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Differential developmental toxicity of crude oil in early life stages of Atlantic halibut (*Hippoglossus hippoglossus*)

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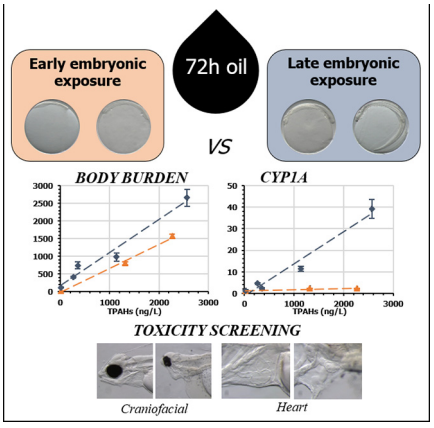
HIGHLIGHTS

- Atlantic halibut displayed latent toxic effects after crude oil embryonic exposures.
- Metabolic *cyp* induction was higher when fish were exposed during organogenesis.
- Concentration dependent decrease of condition and fitness during yolk-sac stage
- Oil exposure during organogenesis induced greater teratogenic effects in halibut.

GRAPHICAL ABSTRACT



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ABSTRACT

To further understand the complexity of developmental toxicity of dispersed oil and importance of exposure timing on fish early life stages, Atlantic halibut (*Hippoglossus hippoglossus*) were exposed to environmentally relevant concentrations through two embryonic developmental windows: the first period occurred during the epiboly process (named as "early embryonic exposure") and the second period overlapped the ontogenesis and cardiogenesis processes (named as "late embryonic exposure"). Following 72 hour oil exposure, embryos were transferred to clean seawater and a toxicity screening was performed in the yolk-sac larvae until first-feeding stages (56 days). The current study demonstrated that the exposure timing is essential for the development of toxic effects of crude oil in Atlantic halibut. Neither embryonic exposures (early or late) showed notable acute toxicity during exposure, yet both showed global latent teratogenic effects during yolk sac stages. Fish exposed during organogenesis (late) displayed stronger and more severe toxic effects than fish exposed during epiboly process (early), including reduced condition, severe craniofacial deformities and cardiovascular disruptions. The uptake level of polycyclic aromatic hydrocarbons into larval tissue and metabolic activity were greater following the late embryonic exposure and remained high during the depuration period at the highest exposure concentration. Overall, the long yolk sac stage development timing of Atlantic halibut makes this species a good candidate for evaluation of embryonic crude oil toxicity and its mechanisms.

1. Introduction

Oil pollution has been highlighted as one of the most pervasive human impacts and represents a strong threat for the marine ecosystems. Around

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the world, common accidents such as the *Amoco Cadiz* (1978, France), the *Exxon Valdez* (1989, Alaska), the *Erica* (1999, France), the *Prestige* (2002, Spain) or the *Deepwater Horizon* oil drilling explosion (2010, Gulf of Mexico) resulted in disastrous consequences on the local ecosystems and natural fisheries (Barron et al., 2020; Beyer et al., 2016; GESAMP, 2007; Moreno et al., 2013; Peterson et al., 2003; Rohal et al., 2020). The toxicity of the petroleum compounds and its effect on teleost fishes have been thoroughly documented through field and laboratory studies (Burggren et al., 2015; Claireaux et al., 2004; Incardona, 2017; Incardona et al., 2013, 2014; Jones et al., 2020; Jung et al., 2017; Pasparakis et al., 2019), although the damages caused differ based on the composition and the environment that the oil is released into.

Crude oil is a complex mixture of chemical compounds, and only a fraction of these compounds is amenable to characterization using current analytical techniques. Oil toxicity is a combination of effects of multiple chemical compounds, both polar (Melbye et al., 2009) and hydrophobic (Adams et al., 2014). Although the aromatic fraction is not the only class of toxic compounds present in petroleum, polycyclic aromatic hydrocarbons (PAHs) are strongly correlated with the toxicity of oil products (Adams et al., 2014; Kamelia et al., 2019). Metabolism of the larger PAHs is initiated by activation of the aryl hydrocarbon receptor (AHR), which in turn induces the xenobiotic biotransformation battery, such as Cytochrome P4501A (CYP1A). Accordingly, induction of the CYP1A paralog has for a long time been used as a sensitive biomarker for crude oil toxicity (Goksøyr, 1995). Other CYP1 paralogs such as CYP1B and CYP1C are also involved in the biotransformation phase of PAHs, however, the induction response is dependent on developmental stage (Kühnert et al., 2017; Sørhus et al., 2021).

There is a general understanding that it is primarily the water-soluble oil compounds that provide the largest contribution to the toxicity to fish, as they are more readily bioavailable (Carls et al., 2008; Redman and Parkerton, 2015). However, microdroplets of dispersed oil are shown to increase the effects of embryonic oil exposure for some fish, like Atlantic haddock (*Melanogrammus aeglefinus*) (Hansen et al., 2019a; Sørensen et al., 2017; Sørhus et al., 2015, 2016b) and medaka (*Oryzias latipes*) (González-Doncel et al., 2008). Therefore, dispersed oil should be included in realistic oil dosing (Nordtug et al., 2011). Early life stages of fish are particularly vulnerable to crude oil due to their critical developmental window, limited mobility, transparency, high relative content of lipids and primitive metabolic capacities (Petersen and Kristensen, 1998). It is now well established that fish embryos exposed to trace levels of crude oil exhibit a common syndrome of developmental toxicity (Incardona, 2017; Pasparakis et al., 2019).

The cardiovascular system of fishes at the onset of organogenesis is a major primary target of crude oil exposure (Edmunds et al., 2015; Esbaugh et al., 2016; Incardona et al., 2004, 2013; Incardona and Scholz, 2016; Khursigara et al., 2017; Lema et al., 2007; Mager et al., 2014; Perrichon et al., 2016, 2018; Xu et al., 2016). PAHs and crude oil cause a suite of pathologies related to embryonic heart failure, mostly including bradycardias and arrhythmias (Edmunds et al., 2015; Incardona et al., 2005, 2009, 2013; Perrichon et al., 2016; Sørhus et al., 2016b). These functional cardiac impairments are often accompanied with improper cardiac chamber looping, shape, orientation or contractility (Perrichon et al., 2018; Sørhus et al., 2017). The primary etiology of defects induced by crude oil compounds on cardiovascular function have shown secondary consequences on osmoregulation processes (edema formation) and neural tube structure and formation of craniofacial skeleton (eye, jaw, finfold) in a variety of fish species (Adeyemo et al., 2015; Cypher et al., 2017; de Soysa et al., 2012; Edmunds et al., 2015; Hansen et al., 2019b; Incardona et al., 2014; Khursigara et al., 2017; Lema et al., 2007; Pasparakis et al., 2016; Perrichon et al., 2018; Sørhus et al., 2016b, 2021). At high doses, these impairments may induce increased injuries, developmental delay or mortality (Heintz et al., 2000; Incardona et al., 2009, 2013; Incardona and Scholz, 2016).

Multiple studies over the last two decades have shown that developing fish are highly susceptible to crude oil exposure, however several factors impede direct comparison between species: variation in preparation of oil mixture, exposure duration, differences in species sensitivity, habitat differences or developmental window used in the toxicity testing. In particular, cold-water fishes have been suggested to be more sensitive to oil-induced toxicity than sub-tropical-water fishes (Edmunds et al., 2015; Hansen et al., 2019b; Incardona et al., 2014; Incardona and Scholz, 2016; Laurel et al., 2019; Sørhus et al., 2015, 2016b). Recently, the opening for oil drilling in the sensitive areas along the Norwegian coast, the Barents Sea and the Arctic poses great concern making the spawning grounds and larval-drift areas highly vulnerable to oil spills which may damage harvestable fish populations (Olsen et al., 2010; Ottersen et al., 2014; Stige et al., 2018). Consequently, a large effort has been made to develop risk assessment tools to document the potential impact of dispersed oil on these spawning areas and ecophysiological characteristics of several commercially important cold water species, including haddock (*Melanogrammus aeglefinus*), cod (*Gadus morhua*), Polar cod (*Boreogadus saida*), saithe (*Pollachius virens* L.), Atlantic halibut (*Hippoglossus hippoglossus*), and herring (*Clupea harengus*) (Hansen et al., 2019b; Laurel et al., 2019; Lie et al., 2019; Sørensen et al., 2017; Sørhus et al., 2016a, 2016b, 2021).

The Atlantic halibut is a large, long-lived, cold-water flatfish inhabiting the boreal and subarctic waters and is an attractive demersal species of high commercial interest for the Northern European industry (Haug, 1990). The early developmental stages of halibut are pelagic with larger eggs (~2.9 mm) and greater yolk reserve than cod or haddock fishes. The halibut embryos develop slowly; hatching occurs at ~15 days post-fertilization (dpf, 6 °C) and the premature yolk-sac stage lasts for 35 days until first-feeding stages. The advantage of using Atlantic halibut, is their optic transparency until metamorphosis, giving opportunities for direct and visual observation of the long developmental physiological processes. The aim of the present work was to explore the extent of developmental patterns in Atlantic halibut following short-term exposures of crude oil through two critical embryonic developmental windows (early embryonic exposure: from 5- to 8-dpf; late embryonic exposure: from 10- to 13-dpf). The choice for exposure timing was intended to cover two major processes of embryonic development. First, the early embryonic experiment overlapped during the gastrulation/segmentation transition stage when the epiboly process occurs, with appearance of neural groove, the otic placode and the optic primordium. Second, the late embryonic exposure overlapped during the somitogenesis and the prehatching stages, therefore when organogenesis occurs in the embryo. This work will enable us to understand how timing may affect the morphology, condition and cardiac function of early life stages of halibut.

2. Materials and methods

2.1. Halibut egg production

The Atlantic halibut embryos (*Hippoglossus hippoglossus*) for the exposure assays originated from a single spawning of one female (wild origin) and one neomale from hand-stripped broodstock (Norberg et al., 1991) held at the Institute of Marine Research, Austevoll Research Station, Storebø, Norway. Fertilized eggs were separated into two batches (fertilization rate = 90%) and were then incubated in 250 L tanks at 6 °C, salinity 34‰ and in total darkness until transfer to the exposure tanks.

2.2. Experimental design

Crude oil was obtained from SINTEF Materials and Chemistry (Trondheim, Norway) and originates from the Trollfield in the North Sea. Before exposure, the crude oil was heated to 200 °C to remove light and volatile substances present in crude oil, similar to what would evaporate from the sea surface after 2–7 days at around 10 °C air temperature (Nordtug et al., 2011).

The principle of the exposure system and procedures are described in detail in Nordtug et al. (2011) and Sørhus et al. (2016a, 2016b). Oil exposures were performed with an oil dispersion system and twelve 50 L circular tanks. The oil was pumped into the dispersion system using a HPLC pump (Shimadzu, LC-20AD Liquid Chromatograph Pump) with a flow of 5 μL/min together with a flow of seawater of 180 mL/min. The system generates an oil dispersion with oil droplets in the low μm range with a nominal oil load of 31 mg/L (stock solution). The exposure dose to the tanks was regulated by a parallel pipeline system with one line of flowing clean seawater and the other line containing a flow of the stock solution. The 2 pipelines were connected by a 3-way magnetic valve allowing water to be collected from both lines. Different dilutions were made by controlling the relative sampling time from the oil stock solution and clean water, respectively.

The experimental set up (Fig. 1) consisted of two exposure experiments, hereafter referred as "early embryonic exposure" and "late embryonic exposure". The exposure time periods covered two distinct embryonic developmental windows. The early embryonic exposure (from 5- to 8-dpf) overlapped with the epiboly process and the somitogenesis, while the late embryonic exposure (from 10- to 13-dpf) coincided with the end of somitogenesis and the prehatching stages, therefore further in the organogenesis. During the late developmental period, the myotome is forming, the eye is developing with apparent lens and retina and the onset of heartbeat begins (~10.5–11-dpf) with irregular peristaltic movements.

The applied exposure doses consisted of two treatments (150 and 300 μg oil/L) for the early embryonic experiment and five treatments (38, 75, 150, 300 and 600 μg oil/L) for the late embryonic experiment. The experiments shared two concentrations (150 and 300 μg oil/L) in common to enable the comparison. All doses were based from previous studies using cod and haddock to enable the comparison between species (Sørensen et al., 2017; Sørhus et al., 2015, 2016b, 2021). Each experiment had a control with clean seawater.

For each treatment, ~3000 halibut eggs were transferred into midwater nets settled into the circular exposure tanks. These nets were made to simulate exposure in the water column and were positioned below the water surface to keep eggs underneath the concentrated surfaced oil. Exposures were performed during 72 h at 6 ± 0.5 °C and in total darkness. Following the 72 h oil exposure (early exposure: 8 dpf; late exposure 13 dpf), 180 embryos per treatment were transferred individually into 6 well Nunc™ cell culture plates containing 8 mL clean seawater and maintained at 6 ± 0.5 °C in the dark for further monitoring (Fig. 1). Water was renewed (3 mL) on a weekly basis to maintain good oxygenation. Hatching period occurred between 14 and 16 dpf. Hatching success was recorded. Survival of individuals was monitored until the first-feeding stages (56 dpf = 42 dph, days post-hatching). Embryos were subsampled at different developmental time points for morphological analyses, condition and gene expressions (see the timeline Fig. 1).

2.3. Water chemistry

Water samples (1 L) were collected from each exposure tank in 1 L glass bottles at 0- and 72-hour post-exposure during the both experiments. Samples were preserved by acidification (HCl, pH < 2) and stored at 4 °C in the dark until chemical analysis. Deuterated internal standards (naphthalene-d8, biphenyl-d8, acenaphtylene-d8, anthracene-d10, pyrene-d10, perylene-d12 and indeno[1,2,3-c,d] perylene-d12) were added prior to extraction. An aliquot of 1 mL from each sample was then extracted twice by partitioning to dichloromethane and dried with Na_2SO_4 (0.2–0.4 g). Extracts were concentrated to 200 μL and solvent exchange into isooctane. PAHs were then quantified by using Agilent 7890 gas chromatograph coupled to an Agilent 7010c triple quadrupole mass detector (GC/MS-MS) as described in Sørensen et al. (2016a, 2016b). Blank analysis was carried out to ensure the absence of contamination prior to and during analysis. Reported TPAH values represent the total sum of 47 PAHs analytes and alkyl-clusters selected for standard analysis (Table S1) at initial (0 h) and final (72 h) concentrations and their respective geometric means.

2.4. PAHs body burden

Halibut egg samples were collected (5–20 eggs per replicate) at 7 (early exposure) and 10 (late exposure) time points and snap frozen in liquid nitrogen until extraction (Fig. 1). Sample replicates (N = 1–3) were taken from each time point in both experiments. Final timepoint and early recovery period (late exposure) were only analyzed for the concentration 38, 75 and 600 μg oil/L. Samples were homogenized after adding internal standard and extracted after Sørensen et al. (2016a, 2016b) using (1:1, v:v) *n*-hexane:dichloromethane and dried with 0.2 g Na_2SO_4. Sample clean-up was done with Chromabond SiOH solid phase extraction columns (3 mL, 500 mg, Machery-Nagel, Germany) and eluted with 9:1 *n*-hexane:dichloromethane. Extracts were reduced to 50–100 μL. PAHs were then quantified by using Agilent 7890 gas chromatograph coupled to an Agilent 7010c triple quadrupole mass detector (GC/MS-MS) as described in Sørensen et al. (2016a, 2016b). Blank analysis was carried out to ensure the absence of contamination prior and during analysis. Reported values represent the total sum PAHs per individual (pg PAH/individual) and g of wet weight (ng PAH/ g ww).

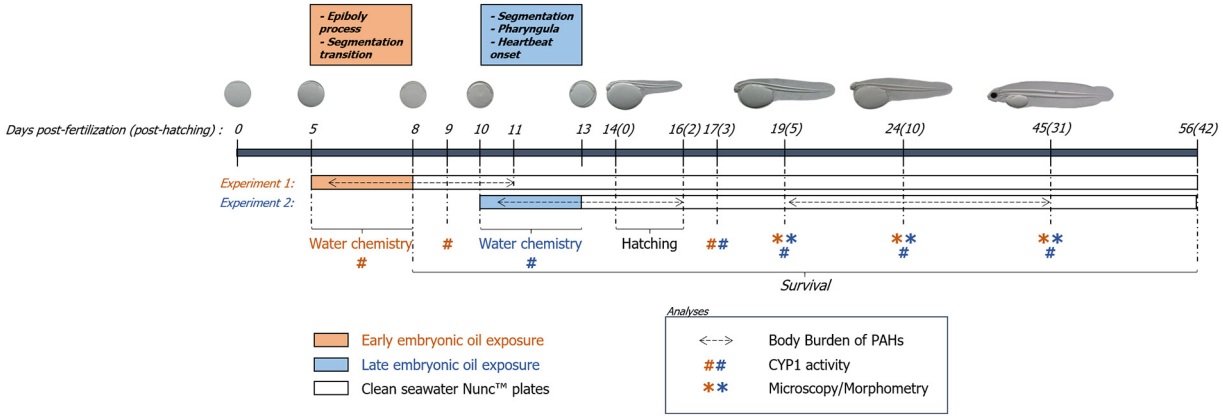

Fig. 1. Experimental design. Developmental timeline and the embryonic oil exposure periods are indicated in orange (Experiment 1: Early embryonic exposure) and in blue (Experiment 2: Late embryonic exposure). Sampling and biological analyses are represented by the symbols and the reference colors.

2.5. Gene expression of cyp 1a, b and c

Halibut eggs were sampled at 6 (early exposure) and 8 (late exposure) time points (Fig. 1). Triplicates of 10 eggs were collected during the exposure while individual samples were collected after exposure. A single sample per treatment was analyzed at 96 h (early exposure) and 3 dph (early and late exposures). Once collected, eggs and larvae were snap-frozen in liquid nitrogen and stored at −80 °C until extraction. RNA was extracted using Maxwell® HT SimplyRNA kit (Promega, Madison, WI, USA) and Biomek® 4000 automated liquid handler (Beckman Coulter, Indianapolis, IN, USA) following the manufacturer's instructions. Samples at 5, 10 and 31 dph in the late experiment were extracted using TRIzol™ Reagent (Invitrogen, Life Technologies, Oslo, Norway) and RNeasy mini Kit (Qiagen, Oslo, Norway) according to the manufacturer's instructions. The procedures included a DNase treatment step using DNase I combined. The amount of RNA and purity was quantified using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

First-strand cDNA was synthetized from 1 μg total RNAs. Reaction mix included 3 μL of RT buffer, 6.6 μL of 25 mM magnesium chloride, 6 μL of 10 mM deoxyribonucleotide triphosphate (dNTPs) and 1.5 μL of 50 μM of oligo(dT)16 primer for a final volume of 30 μL. cDNA was normalized to obtain a concentration of 50 ng/μL. and 0.6 μL of RNase inhibitor (20 U/μL). The reaction was initiated using 2.5 μL of Multiscribe Reverse Transcriptase 50 U/ μL and 0.6 μL of 20 U/μL RNase inhibitor (Applied Biosystems™, Madison, USA). Reverse transcription reaction was carried out for 1 h at 48 °C in an Eppendorf Mastercycler and inactivated by heating for 5 min at 95 °C. The cDNA mixture was stored at −20 °C until real-time PCR analysis.

Quantification was performed using SYBR® Green methodology. The reaction mixture included 5 μL Fast SYBR® Green Master Mix 5× (Roche Norge A/S, Oslo, Norway), 0.1 μL of primers (F and R) at 50 μM (ThermoFisher, Life Technologies A/S), 2.8 μL of Milli-Q water and 2 μL of cDNA. PCR primers of four selected genes were designed with GenScript® software and are reported in Table S2. SYBR Green PCR assays were performed in duplicate, using QuantStudio™ 5 Real-Time PCR System (Applied Biosystems™, Carlsbad, California, USA). Thermal cycling conditions were: 5 min at 95 °C, followed by 45 cycles at 95 °C for 10 s, 10 s at 60 °C and 10 s at 72 °C. The specificity of the process was verified after completion of the PCR run, by testing the nature of the amplified product with gel electrophoresis and melting curves. Gene expression data were normalized to one housekeeping gene *Gtf3c6* (Edvardsen et al., pers. comm.) and calculated relative to the respective control using the ΔΔCt method (Livak and Schmittgen, 2001).

2.6. Morphometry and condition

Digital images of hatched larvae were recorded using video cameras Moticam 1080 (Motic®, Richmond, BC, Canada) mounted on Olympus SZX10 stereomicroscope. Unanaesthetized larvae were individually immobilized in a Petri dish containing 2% methylcellulose/98% seawater and positioned on a thermally regulated microscope stage (Brook Industries, Lake Villa, IL) at 6 °C. Images and twenty-second live videos were digitized at 30 frames.s^{-1} using Motic Live Imaging Module. Larvae were oriented in left lateral view for image capturing and in ventral view for video capturing at 5- and 10- dph. At 31 dph, all images and videos were captured in left lateral view due to the migration of the cardiac chambers in lateral position.

Morphometric measurements were processed by ImageJ software (Schneider et al., 2012). Standard length (mm), myotome height (mm), fluid area of the yolk sac (mm^2), pericardial area (mm^2), finfold thickness (mm), opening mouth angle (°), distance eye-premaxillary (mm) and left eye area (mm^2) were measured in larvae at different developmental stages (Fig. 1). All imaging measurements were performed blind to treatment.

Morphometric measurements were additionally supplemented by scoring of craniofacial deformities regarding jaw structures. Table S3 summarizes the five categories of jaw phenotypic deformities scored in 31 dph larvae.

2.7. Ventricular cardiac function

Heart rate and stroke volume were determined from video sequences of the ventricle during halibut development and used for cardiac output calculation according to the methodology reported in Perrichon et al. (2017). Heart rate was determined by manually counting the number of ventricular contractions in each 20s-video collected through development. Stroke volumes were determined by outlining the ventricular perimeter during end-diastole and end-systole (Perrichon et al., 2017, 2018). Ventricular perimeter was fitted to the image with an ellipse using ImageJ software (Schneider et al., 2012), where major and minor axes were then extracted. End-diastolic and end-systolic volumes of the ventricle were calculated using the conventional prolate spheroid (PS) formula (Perrichon et al., 2017):

$$V_{PS} = \frac{\pi}{6} ab^2$$

where *a* represents the major (longitudinal) semi-axis and *b* the minor (width) semi-axis. The mean stroke volume (nL) was calculated as the difference between diastolic and systolic ventricular volumes. Cardiac output (nL/min) was calculated as the product of heart rate and stroke volume.

Cardiac morphological deformities were assessed through the orientation angle (looping) of cardiac chambers during development. Also, four categories of cardiac phenotypes were scored in 31 dph larvae. Further details are described in Table S3.

2.8. Statistical analyses

Statistical analyses were made using R statistical software. Water chemistry, body burden, survival and hatching success are expressed as mean ± standard error of mean (SEM). Linear regressions and slopes differences were tested for the metabolic activity (bioconcentration and activity of *cyps*). Morphometry, condition of larvae, craniofacial measurement and cardiac function are shown by boxplots with the median (horizontal line), Q1-Q3 quartiles (box), the mean (black point) and the non-outlier range (whisker). Concentrations effects were evaluated with one-way and two-way ANOVAs, followed by Tukey multiple comparisons post hoc test. Jaw and heart phenotype data are shown as percent of each category, and differences were tested through Pearson Chi-Squared test. A significance level of 5% was used for all analyses.

3. Results and discussion

Atlantic halibut displayed a concentration dependent latent decrease of larval condition and fitness following embryonic oil exposure. Dispersed oil induced functional and morphometric symptoms of crude oil syndrome, including yolk sac edema, reduction of pericardial chamber, cardiomorphological failures (delayed looping, reduced size chamber, absence of atrio-ventricular junction) and cardiac function disruptions (bradycardia, silent ventricle, contractility reduction). Milder secondary phenotypes downstream of cardiac failure included craniofacial deformities (jaw, eye, finfold). When comparing between exposure windows, consequences of embryonic oil exposure on morphology and cardiac function in halibut appeared to be more severe in the late embryonic experiment and notably in the highest exposed doses. The late exposed fish had also higher uptake of polycyclic aromatic hydrocarbons and greater metabolic capacity (*cyps*). This may

be explained by the higher sensitivity of embryos when organs begin to develop.

3.1. Differential PAH uptake and metabolism between early and late embryonic exposures

Concentration dependent increase in body burden levels were observed in both experiments during the exposure period (Table 1, Fig. 2A). Uptake of PAHs during the late embryonic exposures was significantly higher than the early embryonic exposure (Fig. 2A). An immediate PAHs uptake were observed after just 12 h of exposure in both experiments. Body burden were approximately 12 to 21- fold higher at 300 μg/L compared to the control during the early and late embryonic exposures, respectively. After 72 h of exposure (end), the total PAH tissue concentrations increased 3.2- and 3.6-fold higher at 150 and 300 μg/L during early embryonic exposure, and a concentration 4.0-fold higher for similar concentrations during late embryonic exposure compared to 12 h (Table 1, Fig. 2A). At the end of exposure period (72 h), TPAHs concentration in embryos exposed to 300 μg/L were 1.7-fold higher in late embryonic exposure (2659 ± 235 ng/g ww) than in early embryonic exposure (1570 ± 61 ng/g ww) (Table 1, Fig. 2A). Embryos exposed to 600 μg/L reached a total body burden level at 41717 ± 9467 ng/g ww at the end of late exposure (Table 1). This corresponds to a concentration of 355- and 15.7-fold higher compared to control and 300 μg/L, respectively. While the concentration at this dose should be relatively high, we suspect a measurement bias has been added in this concentration and possibly residual oil adhered on the chorion might have been accumulated in this time point, although precautions and clean water rinsing were undertaken before sampling.

PAH concentrations in halibut embryos during early or late embryonic oil exposure were comparable to the concentrations found in cod or Polar cod species exposed in the same dispersed oil exposure system (Laurel et al., 2019; Sørensen et al., 2017). However, notably higher body burden concentration was found in haddock early life stages with a differential of 2–3-fold higher (Sørensen et al., 2016b, 2017; Sørhus et al., 2021). Differential PAH uptake was also observed depending on the fish developmental stage. Haddock embryos (2961 ± 1258 ng/g ww) accumulated 3.7-fold more PAHs in tissue than larval exposed to high dose concentrations of dispersed oil (Sørhus et al., 2016b). Those differences can be partly explained by the specificity of haddock eggshell that lead to oil droplet binding making the embryo more susceptible to a longer exposure (Hansen et al., 2019b; Sørhus et al., 2016b). Halibut embryos were not physically in contact with oil droplets but were exposed to compounds from the water-soluble fractions. Surprisingly, fish exposed during late embryonic exposure expressed significantly higher uptake while metabolic activity should be higher, therefore reducing PAH tissue uptake. Halibut chorion might be the main explanation and might offer a greater degree of protection to the embryo during early developmental stages until the most of organs develop. Development of main organs is achieved at the end of exposure period during the late experiment and fish are getting ready to hatch by producing hatching enzymes (Yamagami, 1988). These hatching enzymes will help chorion digestion therefore it will become more permeable to waterborne compounds from surrounding environment.

Table 1
Body burden in halibut embryos during and after embryonic oil exposure. Body burden (pg/individual and ng/g wet weight) of PAHs is measured in embryos from treatment groups during the both early and late embryonic exposures. Data are mean ± SEM (N = 1–3).

Experiment	Period	Time	~0 μg/L	38 μg/L	75 μg/L	150 μg/L	300 μg/L	600 μg/L
Body burden (pg PAH/individual)								
Early exposure	Oil exposure	12 h	164 ± 59^{a}			1119 ± 107^{ab}	1954 ± 252^{b}	
		24 h	194 ± 148^{a}			1903 ± 160^{b}	3682 ± 371^{c}	
		48 h	165 ± 85^{a}			3499 ± 228^{b}	6254 ± 259^{c}	
		72 h	92 ± 9^{a}			3617 ± 198^{b}	7065 ± 273^{c}	
	Recover	96 h	756			3515	6169	
		120 h	793			3399	1707	
		144 h	21			2796		
Late exposure	Oil exposure	12 h	142 ± 96^{a}			1103 ± 36^{b}	3057 ± 272^{c}	
		24 h	28 ± 22^{a}			1690 ± 27^{b}	4854 ± 468^{c}	
		48 h	223 ± 76^{a}			2898 ± 331^{b}	9925 ± 239^{c}	
		72 h	529 ± 35^{a}	1826 ± 119^{ab}	3342 ± 423^{b}	4403 ± 510^{b}	11,964 ± 1058^{c}	187,725 ± 42600^{d}
	Recover	96 h	155	208	135	362	833	7689
		120 h	94	247	183	106	1046	4639
		144 h	46	75	99	517	1106	2743
		5 dph	121			26	107	1427
		10 dph	56			29	62	727
		31 dph	*nd*			30	73	183
Body burden (ng PAH/ g ww)								
Early exposure	Oil exposure	12 h	37 ± 13^{a}			249 ± 24^{ab}	434 ± 56^{b}	
		24 h	43 ± 33^{a}			423 ± 36^{b}	818 ± 82^{c}	
		48 h	37 ± 19^{a}			778 ± 51^{b}	1390 ± 57^{c}	
		72 h	20 ± 2^{a}			804 ± 44^{b}	1570 ± 61^{c}	
	Recover	96 h	168			781	1371	
		120 h	176			755	379	
		144 h	5			621		
Late exposure	Oil exposure	12 h	32 ± 21^{a}			245 ± 8^{b}	679 ± 60^{c}	
		24 h	6 ± 5^{a}			376 ± 6^{b}	1079 ± 104^{c}	
		48 h	50 ± 17^{a}			644 ± 74^{b}	2206 ± 53^{c}	
		72 h	117 ± 8^{a}	406 ± 26^{ab}	743 ± 94^{b}	978 ± 113^{b}	2659 ± 235^{c}	41,717 ± 9467^{d}
	Recover	96 h	34	46	30	80	185	1709
		120 h	21	55	41	23	232	1031
		144 h	10	17	22	115	246	609
		5 dph	21			1	5	61
		10 dph	5			2	2	45
		31 dph	*nd*			2	4	12

nd: no data available due to analyses issue; h: hours during experiment; dph: days post-hatching.
Significant differences between oil-exposed groups and the respective control are indicated by lowercase letters (ANOVA and *posthoc* test, $p < 0.05$).

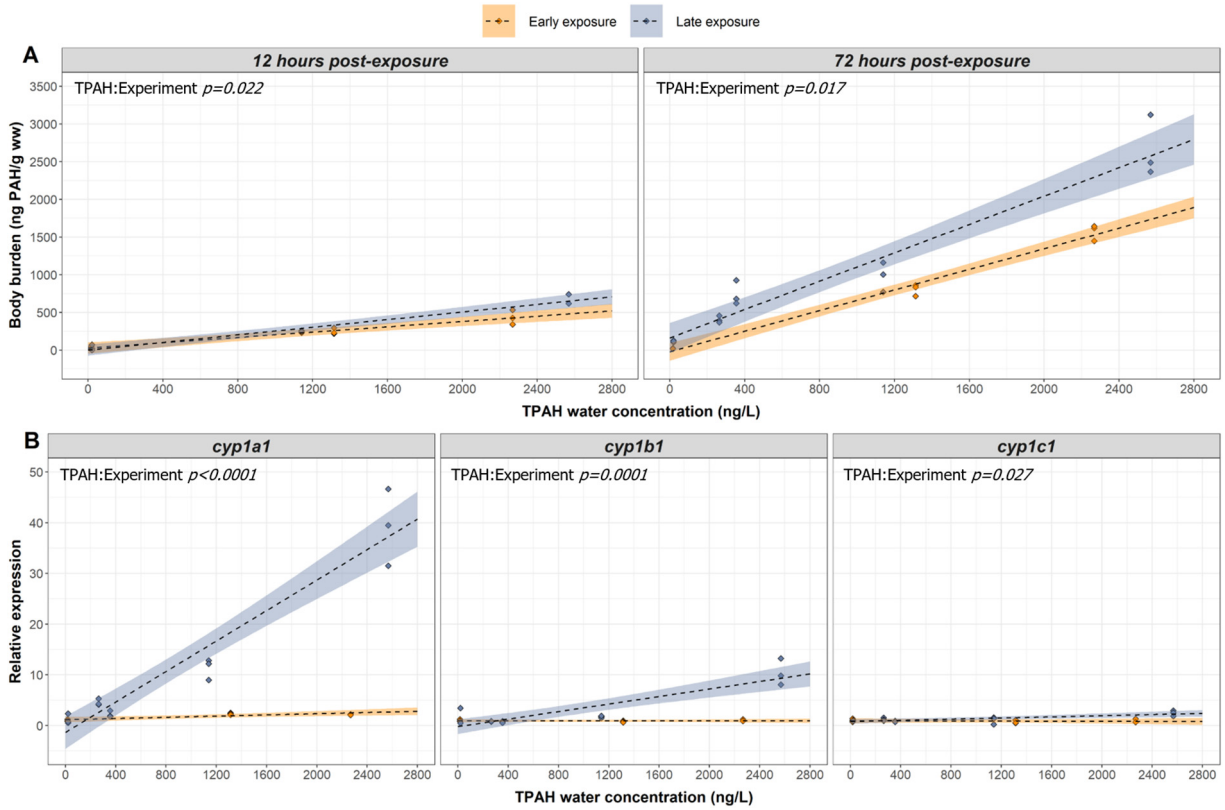

Fig. 2. Metabolic activity in halibut embryos expressed as a function of water exposure concentration (ng/L). Body burden (ng/g ww) is represented after 12 h and 72 h of oil exposure (end exposure) (A) and relative expression of cyp1s activity at the end of exposure (B). Data are mean ± SEM ($N = 1–3$). Dashed lines represent linear regressions (early experiment in orange and late experiment in blue) and bands represent standard errors. The 600 μg/L concentration was omitted in the late embryonic exposure due to erroneous/biased values.

Following one incubation day in clean seawater, PAH levels in halibut dropped drastically (by 86 to 97%) for all doses, consistent with metabolic depuration (Table 1). Despite this, PAH levels appeared to be dose dependent during depuration period with persistently notable higher levels from 150 to 600 μg/L (from 11- to 60-fold higher) after three depuration days (144 h). PAH tissue concentration continues to be higher in 600 μg/L larvae during yolk sac development even after 10 dph (9-fold higher compared to control), indicating a potential dysregulation in the metabolic capacity of these larvae. Halibut larvae have relatively big yolk sac that might act as a "toxicant sink" by storing compounds into the yolk lipids, making them less available for mobilization (Van Leeuwen et al., 1985).

The patterns of *cyp* induction were unique among the different *cyp* genes and developmental stages (Figs. 2B and 3). Activity of Cyps during the late embryonic exposures was significantly higher than the early embryonic exposure (Fig. 2B). During early embryonic exposure (Fig. 3A), *cyp1a1* was not induced until 48 h post exposure. At the end of exposure, *cyp1a1* showed a slight 2.2-fold induction in exposed larvae (150 and 300 μg/L) compared the respective control (Fig. 3A). An early transcriptional signal (12*h*) of basal *cyp1b1* was observed in early embryonic oil exposure but became non-significant at later time points. *cyp1c1* was not significantly up-regulated during the early embryonic exposure (Fig. 2B). During the late embryonic exposure (Figs. 2B and 3B), the three *cyps* were significantly induced during exposure period. *cyp1a1* showed a small induction after 12 h of exposure, which became statistically significant after 24 h (300 μg/L) (Fig. 3B). At the end of 72 h exposure (before hatching process), *cyp1a1* was 11-fold higher at 150 μg/L and 39–36- fold higher at 300 and 600 μg/L compared the control. Given the results a *cyp1a1* induction plateau could be observed in larvae exposed to a concentration greater than 300 μg/L. During the long-term depuration period, *cyp1a1* transcription dropped to the basal level, except for the highest concentration (600 μg/L) where *cyp1a1* was still 16-fold upregulated at 31 dph. The sustained *cyp1a1* expression in the 600 μg/L treatment aligns with the body burden levels that remained high during the depuration period. Induction of *cyp1b1* was delayed and was not significantly upregulated until 48 h at 300 and 600 μg/L. *cyp1b1* expression remained high in the 600 μg/L treatment during the whole depuration period. The magnitude of the *cyp1c1* transcription was low during the entire experiment, with a slight late induction (5-fold) at the end of exposure (72 h) at 600 μg/L.

Our results showed that the level of *cyp* induction in halibut was also dependent on the developmental stage. The magnitude of the induced *cyp* transcription was notably low during the early embryonic experiment (5–8 dpf) compared the late embryonic experiment (10–13 dpf) (Fig. 2B). The potential of fish to synthesize CYP was more pronounced during the organogenesis process in halibut (late embryonic exposure) and this indicates a possible immature biotransformation capacity in earlier development in halibut. The *cyp* induction was dose dependent, up to a plateau at 600 μg/L. The temporal patterns of *cyp* are coherent with other studies in literature showing that the differences in synthetizing capabilities are dependent on developmental window and on the duration of exposure (Braunbeck et al., 2015; Kühnert et al., 2017; Otte et al., 2010, 2017). Xenobiotic induced gene transcription may also be modulated by ontogenic transcription patterns. For example, it is shown that the basal expression of genes encoding cyp can vary during zebrafish development (Kühnert et al., 2017; Scornaienchi et al., 2010). Recently, Sørhus et al. (2021) showed the same induction pattern in oil-exposed haddock, where *cyp1a* was the main xenobiotic activity in early embryonic development (end gastrulation/end organogenesis). A delayed expression of *cyp1b* and *cyp1c* similar to halibut late

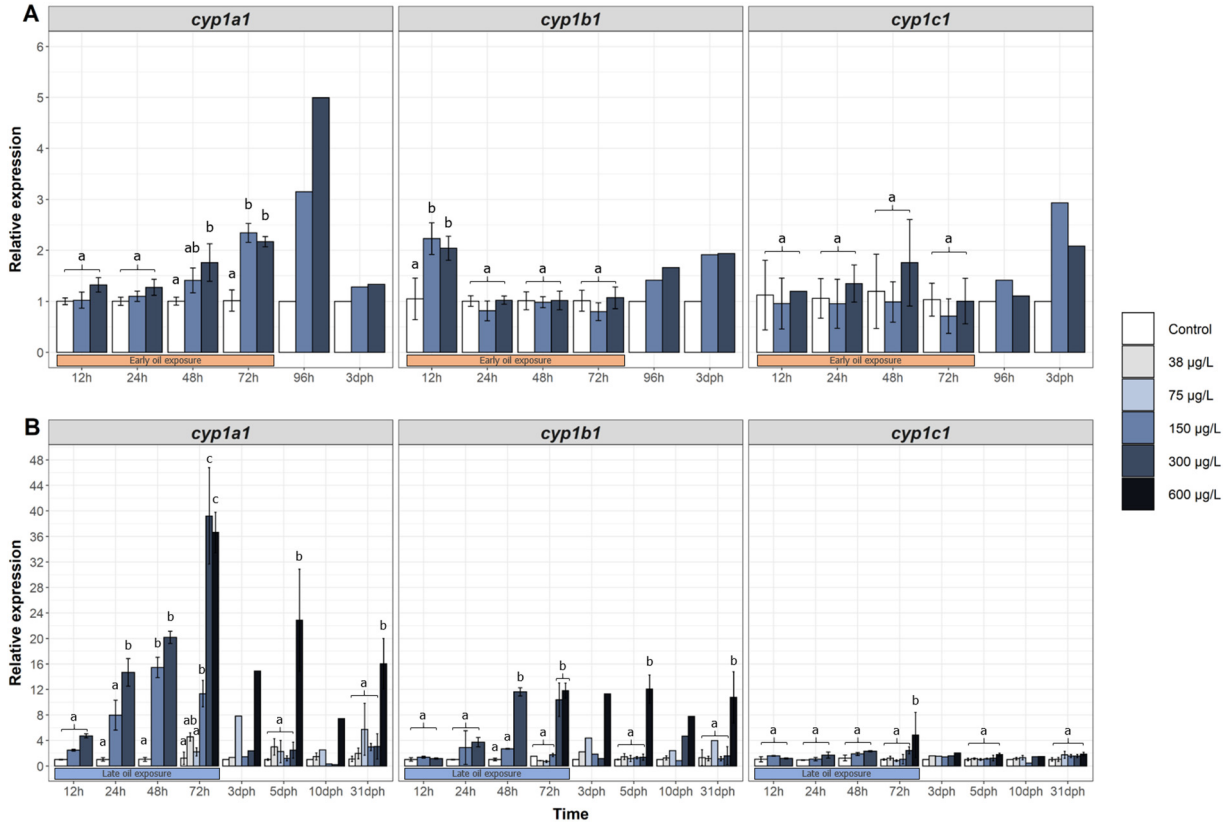

Fig. 3. Relative expression of *CYP1* activity. Relative expression of *cyp1a1*, *cyp1b1* and *cyp1c1* following early embryonic (A) and late embryonic (B) oil exposures. Relative expression of genes was compared to respective control at each time point. Significant differences between oil-exposed groups are indicated by lowercase letters (ANOVA and Tukey's *posthoc* test, $p < 0.05$). A single sample per condition was analyzed at 96 h (early) and 3 dph (early and late). h: hours during oil exposure, dph: days post-hatching.

embryonic experiment or other studies in zebrafish has been shown (Kühnert et al., 2017). Expression level of those *cyp1* paralogs might contribute also to the oxidative biotransformation of xenobiotics and determine the cellular and organ targets of toxic substrates. Teratogenic effects might be increased if any disruption is observed in the homeostasis balance, steroidogenesis, vitamin pathway or the catabolism of many hormones (Godard et al., 2005; Goldstone et al., 2010; Otto et al., 2003).

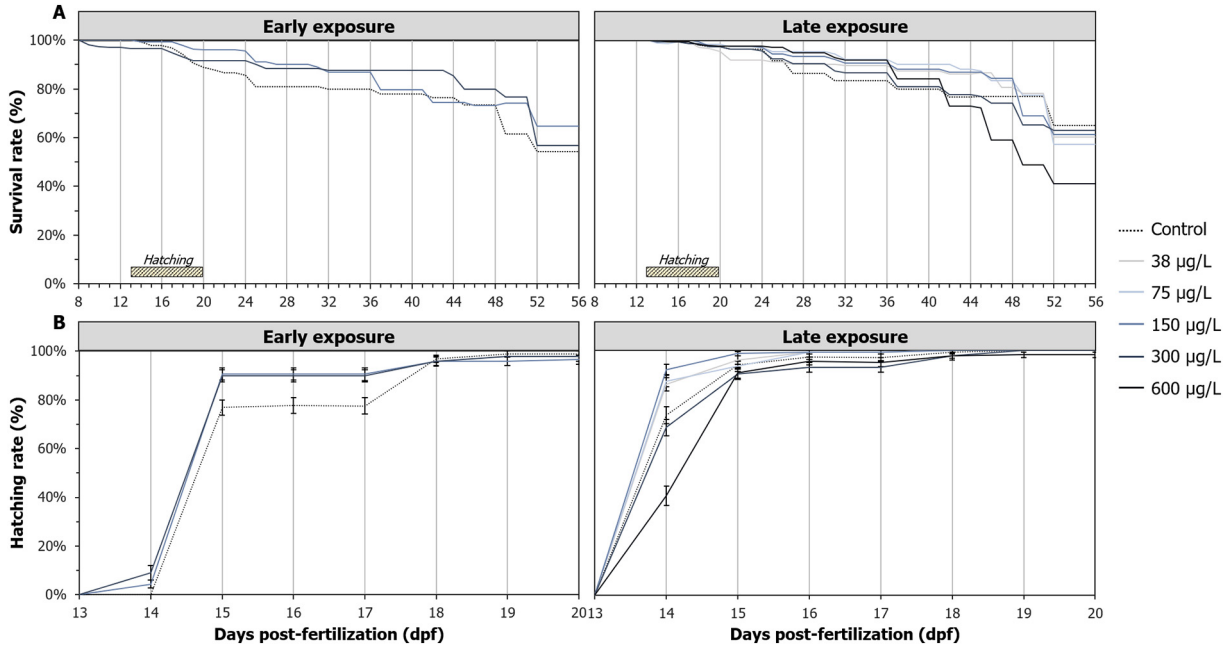

Fig. 4. Survival monitoring (A) and hatching fractions (B) after embryonic oil exposure (early and late experiments). Survival has been monitored during the depuration period and until the first-feeding stages (early experiment: from 8 to 56 dpf; late experiment: from 13 to 56 dpf). Data are mean ($N = 30, n = 180$). Standard error for survival rate did not exceed ±6% and error bars are omitted for better clarity in the plot.

3.2. Late embryonic oil exposure produced latent acute effect

No acute mortality was observed during the exposure period for either of the embryonic experiments, but some delayed mortality was observed. In both experiments (Fig. 4A, early and late embryonic exposures), larvae displayed a quite similar survival pattern to 41 dpf, with over 75% survival regardless the exposure treatment. After 41 dpf, a significant increased mortality was observed at 600 μg/L. Survival declined from 73 ± 0.05% at 42 dpf (28 dph) to 41 ± 0.08% at 56 dpf during the depuration period. A long-term monitoring period was used to understand the resilience of halibut larvae after an embryonic oil exposure period and ability to perform in time until the need for exogenous nutrition (first-feeding stages).

A slight delay in the whole hatching process was observed during the early embryonic exposure compared to the late embryonic exposure (Fig. 4B). In the early experiment, all fish succeeded over 96% of hatching success in all three treatments from 18 dpf (=4 dph), although a surprising slight delay in time was observed in the control larvae. Regarding the late embryonic exposure, hatching success patterns were nearly similar in all treatment groups, where all larvae reached 95–98% of hatching success from 15 dpf (=1 dph). Although, on the first day of hatching process (14 dpf) only 40% of larvae hatched in 600 μg/L group compared to the other treatment groups (70%).

Overall, oil embryonic exposures did not show notable lethal toxic effect in Atlantic halibut. Atlantic halibut appeared to be more resilient than other cold-water fishes at similar oil exposure concentrations and developmental window (Hansen et al., 2019b; Sørensen et al., 2017; Sørhus et al., 2015). Hansen et al. (2019a, 2019b) showed a mortality differential between cod fish exposed in early developmental stages (gastrulation) and late developmental stages (segmentation and organogenesis) and during the recovery period. A significant lethality of cod embryos was recorded during early oil exposure unlike the late exposure, whose mortality is recorded solely during the recovery period. In the present work, only the highest concentration induced a latent lethal effect following the late embryonic exposure. This latent mortality goes along with high upregulation of biotransformation processes (*cyp1a1* and *cyp1b1*) during the depuration period and might indicate strong developmental toxic effects in yolk-sac stage larvae. Halibut differ from most other studied marine fish by having a long embryonic development and long yolk sac stage of approximately 45 days at 6 °C. Observed differences in sensitivity between halibut and other cold water fishes may be due to the long developmental timing, but also to its larger egg surface/volume ratio making them more resilient to oil component. It has been demonstrated that smaller embryos (with larger relative surface area) accumulate higher tissue PAH concentrations more quickly (Edmunds et al., 2015).

3.3. Latent decrease of larval condition following late embryonic exposure

Larval condition (standard length) was only significantly affected at the highest dose in late experiment beginning at 10 dph (Fig. S1A). Larvae were significantly shorter at 600 μg/L at 31 dph compared the other treatment groups ($p < 0.001$). No significant differences in the myotome height were found in larvae in either experiments (Fig. S1B). Similar to larval condition factors, edema was only significantly observed at the highest dose in late experiment beginning at 10 dph (Fig. S2A). However, data suggested a trend of dose dependent increase of fluid accumulation into the yolk sac at 10 and 31 dph. No change in the pericardial area ($p = 0.544$) were found in the same larvae during development (Fig. S2B). Pericardial area was not clearly delineated from still images at 5 dph in halibut larvae due to development and morphology, so measurement at this developmental time was not performed. In larvae exposed during late embryonic exposure, no evidence of edema formation into the yolk sac was noted at 5 dph (Fig. S2A, B). Higher variability between individuals were found at 10 dph and 31 dph when exposed to 300 and 600 μg/L in the late embryonic experiment compared to the early embryonic experiment. Larvae displayed high fluid accumulation in the yolk sac at 10 and 31 dph when exposed to 600 μg/L ($p < 0.001$) compared the other treatment groups, which clearly demonstrated a severe fluid imbalance into the body. No fluid accumulation was observed into the pericardium of those larvae. However, a reduction of the pericardial area was shown in 31 dph larvae at 600 μg/L ($p < 0.001$), which might be correlated together with the decrease in larval size as well as the presence of strong yolk edemas. Fish exposed during organogenesis (late exposure) showed latent decrease of condition and potential osmoregulation disruption in yolk sac stage.

Fluid accumulation into the pericardium or the yolk sac during the heart development is by far the most frequently reported downstream effect of cardiac dysfunction recorded after oil exposure in variety of fish species (Adeyemo et al., 2015; Edmunds et al., 2015; Hansen et al., 2019b; Incardona et al., 2004; Incardona and Scholz, 2016; Khursigara et al., 2017; Pasparakis et al., 2019; Perrichon et al., 2016, 2018; Sørhus et al., 2016b). Pericardial edemas are generally correlated with decreased cardiac output in fish larvae (Khursigara et al., 2017; Perrichon et al., 2018). In halibut (late experiment), oil induced fluid accumulation into the yolk sac which may lead to a constriction of the pericardium at the highest concentration. Pericardial fluid outside the heart moves cyclically with the beating of the heart, and the flow generated contributes to cardiac morphogenesis, so edema (increased hydrostatic pressure) or effusion into the pericardium might induce heart shape, growth and function defects (Andrés-Delgado and Mercader, 2016). Hydrostatic pressure into the yolk might also be associated with yolk utilization defects, malabsorption and osmoregulation disruption. Studies on Atlantic haddock indicate oil exposure may interfere with the larvae ability to mobilize lipids from the yolk during the critical transition to the first-feeding stages. Upregulation of intrinsic cholesterol biosynthesis may have reflected deprivation in larval tissues as a consequence of the heart and circulatory system failing to deliver lipoproteins from the yolk (embryos) and intestine (larvae) (Sørhus et al., 2017). Recently, it was reported that exposure to low levels of oil caused a dysregulation of lipid metabolism and under-utilization of yolk lipids, which were associated to decreased growth in larval polar cod (Laurel et al., 2019). Perturbation of the yolk utilization and content as early embryonic nutrition proxy may potentially be indicative of later life metabolic conditions of larvae and should be further examined.

3.4. Late embryonic exposure induced greater incidence of craniofacial deformities

Late embryonic exposure induced craniofacial deformities in yolk sac larvae, while none were observed in early embryonic exposure. Following the early embryonic exposure, no change in the finfold thickness ($p = 0.876$), the opening mouth angle ($p = 0.717$), the distance eye to the premaxillary ($p = 0.129$) and the eye area ($p = 0.180$) was shown in larvae whatever the oil concentration (Fig. S3A–D).

Following the late embryonic exposure, several craniofacial endpoints were affected. The finfold thickness tended to be smaller in oil exposed larvae (Fig. S3A). Significantly smaller finfold was reported in larvae exposed to 300 μg/L (0.04 mm, $p = 0.002$) compared to control larvae (0.085 mm). A nearly total absence of finfold in larvae exposed to 600 μg/L (0.005 mm, $p < 0.001$) was also shown. Larvae exposed to 600 μg/L displayed an opening mouth angle 3- to 4-fold higher (71.7°, $p < 0.001$) than the other groups (Fig. S3B). The eye-premaxillary distance (Fig. S3C) was also 2.2 to 2.5-fold smaller ($p < 0.001$) in this 600 μg/L group compared to the other groups. Mouth angle and eye-premaxillary distance disruptions indicate some severe disruptions in jaw structures. Regarding the eye morphometry (Fig. S3D), no change in the eye area was found in 5 dph larvae. However, a size reduction trend appeared in 300 μg/L and was significant in 600 μg/L larvae ($p = 0.009$) compared the other treatment groups. The eye size was further reduced later during the development (31 dph).

Five categories of jaw phenotypes were found in exposed larvae and are described in Table S3. Jaw malformations were consistently observed in both control groups, where 36 and 25% of larvae had malformations (Fig. 5), including phenotypes 2 and 3. Overall, an increased incidence of jaw deformities with increasing oil concentration was observed in both experiments (from 36 to 62% in early exposure and from 25 to 100% in late exposure). Larvae from both experiments presented similar proportion of phenotypes in 150 μg/L (early exposure: 55%; late exposure: 50%) and 300 μg/L (early exposure: 62%; late exposure: 60%) groups. Nevertheless, larvae from the late exposure presented higher incidence of phenotype 5 ("*screamer*" or high severe locked open mouth phenotype). An incidence of 10% and 100% of this jaw phenotype 5 were found in 300 and 600 μg/L groups, respectively. The high incidence of jaw deformities was in accordance with the measured craniofacial proxies: the abnormal/absence of dorsocranial finfold, the higher opening mouth angle (especially for the "screamer" phenotype), the reduction of the eye-premaxillary distance and/or the abnormal preorbital structures.

Craniofacial results showed again that timing of exposure is important. Fish exposed during the organogenesis (late) showed more severe craniofacial impairments than fish exposed during epiboly process. Craniofacial phenotypes observed in our halibut experiments are in accordance with those reported in literature after oil exposure in different fish species (Incardona et al., 2004; Khursigara et al., 2017; Magnuson et al., 2018; Perrichon et al., 2016; Sørhus et al., 2015, 2016b). Craniofacial phenotypes were shown to be concentration dependent, and many of those were thought to be secondary to reduced cardiac function (Incardona et al., 2004, 2013). Jaw malformations were caused mainly by disruption of cartilage development (Hansen et al., 2019b; Incardona et al., 2004; Khursigara et al., 2017; Pollino and Holdway, 2002; Sørhus et al., 2016b). Irregular location and development of jaw bones and lower quadratal angle value (head proportions proxy) were recorded in oil exposed cod and haddock (Hansen et al., 2019b; Sørhus et al., 2016b). These studies also showed a severity differential of jaw malformation depending on exposure timing. Zebrafish embryos exposed to *Macondo* crude oil showed dramatic reduction in all pharyngeal arch cartilage elements (ceratobranchial) and lack of anterio basihyal cartilage (de Soysa et al., 2012). These malformations were demonstrated to be associated with defects in early cranial neural crest cell differentiation which induced more visible defects in the heart, arch vasculature and may be activated directly or independently of the AhR (de Soysa et al., 2012). Impairment in craniofacial development results widely in reduced head growth and is closely associated to eye developmental defects (Incardona et al., 2004; Perrichon et al., 2016).

Visual development (in regard to morphometry) appeared to be impaired solely at high concentrations in halibut. Smaller and more deformed eyes in both halibut and haddock have been shown, but only when oil exposure overlapped with organogenesis and was close to hatching (Lie et al., 2019; Sørhus et al., 2021). Different aspects of eye size (retina diameter, photoreceptor layers) were reduced in both larval red drum and sheepshead minnow exposed to *Deepwater Horizon* oil (Magnuson et al., 2018). Similarly, Xu et al. (2017) demonstrated inhibition of eye formation and retinal degeneration ribosome biosynthesis in red drum larval exposed to slick oil. Disruption of retinoid signaling with subsequent downregulation of genes related to eye development (e.g. *stra6*) were part of the disruptive effects causing the observed eye abnormalities and might be linked to circulation defects (Golzio et al., 2007; Lie et al., 2019). Abnormal *cyp* balance was suggested also to contribute to disruption of retinoid signaling resulting in greater incidence of eye abnormalities (Lie et al., 2019; Xu et al., 2016, 2017). Craniofacial abnormalities in halibut were mostly shown in yolk sac stage larvae following late embryonic oil exposure, in which *cyp1s* were strongly upregulated. Relative expression of *cyps* (*cyp1a1*, *cyp1b1*) was still high through development until 31 dph and might contribute to observed craniofacial abnormalities.

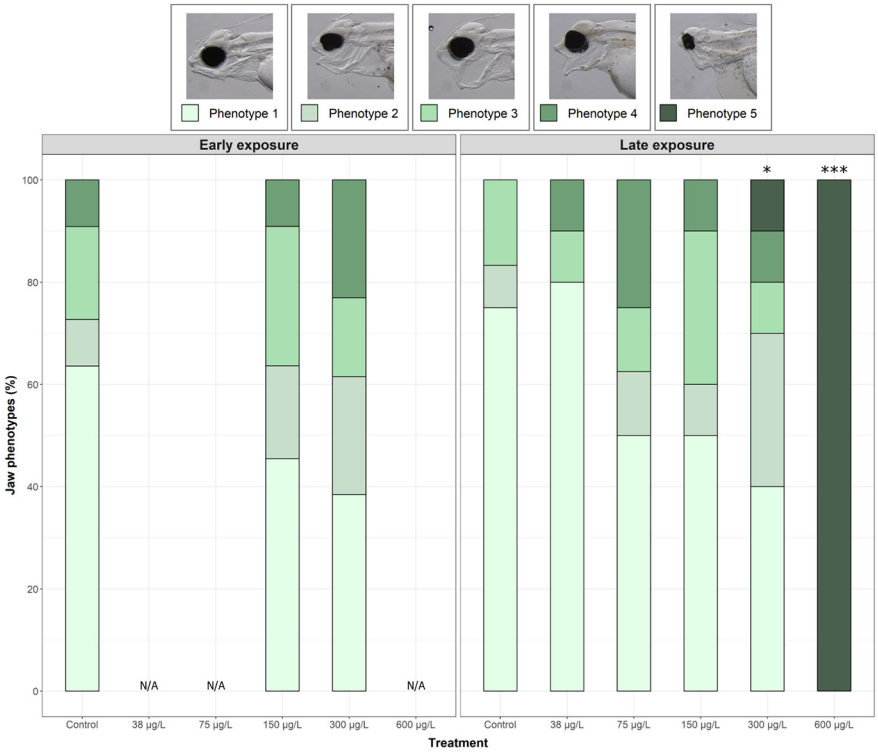

Fig. 5. Jaw phenotypes in 31 dph larvae after oil exposure. Developmental responses in 31 dph larvae scored according four categories of cardiac phenotype from all treatment groups in early and late exposures Data are percentage of each score. Asterisks indicate significant differences with the respective control between the total proportion of malformed and non-malformed jaw (Pearson Chi-Square, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Overall, morphological (finfold) and craniofacial (jaw, eye) abnormalities will most likely result in decreased feeding capacity, competing, escaping, migration response and survival over time (Tilseth et al., 1984). These abnormalities were shown to be downstream of steroid biosynthesis perturbation, circulation defects and altered cytochrome P450 suggesting other targets besides AhR pathways or the heart may be involved in the developmental toxicity (Xu et al., 2016).

3.5. Both embryonic exposures induced latent decrease of ventricular cardiac function

The cardiovascular system is critical, and it is the first to organ system to begin functioning in larval fishes. Here, we explore the extent of developmental patterns and provide a phenotypic map of conventional indicators of abnormal heart development in the yolk sac stage of halibut following embryonic oil exposures. No significant change in the heart orientation angle (Fig. 6) was observed in larvae from the early embryonic oil exposure, although a trend of a higher looping angle was shown in 31 dph larvae ($p = 0.056$). Regarding the late embryonic exposure, heart of 10 dph larvae exposed to 300 and 600 μg/L displayed a significantly higher angle ($p = 0.038$ and $p = 0.001$, respectively) suggesting a possible migration and positioning disruption of cardiac chambers or developmental delay. The severity of these morphological damages greatly increased during development (31dph, $p < 0.001$).

Four categories of morphological and functional phenotypes were scored at 31 dph and are described in Table S3. Larvae at both control groups presented with 8–10% of mild deformities including size failure in both chambers (Fig. 6B). Larvae presented a concentration dependent increase of heart deformities in both experiments (from 10 to 46% in early exposure and from 8 to 100% in late exposure). Similarly, severity of heart damages also increased with oil concentration. In early embryonic exposure, 46% of the hearts from larvae exposed to 150 and 300 μg/L were abnormally shaped and experienced non contracting ventricle (silent ventricle), however the highly severe deformities were only observed in 300 μg/L. Following the late embryonic exposure, 10 to 64% (38–600 μg/L) of developing hearts had severe and highly severe deformities. All the deformities observed, including chambers

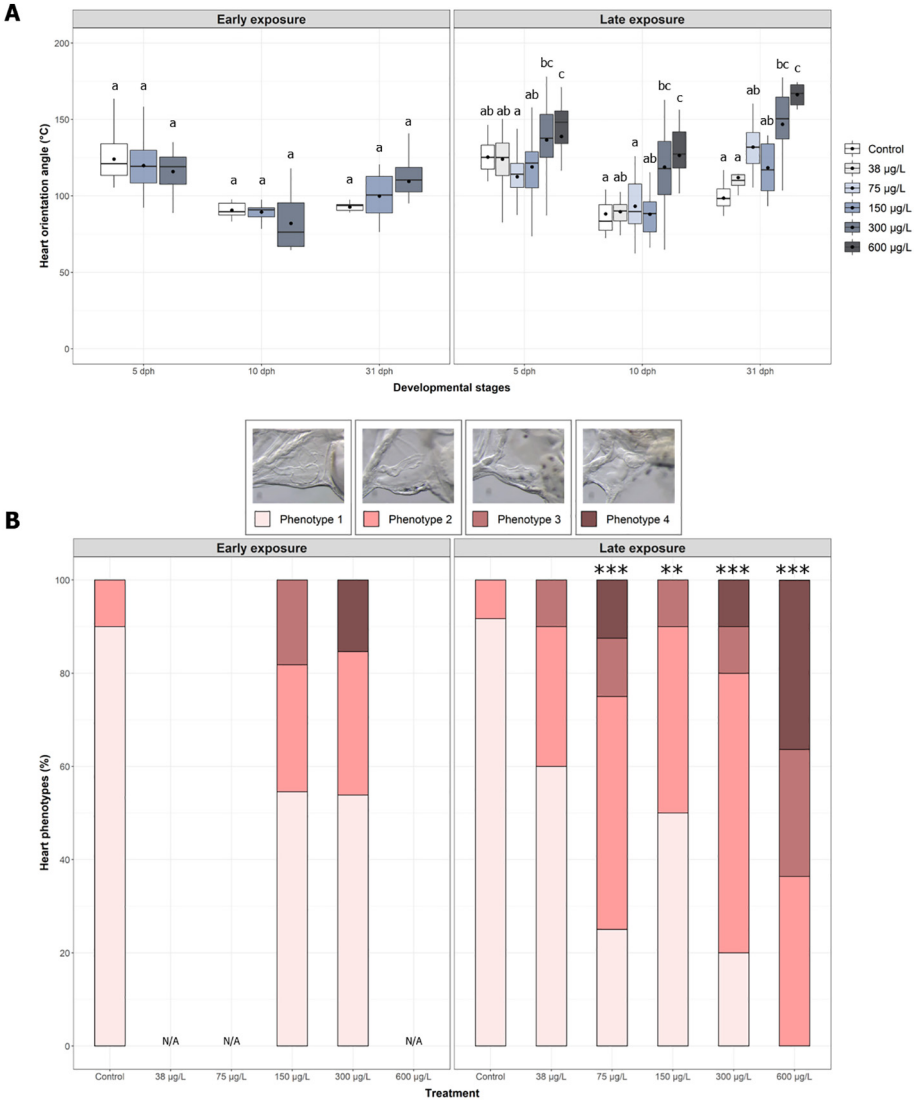

Fig. 6. Heart developmental phenotypes to oil exposure. Orientation angle of cardiac chambers (A, $N = 15–30$) is measured through the development from all treatment groups. Boxplots represent the median (horizontal line), Q_1-Q_3 quartiles (box), the mean (black point), and the non-outlier range (whisker). Significant differences between oil-exposed groups are indicated by lowercase letters (ANOVA and Tukey's *posthoc* test, $p < 0.05$). Four categories of cardiac phenotype (B) were specifically scored in 31 dph from all treatment groups in early and late exposures. Data are percentage of each score. Asterisks indicate significant differences with the respective control between the total proportion of malformed and non-malformed heart (Pearson Chi-Square, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

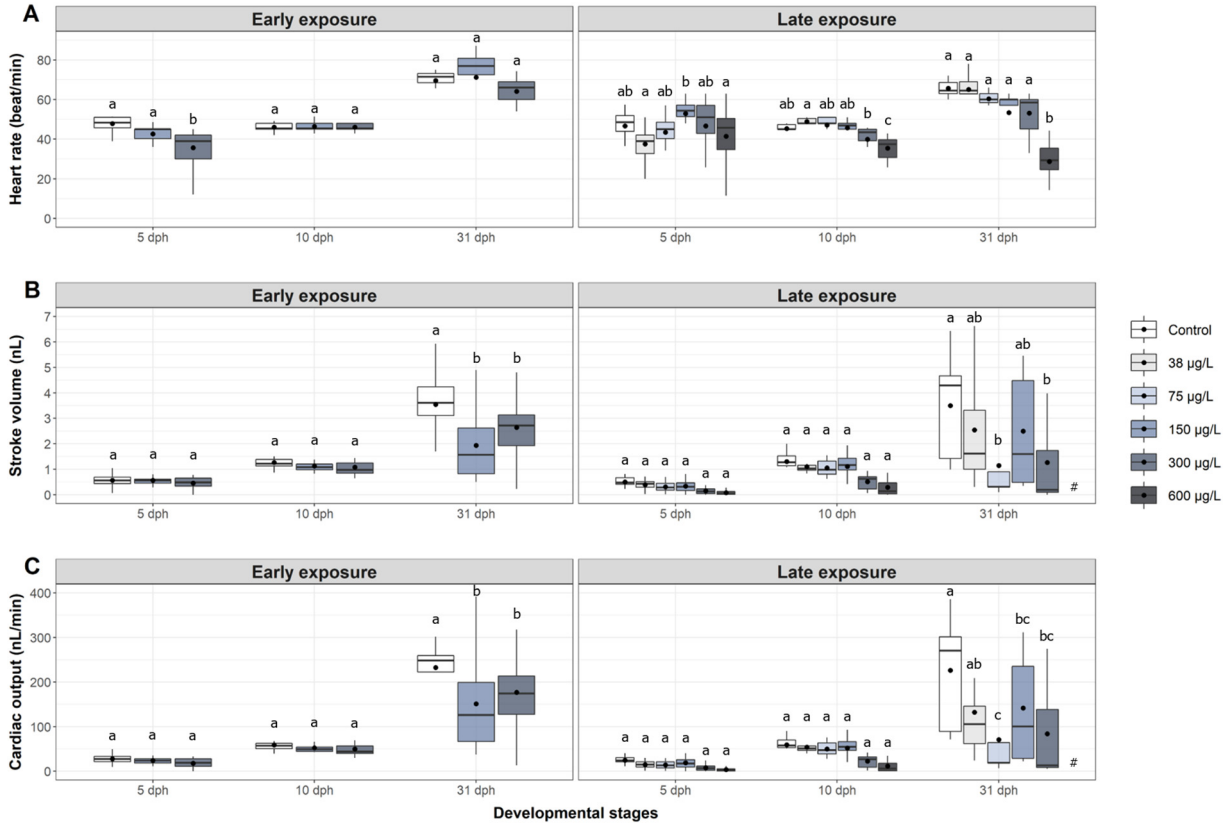

Fig. 7. Larval cardiac function assessed after both embryonic oil exposures (early and late exposures). Ventricular cardiac function is measured from all treatment groups through development using different proxy such as the heart rate (A, N = 15–30), the stroke volume (B, N = 15–30) and the cardiac output (C, N = 15–30). Boxplots represent the median (horizontal line), Q_1-Q_3 quartiles (box), the mean (black point), and the non-outlier range (whisker). Significant differences between oil-exposed groups and the respective control are indicated by lowercase letters (ANOVA and Tukey's *posthoc* test, $p < 0.05$). # indicates that the outlining measurement have not been possible due to high deformities of the cardiac chamber.

positioning, migration failure, size, disruption of atrioventricular junction, tubular heart, morphology and silent ventricle might have impacts on proper functioning and contractility mechanisms.

Observed heart developmental phenotypes in halibut are common morphometric symptoms of the crude oil syndrome observed in different tropical and cold-water fish species (Edmunds et al., 2015; Incardona et al., 2004, 2014; Jung et al., 2013; Perrichon et al., 2018; Sørhus et al., 2016b, 2017). Although halibut larvae from early embryonic oil exposure displayed abnormal developing heart phenotypes, the morphology was nearly not as impacted compared to larvae from the late embryonic exposure. Pervasive effects in the late exposure were found already at 5 dph, which indicates an early internal injury of halibut fish. These results are in accordance with studies in literature demonstrating that visible forms of injuries (craniofacial abnormalities, edemas, etc.) are downstream of cardiac failure (Incardona and Scholz, 2016). Calcium plays a central role in signal transduction in cells, thereby activating cellular growth and development. Well-functioning calcium activity during embryonic development is essential in cardiomyocyte proliferation and leads to proper cardiac form and function (Clapham, 1995; Ebert et al., 2005; Rottbauer et al., 2001). Cardiomyocytes originate soon after gastrulation, and this might explain the presence of more severe heart failures in halibut larvae from the late embryonic exposure. Cardiac myogenesis specification and morphogenesis are subject to regulation by protein factors, including bone morphogenetic proteins, that are secreted from adjacent endoderm (Chen et al., 2004; Srivastava and Olson, 2000). Crude oil components were shown to affect the bone morphogenetic proteins in different fish species (Incardona, 2017; Sørhus et al., 2017; Xu et al., 2016). The direction of cardiac looping is determined by an asymmetric axial signaling system established just before organogenesis onset (Chen et al., 1997), which was also seen to be altered by oil exposure in haddock fish (Sørhus et al., 2017). Overall, crude oil disrupts cardiac morphogenesis in early life stages of fishes through intracellular calcium cycling. As a consequence, the ventricle fails to grow and may induce subsequent cardiac contractility failure and occasionally become electrically silent (Khursigara et al., 2017; Perrichon et al., 2018; Sørhus et al., 2016b, 2017; Xu et al., 2016).

Crude oil exposure differentially impacted the cardiac function in halibut larvae depending on the targeted exposure window. Heart rate (Fig. 7A) was significantly reduced in 5 dph larvae at 300 µg/L following the early embryonic oil exposure ($p < 0.001$). While a rhythm change was found in those 5dph larvae (300 µg/L), no changes in contractility via stroke volume and cardiac output were found ($p > 0.05$; Fig. 7B and C). No change in rhythm and functional contractility were noted in 10 dph larvae. However, larvae at 31 dph displayed significant reduction in stroke volume and cardiac output in both oil concentrations ($p < 0.001$; Fig. 7B and C), while the heart rate still unchanged (Fig. 7A). Regarding the late embryonic oil exposure, no notable change in heart rhythm and in contractility were found in 5 dph larvae (Fig. 7A). A trend of decreasing heart rate with oil concentration was observed at 10 and 31 dph. Significant bradycardias were observed at 10 and 31 dph at the highest concentration, compared to other treatment groups. Similar trends of depletion (but not statistically significant) in stroke volume and cardiac output were observed at 300 and 600 µg/L in 10 dph larvae (Fig. 7B and C). However, bradycardias were paired with severe reduction of contractility in 31 dph larvae. The measurement of contractility was not possible in larvae at the highest concentration due to the high incidence of silent ventricle phenotype (64%, Fig. 6B), and difficulty delineating between the two cardiac chambers with high morphological defects. Animals

with silent ventricle phenotype have a stroke volume of nearly 0 nL and they were not included in the calculation. Considering this, a high reduction of cardiac output in oil-exposed larvae was noted. As well as the visible forms of injury, reduction in contractile function was more prominent as larval development progressed and concentration increased.

Recently, Sørhus et al. (2021) showed similar cardiac functional defects linked to reversible or irreversible morphological changes in haddock fish following early and late embryonic oil exposures. They suggested that latent changes in contractility might also be due to differences in metabolic modifications and accessibility of toxicants (storage in the yolk sac) during early stages (Sørhus et al., 2016b). While the major initiating events associated to cardiac defects are well known (chemical blockade of repolarizing potassium currents and disruption of intracellular calcium), a calcium depletion in the sarcoplasmic reticulum through ryanodine receptor or SERCA pump might also play a role in arrythmia or contractility defects in fish (Brette et al., 2014; Incardona et al., 2009; Incardona, 2017; Sørhus et al., 2016b).

The contractile deficiency of the ventricle may be also due to mechanical deficiencies (e.g. sarcomere defects) or failure of conduction from the atrium (Ebert et al., 2005). Heartbeat drives epicardium formation through the effect of pericardial fluid advections, which contribute to myocardial growth and maturation, and vasculature development in later stages. Cardiac contraction might also be needed to promote complete epicardial layer formation through the promotion of cell adhesion or migration. Impaired epicardial development has been associated with defects in valve development, cardiomyocyte proliferation and alignment, cardiac conduction system maturation and adult heart regeneration (Andrés-Delgado and Mercader, 2016; Carmona et al., 2010; Peralta et al., 2014). Taken together, oil affects rhythm and contractile function of the halibut heart, which in turn guide the shape of the heart through different mechanisms. Cardiac failures lead to secondary reversible and irreversible morphological defects when severity is increased, thereby decreasing condition and might warn on how fish will perform in later stages.

4. Conclusions

The current study demonstrates that the exposure timing is essential for the development of toxic effects of crude oil in Atlantic halibut. Neither embryonic exposures (epiboly and organogenesis) showed notable acute toxicity during exposure, yet both showed global latent toxicity during yolk sac stages. Toxicity included common craniofacial and cardiac symptoms of crude oil syndrome. Halibut displayed a concentration dependent latent decrease of larval condition and fitness following embryonic oil exposure. Fish exposed during organogenesis (late embryonic exposure) displayed stronger and more severe teratogenic effects than fish exposed during gastrulation/epiboly process (early embryonic exposure). Fish in late embryonic exposure displayed also greater PAH uptake and metabolic capacity (*cyp1s* upregulation). The differences in sensitivity between developmental windows and the long yolk sac stage development timing of Atlantic halibut make this species a good candidate for discovering new mechanisms of oil toxicity in developing fish.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.145349.

CRediT authorship contribution statement

Prescilla Perrichon: Conceptualization, Investigation, Formal analysis, Writing – original draft, Visualization. **Carey E. Donald:** Conceptualization, Investigation, Writing – review & editing. **Elin Sørhus:** Conceptualization, Writing – review & editing. **Torstein Harboe:** Writing – review & editing. **Sonnich Meier:** Conceptualization, Investigation, Writing – review & editing, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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