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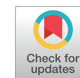
Chemically-dispersed crude oil and dispersant affects sperm fertilizing ability, but not sperm swimming behaviour in capelin (*Mallotus villosus*)☆



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ABSTRACT

The effects of petroleum aromatic hydrocarbons (PAHs) on the embryonic and larval life stages of teleosts have been extensively examined. However, very little work has been conducted on how spilled oil affects fish sperm and there is no related knowledge concerning oil dispersing agents. The objective of our study was to determine sperm performance of a teleost fish under direct exposure to different concentrations of WAF (water accommodated fraction) and CEWAF (chemically enhanced water accommodated fraction). Capelin sperm motility, swimming behaviour, and sperm fertilization ability were evaluated in a scenario of an oil spill untreated (WAF) and treated (CEWAF) with the dispersant Corexit® EC9500A. Sperm fertilizing ability was lower when exposed to CEWAF concentrations of 16.1×10^3 µg/L total petroleum hydrocarbons and 47.9 µg/L PAH, and when exposed to the dispersant alone. The mechanism responsible for this reduced fertilizing ability is not clear. However, it is not related to the percentage of motile sperm or sperm swimming behaviour, as these were unaffected. WAF did not alter sperm swimming characteristics nor the fertilizing ability. We suggest the dispersant rather than the dispersed oil is responsible for the decrease in the sperm fertilizing ability and hypothesize that the surfactants present in the dispersant affect sperm membrane functionality.

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1. Introduction

Growing offshore oil activities, such as drilling operations and transport have increased concerns about accidental marine oil spills and the potential acute effects on aquatic organisms (Beyer et al., 2016; Duran and Cravo-Laureau, 2016; Mearns et al., 2015). In the event of a major oil spill, oil dispersants like Corexit® EC9500A are often used in large amounts to disperse floating oil, increase microbial degradation, and reduce the residence time of the spilled oil components (Prince, 2015). As an example, in the recent BP Deepwater Horizon oil spill, a total of 1.84 million gallons of dispersants were used (Rufe et al., 2011); Corexit® EC9500A and EC9527A.

Dispersants contain both solvents and surfactants that facilitate oil breakdown into tiny droplets that are more rapidly diluted in water and become more available for biodegradation (John et al., 2016; Major et al., 2012; Word et al., 2015). Due to their mode of action, dispersants can (1) directly cause the disruption of biological membranes, and thus, are potentially dangerous for aquatic life (Könnecker et al., 2011; Word et al., 2015) and (2) indirectly cause problems by interacting with oil, releasing greater amounts of toxic components of oil into water, resulting in higher toxicity as compared to untreated oil (Couillard et al., 2005; Adams et al., 2014; Hansen et al., 2014). However, the potential impacts of dispersants on marine organisms are poorly understood.

Early life stages are usually considered to be most vulnerable to contaminants, such as petroleum aromatic hydrocarbons (PAHs). Hence, much attention has been placed on these stages, particularly embryos (embryotoxicity) and larvae of species with external fertilization like most teleosts (e.g., Adams et al., 2014; Frantzen et al., 2012; Incardona and Scholz, 2016; Martin et al., 2014). Nevertheless, as reviewed by Hatef et al. (2013), spermatozoa are also affected by environmental contaminants, and the term

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spermiotoxicity has appeared in the literature. There are two potential routes of sperm cell exposure to any type of contaminant in external fertilizers. First, indirectly via bioaccumulation in adult tissues. For example, Sammarco et al. (2013) observed concentrations of 3.97×10^6 μg/L of TPH (total petroleum hydrocarbons) and 129 μg/L of PAH in various tissues of commercial marine species (mollusks, crustaceans and teleosts) captured in the area of the BP Deepwater Horizon oil spill. This accumulation of contaminants in tissues can cause disruption of testicular function (Hatef et al., 2013 and references within). Second, directly via post-ejaculation exposure of sperm to contaminants prior to fertilization. For example, Hatef et al. (2011) observed that perch (*Perca fluviatilis*) sperm motility was totally suppressed when activated in a medium containing 250 μM of $HgCl_2$. In both exposure routes, the negative effects associated with the presence of contaminants can range from a slight decrease in sperm motility to complete loss of fertilizing ability (Hatef et al., 2013).

No work has been conducted on the direct effects of dispersants on fish sperm. This maybe particularly problematic because there is potential for dispersants to instantly affect membrane function of sperm cells and by doing so, affect fertilizing ability. Knowledge of direct effects of oil on sperm function is also lacking. To date, only Hamoutene et al. (2010) and Bender et al. (2016) have tested the effects of petroleum hydrocarbon pollution on sperm of a teleost with external fertilization. Bender et al. (2016) analyzed the bioaccumulative effects through dietary crude oil exposure in polar cod (*Boreogadus saida*). Although they observed certain changes in sperm motility parameters, these authors collected sperm one month before the spawning season and attributed their results to the presence of immature sperm cells. Hamoutene et al. (2010) evaluated the direct effect of produced water (i.e., water produced during offshore oil and gas drilling) on Atlantic cod (*Gadus morhua*) sperm. These authors observed subtle effects in sperm metabolism (e.g., increase in sperm proteins and citrate synthase activity); however, exposure to produced water did not affect the sperm fertilization ability. Nonetheless, in the event of an oil spill, sperm cells will be exposed to considerably higher concentrations of PAHs than the concentrations found in offshore oil and gas drilling produced water.

Here, for the first time we test fish sperm performance in direct exposure to dispersant, and concentrations of WAF (water accommodated fraction) of oil and CEWAF (chemically enhanced water accommodated fraction) resultant of the mix of oil and dispersant, that are realistic for a marine oil spill event. Commercially-exploited capelin (*Mallotus villosus*) are the most important fish species in the northwest Atlantic food web (and of significance in the Arctic and Pacific) being major forage for top predators, such as cod, marine mammals, and sea birds (e.g., Mullowney and Rose, 2014). Capelin recruitment has huge implications for the functioning of the marine food web in Atlantic Canada. However, their reproductive behaviour puts them at higher risk to oil spills than other fish. Some capelin populations (including that near the Newfoundland offshore oil production platforms) form dense schools, and then migrate inshore, where they then spawn either in demersal sites or on beaches (Penton and Davoren, 2013) over a period of a few weeks. This makes them particularly susceptible to the potential effects of oil spills that concentrate nearshore. Two studies have evaluated the effects of crude oil on capelin embryos and larvae (Paine et al., 1992; Frantzen et al., 2012) and found lethal effects to be higher at the larval stage than at the embryonic stage and sub-lethal effects were detected at concentrations as low as 10% of the lethal dose. We report on how sperm swimming behaviour and sperm fertilization ability are affected by mechanically- and chemically-dispersed crude oil.

2. Materials and methods

2.1. WAF and CEWAF production

Crude oil (ρ = 0.759 g mL-1) was obtained from the Hibernia offshore production platform. Exposure solutions (WAF and CEWAF) were prepared according to Martin et al. (2014) that largely follows Singer et al. (2000) recommendations. Briefly, WAF was prepared by adding crude oil at an oil-to-water ratio (OWR) of 1:9 (v:v), using 15 psu water prepared with Instant Ocean® sea salt and distilled water, corresponding to 84 g of oil per L. 15 psu water was selected to conduct these trials because Beirão et al. (2018) showed that higher salinities cause a decrease in the percentage of motile sperm and sperm velocity in beach-spawning capelin. This solution was left stirring at room temperature in the dark, with a mixing vortex equivalent to 25% of water depth for 18 h, in an unsealed container. After the mixing time, the solution was left to settle for 2 h. CEWAF preparation followed the same steps, but after the 18 h mixing time, Corexit® EC9500A (Nalco) was added to the center of the vortex at a dispersant to oil ratio (DOR) of 1:20 (v:v). Stirring continued for another hour after which the solution was left to settle for 1 h. The WAF and CEWAF solutions were extracted from the bottom of the glass beaker using a syringe with a needle, avoiding the surface. It is possible that these WAF and CEWAF preparations could contain some amounts of microdroplets. The WAF and CEWAF stocks were then diluted to reach 1, 5, and 10% with 15 psu water. A treatment with dispersant alone was prepared by adding dispersant to water in the same proportion used in the CEWAF solution preparation (5 ml L-1). The solution was left stirring for 1 h and rested for another hour before mixing 1:9 with 15 psu to obtain the corresponding concentration of dispersant to the concentration in the 10% CEWAF. All solutions were freshly prepared each day, in total four different days, and were kept at 4–5 °C until used.

2.2. Chemical analysis of solutions

Samples of the undiluted stock solutions were collected immediately after preparation and kept refrigerated (4–5 °C in the dark) until analysis at the stable isotope laboratory of Memorial University. The samples were analyzed by gas chromatography-mass spectrometry (GC-MS) using a C7-C40 saturated alkanes standard (Sigma Aldrich) in a combined scan/selected ion monitoring mode. TPH were measured by GC – flame ionization detection (see Table S1, S2 and S3 – Supplementary material for a detailed overview of the methods). For the negative control (15 psu water only), there was no Unresolved Complex Mixture (UCM), thus TPH equals the sum of the individual n-alkanes. Parent PAHs were quantified using CRM48905 mix (Sigma Aldrich). We assumed that the TPH and PAH concentrations of the tested treatments were proportional to the stock solution's dilutions. For the dispersant alone treatment there was visible breakthrough during the silica gel separation step and, for the fraction containing the PAHs there was co-elution of the internal standard with an unknown compound, so that no concentrations can be reported.

2.3. Capelin sampling; sperm, and egg collection

Groups of beach spawning capelin (2–3 years old inferred by their length (DFO, 2015)) were captured repeatedly in July and August 2016 from different locations on the Avalon Peninsula, Newfoundland, Canada (males n = 13 for sperm behaviour and n = 38 for fertilization experiment, total length = 166 mm ± 8.3; females n = 25, total length = 151 mm ± 11.1, mean ± s.d.). Fish were transported 10–90 min in aerated coolers and then kept in a

400 L flow-through sea water tank at 7–10 °C and ~32 psu, until the next day. A maximum of 40 fish (both males and females) was kept in a tank. Gametes were collected within 24 h of capture. Fish were euthanized with an overdose of MS-222 and then, thoroughly dried. Eggs were collected in a plastic dish by gently pressing on the female's abdomen. Semen was collected from the urogenital pore with a pipette. Capelin sperm are motile upon stripping, thus immediately after collection, the sperm were diluted 1:50 in a non-activating solution as described by Beirão et al. (2018). Sperm samples were kept at 5 °C until analyzed (within 1 h). All procedures followed Canadian guidelines for the use of research animals (Memorial University protocol 16-19-CP).

2.4. Sperm behaviour experiment

Among-individual replicates for this particular experiment were conducted over two days. Sperm from different males (n = 13) that were pre-diluted in the non-activating solution (0.2 μL) were re-activated by adding 4 μL of one of the eight different treatments (WAF 1%, WAF 5%, WAF 10%, CEWAF 1%, CEWAF 5%, CEWAF 10%, dispersant alone, negative control – 15 psu water) with 0.1% BSA to prevent sperm cells from adhering to the slide chamber (MicroTool 2 chambers, Cytonix) as described by Beirão et al. (2014). The microscope plate was pre-chilled to 10 °C with a customized Physitemp TS-4 system. Videos were acquired with an inverted Leica DM IL LED microscope (Leica microsystems, Concord, Ontario, Canada), using a 20 × phase contrast objective, and a Prosilica GE680 monochrome camera (Allied Vision Technologies, Burnaby, British Columbia, Canada) set at 100 frames per s. Videos were analyzed with a computer assisted sperm analysis (CASA) plugin (Wilson-Leedy and Ingermann, 2007 and modified by Purchase and Earle, 2012). Beirão et al. (2018) previously described the plugin input parameters for capelin, and showed that in capelin sperm few cells are motile at 15 s and after 30 s virtually all have stopped moving, thus we chose to use 5 and 10 s after sperm:water mixing to evaluate sperm behaviour. The following sperm swimming parameters were selected from the different CASA outputs for analysis: the percentage of motile sperm cells (% motile), the curvilinear velocity (VCL) that corresponds to the sperm cells' velocity along the actual path and the linearity (LIN) of the sperm cells trajectory. These parameters have been shown to be associated with fertilization success in other fish species. As a technical replication, two subsamples of each animal's semen were recorded for each treatment. These were averaged and the means of these means are presented among individuals.

2.5. Fertilization experiment

This experiment was repeated four times, over two days, with unique pools of sperm and eggs from different fish (see Fig. 1 for a schematic representation of the experiment). The number of males (between 9 and 10) and females (between 3 and 10) in each pool was adjusted accordingly to the volume of semen and eggs stripped from each individual in order to have enough volume for the experiment. Pooled sperm that were pre-diluted in the non-activating solution were re-activated in a falcon tube by dilution 1:19 in one of the different treatments (WAF 1%, WAF 5%, WAF 10%, CEWAF 1%, CEWAF 5%, CEWAF 10%, dispersant alone, negative control – 15 psu water) for 10 s. After this time, the sperm were immediately added to one of eight Petri dishes (one Petri dish per treatment) with 500 eggs (estimated by weight) from a pool of eggs across females (equal contributions). The semen volume (≈1 ml) added to the eggs was adjusted to obtain a sperm:egg ratio close to 4000, as preliminary trials indicated that this ratio was limiting in terms of fertilization success, as higher ratios (ceiling effect) could mask a significant effect on the sperm fertilizing ability. After 30 s of contact time, the eggs were rinsed with abundant 15 psu water to remove any remains of sperm or treatment solution. Most fertilizations likely happened within 2–3 s of egg exposure to sperm. The sperm and egg containers were kept on ice at all times to control temperature. After fertilization, the eggs were incubated at 4 °C in 15 psu water. The 15 psu salinity was chosen because Purchase (2018) showed beach-spawning capelin embryos perform well from 2 to 28 psu but present low hatching success at higher salinities. Fertilization rate was assessed after 20 h by counting the number of fertilized (8- and 16-cell stage embryos) and unfertilized eggs (no cell division). To summarize, sperm were exposed to treatment for 10 s, then massively diluted in egg volume for 30 s, then thoroughly rinsed to remove any contaminant. Thus, any effects on embryos were assumed to occur as a result of the 10 s sperm exposure period. The entire process was repeated four times using different groups of adult fish.

2.6. Data analyses

Statistical tests were conducted using R 3.1.2 (R Development Core Team, 2014).

In the sperm behaviour experiment, differences for the sperm swimming parameters (% motile, VCL, and LIN) when the samples were activated in the different treatments (fixed effects) were analyzed with a repeated measures ANCOVA, using the 'aov'

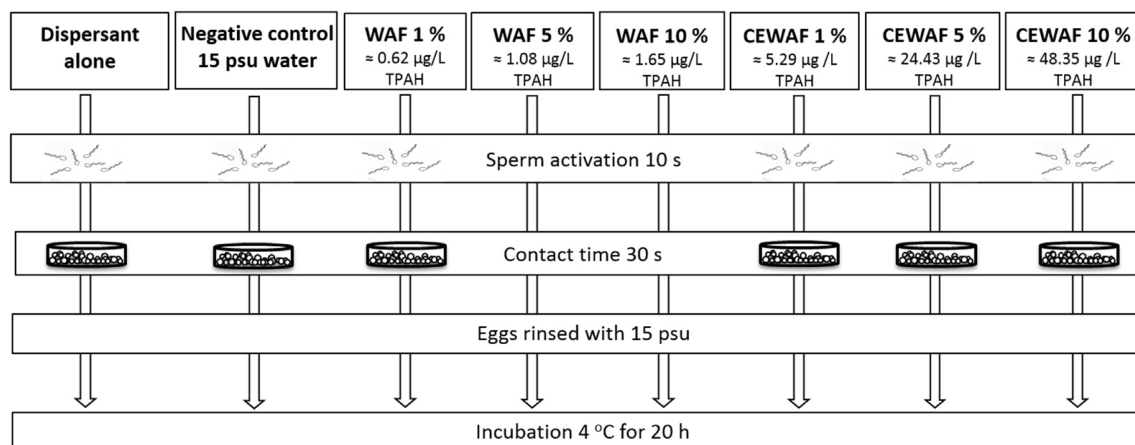

Fig. 1. Schematic representation of the fertilization trial. The top row represents the eight treatments with the expected TPAHs concentration. The dispersant alone treatment corresponds to the same proportion used in the CEWAF 10% solution (0.5 ml/L). The experiment was repeated four times using four pools of sperm and eggs from different fish.

function of the R stats package. Male was considered repeated (random effects) and time after sperm activation was considered as a covariant.

Differences in the percentage of fertilized eggs were analyzed with repeated measures ANOVA, using the 'aov' function, where pool was considered repeated (random effect) in each treatment (fixed effects). Post-hoc analyses were conducted for multiple comparisons with Tukey's Honestly Significant Difference (HSD) test.

The underlying assumptions of our parametric tests were checked with the 'Bartlett.test' function for homogeneity of variances whereas the different model's residuals' normal distribution was verified with histograms. In all cases, results were considered significantly different for $p < 0.05$.

3. Results

The dispersant had a profound effect on the composition of test solutions. The values presented in Tables 1 and 2 represent concentration values for the WAF and CEWAF stock solutions before conducting the dilutions to obtain the different treatments (1%, 5% and 10%). The TPH concentration increased more than 80 times in the CEWAF compared to WAF, 161×10^3 vs 1.85×10^3 μg/L TPH respectively (Table 1). The total concentration of PAHs (TPAH) dissolved in the solutions was about 40 times higher in the CEWAF compared to WAF, 479 vs 12.0 μg/L TPAH respectively (Table 2). The individual n-alkanes in the CEWAF solution were between 50 and 100 times higher than the WAF solution (Table 1). Not all n-alkanes presented in the CEWAF were detected in the WAF solution. Also in the case of the PAHs detected in the CEWAF solution, not all were present in the WAF or else they were below the detection limit, such as pyrene, which was only detected in the CEWAF stock solution, 6.05 μg/L (Table 2).

In the sperm behaviour experiment, none of the measured parameters (% motile, VCL or LIN) were affected by the different treatments (Fig. 2), even at the highest CEWAF concentrations of 10%, corresponding to ~16.1×10^3 μg/L TPH and ~48.6 μg/L TPAH ($F_{7, 83} = 1.946$, $p = 0.072$ for % motile; $F_{7, 83} = 1.201$, $p = 0.312$ for VCL and; $F_{7, 83} = 1.349$, $p = 0.238$ for LIN).

Nonetheless, there was a significant effect of the treatments on sperm fertilizing ability ($F_{7, 21} = 33.94$, $p < 0.001$). The percentage of fertilized eggs was significantly impaired when the sperm were activated in either the highest CEWAF concentration (~48.6 μg/L TPAH) or with the dispersant alone at a similar concentration (Fig. 3), but not by any of the tested WAF concentrations. The greatest effect was from dispersant alone, where fertilization rates were $19.5 \pm 10.3\%$, compared to $74.1 \pm 5.8\%$ for the negative control and $34.0 \pm 16.1\%$ for the highest CEWAF concentration.

Despite the low fertilization rates in the treatments with higher CEWAF concentrations and with dispersant alone, the eggs did not degrade (they were not exposed to contaminants), but rather had no cell division and were relatively transparent. This is in contrast to observations of eggs which degraded and became opaque when chronically exposed to higher concentrations of toxicants in a parallel experiment testing CEWAF toxicity in capelin embryo development (unpublished results).

4. Discussion

Unlike the long-lived sperm of marine invertebrates such as oysters, urchins and corals, in most teleosts with external fertilization sperm enter the egg within a few seconds after ejaculation and thus a sperm's direct exposure to any environmental contaminant is very short. However, sperm are very sensitive to some chemicals because they mediate natural- (Reinhardt et al., 2015) and sexual-selection (Evans and Garcia-Gonzalez, 2016) on

Table 1
Concentrations in μg/L of petroleum hydrocarbons (PH) in the negative control (15 psu water), water accomodated fraction (WAF), and chemically enhanced WAF (CEWAF) undiluted stocks, analyzed using GC – flame ionization detection, and used for the sperm exposure experiments after dilution (1%, 5% and 10%). TPH: Total petroleum hydrocarbons.

n-alkane	Negative control (μg/L)	WAF (μg/L)	CEWAF (μg/L)
C11			606
C12			1576
C13			1866
C14			2107
C15		2.4	2273
C16		13.8	1997
C17		23.3	1991
C18		23.4	1578
C19		24.1	1415
C20		24.7	1344
C21		22.8	1181
C22	0.7	21.9	1087
C23	2.0	21.5	1046
C24	2.9	21.0	957
C25	4.5	23.2	946
C26	3.8	20.0	850
C27	4.2	19.5	801
C28	4.4	16.3	659
C29	4.8	16.4	602
C30	4.8	14.5	491
C31	5.1	12.9	422
C32	5.2	11.3	327
C33		9.8	236
C34			199
Sum	**43**	**352**	**26 560**
Pristane	-	13.6	1226
Phytane	-	17.8	1272
TPH	43	1.85×10^3	161×10^3

Table 2
List of polycyclic aromatic hydrocarbon (PAH) concentrations in μg/L in the negative control (15 psu water), water accomodated fraction (WAF) and chemically enhanced WAF (CEWAF) undiluted stocks, and used for the sperm exposure experiments after dilution (1%, 5% and 10%).

PAHs[a]	Negative control (μg/L)	WAF (μg/L)	CEWAF (μg/L)
Naphthalene	ND	< DL[b]	1.05
C-1 Naphthalenes	ND	0.52	2.75
C-2 Naphthalenes	ND	0.54	10.3
C-3 Naphthalenes	ND	0.53	15.8
C-4 Naphthalenes	ND	0.48	6.04
Fluorene	ND	2.37	99.3
C-1 Fluorenes	ND	0.68	14.5
C-2 Fluorenes	ND	0.67	12.6
Phenanthrene[c]	0.50	4.64	185
C-1 Phenanthrenes	ND	0.87	33.9
C-2 Phenanthrenes	ND	0.70	25.0
C-3 Phenanthrenes	ND	ND	9.50
Pyrene	ND	ND	6.05
Chrysene	ND	ND	42.4
C-1 Chrysenes	ND	ND	4.30
Benzo(b)fluoranthene[d]	ND	ND	5.12
Dibenzo(a)anthracene[d]	ND	ND	2.30
Benzo(ghi)perylene[d]	ND	ND	2.68
Sum of all PAHs	**0.5**	**12.0**	**479**

[a] Detection limits are estimated to be 0.1 μg/L for the 15 psu and WAF and 0.5–1 μg/L for the CEWAF.
[b] Trace amounts present, but lower than the detection limit (DL).
[c] Phenanthrene was present in dihydrophenanthrene, which was one of the surrogate standards added to all the samples. This added a background level of approximately 0.5 μg/L to all the samples.
[d] For these compounds, peaks were present at the expected retention time in the selective ion monitoring trace, but their intensity in the scan trace was too low to allow for verification of identity by their mass spectrum.

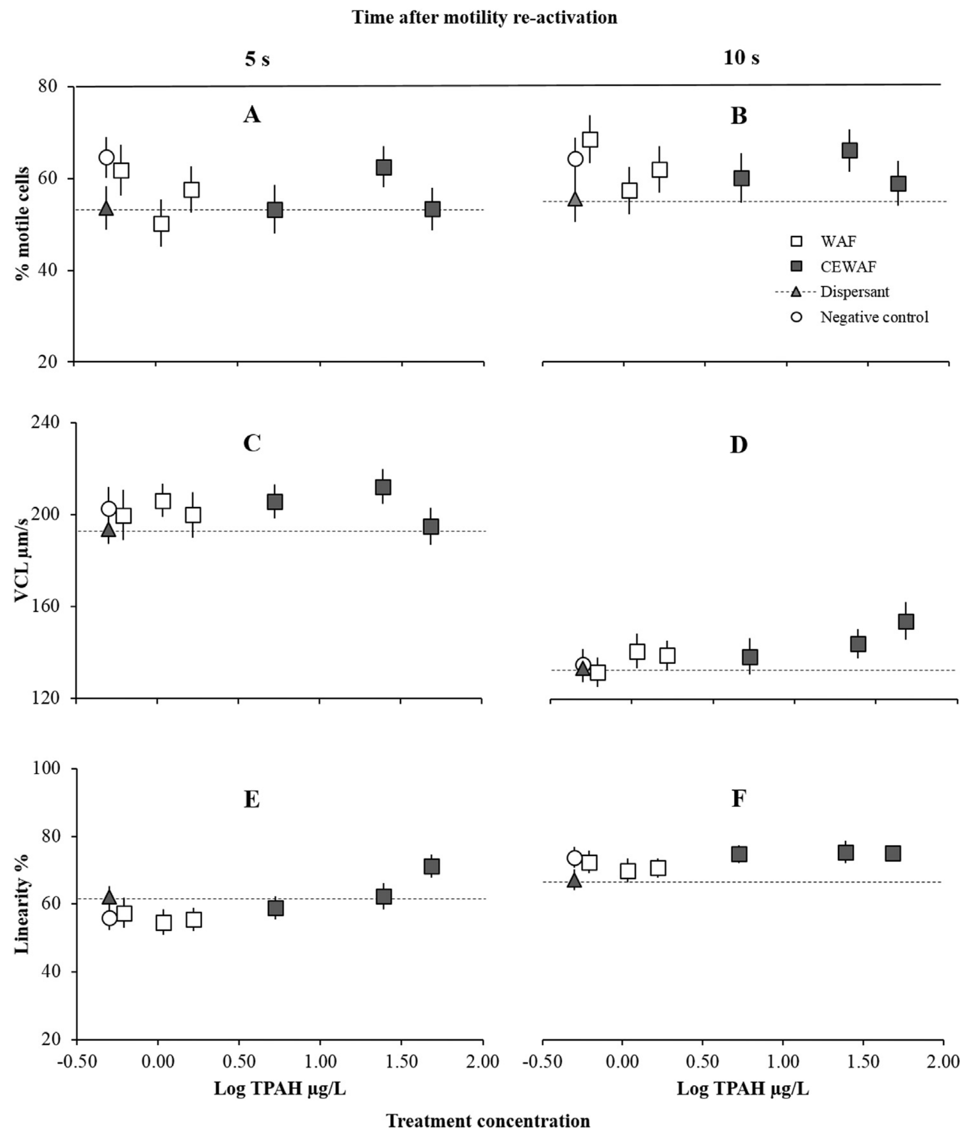

Fig. 2. Sperm behaviour: percentage of motile cells (A, B), curvilinear velocity (VCL) in µm/s (C, D), and linearity in percentage (E, F) for capelin sperm in 15 psu water (negative control), WAF (1%, 5%, 10%), CEWAF (1%, 5%, 10%) and dispersant Corexit® EC9500A alone at the same dispersant concentration as CEWAF 10%. The data in the independent axis is ploted according to the total polycyclic aromatic hydrocarbon (TPAH) content of each treatment. Dispersant alone treatment is represented by a dotted line placed horizontally. For easier vizualization the symbol for this treatment and the correspondent error bar appear in line with 15 psu water (negative control). Graphs on the left side (A, C and E) are for sperm 5 s after motility re-activation, while those on the right (B, D and F) are for 10 s. Error bars represent SEM among individual males (n = 13). No significant differences were observed among the different treatments for the different measured variables with the ANCOVA model used.

swimming performance that influences fertilization. Therefore, the potential fitness effect of direct contaminant exposure to sperm is great. Ours is the first study to evaluate how mechanically- or chemically-dispersed crude oil affects sperm swimming behaviour and fertilization ability in a teleost. The key finding from our work was that after only 10 s of exposure, chemically-dispersed crude oil acutely impairs fish sperm fertilizing ability. Although uncertainty exists in the underlying process, this seems to be more likely caused by direct dispersant effects rather than crude oil components.

The mechanism responsible for the lower fertilizing ability is not clear. There was no effect from WAF (oil alone). CEWAF contains dispersant and higher concentrations of oil compounds than WAF. Interestingly, the lowest fertilization rates were with dispersant alone rather than dispersed oil, contrary to that normally observed in fish embryos (e.g., Adams et al., 2014). We did not expect this and thus did not measure the exact amount of free dispersant in the different solutions, but it suggests there may be some direct effect of dispersant on an aspect of the fertilization process. The percentage of motile sperm cells and their swimming characteristics is correlated with fertilization success in teleosts (e.g., Beirão et al., 2011). However, we show that the lower fertilizing ability was not due to a reduction in swimming performance. As expected, there was a major decrease in the sperm velocity between 5 and 10 s in all treatments (Beirão et al., 2018), but there was no difference in sperm swimming across treatments.

We consider five other possible mechanisms to explain the reduced fertilization rate in CEWAF and dispersant alone treatments: the first is physical; where 1) the spermiotoxicity may have been facilitated by the presence of oil microdroplets caused by the WAF and CEWAF production techniques. However, the presence of these droplets is expected to be higher in the WAF solution, as the dispersant would have contributed to dissolve them. Indeed, Vignier et al. (2017) observed a reduction of the number of sperm available for fertilization in a treatment without dispersant, that

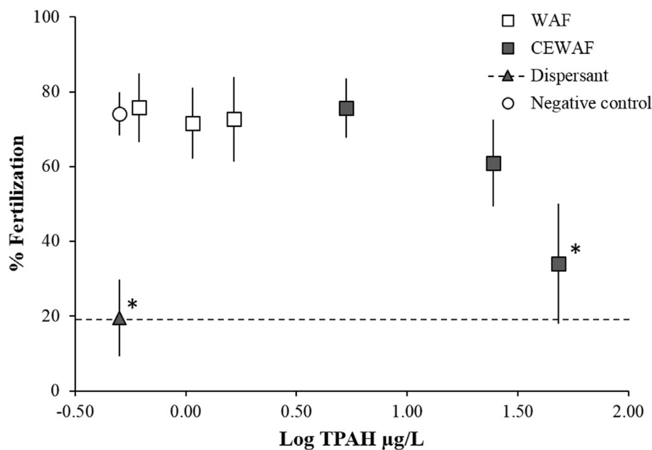

Fig. 3. Fertilization success: capelin sperm fertilization capacity in 15 psu water (negative control), WAF (1%, 5%, 10%), CEWAF (1%, 5%, 10%) and dispersant alone at the same concentration as CEWAF 10%. The data in the independent axis is ploted according to the total polycyclic aromatic hydrocarbon (TPAH) content of each treatment. Dispersant alone treatment is represented by a dotted line placed horizontally. For easier vizualization the symbol for this treatment and the correspondent error bar appear in line with the 15 psu water (negative control). Treatments with significantly lower fertilization are signed with a * ($p < 0.05$). Error bars represent SEM among pools ($n = 4$) of fish.

they attributed to the higher occurrence of oil droplets that aggregates sperm cells. However, this was not the case in our WAF treatments, thus, dissolved components and not oil droplets seem to be main cause for toxicity in our experimental set up.

Other potential mechanisms are physiological. 2) In an oyster, Volety et al. (2016) observed a decrease in fertilization after 30 min exposure of gametes to high concentrations of CEWAF and dispersant alone. Volety et al. (2016) found a reduction in the reactive oxygen species (ROS) production in sperm and attributed these modifications to a disruption of the mitochondria functionality, which can affect ATP synthesis. We did not measure ROS production nor the mitochondrial membrane potential, thus we cannot discard this effect. 3) Another potential route is by affecting other aspects of sperm metabolism. When Hamoutene et al. (2010) tested Atlantic cod sperm performance in produced water they observed subtle effects on sperm total proteins and levels of the enzyme citrate synthase (at 0.034 µg/L PAH), even though Atlantic cod sperm fertilizing ability was not affected. Whereas the concentrations tested by Hamoutene et al. (2010) were realistic for contamination resulting from produced water, in the event of an oil spill and treatment with dispersants the contamination by petroleum hydrocarbons would be in the order of 1000 times higher and so these effects can also be much higher and affect fertilization ability. 4) Although less likely due to the short (10 s) exposure time, environmental contaminants could cause modifications or damage to sperm DNA/RNA (reviewed by Hatef et al., 2013). In adult sheepshead minnows (*Cyprinodon variegatus*) 7 days exposition to CEWAF and dispersant alone caused gene expression modifications (Jones et al., 2017), whereas, in humans, both Hsu et al. (2006) and Gaspari et al. (2003) observed that long-term exposure to PAHs affects sperm function by damage to DNA.

Lastly, because of the mode of action of dispersants we hypothesize 5) that the decrease in fertilizing ability could be at least partially be caused by toxic effects on the sperm head membrane. Dispersants contain both solvents, such as propylene glycol and petroleum distillates, and non-ionic and anionic surfactants. Some concern exist that the use of dispersants contribute to the insertion of hydrocarbons in the ecosystem, but these ones have relatively low toxicity compared with PAHs. Therefore most of the dispersants' toxicity is attributed to the surfactants, namely the anionic surfactant bis (2-ethylhexyl) sodium sulfosuccinate (DOSS) that degrades at a very slow rate compared with the non-ionic components (John et al., 2016). Testing in cell lines, Dasgupta and McElroy (2017) have evidenced DOSS to be responsible for most of the Corexit® EC9500A cytotoxicity. In recent work, Dasgupta et al. (2018) observed that DOSS altered the ROS production and increased lipid peroxidation, although most of this toxic effect seem to be prevented by the remaining Corexit components. In our work we followed the method used by Martin et al. (2014) that has been adopted by other authors (e.g., Madison et al., 2015) and mixed the dispersant with the oil for an hour, after the initial 18 h of stirring. Another approach is to add the dispersant immediately after the oil vortex is established (see the review by Adams et al., 2017). During the CEWAF preparation we were careful to only add the dispersant in the center of the vortex on top of the oil, however the short mixing time of 1 h, could decrease the oil chemical dispersion and increase the amount of Corexit components free in the solution. In this context, Corexit® EC9500A can directly (and instantly) reduce sperm membrane lipid functionality, and its capacity to fuse with the egg plasma membrane. This may or may not be different for species with sperm acrosomes (e.g., oysters) versus those without (e.g., teleost fishes). These mechanisms would help explain why the greatest effects were observed in the treatment with dispersant (free in water), and not with chemically dispersed oil (same amount of dispersant used, but not all would be freely available). However, the exact mechanism by which the CEWAF and dispersant alone impaire sperm fertilizing ability deserves further attention.

Our study purposely isolated direct contaminant exposure to sperm. In the real world this would be combined with indirect exposure of the adult male, which can lead to cumulative effects. In polar cod, Bender et al. (2016) observed some disruption of sperm motility parameters in fish fed with diets mixed with crude oil. In capelin, Khan and Payne (2005) showed that concentrations of TPH lower than the ones we tested with our CEWAF treatments killed more than 50% of mature capelin within 4 days of exposure. Our highest treatments of TPH (~16.1×10^3 µg/L) and TPAH (~48.6 µg/L) may therefore not be relevant for direct sperm exposure (they had no effect on sperm swimming behaviour, despite being higher than fish survive), but much higher concentrations have been observed after spill events. For example, during the BP Deepwater Horizon oil spill, where the Corexit® EC9500A dispersant was used at unprecedented levels, Sammarco et al. (2013) reported values as high as 11.4×10^6 µg/L of TPH and 1.23×10^3 µg/L of PAH.

Our experiments were conducted with 15 psu, since higher salinities in beach-spawning capelin were previously shown to negatively affect sperm motility (Beirão et al., 2018). Even though the solubility of PAHs is lower at higher salinities and thus the toxic effects of WAF over sperm should be lower in full strength seawater (Shukla et al., 2007), the dispersant's effectiveness is the opposite: normally increasing with salinity (Chandrasekar et al., 2006). Thus, the effects observed in this study, which used 15 psu, would likely be higher for offshore spawning capelin and other species that spawn in full strength sea water (30–35 psu). More studies testing different variables will certainly improve our understanding of the potential impact of crude oil pollution and the use of dispersants on fish sperm performance. Nonetheless, our study clearly indicates that an oil spill event and the use of dispersants during the capelin spawning season can affect fertilization success, and this could have serious consequences on recruitment of the primary forage fish in the northwest Atlantic food web.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.05.080.

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