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Developmental effects in fish embryos exposed to oil dispersions – The impact of crude oil micro-droplets

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ABSTRACT

During accidental crude oil spills and permitted discharges of produced water into the marine environment, a large fraction of naturally occurring oil components will be contained in micron-sized oil droplets. Toxicity is assumed to be associated with the dissolved fraction of oil components, however the potential contribution of oil droplets to toxicity is currently not well known. In the present work we wanted to evaluate the contribution of oil droplets to effects on normal development of Atlantic cod (*Gadus morhua*) through exposing embryos for 96 h to un-filtered (dispersions containing droplets) and filtered (water soluble fractions) dispersions in a flow-through system at dispersion concentrations ranging from 0.14 to 4.34 mg oil/L. After exposure, the embryos were kept in clean seawater until hatch when survival, development and morphology were assessed. The experiment was performed at two different stages of embryonic development to cover two potentially sensitive stages (gastrulation and organogenesis). Exposure of cod embryos to crude oil dispersions caused acute and delayed toxicity, including manifestation of morphological deformations in hatched larvae. Oil droplets appear to contribute to some of the observed effects including mortality, larvae condition (standard length, body surface, and yolk sac size), spinal deformations as well as alterations in craniofacial and jaw development. The timing of exposure may be essential for the development of effects as higher acute mortality was observed when embryos were exposed from the start of gastrulation (Experiment 1) than when exposed during organogenesis (Experiment 2). Even though low mortality was observed when exposed during organogenesis, concentration-dependent mortality was observed during recovery.

1. Introduction

In the event of oil spills in the marine environment, oil undergoes a number of weathering processes altering the physical and chemical nature of the oil. One of the most important processes in weathering is the formation of oil-in-water dispersions, as this increases the surface to volume ratio of the oil. Formations of oil droplets will naturally occur when oil is released from the seabed during blowouts, and when surfaced oil is dispersed into the water column by wave energy during heavy weather. Even though oil is lighter than water, micro-sized droplets may persist in the water column due to very low surfacing velocity. Oil components such as polycyclic aromatic hydrocarbons (PAH) will equilibrate between the bulk oil matrix of each droplet and the surrounding water, and in proportions largely determined by their water solubility and the droplet surface-to-volume ratio. This may also vary with time, type of oil, magnitude/location/depth of the release, as well as physical parameters such as temperature, pressure, light conditions and currents (NRC, 2005). However, oil droplets will contain most oil components present in an oil dispersion, and only a small fraction will be dissolved.

It is well known that there is a high correlation between abnormal fish embryonic development and pollution load in the environment (Cameron et al., 1990; Westernhagen et al., 1988). Developmental malformations in fish embryos exposed to PAHs have been reported in connection with accidental oil spills following train derailment (Debruyn et al., 2007) and surface oil spills at sea (Incardona et al., 2012). Laboratory studies of exposure to PAHs and crude oil have reported developmental effects on fish embryos and larvae that include

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failed inflation of the swim bladder, anemia, pericardial and yolk sac edema (Incardona et al., 2004), dorsal curvature (Li et al., 2011) and craniofacial skeleton malformations (de Soysa et al., 2012; Shi et al., 2012). Different PAH components have also have been reported to have specific effects on early life stages in laboratory studies (Incardona et al., 2004).

Toxicity of oil dispersions is believed to be largely attributed to the dissolved phase because of increased bioavailability (Carls et al., 2008; Di Toro et al., 2000; French-McCay, 2002; Nordtug et al., 2011b; Olsvik et al., 2011). However, it is currently not well known if oil droplet adhesion to the surface of fish eggs may contribute an additional source of PAH uptake and subsequent developmental toxicity to the embryos (Hansen et al., 2019; Sørensen et al., 2019; Sørhus et al., 2015), and the potential contribution of oil droplets to toxicity remains to be quantified. In addition, qualitative and quantitative differences in chemical and physical behavior between crude oils impede comparison of effects, justifying oil-type specific studies (Neff et al., 2000; Philibert et al., 2019). Furthermore, field studies commonly fail to provide sufficiently detailed data on physico-chemical composition of the oil in time and space, and laboratory study data are generally used in modelling and contingency planning (Aamo et al., 1997; De Hoop et al., 2013; Olsen et al., 2013b). However, to predict the effect of oil dispersions in oil spill modelling based on data generated by experimental exposures, observed effects must be related to parameters that can be predicted in time and space, such as concentration and chemical composition of total and dissolved hydrocarbons, and size distribution of oil droplets. In the present study, we have therefore focused on evaluating the potential contribution of oil droplets to known developmental effects related to oil exposure. We used a standardized flow-through exposure system, where effects were compared between an oil dispersion consisting of micro-droplets and the dissolved water-soluble fraction (WSF). This corresponding WSF was filtered, therefore comparison of exposure media without oil droplets can be made in a controlled manner (Nordtug et al., 2011a). We have previously used this system successfully to isolate the contribution of oil droplets to effects in first-feeding fish larvae (Nordtug et al., 2011b; Olsvik et al., 2010, 2011) and copepods (Hansen et al., 2009). In addition, the contribution of chemical dispersants to the effects of oil dispersions were also evaluated (Hansen et al., 2012, 2015, 2016; Olsvik et al., 2012b). For fish larvae, limited contribution of oil droplets to toxicity was observed.

The Atlantic cod (*Gadus morhua* L.) embryo is one the most sensitive species for exposure to hydrocarbons, and the earliest stages of fish embryo development are generally more sensitive than the late embryonic stage (Kjørsvik, 1986). The embryo stage of *G. morhua* extends over a period of approximately 18–20 days at 5–6 °C (Geffen et al., 2006; Hall et al., 2004; Kjørsvik et al., 1984), and in the present work we aimed to i) assess the potential contribution of oil droplets to effects on normal development, and to ii) determine how timing of exposure may have an impact on developmental anomalies in the embryos and yolk-sac larvae. This was achieved by exposing embryos from the same batch of eggs to identical exposure scenarios (4-d exposure to dispersions and corresponding WSF) at different developmental stages; the first during early development (during gastrulation; days 3–7 days post fertilization) and the second during late development (after gastrulation, during organogenesis; days 9–13 post fertilization). After the exposure, embryos were transferred to clean sea water, and potential effects on survival, larval condition (body size, myotome height, yolk sac size) and morphology (spinal deformation, head cranial cartilage development) were recorded of yolk-sac larvae 2–3 days post hatch (dph).

2. Materials and methods

2.1. Cod embryo supply and maintenance

Eggs were collected from a broodstock spawning tank at the National cod breeding center (NOFIMA, Tromsø, Norway). Natural spawning took place among several broodstock fish in each tank, and newly fertilised eggs were collected every morning. The eggs were sent to the lab by air cargo in a temperature stabilized container within 24 h after fertilization. On arrival to the test lab the eggs were acclimatized and transferred to a 150 L tank with flow-through sea water and aeration inside a temperature-controlled room. They were kept in darkness at a temperature of 5.2 ± 0.4 °C (stdv). The use of any disinfectants was avoided during the handling of the eggs. The water exchange was set at 12 L/hour. Sea water at approximately 8 °C was supplied from 70-m depth in the Trondheimsfjorden, filtered through a sand filter and patron filters (1 μm) to a holding tank with residence time of approximately 2 days. Before entering the system for holding and exposure the temperature was adjusted with heat exchangers. A gas exchange system is incorporated in the water treatment system to avoid supersaturation and maintain the saturation at safe levels.

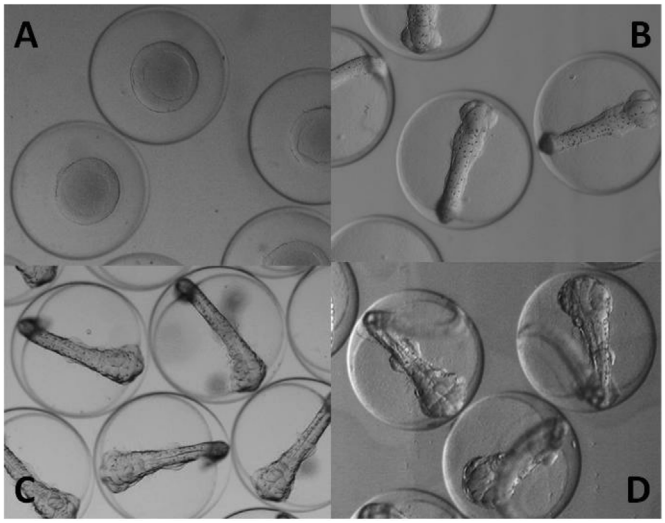

Fig. 1. Developmental stages of cod embryo before and after exposure. A: Early gastrula embryos (germ ring visible) at onset of exposure in experiment 1. B: Embryos after 4 days exposure in experiment 1, just after blastopore closure and end gastrulation. C: Embryos at onset of exposure experiment 2, after gastrulation and during the period of organogenesis. D: Embryos after 4 days exposure in experiment 2. One egg is approximately 1.2 mm in diameter. Photo: Per-Arvid Wold.

Two distinct developmental stages were used in the two experiments (staging after Hall et al. (2004)). The first experiment (Experiment 1) exposed eggs from the start of gastrulation (appearance of germ ring; Fig. 1A) to completion of epiboly (closure of the blastopore; Fig. 1B) at days 3–7 post fertilization. In the second experiment (Experiment 2), eggs were exposed at days 9–13 post fertilization during the segmentation period and further organogenesis (Fig. 1C–D).

2.2. Exposure and recovery systems

The exposure system used is described in detail in Nordtug et al. (2011a). 1000 eggs (with a total biomass of approximately 1 g) were introduced to each chamber. In order to simulate exposure in the water column, a net (300 μm mesh size, 254 cm^2) was positioned horizontally just below the water surface to keep the slightly positively buoyant eggs below the water surface layer. This ensured the eggs were only exposed to oil droplets in the water column, and not to surfaced oil at the water surface. The average flow rate into the exposure chambers was 15.7 mL/min, resulting in 5 exchanges of the whole water volume (4.5 L) of each chamber daily.

After the exposure period, the eggs were transferred to a recovery system consisting of a 0.5 L glass bottle with sea water flow-through (approximately 25 mL x min^{-1}, mean residence time 20 min), containing a large (10 cm^2, 300 μm) mesh at the outlet. The eggs/larvae

were kept in this system until 2–3 days post hatch (dph) when the experiment was terminated.

The oil used for the experiments was a naphthenic crude oil (Troll) slightly weathered using one step distillation procedure at 150 °C for 1 h (Stiver and Mackay, 1984), and we used the 150 °C residue to generate oil dispersions. The method for generating oil dispersions and the technical details are described in Nordtug et al. (2011a). Briefly, oil dispersions of 10 mg/L were generated continuously by injecting 10 mg of oil per liter seawater in an oil droplet generator with a series of 0.5 mm nozzles generating repeated turbulence to break up the oil into small droplets with a mean size between 10 and 14 µm. This "stock" dispersion (10 mg oil/L) was diluted in-line with sea water at dispersion-to-water ratios of 1:1 (5 mg/L), 1:5 (1 mg/L) and 1:25 (0.2 mg/L) by computer-controlled pulsing of 3-way solenoid valves. At each dilution step the stream of diluted dispersion was split in two with one half fed directly to the exposure chamber and the remaining half filtered to extract the WSF into a parallel exposure chamber. The filtration device consisted of a custom-made filter container (250 mL, Fig. S2) containing loosely packed fine glass wool (10 g; Assistent, Germany) on top of two stacked Whatman GF/C and GF/F Glass Microfiber Filters (Whatman Ltd., Maidstone, UK) with particle retention 1.6 and 0.7 µm, respectively.

2.3. Experimental design and exposure levels

The experimental timeline is given in Fig. 2. The experiments were performed on a single batch of eggs that where split in two groups. One group (Experiment 1) was exposed for four days during gastrulation (3–7 dpf) in a period where the earliest cell differentiation and formation of the embryo axis occurred. The second group (Experiment 2) were exposed right after gastrulation (9–13 dpf), when neurulation and organogenesis took place. Both groups were transferred to clean sea water after exposure (96 h) and kept until 2–3 days post hatch (dph), when the experiment was terminated. Survival was monitored after exposure and at the end of the experiment (after the recovery period). Particle analysis was performed daily from all exposure solutions, and water samples for chemical analyses were taken on day 1 and 3 of the exposure periods. All exposure concentrations were performed with 4 replicate units split into pairs and positioned at different locations in the experimental set-up. Experiment 1 was terminated at 2 dph and experiment 2 was terminated at 3 dph. The experimental set-up and positioning of exposure chambers in the exposure rig is given in Supporting Information (Fig. S1).

2.4. Documentation of exposure

Samples of the exposure media for chemical analysis of semi-volatile organic compounds (SVOC, approximately 800 mL) were sampled in 1 L glass bottles and acidified with diluted hydrochloric acid to pH < 2. Acidified water samples were extracted with dichloromethane (DCM), dried over Na_2SO_4, and concentrated to 1 mL. Water samples for volatile organic compounds (VOC) were sampled in 40 mL vials pre-added hydrochloric acid for acidification and capped without headspace.

Determination of the total extractable organic compounds (total hydrocarbon content with carbon numbers from 10 to 36; THC (C10–C36) was performed on dichloromethane (DCM) extracts by Gas Chromatography–Flame Ionization Detector (GC-FID) (US-EPA, 2013). The system comprised of an Agilent6890 N GC fitted with an Agilent 7683B Series autosampler. The column was an Agilent J&W HP-5 fused silica capillary column (30 m × 0.25 mm ID × 0.25 µm film thickness). The carrier gas was helium at a constant flow of 1.5 mL x min^{-1}. 1 µL samples were injected into a 310 °C split/splitless injector. The oven temperature was heated to 40 °C for 1 min, then heated to 315 °C at 6 °C/min and held at 315 °C for 15 min. THC measurements are highly dependent on baseline-corrections which make the THC-values at low concentrations unreliable. In order to compensate for this the correlation between individual components (naphthalenes) quantified by the more sensitive GC-MS in the same samples, and THC, were used to offset the THC values for the whole dataset to make the linear regression lines pass through the origin of the axes. The maximum offset was 1.4% of the highest dispersion concentration.

Volatile organic compounds (C5–C10) were quantified by Purge and Trap Gas Chromatography/Mass Spectrometry, using a modified US Environmental Protection Agency EPA-Method 8260 (US-EPA, 2006), with a 50 m (0.20 mm ID, 0.50 µm film thickness) Supelco Petrocol capillary column. Target analyses were detected with an Agilent 5973B MSD and the data were acquired using the Agilent EnviroQuant Chemstation software.

Analysis for semi-volatile organic compounds including decalins, naphthalenes, and 3 to 5-ring polycyclic aromatic hydrocarbons were performed by Gas Chromatography–Mass Spectrometry (GC-MS) operated in selected ion monitoring mode according to US-EPA method (USEPA, 2007). The system comprised of an Agilent 6890N GC with an Agilent 5975B quadrupole Mass Selective Detector. The column was an Agilent J&W HP-5MS fused silica capillary column (60 m × 0.25 mm ID × 0.25 µm film thickness). The carrier gas was helium at a constant flow of 1.2 mL x min^{-1}. A 1 µL sample was injected into a 310 °C split/splitless injector. The oven temperature was heated to 40 °C for 1 min, then heated to 315 °C at 6 °C x min^{-1} and held for 15 min. Data and chromatograms were monitored and recorded using MSD ChemStation

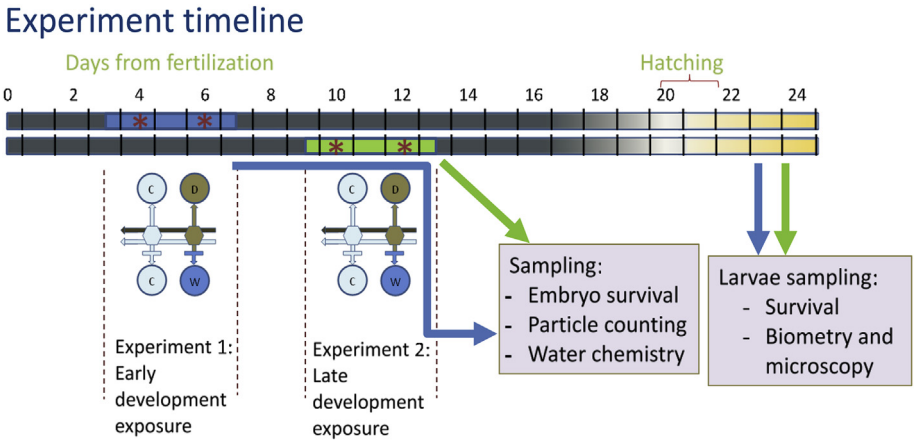

Fig. 2. Experimental time line. The exposure periods are indicated in blue (Experiment 1) and green (Experiment 2), and timing of water sampling is indicated with asterisks (*). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(version D.03.00.611) software. The MSD ion source temperature was 230 °C.

Particle counting with Coulter Counter Multisizer 3 (Beckman Coulter Inc., US) was used for monitoring of oil droplets in the exposure tanks. The Multisizer 3 was equipped with an aperture of 100 µm. All samples measurements were collected in 25 mL polystyrene vials (Kartell) and the oil dispersion samples were analyzed immediately after sampling in order to avoid loss of droplets due to surfacing. All samples were analyzed with 3 consecutive runs on the Coulter Counter. The results were processed and plotted with the Beckman Coulter particle characterization software (Beckman Coulter inc. ver. 3.51, 2002 and ver. 4.01, 2008).

2.5. Microscopic imaging and biometry of hatched cod larvae

All hatched larvae sampled during the experiment were anaestethised with Tricaine Methanesulfonate (MS222) before further treatment. Larvae for biometric analyses were sampled at the end of the experiment. They were fixed in 2.5% glutardialdehyde in 0.11 M hepes buffer, and stored in vials at 4 °C until they were photographed using a dissecting microscope (Leica M205, Leica Microsystems, Germany) and a CCD camera (Nikon DS-5M/L2, Nikon Corporation, Japan). Pictures were used for analyses of biometric endpoints (standard length, myotome height, body area and yolk sac length) of larvae (Fig. 3A), which were measured by ImageJ (ImageJ v2013_2, National Institutes of Health (NIH), US). Care was taken to image larvae in one straight plane keeping the eyes on top of each other and the whole body in focus, but some larvae measurements may be slightly biased in some highly deformed larvae, e.g. for larvae suffering from scoliosis (example in Fig. 3B, R3 example). For spinal deformations, the larvae were divided into six groups (Fig. 3B) where normal (N) displayed a straight spinal shape. With increasing degree of spinal kyphosis curvature larvae were denoted R1-R4, and larvae which displayed an opposite curvature (lordosis) were denoted S.

2.6. Cranial cartilage analyses of yolk sac larvae

For larval analysis of jaw development, the larvae were fixed in 4% formaldehyde in phosphate-buffered saline (PBS), and stored in vials at 4 °C until further processing. Larvae from experiment 1 were sampled at

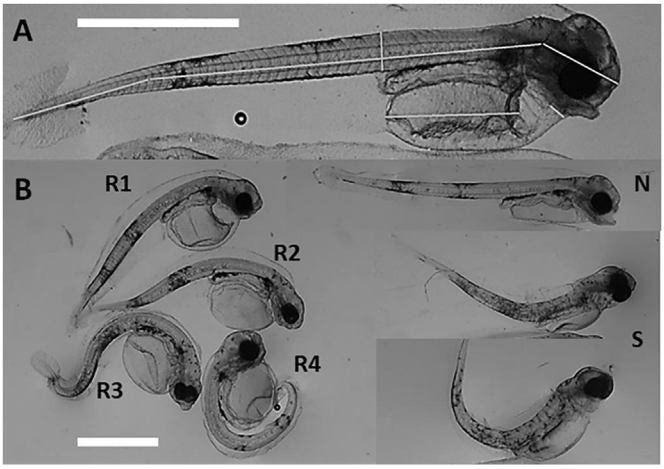

Fig. 3. Biometric analyses of hatched cod larvae. A: Measurements of standard length (from tip of the snout to the end of the notochord), myotome height (behind the anus) and length of yolk sac indicated as lines. B: Characterisation of spinal shapes. N: normal shape, R1: mild spinal curvature, R2: moderate curvature, R3: severe curvature, R4: very severe curvature, S: two examples of reversed curvature. R1-R4 is a kyphosis curvature, whereas S is characterized as representing lordosis. Scale bars (1 mm) are added as white horizontal bars representative for A and B. Photo: Kari Ella Read.

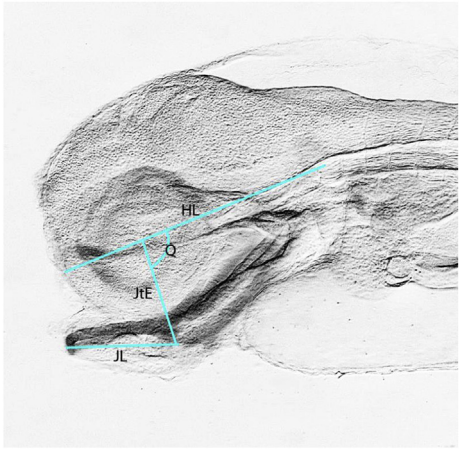

Fig. 4. Craniofacial endpoints. Craniofacial endpoint measurements made for all 2–3 day old cod larvae: Head length (HL), jaw point angle (Q), jaw length (JL) and Jaw-to-Eye length (JtE). Photo: Tora Bardal.

2 dph, and from experiment 2 at 3 dph. Up to 25 larvae from each treatment were stained for cartilage with Alcian blue according to Balon (1985) and Gavaia (Gavaia et al., 2000), and photographed with a dissecting microscope (Leica M205, Leica Microsystems, Germany) equipped with a CCD camera (Nikon DS-5M/L2, Nikon Corporation, Japan). Skeletal measurements were made according to Herbing et al. (Herbing, 2001; Herbing et al., 1996), and Fig. 4 displays the morphological endpoints measured in the cranium-skeletal region. Head length (HL) was measure from the front of the skull through the center of the eye, to the first vertebra. Jaw length (JL) was measured from the extreme caudal end to the tip of the Meckel's cartilage. The Jaw-to_Eye (JtE) length was the distance between the jaw joint and the center of the eye (Herbing, 2001). The “jaw joint” quadratal angle Q was angle measured as the angle between the lines HL and JtE. All analyses were performed using ImageJ (ImageJ v2013_2, National Institutes of Health (NIH), US).

2.7. Statistics

Student t-test was used for pairwise comparisons of controls with exposed cod, and of WSF-exposed with dispersion-exposed cod, where variances were homogenous; otherwise Mann-Whitney *U* test was applied. GraphPad Prism 6 (Version 6.03, GraphPad Software Inc.) was used for all statistical analysis.

3. Results and discussion

3.1. Exposure validation

Surfacing velocity of oil droplets is a function of droplet size, oil density and turbulence in the system. All droplets (from an oil with density < 1) will eventually reach the surface in a non-turbulent system. However, in the event of an oil spill wave action causes turbulence that keeps smaller oil droplets from reaching the surface. The mean particle size (diameter) in the oil dispersions was 10,9 µm (± 0,39) in the early exposure and 10,8 (± 0,27) in the second experiment (Supporting information 3). The density of the original oil was 910 kg/m^3. According to Stokes' Law, the theoretical rise velocity of this droplet size in stagnant seawater is 1.7 cm/h. The size at d_{90} is about 21 µm corresponding to a theoretical rise velocity of 6.5 cm/h. Due to a slight turbulence created by the “jet” capillary inlet and a constant circular air stream applied to the surface we expect the rise velocity to be less than the theoretical value. All droplets were drained by the outlet that skims the water surface, so there should be no real separation of droplet sizes in the system (see Supporting Information 1,

Table 1
Chemical composition in the exposure media. Concentrations (given in μg/L) of particulate mass, C9–C36-total hydrocarbon content, volatile organic compounds (C5–C9, VOC), naphthalenes (C0–C4), 2–3 ring PAHs, 4–6 ring PAHs, total-PAH (T-PAH) and hopane are given as mean ± average (N = 8, except for particle mass where N = 4). Naphthalenes were not included in the sum of 2–3 ring PAHs.

Experiment 1		Low		Medium		High	
	Ctrl	Dispersion (LD)	WSF (LW)	Dispersion (MD)	WSF (MW)	Dispersion (HD)	WSF (HW)
Particle mass	12,5 ± 9,7	142,7 ± 23,8	3,3 ± 2,7	668,3 ± 140,4	3,0 ± 2,2	3532,2 ± 336,0	6,4 ± 3,2
C9–C36-THC	0,01 ± 2,76	77,44 ± 10,35	25,04 ± 14,06	414,63 ± 30,60	24,52 ± 4,59	2403,12 ± 165,92	99,84 ± 22,81
VOC	0,33 ± 0,14	3,18 ± 2,04	5,37 ± 1,65	28,85 ± 8,71	28,14 ± 7,67	149,04 ± 24,12	113,17 ± 24,02
C5–C36-THC	0,34 ± 7,53	80,62 ± 20,30	30,41 ± 20,85	443,47 ± 115,20	52,66 ± 22,70	2552,17 ± 511,36	213,01 ± 48,23
Naphthalenes	0,18 ± 0,05	1,64 ± 0,26	1,55 ± 0,42	7,78 ± 1,74	6,48 ± 1,12	36,07 ± 7,39	22,74 ± 3,38
2-3 ring PAHs	0,02 ± 0,01	0,71 ± 0,12	0,54 ± 0,07	3,33 ± 0,72	1,75 ± 0,13	14,43 ± 2,70	4,25 ± 0,29
4-6 ring PAHs	0,00 ± 0,00	0,09 ± 0,01	0,03 ± 0,00	0,71 ± 0,19	0,06 ± 0,01	3,47 ± 0,65	0,07 ± 0,01
T-PAH	0,20 ± 0,05	2,44 ± 0,38	2,12 ± 0,48	11,82 ± 2,61	8,30 ± 1,25	53,97 ± 10,71	27,06 ± 3,64
Hopane	N.D.	0.02 ± 0.002	N.D.	0.11 ± 0.009	N.D.	0.53 ± 0.031	N.D.

Experiment 2	**Ctrl**	**Low Dispersion (LD)**	**WSF (LW)**	**Medium Dispersion (MD)**	**WSF (MW)**	**High Dispersion (HD)**	**WSF (HW)**
Particle mass	12,2 ± 4,3	176,3 ± 5,3	11,5 ± 0,8	854,3 ± 40,8	6,8 ± 0,5	4342,5 ± 244,9	10,1 ± 5,3
C9–C36-THC	0,07 ± 19,33	86,13 ± 20,55	28,22 ± 32,64	486,41 ± 12,38	38,52 ± 7,52	2692,42 ± 424,33	123,11 ± 15,77
VOC	2,94 ± 2,02	14,51 ± 8,97	6,48 ± 2,70	48,94 ± 18,27	36,94 ± 3,70	207,17 ± 14,07	166,95 ± 16,84
C5–C36-THC	3,01 ± 23,94	100,65 ± 36,19	34,71 ± 34,22	535,34 ± 34,80	75,46 ± 27,84	2899,59 ± 650,70	290,06 ± 24,60
Naphthalenes	0,30 ± 0,16	2,16 ± 0,18	2,22 ± 0,16	10,51 ± 0,46	9,03 ± 1,10	47,66 ± 3,40	31,32 ± 3,69
2-3 ring PAHs	0,03 ± 0,02	0,89 ± 0,09	0,66 ± 0,05	4,18 ± 0,14	2,14 ± 0,12	19,75 ± 1,32	4,98 ± 0,38
4-6 ring PAHs	0,00 ± 0,00	0,15 ± 0,03	0,03 ± 0,01	0,89 ± 0,07	0,06 ± 0,01	4,92 ± 0,34	0,09 ± 0,01
T-PAH	0,33 ± 0,18	3,20 ± 0,25	2,90 ± 0,16	15,58 ± 0,41	11,22 ± 1,19	72,33 ± 4,89	36,40 ± 4,07
Hopane	N.D.	0.03 ± 0.004	N.D.	0.15 ± 0.003	N.D.	0.75 ± 0.012	N.D.

Fig. S1). Since the inlet is at the bottom and the outlet on top, the only difference would be that the larger droplet fraction has a slightly shorter residence time than the smaller droplet fraction. In these experiments, our exposure chambers (4,5 L) had an average flow rate of 15.7 mL/min and the theoretical mean residence time of the water (assuming full mixing) is 4 h and 47 min.

There was no evidence of oil droplets in the WSFs as indicated by particle measurements as well as chemical characterization (Table 1 and Supporting Information 3 Fig. S3). The particle content of all filtered solutions did not exceed that of the controls indicating clearly that the oil content in the filtered solutions was extremely low or absent (Supporting Information 3, Figs. S4 and S5). We also measured hopane, an oil component with extremely low solubility and expected to be present only in droplet oil. This component was detected in all dispersions, but not in any of the WSF samples (Table 1).

Three nominal concentrations were chosen based on previous experiments using the same oil type (Troll) and experimental design on cod larvae (Nordtug et al., 2011b; Olsvik et al., 2011, 2012b) and copepods (Hansen et al., 2012; Olsen et al., 2013a). The characterizations of the exposure media are given in Table 1. Based on particle mass, the low, medium and high dispersion concentrations consisted of 0.14–0.18, 0.67–0.85 and 3.53–4.34 mg oil/L, respectively. The measured concentrations were lower than the nominal, and this is probably caused by loss of larger oil droplets in the settling chamber and adhesion of particulate oil to tubes and fittings. Although it was aimed to perform two identical experiments, the oil exposure concentrations of Experiment 1 were approximately 25% lower than in Experiment 2. This may be caused by small grains partially clogging the tubes as oil is being pumped through into the oil droplet generator. This variation does not affect the assessment of the contribution of oil droplets to toxicity but may interfere slightly when comparing the differences in response between developmental stages. Based on the THC measurements, the WSFs consisted of concentrations approximately 11% of the concentrations in the dispersions, thus the droplet fraction in oil dispersions constituted approximately 89% of the hydrocarbons.

The concentrations used were comparable to those previously reported to cause spinal deformations and craniofacial effects in gadid fish (Sørhus et al., 2015), but effect levels for the studied endpoints may vary based on species sensitivity and oil type. Based on T-PAH, the concentrations (> 2 μg T-PAH/L) used in our experiments are in the high range of concentrations measured during oil spills, including the Deepwater Horizon incident, where the majority of water samples consisted of T-PAH concentrations < 1 μg/L (Echols et al., 2015). The aim of the experiments was, however, not to identically mimic an oil spill event in the laboratory, but rather to parameterize experimental conditions to provide direct information about the contribution of oil droplets to the toxicity of crude oil-in-water dispersion. Our flow-through experimental system generated two parallel types of exposure media; one with oil droplets (dispersion) and the other without (WSF). The exposure concentrations, droplet concentrations and droplet size ranges (for the dispersions) were relatively constant over the 96 h exposure periods (Supporting Information 3, Figs. S3–S5). Differences in effects between parallel treated groups (LD and LW; MD and LW; HD and HW) would indicate that oil droplets contributed to the observed effects (Nordtug et al., 2011a).

Based on THC, the concentrations of extractable oil components in the dispersion is in the range 5–11 times higher than in the WSFs. The partitioning of PAHs and other oil soluble oil components between water and oil is determined by their solubility in water and their molar fraction in the oil phase (Raoult's Law) (Guggenheim, 1937). Since the solubility for PAHs in general decreases with molecular weight (and Log Kow), the lighter components, such as naphthalenes, are predominantly in the water phase, whereas the heavier components, such as 4-6-ring PAHs, are predominantly found in the oil phase (Table 1). Thus, the contribution of oil droplets to toxicity may be caused by mechanical properties or by the droplets acting as a reservoir for harmful substances that are made bioavailable ether by direct contact with the cod eggs or by replenishing the pool of dissolved components as they are accumulated by the embryos.

3.2. Acute and delayed mortality

Survival assessed at end of the 96 h exposure period (7 dpf for Experiment 1 and 13 dpf for Experiment 2) and after hatching (2 dph

Table 2
Surviving fraction. Fraction of surviving cod after exposure (Exposure), from exposure to end of recovery (Recovery) and for the whole period (Total). Values are given as average ± standard deviation (N = 4 for all). Notation * denotes significant differences between exposed and control, and ¥ denotes significant differences between parallel treatments to dispersion and WSF. *0.05, ***0.001, ****0.0001, ¥¥¥0.001, ¥¥¥¥0.0001.

Experiment 1 (early exposure)

Group	Exposure (3–7 dpf)	Recovery (2 dph)	Total (3 dpf-2 dph)
C	0,90 ± 0,02	0,46 ± 0,19	0,41 ± 0,16
LD	0,90 ± 0,01	0,42 ± 0,20	0,38 ± 0,18
MD	0,89 ± 0,01	0,50 ± 0,04	0,45 ± 0,04
HD	0,05 ± 0,04****,¥¥¥¥	0,05 ± 0,02***, ¥¥¥	0,00***
LW	0,89 ± 0,01	0,42 ± 0,09	0,37 ± 0,08
MW	0,89 ± 0,01	0,44 ± 0,09	0,39 ± 0,09
HW	0,38 ± 0,14****, B	0,44 ± 0,10	0,17 ± 0,09*

Experiment 2 (late exposure)

Group	Exposure (9–13 dpf)	Recovery (3 dph)	Total (9 dpf-3 dph)
C	0,98 ± 0,01	0,66 ± 0,20	0,65 ± 0,20
LD	0,98 ± 0,00	0,58 ± 0,04	0,57 ± 0,04
MD	0,98 ± 0,01	0,25 ± 0,10***	0,25 ± 0,10***
HD	0,99 ± 0,01	0,05 ± 0,02****	0,05 ± 0,02****
LW	0,98 ± 0,00	0,56 ± 0,15	0,55 ± 0,15
MW	0,99 ± 0,01	0,40 ± 0,10*	0,39 ± 0,10*
HW	0,99 ± 0,00	0,22 ± 0,04***	0,22 ± 0,04***

for Experiment 1 and 3 dph for Experiment 2), demonstrated clear differences in mortality patterns between exposure in early or late developmental stages (Table 2). After exposure of early stage embryos (Experiment 1), a clear concentration-dependent increase in mortality was observed for both WSF and dispersion, with highest mortality for the dispersion treatment. After exposure, and through to hatching, very low post-exposure mortality was observed, with no significant differences in the treated groups compared to controls. For the late exposure (Experiment 2), the opposite trend was observed; very low mortality in all groups during exposure, and no significant difference from the control group. However, during the recovery period, lasting until after hatching (3 dph), a concentration-dependent decrease in survival was observed, and very few hatched larvae from the highest dispersion treatment were available for analyses because of high mortality.

The choice for timing of exposure was intended to cover two major processes of embryonic development; firstly, the gastrula stage, when the first cell differentiation, tissue layers, and embryo axis is formed, and secondly after gastrulation and closure of blastopore, when major organogenesis and neurulation occur in the embryo. During the early-stage embryonic exposure, mortality was concentration dependent and close to 100% for the highest dispersion exposure, whereas during the late-embryonic exposure experiment, almost all embryos (> 95%) survived in all groups. This may be explained by the higher sensitivity at the gastrula stage, when blastodermal cells spread laterally and migrate towards the vegetal pole, and tissues and organs begin differentiation as epiboly proceeds. This is when the switch from maternal to embryonic gene regulation sets in (Drivenes et al., 2012), and the gastrula period is regarded as a very vulnerable stage in fish development. In terms of acute mortality, embryos exposed at an early stage were more sensitive to exposure than those exposed at a later stage. This may be caused by oil components disrupting cell signals and gene activation patterns related to e.g. cell-cell adhesion during early development (Kjorsvik et al., 1982) and/or disruption of embryo surface membrane integrity and adhesion-dependent morphogenesis during epiboly (Drivenes et al., 2012; Shimizu et al., 2005). Developing cod embryos are hyposmotic to sea water and are dependent on the integrity and low permeability of the surface membrane to preserve water and remain in osmolytic balance, but previous studies have shown that their membrane permeability was not affected by exposure to WSFs of North Sea crude oil at concentrations of 50–150 µg/L (Mangor-Jensen and Fyhn, 1985). In contrast to the group exposed early in their development, the groups exposed after gastrulation showed a concentration dependent mortality during the recovery after the exposure with only approximately 5% survival of the embryos subjected to the high dispersion treatment. This clearly suggests different modes of toxic action from similar exposure at two different stages of the cod embryonic development. This may be due to a lower ability of the embryos to activate biotransformation enzymes (e.g. CYP1A) through the aryl hydrocarbon receptor (AHR) during earlier development. Fish embryos have shown to have lower CYP1A activity from a given level of CYP1A mRNA than larval stages (Mattingly and Toscano, 2001). Cod embryos exposed to crude oil dispersions have displayed elevated CYP1A expression at 10 dpf (Sørhus et al., 2015). Although not measured in the current work, AHR-activation plays a role in developmental effects in early life stages of fish (Billiard et al., 2006), and could explain differences in developmental toxicity observed in the present work. In addition, exposure to heavier (4–5 ring PAHs) and alkylated 3-ring PAHs in the dispersion treatments may enhance developmental effects compared to WSF treatments as they may serve as better AHR ligands than lighter PAHs, e.g. unsubstituted 2-3-ring PAHs (Barron et al., 2004; Zhang et al., 2012).

3.3. Condition of the larvae

The biometric measurements of the yolk-sac larval condition factors (standard length, myotome height, body surface and yolk sac length) is illustrated in Fig. 3. Effects are plotted in Fig. 5 as a function of the T-PAH concentrations in the WSFs (not dispersions). The reasoning behind this is that if oil droplets do not contribute to any of the observed effects, and as such, function only as inert particulate matter in the water, effects data from parallel treatments with dispersion and corresponding WSF should overlap. If, however, both effects data from the parallel treatments were plotted as a function of their actual PAH concentrations, the concentration of the dispersion will be considerably higher, because the PAHs contained within the oil droplets, and presumably less bioavailable, will add to the water concentration.

For both experiments, a general reduction in length was observed as a function of exposure concentration (Fig. 5A–B), although no larvae hatched in the highest dispersion exposure in Experiment 1. Compared to controls in Experiment 1, the mean larval length was significantly shorter for larvae in medium and high concentrations (MD: $p < 0.01$, MW: $p < 0.05$, HW: $p < 0.0001$), and with no significant difference between dispersion and the WSF in the medium exposure. In Experiment 2, all treatments resulted in significantly shorter larvae than the control (LD: $p < 0.001$, MD: $p < 0.0001$, HD: $p < 0.0001$, LW: $p < 0.05$, MW: $p < 0.0001$ and HW: $p < 0.0001$), and significant difference were observed between the medium treatments with or without oil droplets. Smaller size in developing cod larvae exposed to oil have previously been reported in a concentration-dependent manner. Tilseth et al. (1984) exposed developing embryo and larvae for three weeks to WSF from North Sea crude oil starting one week prior to hatching, resulting in reduced lengths of exposed larvae from hatch until end of exposure for concentrations ranging 45–245 ppb. In our experiments, the lengths of larvae exposed to dispersions were shorter than the parallel WSF-treatment, however significantly shorter larvae were observed only for the medium parallel treatments (Fig. 5B) suggesting that the contributions of oil droplets to these effects are minor.

Similarly, no significant differences between parallel treatments were observed for larvae myotome height from the two experiments. Larvae myotome height is often correlated with larvae length as growth metrics, and, significantly increased myotome heights compared to controls were observed for the medium and high concentrations (early exposure: MD: $p < 0.05$, LW: $p < 0.05$, MW: $p < 0.05$; late exposure: MD: $p < 0.001$, HD: $p < 0.05$, MW: $p < 0.01$, HW: $p < 0.01$), and

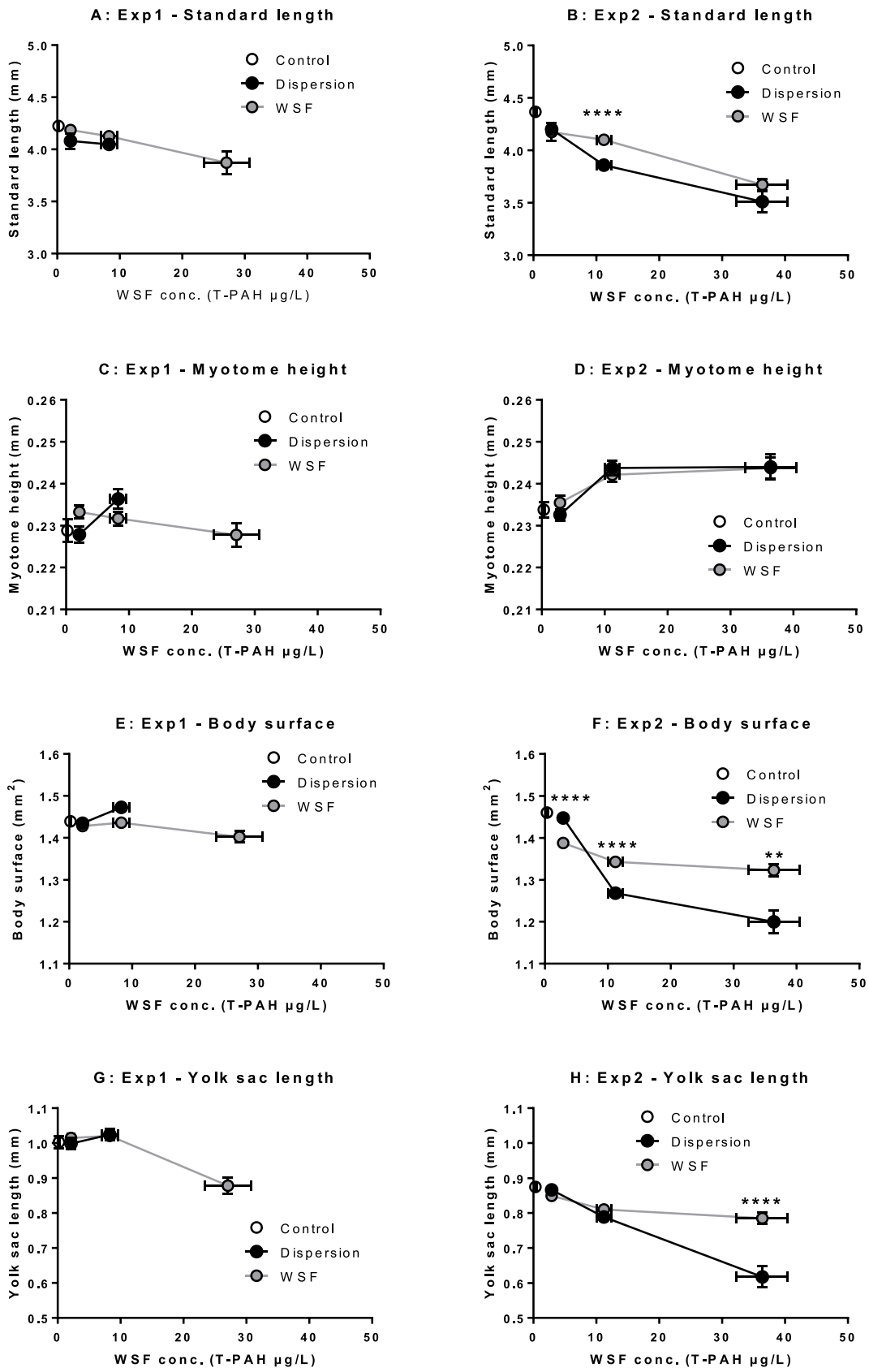

Fig. 5. Condition of hatched larvae. Condition of hatched larvae from all treatment groups assessed as mean standard length (A and B, N = 15–25), myotome height (C and D, N = 11–98), body surface (E and F, N = 11–98) and yolk sac length (G and H, N = 11–98). Data are given as mean ± SEM (N = 11–98). Significant differences between parallel groups treated with dispersion and WSF are given as asterisk **p < 0.01 and ****p < 0.0001.

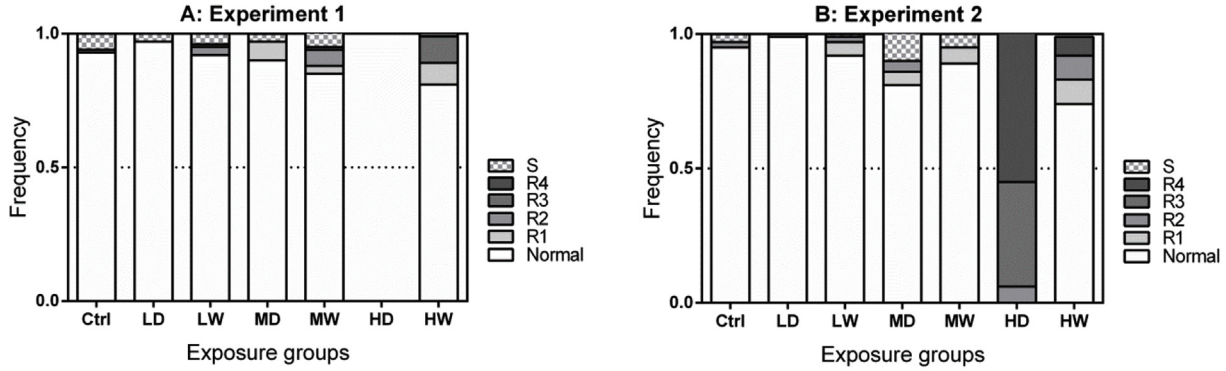

Fig. 6. Frequency of abnormal spinal curvatures in hatched larvae treated at early (A) and late (B) embryonic development.

for late exposure this also displayed a concentration dependent response. The reason for increased myotome height in exposed fish is unknown, however, it may be related to the development of spinal deformations. This effect does not appear to be related to oil droplet exposure as the curves in Fig. 6D overlap. For body surface areas of the larvae (Fig. 6E–F), both experiments displayed treated larvae with significantly reduced body areas when comparing to the corresponding controls (early exposure: HW: $p < 0.05$; late exposure: MD: $p < 0.0001$, HD: $p < 0.001$, LW: $p < 0.0001$, MW: $p < 0.0001$, HW: $p < 0.0001$). However, the effects were more pronounced in yolk-sac larvae exposed at the late stage of their embryonic development. For late exposure, oil droplets may also contribute to this effect, as body areas were significantly different between parallel treatments (Fig. 6F). Yolk sac length (Fig. 6G–H) decreased in treated larvae compared to controls as a function of exposure concentration (early exposure: HW: $p < 0.0001$; late exposure: MD: $p < 0.0001$, HD: $p < 0.0001$, MW: $p < 0.001$, HW: $p < 0.0001$). We also observed that smaller yolk sacs for larvae treated with the highest dispersion (HD) compared to parallel WSF (HW) (Fig. 6H; $p < 0.0001$), indicating a contribution of droplets to this effect. The yolk sac represents an energy storage, which is being utilized by the embryo/larvae for growth until they reach the first-feeding stage. The yolk consumption was expected to be correlated to energy spent by the developing embryo/larvae, and as such a proxy for cost of growth and maintenance. Thus, a smaller yolk sac as well as the observed smaller larvae in the exposed groups, will indicate an increased energy demand due to chemical stress imposed by the oil treatment. Cod embryos have displayed the capacity to express biotransformation enzymes already at the blastula stage of embryonic development (Olsvik et al., 2012a), and this may cause exhaustion of the yolk sac. Another reason for reduced yolk sac length may be oil-enhanced development of pericardial edema pushing the yolk sac backwards (Incardona et al., 2004).

3.4. Spinal deformities

Larvae displaying a straight spine were counted as “normal” and larvae with vertebral curvatures were characterized based on the type and severity of the deformation, as illustrated in Fig. 6. In our experiments, 7 and 5% of the control larvae in early and late exposure, respectively, displayed abnormal spinal curvatures according to our criteria. Vertebral deformities is a common problem in juvenile cultivation of marine fish species (Boglione et al., 2013), and Fjelldal et al. (2009) found that about 6% of wild juvenile Atlantic cod had some vertebral deviations.

A concentration-dependent increase in the fraction displaying spinal curvatures was observed for WSFs and dispersions in both experiments. For WSF early treated embryos (Experiment 1), a concentration dependent increase (from 8 to 19%) in spinal deformations were observed. For the dispersion-treated cod embryos, the percentage displaying deformations increased from 3 to 10% between low and medium treatment (Fig. 5A). As mentioned above, very few embryos survived until hatch in the high dispersion treatment (HD) for the early-stage embryonic exposure, thus we have no data for this group. No clear trend for contribution of droplets was observed for the low and medium concentrations, with no significant difference between the treatments. However, the almost total mortality in high dispersion compared to 19% deformed larvae in the high WSF suggested significant negative effect from the oil droplets. For the late exposure period after gastrulation, a concentration-dependency was observed for both treatments, increasing by concentration from 1 to 100% and 8–26% for dispersion and WSF treatments, respectively (Fig. 5B). The percentages of larvae displaying spinal deformities were overall higher for the dispersion treatments than for the corresponding WSF treatments, suggesting a contribution from droplets to this effect. Developmental deformations have previously been shown to occur in cod larvae subjected to produced water during the embryonic period (Meier et al., 2010).

The occurrence of deformations is similar in the two experiments for the WSF gradients, and the data for the low and medium concentrations did not provide evidence that exposure at the different developmental stages caused different frequency and severity of spinal deformations. However, for the highest exposure concentrations, there was a highly significant negative effect from the oil droplets in both experiments. The mortality was acute in the early exposure period, and while embryos survived after the late exposure period, all hatched larvae had abnormal spinal curvatures and thus could be considered moribund.

3.5. Jaw morphology and development

A number of studies have shown that exposure to oil components results in disruption of jaw and cartilage development causing craniofacial deformities in fish larvae (He et al., 2011; Incardona et al., 2004; Olsvik et al., 2011; Pollino and Holdway, 2002; Shi et al., 2012; Sørhus et al., 2015). Our experiments also demonstrated that embryos exposed to oil developed deformities in jaw formation and cartilage structures (Fig. 7).

The three measurable endpoints reflecting this were the quadratal angle Q (Fig. 8A–B), jaw length (Fig. 8C–D) and jaw-to-eye (Fig. 8E–F) distance. As larvae from Experiment 2 were sampled one day after those in Experiment 1 (3 vs 2 dph), their jaw cartilage formations were more developed and stained more clearly than in larvae from Experiment 1. They are therefore not directly comparable, and only larvae from Experiment 2 are used to exemplify the effects on cartilage formation in Fig. 7.

In the WSF, jaw cartilage development was observed in all larvae. However, a clear gradient effect was observed for the jaw cartilage normal development. In Experiment 2, the LW larvae had jaw length and visible cartilage development as in the control larvae (Fig. 7B), whereas the developing jaw in larvae from the MW treatment had irregular location and development of the jaw bones (Fig. 7C), and the HW larvae had very abnormal jaws and much less developed cartilage

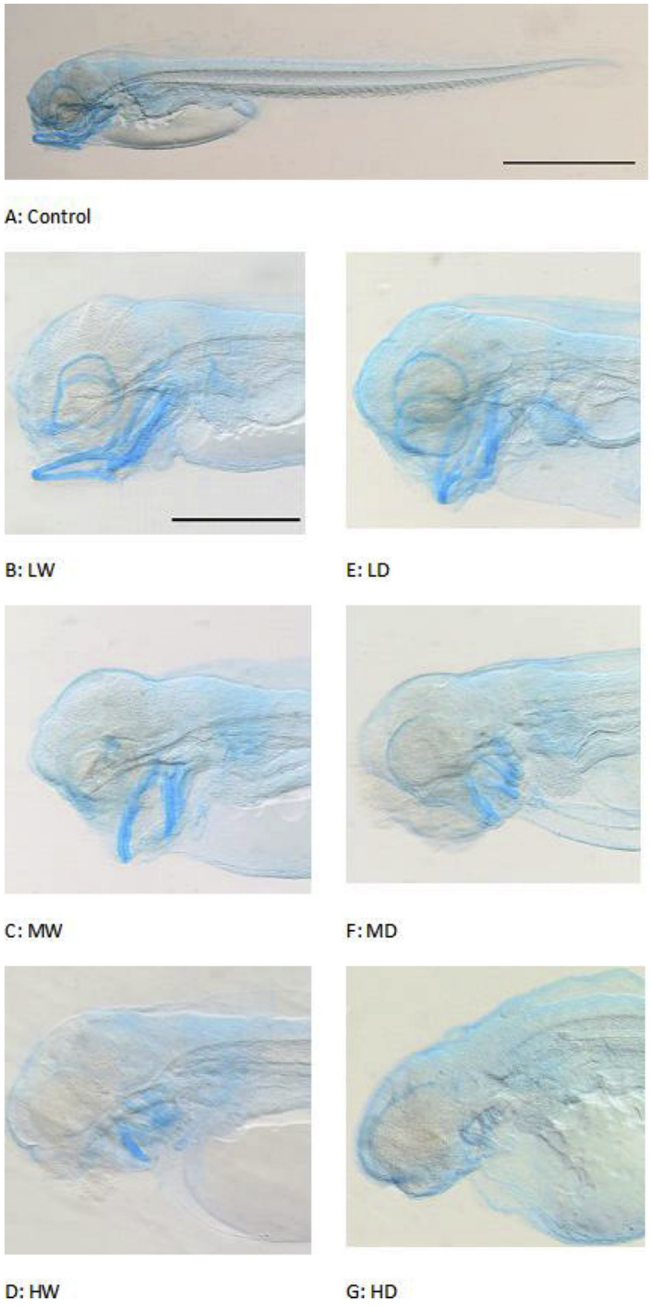

Fig. 7. Craniofacial deformations. Example of typical effects on jaw cartilage development in 3 day old cod larvae, after oil exposure for 4 days during late embryonic stage (from experiment 2), the larvae were stained with Alcian blue. Control (A), and embryos treated with low (B), medium (C) and high (D) concentrations of WSF and the parallel low (E), medium (F) and high (G) concentrations of dispersions. Bar = 1 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

formation (Fig. 7D). In the dispersion treated group a similar gradient could be observed, although in the HD groups, no larvae hatched after early exposure (Experiment 1), and only 1 of 15 stained larvae had developed any cartilage at all after late exposure (Experiment 2). No statistical data treatment was therefore done for these groups (Fig. 7E–G), although a clear effect from the oil droplets could be seen on the developing HD-larvae.

For the quadratal angle Q (Fig. 8A–B), significantly ($p < 0.05$) reduced values and clear gradient effects were obtained for all oil treatments, suggesting disruption of jaw and cartilage development. In cod larvae, the angle Q increases from 85° to 165° from hatching up to 35 dph, and subtle changes in Q reflects changes in head proportions caused by allometric growth, whereas larger shifts in Q indicate shifts in kinematic mechanisms for mouth opening (Herbing, 2001). Angle Q differed significantly between the WSF and dispersion treatments in the early exposure group, with much lower Q value for the medium dispersion exposure ($p < 0.05$), and with no hatching for the high dispersion group, demonstrating a strong contribution from oil droplets to this effect after exposure during early embryonic development. No significant difference in angle Q was found between the treatments of Experiment 2 in medium exposures. However, a strong effect was seen after high dispersion exposure, as almost no larvae developed cartilage structures in the HD-group in Experiment 2.

Similar to responses in Q, reduced jaw lengths (Fig. 8C–D) were observed as a function of exposure concentrations, except for LW in the first experiment, where the average jaw length was not significantly different from the control (Unpaired *t*-test: $p = 0.2565$). We also observed significantly shorter jaw lengths from the dispersion exposure than from WSF in low (Experiment 1) and medium (both experiments) concentrations, suggesting that oil droplets contributed to this effect. Significant differences between parallel treatments were not found for jaw-to-eye measurements, but the data (Fig. 8E–F) suggested different responses between the two experiments. Hatched larvae exposed early displayed significantly longer distance for LW (Mann Whitney test: $p = 0.0033$), LD (Unpaired t-test: $p = 0.0039$) and MW (Unpaired *t*-test: $p = 0.0236$) compared to controls, and no significant changes were observed for the HW group (Fig. 8E). For fish exposed after gastrulation, hatched larvae displayed significantly shorter distance between jaw and eye for LD (Unpaired *t*-test: $p = 0.0097$), MW (Unpaired *t*-test: $p < 0.0001$), MD (Unpaired *t*-test: $p < 0.0001$) and HW (Mann Whitney test: $p < 0.0001$) (Fig. 8F) compared to controls, and with no significant difference between the parallel treatments.

Continuous exposure to 0.25 ppm WSF of a North Sea crude oil over a 14-day period caused malformations in the head and jaw regions in cod larvae (Tilseth et al., 1984). A 7-day exposure of rockfish (*Sebasticus marmoratus*) embryos to the PAH benzo(a)pyrene (concentration range 0.5–50 nM) resulted in dose-responsive impaired craniofacial development, manifested as shortened lower and upper jaw with a severe malformation in Meckel's cartilage at the highest exposure (Shi et al., 2012), comparable to the results obtained in our experiments on cod embryos. Rockfish also displayed similar dose-responsive craniofacial effects when exposed at the larval stage to pyrene, phenanthrene and benzo(a)pyrene in concentration ranges of 0.01–1.0 μg/L (Li et al., 2011). Reduced head growth was also apparent in zebrafish newly hatched larvae following exposure to single PAHs and mixtures at the embryo stage (Incardona et al., 2004). Importantly, in this zebrafish study, different PAHs caused slightly different effects. No morphological features were affected by naphthalene, anthracene and chrysene, in contrast to fluorene, dibenzothiophene and phenanthrene which caused spinal curvature (dorsal curvature of trunk and tail). Our studies used crude oil consisting of a mixture of these PAHs (among other not identified oil compounds) with individual PAH concentrations in the low range of reported values in the studies on rockfish and zebrafish (Table 1). The implications of jaw malformation due to oil exposure would be reduced uptake of food and high mortalities, which has been shown in cod continuously exposed to WSFs (0.25 ppm) during the embryonal and larval phase (Tilseth et al., 1984). Cod larvae start first feeding at 3–5 dph, and the concentration-dependent responses in endpoints related to jaw development (Fig. 8) in our experiments, suggest that the oil impact will reduce the ability of larvae to consume food when they start feeding. Significant impacts on jaw developmental endpoints were observed even at the lowest concentrations used in the experiments. Several genes related to formation of muscle, bone formation and mineralization are activated in cod during the latest exposure period (Drivenes et al., 2012). The underlying molecular mechanisms of PAH-mediated effects on skeletal development is not fully

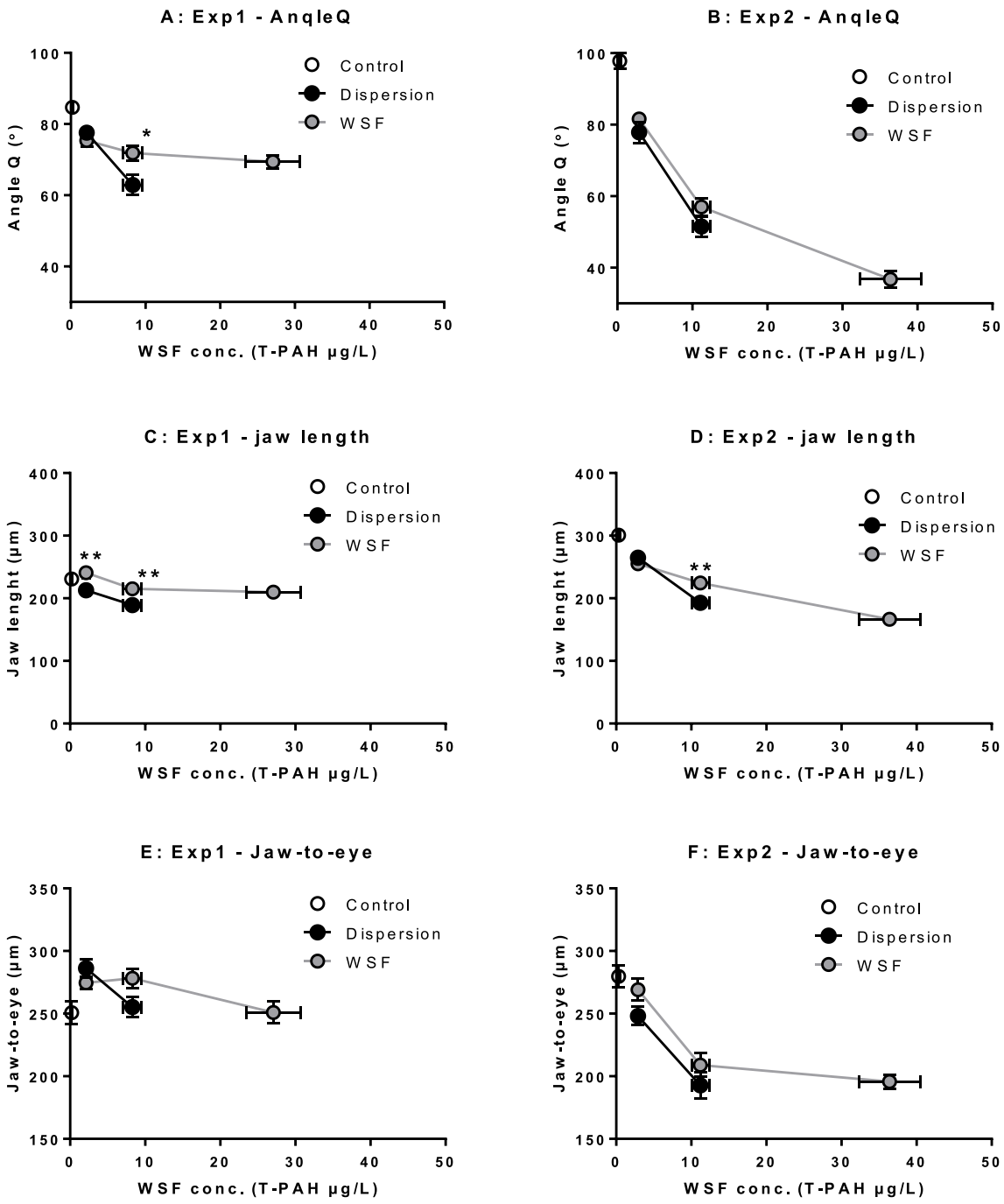

Fig. 8. Craniofacial developmental responses to oil exposure. Jaw developmental effects following exposure of embryos to dispersions and WSFs. Data are given as mean ± SEM (N = 14–24). Significant differences between parallel groups treated with dispersion and WSF are given as asterisk *p < 0.05 and **p < 0.01.

understood. However, it has been shown that PAHs inhibit Na+/K + -ATPase and Ca2+-ATPase activity (Li et al., 2011), reduce cell proliferation activity in the craniofacial skeleton and interfere with the expression of signaling molecules (hedgehog gene family) which regulates proliferation of chondrocytes and skeletal development in rockfish (*Sebastiscus marmoratus*) (He et al., 2011). Craniofacial abnormalities will most likely result in decreased feeding capacity and survival over time, as observed for zebrafish exposed to TCDD during the embryo stage (Chollett et al., 2014).

4. Conclusions

Exposure of cod embryos to crude oil dispersions caused acute and delayed toxicity, including manifestation of morphological deformations in hatched larvae. Oil droplets appear to contribute to some of the observed effects including mortality, larvae condition (standard length, body surface, and yolk sac size), spinal deformations as well as alterations in craniofacial and jaw development. The timing of exposure may be essential for the development of effects as higher acute mortality was observed when embryos were exposed from the start of gastrulation (Experiment 1) than when exposed during organogenesis (Experiment 2). Even though lower mortality was observed when exposed during organogenesis, concentration-dependent mortality was observed during recovery. Our study suggests that, in particular for high dispersion exposures, the contribution of oil droplets to dispersion toxicity should be considered.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2019.104753.

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