.9 +/- 7.0*** | 69 | 23.4 +/- 11.7*** | 78 | 23.5 +/- 6.4 | 78 | 82.8 +/- 4.5 | 78 | 9* | 69 |
| | High surface | 97.5 +/- 9.9 | 19 | 118.8 +/- 14.6 | 19 | 69.6 +/- 7.6*** | 20 | 71.3 +/- 9.7*** | 20 | 17.5 +/- 7.4 | 19 | 2.3 +/- 3.4*** | 20 | 28.3 +/- 8.2*** | 20 | 21.7 +/- 3.7 | 20 | 84.8 +/- 4.8 | 19 | 60*** | 20 |
| | High wsf | 99.9 +/- 10.0 | 18 | 124.4 +/- 8.9 | 18 | 84.1 +/- 9.7 | 21 | 102.2 +/- 11.3 | 21 | 19.7 +/- 6.2 | 18 | 17.6 +/- 5.2 | 21 | 23.0 +/- 8.4*** | 20 | 16.3 +/- 2.8*** | 20 | 90.0 +/- 4.6*** | 19 | 0 | 21 |

*Table 2:* ***Cardiac abnormalities after early and late embryonic exposure.*** *WSF; water soluble fraction, AS; atrial systolic diameter, N; Number of animals analyzed, AD; Atrial diastolic diameter, VS; Ventricular systolic diameter, VD; Ventricular diastolic diameter, AFS; Atrial fractional shortening, VFS; ventricular fractional shortening, BPM; Beats per minute, SiV; Silent ventricle. Statistical significant difference from control is indicated by asterisks; p<0.05 *, p<0.001 **. P<0.0001 ***.*

## 3.6. Temporal expression of genes in response to *early* and *late* oil exposure

621 Generally, the response in gene expression was different for each gene between the two
622 exposures (Fig. 7). Oil-induced differential expression of *bmp10* was independent of circulation
623 with an immediate trend of up-regulation in the *late* exposure . While *rhag* and *abcb1* were
624 delayed, with expression increasing after *cyp1a* expression had increased. Expression dynamics
625 for the control group in all genes are shown in Fig. S8.

626 Exposure-related up-regulation of *bmp10,* a calcium regulated gene involved in
627 cardiogenesis, was observed before first heart beat at the cardiac cone stage (72 hpes), 2.4 fold
628 expression in *H surf* compared to control (Fig. 7). The exposure start in the *early* exposure was
629 at the beginning of gastrulation, prior to or at the time of onset of zygotic transcription. Thus,
630 at exposure start no expression of the potent signaling molecule, Bmp10, was detected at the
631 mRNA level. Induction of *bmp10* (~7 fold) was first initiated at 4 dpf (48 hpes) in all groups
632 including control and showed an increasing expression throughout the measured period.
633 Significant up-regulation in the *H surf* group was observed at 72 and 96 hpes in the *early*
634 exposure (Fig. 7A). At the *late* exposure the expression of *bmp10* was more variable throughout
635 the period. A trend of up-regulation was seen from 4 hpes. In particular, *H sub* and *H surf*
636 groups were significantly different from control overall in the investigated period (Two-way
637 ANOVA $p<0.01$) (Fig. 7B). No significant expression changes were detected at 3 dph after
638 either early or late exposure (Fig S7E).

639 Significant up-regulation of the mRNA encoding the transporter protein Abcb1 was
640 seen after *early* exposure but during *late* exposure (Fig. 7). In the *early* exposure, up-regulation
641 was only seen in *H sub* and *H surf* groups (Fig. 7A). While in the late exposure, up-regulation
642 was observed in medium and all high doses (Fig. 7B) . No differential expression of *abcb1* was
643 seen at 3 dph after any exposure treatment (Fig. S7H).

644 Up-regulation of the mRNA encoding the ammonium and carbon dioxide transporter
645 Rhag, was seen in *H sub* and *H surf* groups at both exposures (Fig. 7). In the *early* exposure, an
646 oil induced up-regulation of 3.5 fold (H surf) and 2 fold (H sub) was observed first at 96 hpes.
647 Differential expression of *rhag* was much more immediate in the *late* exposure (after
648 completion of organogenesis), and significant up-regulation was seen in both *H sub* (2-3 fold)
649 and *H surf* (3-4 fold) groups at 24 hpes and throughout the examined period. Additionally, low
650 dose up-regulation at 24 hpes (Fig. 7B). No differential expression of *rhag* was detected at 3
651 dph in either *early* or *late* exposure (Fig. S7G).

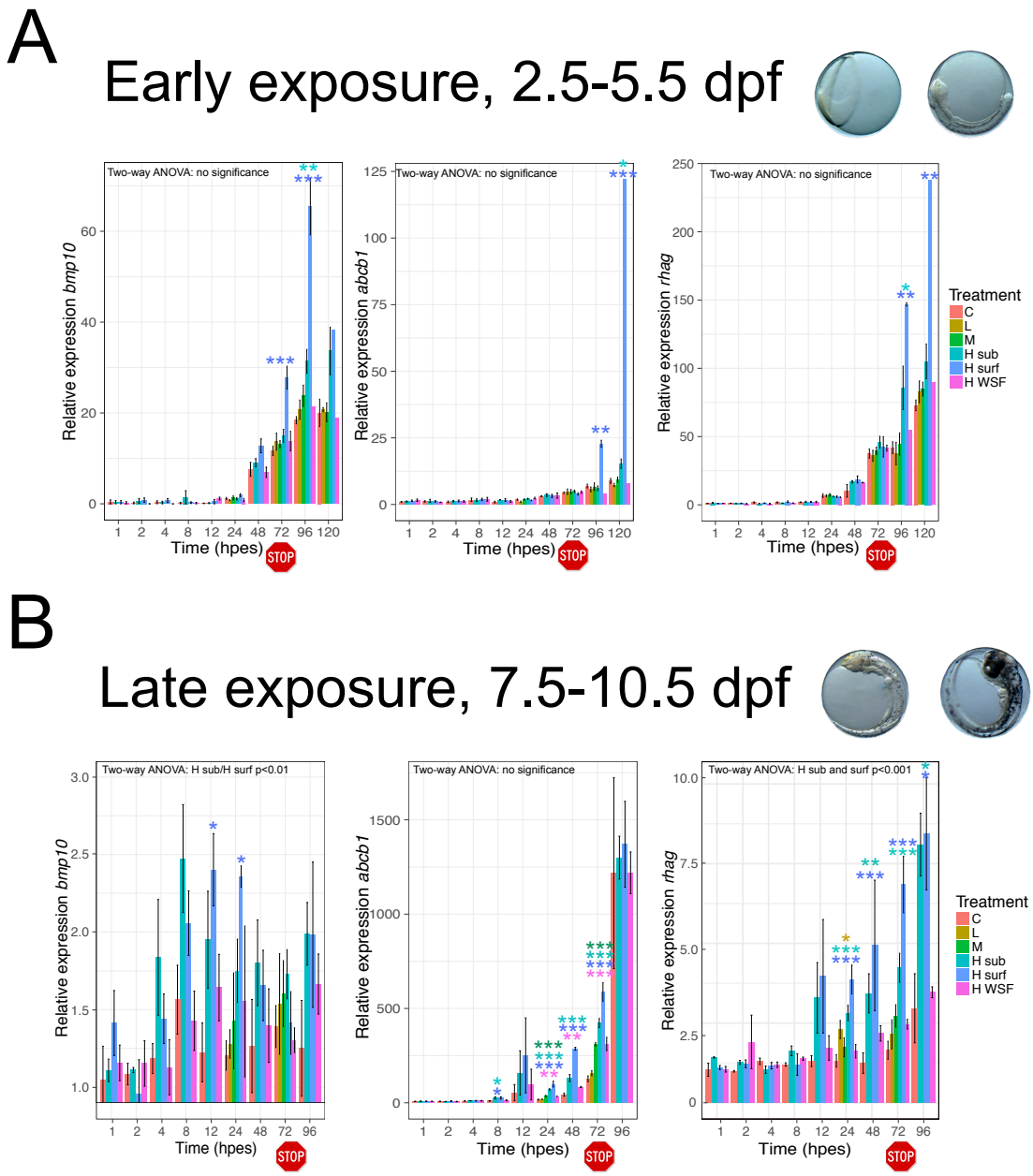



*Figure 7:* ***Relative expression of bmp10, abcb1 and rhag.*** *Relative expression compared to control at exposure start for bmp10, abcb1 and rhag after early (A) and late (B) exposure. Expression was related to control group at exposure start to detect both developmental and treatment related expression. Exposure stop is indicated by a stop icon. Statistical difference to control was tested at each time point with one-way ANOVA with Dunnet´s multiple comparsions, and significant differences are indicated by *=p<0.05 or ***: p<0.001. At 120 hpes in the early exposure, only one sample was collected for H surf and H WSF.Dpf: days post fertilization, hpes: hours post exposure start, C: control, L: low dose, M: medium dose, H sub: high dose submerged, H surf: high dose surface, H WSF: high dose water soluble fraction.*

### *3.6.1. Whole mount in situ hybridization of rhag*

To determine location of *rhag* in early life stages of fish, we performed whole mount in situ (WISH) on unexposed 10 dpf embryos (Fig. 8). In unexposed 10 dpf Atlantic haddock embryos, expression of *rhag* was observed in a line following the branchial arch blood vessels (Fig. 8). No expression was found anywhere else in the embryos, however, due to auto staining, the yolk sac was removed prior to coloration, and thus, the syncytial layer and yolk sac were not available for analysis. The technical negative control (sense) showed no coloration (Fig. 8C).

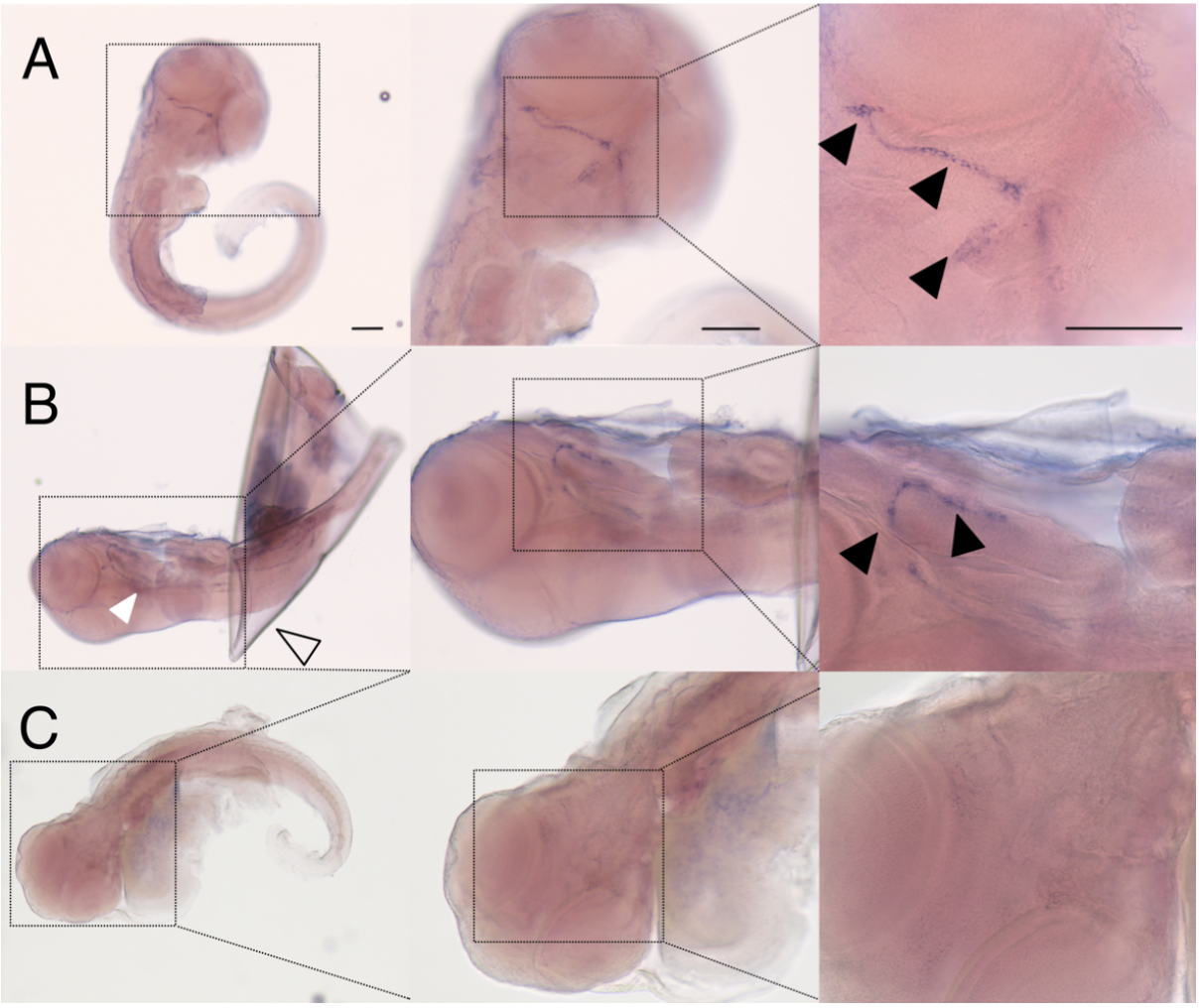



*Figure 8.* ***Whole mount in situ hybridization of rhag.*** *Expression pattern of rhag in ventral (A) and lateral (B) view in 10 dpf unexposed embryos of Atlantic haddock. Expression is following the branchial arch. C) No labeling in sense control. Black arrow head: expression of rhag, open arrow: eggshell, white arrow head: unspecific coloration of otholites.*

## 4. Discussion

679 In this study we aimed to link the consequences of short-term crude oil exposures in
680 two embryonic phases to metabolism and abnormal circulation by investigating morphological
681 and functional phenotypes and related gene expression. In general, the embryotoxic
682 consequences of an oil exposure were more severe in the *early* exposure animals. Severe cardiac
683 and craniofacial abnormalities in the highest doses exposed to micro oil droplets were
684 highlighted. The large difference in phenotypic outcome in *early* vs *late* exposure could be
685 explained both by the prolonged exposure and immature response system. First, particularly in
686 the *early* exposure, the groups exposed to micro oil droplets experienced a prolonged exposure
687 evident by visible oil droplets on the chorion and a prolonged *cyp1a* response. Second, the *early*
688 exposure started prior to or at the time of onset of zygotic transcription, in which the embryo
689 has a less efficient response and detoxification system (Sørhus et al., 2016a). Consequently, we
690 observed a delayed response in gene expression of all genes evaluated, including *cyp1a* which
691 is crucial for the first phase in xenobiotic metabolism (Goksøyr, 1995). Higher oil droplet
692 binding and most likely, accumulation, contributed to a higher body burden of PAHs in the
693 *early* exposure, while the levels of PHE metabolites were 2-3 times higher in the *late* exposure.
694 These results are in line with studies showing that Cyp1a induction has a protective rather than
695 aggravating effect (Incardona et al., 2005; Wells et al., 1997; Wells et al., 2009).
696 Exposing embryos at two developmental time points gave us a unique opportunity to
697 phenotypically anchor temporal gene expression changes and continue to unravel underlying
698 mechanisms of crude oil toxicity. Exposure during early cardiac development, and after onset
699 of heartbeat enabled us to evaluate whether the expression response for the various genes were
700 due to direct effect or secondary to abnormal circulation. Furthermore, we linked gene
701 expression to metabolism by investigating expression in context of Cyp1a regulation, PAH
702 uptake and appearance of metabolites. Mainly, we suggest that *cyp1a*, *cyp1b*, *cyp1c* and *bmp10*
703 are directly induced by parent compounds in the oil. To illustrate, these genes are expressed in
704 the *late* exposure before metabolites are detected. Subsequently, *abcb1* and *rhag,* which are
705 linked to transport of xenobiotic products (Choudhuri and Klaassen, 2006) and nitrogenous
706 waste (Caner et al., 2015), respectively, showed a delayed response corresponding to xenobiotic
707 energy consumption utilizing amino acids (Wang et al., 2019) and transport in xenobiotic
708 metabolism (Kimura et al., 2007).
709
710 **4.1. Oil droplet fouling**
711 Oil droplet fouling occured in both *H surf* and *H sub* groups and consequently increased
712 haddock embryotoxicity by escalating the uptake and prolonging the exposure. Droplet fouling

713 on the submerged exposures show that accumulation of oil droplets was due to the biological
714 characteristics of the haddock embryo and not a surface slick artifact. Unlike other cod fish
715 eggs, haddock have an extra membrane of adhesive material covering the primary egg envelope
716 (Hansen et al., 2018; Morrison et al., 1999; Oppen-Berntsen et al., 1990). The chemical
717 composition of this outer membrane is still unknown. However, it clearly has a hydrophopic
718 interaction with oil, and it is responsible for the accumulation of oil droplets on the eggshell.
719 The outer membrane degrades during the embryo phase, a few days before hatching (Sørhus et
720 al., 2015, 2016). Accordingly, the early egg stages accumulated more oil droplets than the late
721 stages in the present study.

722 Haddock spawn at depths between 30 and 500 meters (Olsen et al., 2010; Solemdal et
723 al., 1997). The eggs have a natural high buoyancy, and all embryonic stages have been observed
724 in the surface at surveillances (Solemdal et al., 1997). Oil droplet fouling can increase the
725 buoyancy of the egg, both by the low density of oil itself, but also by trapping of air bubbles on
726 the chorion (Sørhus et al., 2015). In contrast, we have also observed that haddock eggs with oil
727 droplets become negatively buoyant just before hatching and then sink to the bottom of the
728 exposure tanks. Similar effects have been observed in oil-exposed mahi mahi (*Coryphaena*
729 *hippurus*) (Pasparakis et al., 2017). The mechanism behind this remains unclear, but one
730 possibility is loss of water due to disruption of the perivetiline membrane or disruption of the
731 osmotic control (Pasparakis et al., 2017). Even though we did observe some slick formation as
732 seen in the *H surf* group, we argue that this group is also the more ecologically relevant exposure
733 dose. In a situation where crude oil is discharged at depths like in the Deepwater Horizon
734 accident or in a rough sea, oil droplets will be located throughout the water column (Hansen et
735 al., 2019; Nordtug et al., 2011). Consequently, oil droplet fouling increases buoyancy, that will
736 lead to increased speed to the surface and to even greater exposures in the oil slick.

737 Oil droplet fouling most likely causes transfer of more and higher molecular weight oil
738 components to the embryo. Although the nominal oil water dose in *H WSF* were higher than *M*
739 sub group, both PAH uptake and *cyp1a* induction were mainly higher in *M* sub group compared
740 to *H WSF* group especially in the *early* exposure. Interestingly, the composition of PAHs was
741 also different. Nearly no high molecular weight (5-6-rings) PAHs were found in the PAH tissue
742 of the *H WSF* group in any exposure. Accordingly, the *H WSF* group had very little Cyp1a
743 activity (EROD assay) compared to all other treatments where oil droplets were included.

744 In haddock embryos, oil droplets possibly add a dual toxicity. First, oil droplet fouling
745 results in an increased local concentration of PAHs and other toxic oil components. Second, oil
746 droplets adhered to the chorion may also contribute to transfer of lipophilic compounds that are

747 less available in a general water exposure (Sørensen et al., 2017). The latter may be linked to
748 the high molecular weight PAHs which were not present in the *H WSF.* Enhanced toxicity after
749 direct contact with oil and accumulation on the chorion have also been observed in medaka
750 (*Oryzias latipes*) (Gonzalez-Doncel et al., 2008) and polar cod (Laurel et al., 2019), together
751 with our results it highlight the importance of including dispersed oil in experimental exposure
752 designs and risk assessment models (Nordtug et al., 2011).
753
754 **4.2. PAH uptake, Cyp induction and metabolism**
755 We observed an immature detoxification system in early embryos via delayed
756 expression of *cyp1* paralogs and *abcb1* transporter, higher body burdens of PAHs and lower
757 levels of metabolites. Xenobiotic metabolism is necessary to excrete toxic substances. Among
758 the thousands of potential PAH metabolites, we used PHE as a model compound to study the
759 metabolic capacity of the haddock embryo. Even though PHE is a weak inducer of *cyp1s,* we
760 report PHE metabolite levels as to represent metabolite formation of any kind. The *late*
761 exposure had the more efficient elimination of PAHs evidenced by the immediate strong *cyp1*
762 response resulting in lower peak levels of tissue PAHs and higher levels of metabolites (e.g.
763 PAH levels were approximately 2.5 times lower in *H surf late* compared to *early* exposure). In
764 the *late* exposure *cyp1a, cyp1b* and *cyp1c* mRNA was highly and quickly induced, and already
765 after 24 hours the metabolic capacity surpassed the uptake speed of PHE. This resulted in a
766 reduced body burden even under continuous exposure. However, we did not observe any high
767 accumulation of PHE metabolites, showing that the embryos can effeciently excrete metabolic
768 products during phase III metabolism.
769 Cyp1a mRNA was clearly the first and the main detoxification enzyme mRNA in *early*
770 embryonic development. The other *cyp1* paralogs, *cyp1b* and *cyp1c,* were not induced until later
771 in the *early* exposure. *cyp1b* was upregulated from 3.5 dpf (24 hpes) regardless of exposure
772 including control and emphasizes the other non-detoxifying roles of Cyp1s (e.g. steroid
773 biosynthesis and cardiovascular and eye development (Chaudhary et al., 2009; Li et al., 2017))
774 during early fish development (Goldstone et al., 2010). At 72 hpes a 200 fold developmentally
775 related up-regulation of *cyp1c* was observed and at the same time a strong up-regulation was
776 seen in the treatment groups. These observations suggests that xenobiotic induction of *cyp1c* is
777 occurring as soon as the organism possess Cyp1c acitivity. A delayed expression of *cyp1b* and
778 *cyp1c* underscore the differences in capability for fish embryos to metabolize xenobiotics
779 depending on their embryonic developmental window. Our results coincide with the findings

780 of (Kuhnert et al., 2017) where the expression of *cyp1b* and *cyp1c* is also delayed in zebrafish
781 exposed to benzo(a)pyrene.
782 While Cyp1a appears protective and is necessary for detoxification (Billiard et al., 2006;
783 Mu et al., 2016; Scott et al., 2011; Wincent et al., 2015), it may also result in the production of
784 some reactive toxic metabolites (Wells et al., 1997). Other enzymes can also produce
785 metabolites that can increase the toxicity (Nebert et al., 2004). Several studies have found that
786 hydroxylated PAHs have greater toxic effects than the parent compounds (Chibwe et al., 2015;
787 Diamante et al., 2017; Fallahtafti et al., 2012; Schrlau et al., 2017). Bioactivation of PAHs has
788 also been linked to endocrine disruption (Fernandes and Porte, 2013; Hyzd'alova et al., 2018;
789 Pencikova et al., 2019; Sievers et al., 2013; Van de Wiele et al., 2005). Moreover, electrophilic
790 intermediates of PAH metabolism are well known for the formation of hydrophobic DNA
791 adducts and cancer induction (Moorthy et al., 2015). PAH metabolites may also play an
792 important role in cardiotoxicity. PHE and oil are shown to block currents of $K^+$ through the
793 ERG potassium channel (Brette et al., 2014; Brette et al., 2017). However, the exact mechanism
794 behind how three-ring PAHs are able to block the channel is not well defined (Incardona, 2017).
795 *In silico* docking models suggest that PHE has the necessary three aromatic rings, but lacks any
796 other functional groups essential for strong blocking capacity (Cavalli et al., 2012; Vandenberg
797 et al., 2012). Other types of membrane ion channels have been intensively studied through the
798 use of PAH metabolites: The 9-hydroxy-PHE metabolite (9-phenanthrol) is a selective and
799 sensitive blocker of the transient receptor potential melastatin 4 channels (TRPM4) (Burris et
800 al., 2015; Guinamard et al., 2014; Hou et al., 2018) and anthracene-9-carboxylic acid is a
801 commonly used blocker of the calcium-activated chloride channels (CaCCs) (TMEM16A)
802 channels (Cherian et al., 2015; Ta et al., 2016). The blocking capacity of PAH metabolites to
803 the ERG channels has not yet been studied to our knowledge, but it is possible, if not likely,
804 that critical aspects of crude oil cardiotoxicity are caused by an ERG-blocking PAH metabolite.
805 Taken together we see a need for more studies of the effects of PAH metabolism in oil toxicity,
806 and urge more labs to develope analytical methods able to cover a broad spectra of oil-related
807 metabolites. The latter is a great methological challenge due to extreme complexeity in the
808 number of metabolites and the lack of available standards.
809 We show that *cyp1* mRNA induction, PAH tissue content, and PAH metabolites are
810 related to early life stage toxicity in fish, where the amount of mRNA induction and PAH and
811 metabolite levels is related to the window of exposure. This observation is in line with an
812 established relationship between toxicity and PAHs in crude oil (Carls and Meador, 2009;
813 Hodson, 2017) and petrogenic substances (Adams et al., 2014; Kamelia et al., 2019; Kamelia

et al., 2017). However, there are multiple toxic components in crude oil apart from PAHs that can induce toxicity in early life stages of fish (Meador and Nahrgang, 2019; Sorensen et al., 2019). Therefore, there is a need for more fractionation studies and Effect Directed Analysis (EDA) to increase our understanding of the additive and synergetic effects of the complex oil toxicity.

### 4.3. Morphological abnormalities

The large differences in morphological abnormalities after *early* vs *late* exposure~~,~~ show the importance of timing of exposure. The *early* exposure has major consequences for formation of organs by interfering with the developmental signaling pathways, while *late* exposure mainly shows effect on organism condition possibly downstream of cardiofunctional defects.

The eye abnormalities were most likely downstream of both circulation defects and altered cyp-balance. Crude oil-induced eye developmental defects are well documented. Both reduced eye size (anapthalmia) and reduced size with abnormalities (microphtalmia) are observed (Incardona, 2014; Lie et al., 2019; Magnuson et al., 2018). In the present study, eye size was mainly affected in the *early* exposure, where we find the most severe developmental and functional abnormalities. Zebrafish with no cardiac function (*tnnt* morpholinos) mimic the oil-induced reduction in eye size (Incardona et al., 2004), suggesting that anapthalmia is secondary to circulatory defects. However, it is also suggested that abnormal cyp-balance disrupts retinoic acid signaling pathway through disturbance of Cyp26, leading to abnormal eye development (Lie et al., 2019; Yamamoto et al., 2000). Another Cyp enzyme, Cyp1b, is also involved in eye development (regulating ocular fissure closure) through both retinoic acid-dependent and -independent pathways (Chambers et al., 2007; Williams et al., 2017). Affirmative of a non-toxicological function of Cyp1b was the general increase in expression of *cyp1b* beginning at 3.5 dpf (24 hpes). Overexpression of *cyp1b* causes irregular eye shape referred to as coloboma (Chambers et al., 2007; Williams et al., 2017). The *late* exposure animals possessed less severe abnormalities in general. However, the *H sub* and *H surf* groups still showed excessive incidence of eye abnormalities (irregular eye shape) which were reflected in increased *cyp1b* expression. These findings are in line with findings that proper closure of the ocular fissure is disrupted by overexpression of *cyp1b* (Chambers et al., 2007; Williams et al., 2017).

The craniofacial abnormalities observed in our exposed animals are potentially due to both disruption of jaw and pharyngeal arch formation and reduced outgrowth linked to

848 abnormal craniofacial muscle function. Expression of Cyp1b was found in pharyngeal arches
849 of zebrafish exposed to PCB 126 and TCDD (Timme-Laragy et al., 2008; Yin et al., 2008), and
850 the injection of human *cyp1b* mRNA resulted in disruption of neural crest-derived jaw and
851 pharyngeal arch formation (Williams et al., 2017). Furthermore, muscle contraction is
852 mandatory for proper stacking of chondrocytes and outgrowth of craniofacial cartilages
853 (Shwartz et al., 2012). Crude oil disrupts cardiac muscle function (Brette et al., 2014; Brette et
854 al., 2017; Incardona, 2014), and jaw spasms observed in previous studies suggest a similar
855 effect on the craniofacial muscles. Impairment of craniofacial muscles might explain the high
856 incidences of craniofacial abnormalites in the *late* exposure. In conclusion, the tight correlation
857 between *cyp1b* expression and oil induced eye and jaw deformities suggests that inappropriate
858 expression of *cyp1b* in oil-exposed animals are directly linked to abnormal eye and jaw
859 development.
860
861 **4.4. Cardiac morphological defects**
862 The severely malformed and small hearts after *early* exposure imply multiple impacts
863 on cardiac formation and early development, while the reduced heart size after *late* exposure
864 suggests on late development and especially cardiomyocyte proliferation.
865 A circulation- and time-independent expression of *bmp10* suggests a direct effect of
866 crude oil on calcium homeostasis. Several signaling pathways linked to cardiac development
867 and proliferation are impacted by the complexity of crude oil exposure, including calcium-
868 regulated pathways (Sørhus et al., 2017; Xu et al., 2017), AhR (Yin et al., 2008) and retinoic
869 acid (Lie et al., 2019)- dependent pathways. Disruption of intracellular calcium levels has been
870 shown to reduce cardiomyocyte proliferation leading to smaller ventricles (Ebert et al., 2005;
871 Rottbauer et al., 2001), suggesting that ventricular proliferation is calcium controlled. Bmp10
872 is a calcium-regulated signaling molecule involved in cardiogenesis (Huang et al., 2012;
873 Wamhoff et al., 2004; Wamhoff et al., 2006) and is thought to orchestrate several major key
874 cardiogenic factors and play a part in ventricular cell proliferation (Chen et al., 2004; Shou et
875 al., 1998). In the current study, we observed an up-regulation of *bmp10* regardless of timing of
876 exposure. However, the developmental consequences of timing of an inapropriate gene
877 regulation is essential. In the *early* exposure, the initiation of *bmp10* up-regulation was
878 occurring at cardiac cone stage (72 hpes). At this developmental time point asymmetric Bmp
879 signaling (in particular bmp4) in the cardiac primordium is involved in the looping of the heart
880 (Chocron et al., 2007; Huang et al., 2012; Lombardo et al., 2019). An overexpression of *bmp10*
881 in the cardiac primordium may therefore disrupt left/right looping of the heart. At the start of

882 the *late* exposure, the looping process was already initiated, and the heart was functionally
883 beating. Consequently, we observed less severe effect on looping, while growth of the heart
884 was altered. Reduced size of the ventricle was observed in both *H sub* and *H surf*, as well as an
885 overall up-regulation of *bmp10*. The up-regulation of *bmp10* may be consequential for the
886 reduced ventricle size in exposed larvae from the late embryonic stages. Taken together, the
887 observations from *early* and *late* exposures support that the oil components have a circulation-
888 independent direct effect on calcium homeostasis likely disrupting cardiac development and
889 proliferation indicated by the up-regulation of *bmp10*.
890
891 **4.5. Cardiac functional defects**
892 The cardiac functional defects post-exposure were linked to irreversible
893 morphological changes and reversible effects of circulating crude oil components. Reduced
894 contractility and non-contracting or constantly contracting ventricles (SiV phenotype) were
895 traits found in both *early* and *late* exposures. Functional defects in the *early* exposure could
896 originate from irreversible morphological changes (severly malformed ventricles) but could
897 also stem from consumption of oil components stored in the yolk sac after hatching as
898 observed in previous studies (Sørhus et al., 2016b; Vanleeuwen et al., 1985).. The *late*
899 exposure animals, on the other hand, had normally shaped but often smaller ventricles. The
900 contractility abnormalities most likely stem from components stored in the yolk sac.
901 A suite of defects downstream of cardiac dysfunction has been described for oil-exposed
902 developing fish, including edema accumulation (Incardona, 2017; Incardona and Scholz, 2016).
903 Edema formation measured included both pericardial and yolk sac edemas, and may be due to
904 i) irreversible morphological cardiac deformities that alter the cardiac function later in life and
905 ii) oil components that can directly alter the electrophysiological activity (contractility and
906 rhythm) of the developing heart (Brette et al., 2014; Brette et al., 2017; Incardona, 2017).
907 Circulatory defects may also affect distribution of lipids from the yolk which in turn may affect
908 growth in later stages. Polar cod exposed to transient low levels of oil induced dysregulation of
909 lipid metabolism and reduced growth in morphologically normal juveniles (Laurel et al., 2019).
910 We observed a reduced yolk utilization in *H surf* in the *early* exposure, suggesting that these
911 embryos were deprived of essential yolk-based lipids. Furthermore, the reduced yolk utilization
912 seems to be associated with heart rate, and thus, dependent on circulation. Accordingly, the
913 treatment group~~s~~ with tachycardia had highly increased yolk utilization (*H WSF*), while the
914 treatement group~~s~~ with bradycardia (*H surf*) had severely reduced yolk utilization. Overall,

915 cardiocirculatory defects during embryonic development could be sufficient to impact growth
916 and could be consequential for individual fitness at juvenile and adult stages.
917
918 **4.6. Osmoregulation**
919 Up-regulation of *rhag* suggests that osmoregulatory cells are impacted by oil exposure.
920 Osmoregulation can be disrupted by circulatory defects (Miyanishi et al., 2013), or directly
921 affected by oil components (Sørhus et al., 2017). In adult fish, the main localization for
922 osmoregulation, gas exchange and transport of ammonium are in the gills (Evans et al., 2005)
923 in osmoregulatory cells called mitochondrial rich cells (MRCs) (Evans et al., 2005; Hiroi et al.,
924 2005; Hirose et al., 2003; Shelbourne, 1957). In 10 dpf haddock, expression of *rhag* was found
925 in the branchial arches (primordial gills), in close proximity to expected localization of MRCs
926 (Zimmer et al., 2014; Zimmer et al., 2017). This finding coincides with expression of *rhag* in
927 mahi mahi at approximately the same developmental time point (Wang et al., 2019). In both
928 *early* and *late* exposures, *rhag* seemed to track more with the peak of *cyp1a* induction and
929 *abcb1* transporter. This could indicate that energy for Cyp1a-mediated PAH metabolism is
930 derived from amino acids (Pasparakis et al., 2016), generating nitrogenous waste also suggested
931 by Wang et al. (2019). Thus, this increased amount of ammonium waste can also affect
932 osmoregulation capacity of MRCs and consequently lead to increased edema formation.
933 Additionally, ammonia excess in itself can add to the toxicity. In mouse, ammonium perturbs
934 glial cells ability to remove potassium and are therefore toxic to the brain (Thrane et al., 2013).
935 Consequently, the crude oil exposed embryos could experience an internal synergy between
936 internal ammonium toxicity and PAH toxicity.
937
938 **5. Conclusion**
939 In this study we showed that oil droplet fouling occurred throughout the whole water
940 column and contributed to increased PAH uptake and embryotoxicity. Oil droplet fouling
941 makes the haddock embryo extremely vulnerable to even short, low level oil exposures. The
942 high bioaccumulation of PAHs and lower levels of metabolites in the *early* exposure reflects
943 an immature response system that possibly has an exacerbating toxic effect in early embryos.
944 Short term exposures prior to and after onset of heart beat and frequent sampling regime enabled
945 us to establish direct and downstream effects of crude oil exposure. The relatively rapid and
946 circulatory-independent response in *bmp10* regulation suggested direct effects on calcium
947 homeostasis affecting calcium-regulated developmental pathways, including cardiogenesis.
948 Similarly, we propose that abnormal regulation of *cyp1b* due to oil exposure may affect eye and

jaw development. Expression of *rhag* indicates a direct oil effect on osmoregulatory cells and osmoregulation. We recognize that oil is a complex mixture of many potential toxic components. Yet, the strong correlations among PAH uptake, *cyp1a* induction, metabolite formation and malformations suggest that PAH tissue body burden remains a good metric for oil toxicity.

Our findings are adding more knowledge about development stage-dependent effects of crude oil exposure. Thus, we provide more knowledge and detail to several existing adverse outcome pathways of crude oil toxicity.

## 6. Acknowledgements

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## 7. Author contributions

Conceived and designed the experiments: E.S., S.M., Ø.K. and A.T.
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## 8. Competing interests

The authors declare no competing financial interests.

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