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4 **RUNNING HEAD:** Maternal PAH transfer and effect in copepods

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Maternal PAH transfer and effects on offspring of copepods exposed to dispersed oil with and without oil droplets

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Abstract

Copepods of the genus *Calanus* have the potential for accumulating lipophilic oil components due to their high lipid content, and they have been shown to filter and ingest oil droplets during exposure. As female copepods produce eggs at the expense of their lipid storage, there is a concern for transfer of lipophilic contaminants to offspring. To assess the potential for maternal transfer of oil components, we exposed ovigerous female copepods (*Calanus finmarchicus*) to filtered and unfiltered oil dispersions for 4 days, collected and kept their eggs in clean seawater and studied hatching and gene expression in hatched nauplii. Oil droplet exposure contributed to PAH uptake in dispersion-treated adult copepods, as displayed through PAH body residue analyses and fluorescence microscopy. Applying the latter methodology, transfer of heavy PAH from copepod mothers to offspring was

strongly supported. Subtle effects were observed in offspring with a temporal reduction in hatching success, which appears to be occurring only when mothers were exposed to the unfiltered oil dispersions. Offspring were reared in clean water through to late naupliar stages, collected for RNA extraction and preparation of libraries for high-throughput transcriptome sequencing. Differentially expressed genes were identified through pairwise comparisons between treatments. Among these, several have known roles in responses to chemical stress including xenobiotic metabolism enzymes, antioxidants, chaperones and components of the inflammatory response. While the gene expression results suggest a transgenerational activation of stress responses, the relatively small number of differentially expressed genes suggest a mild long-term effect on offspring following maternal exposure.

Key words: Copepod; dispersion; micro-droplets; maternal transfer; ecotoxicity; oil; petroleum; Arctic

1. Introduction

When oil is released into the marine environment it may disperse into the water, i.e. break up into small oil droplets. This process occurs when surface slicks experience turbulence, such as through breaking waves and/or the use of chemical dispersants, which facilitates the oil dispersion process by reducing the interfacial tension between oil and water. During the Deepwater Horizon incident in 2010 in the Gulf of Mexico, oil was released at the sea floor and dispersed naturally due to the turbulence caused by high pressure and concomitant release of gas. Chemical dispersants were also added during the release to facilitate the process and cause even smaller droplets to form and avoid oil surfacing and impacting shorelines. Also regular discharges of produced water from offshore oil production platforms release dispersed oil droplets, and these are usually regulated and monitored. On the

70 Norwegian continental shelf, the regulations allow dispersed oil concentrations in raw effluents of

71 produced water of up to 30 mg/L.

72 Hence, pelagic organisms may be exposed to dispersed oil during both regular as well as accidental

73 discharges of oil. Due to their higher bioavailability, the dissolved components are expected to be the

74 main driver for toxicity. However, the majority of the mass of the oil is contained in the particulate

75 phase of the dispersions, i.e. the oil droplets. Several studies, focusing mainly on fish eggs (Carls et al.,

76 2008) and fish larvae (Nordtug et al., 2011b; Olsvik et al., 2011; Olsvik et al., 2010), have concluded

77 that oil droplets are not the main driver for toxicity. These assumptions have been challenged recently

78 as fish eggs from some species like Atlantic haddock have displayed a high susceptibility to oil droplet

79 adhesion (Sørhus et al., 2015). In addition, filter-feeding organisms, which filter large volumes of water

80 to acquire food, may be exposed to oil droplets through direct filtration and ingestion (Hansen et al.,

81 2012; Nordtug et al., 2015; Almeda et al., 2014; Almeda et al., 2015; Conover, 1971). Recent studies

82 have shown that exposure to oil dispersions cause up-regulation of stress-associated genes (Hansen et

83 al., 2009), decreased survival (Hansen et al., 2012), reduced food uptake (Hansen et al., 2012) and

84 altered reproduction (Hansen et al., 2015; Olsen et al., 2013) in copepods, however, these publications

85 provide limited insight into the potential contribution of oil droplets to these effects.

86 Sub-Arctic and Arctic copepods from the *Calanus* genus produce and store a large reservoir of lipids,

87 mainly as wax esters, during their copepodite stages serving as an energy reserve for use during

88 overwintering (diapause) and reproduction as adults (Miller et al., 1998; Madsen et al., 2008). Owing

89 to their high lipid content, these copepods have the potential to accumulate large amounts of lipophilic

90 organic toxicants like polycyclic aromatic hydrocarbons (PAHs), as low elimination rates are expected

91 (Hansen et al., 2016). In addition to the passive uptake of dissolved oil components, ingestion of oil

92 droplets may potentially increase the uptake of PAHs further increasing the accumulation in the lipid

93 reservoir. At the expense of the lipid reservoir, eggs and spermatophores are produced in female and

94 male copepods, respectively, during the reproduction period. Hence, theoretically any PAHs

95 accumulated in the lipid reservoir may be transferred into offspring. To the authors' knowledge,
96 transfer of PAHs from parent to offspring has never been studied in copepods before.

97 In the present work, we wanted to investigate the potential for PAH transfer from ovigerous female
98 copepods to their offspring and the potential delayed effects this may have on the offspring during
99 development. We also wanted to isolate and assess the potential contribution of oil droplets to uptake
100 and effects. In order to do this, we exposed ovigerous female copepods to an oil dispersion
101 concentration (2 mg oil/L nominally) that previously has been shown to affect reproductive success,
102 and a filtered dispersion, containing only the dissolved oil components, i.e. water soluble fraction
103 (WSF), of the dispersion. After a 4-day exposure to these treatments, the adult copepods were
104 transferred to clean seawater for egg production. The offspring were monitored for short-term
105 hatching success as well as potential delayed effects by monitoring the differential gene transcription
106 between negative controls (treated with seawater), oil dispersion and WSF. The main aims of the
107 current work were to assess if i) uptake of PAHs was higher in adult copepods when treated with oil
108 dispersions than the corresponding WSF, ii) oil components were transferred between adults and
109 offspring, and finally iii) if potential impacts (based on gene expression) were more severe on offspring
110 from dispersion-treated copepods than WSF-treated copepods.

111

112

113 2. Materials and Methods

114 2.1. Experimental organisms

115 Copepods (*Calanus finmarchicus*) from the continuous lab culture at SINTEF/NTNU Sealab were used
116 for the experiments described here. The culture is routinely maintained at approximately 8-10°C, and
117 details regarding culturing were described previously (Hansen et al., 2007).

118

119 **2.2. Choice of oil**

120 A naphthenic crude oil from the Troll reservoir in the northern part of the North Sea was selected as
121 the representative oil for the studies. The oil was artificially weathered by heating to 200°C
122 (Stiver&Mackay, 1984), and the resulting oil residue, which corresponds to approximately 0.5–1 days
123 of weathering on the sea surface, was collected and used to generate the oil dispersions and filtered
124 oil dispersion (water-soluble fractions (WSF)) (Nordtug et al., 2011a).

125

126 **2.3. Exposure and recovery experiment**

127 An overview of the whole experiment is given in **Error! Reference source not found.**. Ovigerous female
128 copepods (250 individuals in each replicate) were exposed in 5L bottles continuously for 96 h to
129 dispersion or filtered dispersions (WSFs) at 9 ± 1°C. The dispersion had a nominal concentration of 2
130 mg oil/L, and the WSF was a filtered dispersion removing the oil droplets but keeping the water-soluble
131 components (Nordtug et al., 2011a). Females kept in clean seawater were used as negative controls.
132 All three treatments were done in triplicates and had a flow of approximately 15 ml/min. The copepods
133 were continuously fed the unicellular algae *Rhodomonas baltica* at a level of 400 µg C L^{-1} by tubing that
134 pumped the algal culture from a common reservoir. The algae in the reservoir was renewed on a daily
135 basis supplying a fresh stock. After exposure, 200 copepods were transferred to clean seawater in 50L
136 tanks with flow-through seawater (temperature 10 ± 0.5°C) for recovery. During recovery the feeding
137 was maintained as for the exposure period, but due to lower copepod density, food concentration was
138 reduced to 150 µg C L^{-1}. The remaining copepods were either sampled for body burden analyses (25
139 individuals) or photographed for biometry (23-25 specimens per replicate). 7 days after exposure, eggs
140 were harvested from recovery tanks from all treatments/replicates and transferred to PET bottles (1.5
141 L) for hatching and maintenance with food (150 µg C L^{-1}). Nauplii (N5-N6) were sampled 13 days later
142 using a filter (mesh 63 µm) and transferred immediately into RNAlater (Ambion). The samples were

143 flash-frozen in liquid nitrogen and kept at -20°C or colder until they were thawed, extracted and
144 analyzed for gene expression.

145

146 **2.4. Hatching success**

147 Two days after transfer of females to egg laying chambers, a subset of eggs harvested from each tank
148 was transferred to glass vials (40 ml) and kept in darkness at 8°C for hatching. They were terminated
149 after 48, 72 and 96 hours by adding 6 droplets of Lugol's solution to the vial. A Leica MZ125 dissecting
150 microscope (Leica Microsystems, Wetzlar, Germany) was used to image eggs and nauplii, and these
151 images were analyzed using automated classifications using ImageJ (National Institute of Health,
152 Bethesda MD) to quantify the number of eggs and nauplii in each sample. These data formed the basis
153 for determination of time- and exposure-dependent hatching success.

154

155 **2.5. Chemical analyses of water samples**

156 After 24 and 72 h of exposure water samples were taken in 1L glass bottles for analysis of semi-volatile
157 organic components (SVOC; 800 mL) and in sealed glass vials without headspace for analysis of volatile
158 organic components (VOC; 40 mL). All water samples were acidified at sampling.

159 Semi-volatile organic components (SVOC) and volatile organic components (VOC) samples were
160 preserved by acidification with 15% hydrochloric acid. Prior to processing, surrogate internal standards
161 (SIS, *o*-terphenyl, naphthalene-d_8, phenanthrene-d_{10}, chrysene-d_{12}, phenol-d_6, 4-methylphenol-d_8)
162 were added to the water samples. Prior to gas chromatography/flame ionization detection (GC/FID)
163 and gas chromatography/mass spectrometry (GC/MS), analysis recovery internal standards (RIS, 5α-
164 androstane, fluorene-d_{10}, and acenaphthene-d_{10}) were added. Prior to SVOC and total petroleum
165 hydrocarbon (TPH) measurements, water samples were spiked with the appropriate surrogate internal
166 standards and serially extracted with dichloromethane (DCM) using a modification of US EPA method

167 3510C (USEPA, 1996). The combined extracts were dried over sodium sulphate and concentrated to
168 approximately 1 mL using a Zymark Turbovap® 500 Concentrator. The final extract was spiked with
169 the appropriate recovery internal standards and analyzed.

170 Water samples were analyzed for SVOC content (decalins, polycyclic aromatic hydrocarbons (PAHs)
171 and phenols) using GC/MS with a modification of US EPA Method 8270D (USEPA, 2007). The mass
172 spectrometer was operated in the selective ion monitoring (SIM) mode to achieve optimum sensitivity
173 and specificity. The quantification of target compounds was performed by the method of internal
174 standards, using average response factors (RF) for the parent compounds. The PAH and phenol alkyl
175 homologues were quantified using the straight baseline integration of each level of alkylation and the
176 RF for the respective parent PAH compound. The response factors were generated for all targets and
177 surrogates versus fluorene-d_{10}. As an objective way of excluding samples corrupted by the extraction
178 process, all values deviating more than 1.3 x STDEV from the average value were excluded as outliers
179 (corresponding to 80 % or more chance of the values being outliers). The majority of excluded values
180 were from defined samples where irregularities such as excessive evaporation of solvent or low
181 recovery of standards could be identified.

182 Concentrations of TPH in water samples were measured using GC/FID according to a modification of
183 US EPA Method 8100 (USEPA, 1986). Resolved and unresolved TPH (C_{10}-C_{36}) were quantified by the
184 method of internal standards using the baseline corrected total area of the chromatogram and the
185 average response factor for the individual C_{10} to C_{36} n-alkanes. Water levels of 35 target VOC (C_5-C_{10})
186 including BTEX (benzene, toluene, ethylbenzene, and xylenes) were determined by Purge and Trap
187 GC/MS (P&T GC/MS) using a modification of US EPA method 8260C (USEPA, 2006) after spiking the
188 samples with SIS (toluene-d_8 and ethylbenzene-d_8) and RIS (chlorobenzene-d_5). Quantification of
189 individual compounds was accomplished using the RFs of the individual compounds relative to the
190 internal standards. All standards and samples were analyzed in a full scan mode. Total hydrocarbon

191 content (THC, C_5-C_{36}) of water samples was calculated by adding VOC (C_5-C_9) and TPH (C_{10}-C_{36})
192 concentrations.

193

194 **2.6. PAH body burden analyses**

195 After exposure, all copepods were counted, assessed for viability and dead individuals removed. Live
196 copepods (25 individuals) were pooled and sampled from each exposure group and frozen in liquid
197 nitrogen for body burden analysis. Surrogate internal standards (SIS; naphthalene-d8, phenanthrene-
198 d10, chrysene-d12, phenol-d6, and 4-methylphenol-d8) were added to the samples, and the samples
199 were then processed using a microextraction procedure. Recovery internal standards (RIS; fluorene-
200 d10 and acenaphthene-d10) were added prior to analysis. The copepods were weighed into conical,
201 screw-capped sample vials (10 mL) with replaceable Teflon septa, and 3 mL of potassium hydroxide
202 (6.5%) in methanol (80%) and SIS were added to each vial. The mixture was treated for 2 hours in an
203 ultrasonic bath at 80 °C to achieve saponification, followed by filtration and serial extraction with 4 mL
204 of Milli-Q water/2 × 3 mL of hexane/4 mL of Milli-Q water and 0.5 mL of saturated NaCl. The combined
205 organic extracts were dried with sodium sulfate and concentrated to approximately 0.5 mL using a
206 Zymark Turbovap 500 Concentrator. Cleanup of the extracts was performed by solid-phase extraction
207 using 3 mL columns containing 0.5 g of normal-phase silica packing (Superclean LCSi, Supelco Bond
208 Elut, SI, Agilent). The samples were eluted through the column with 3 × 2 mL of DCM:hexane (1:3). The
209 purified extracts were concentrated to 90 µL in an insert GC vial, spiked with RIS components (to a
210 total volume of 100 µL) and analyzed on GC-MS as described above for the water samples.

211

212 **2.7. Sampling and biometry of females**

213 Copepod survival was determined at the end of the 96 hour exposure period, and a sample of surviving
214 copepods (23-25 individuals from each chamber) was photographed during observation in a Leica

215 MZ125 dissecting microscope (Leica Microsystems, Wetzlar, Germany). Pictures were captured with a
216 digital still-video camera (Sony DWF-sx900, Sony Corporation, Tokyo, Japan) operated by Fire-i
217 software (Unibrain, Inc., San Ramon CA, US). Measurements of biometry were performed manually by
218 the use of the software ImageJ (National Institute of Health, Bethesda MD, US) on scaled captured
219 images. 2D projected areas of the lipid storage and the prosome were measured using a graphical
220 tablet (Wacom Cintiq 12wx, Wacom Co., Ltd., Saitama, Japan).

221

222 **2.8. Fluorescence microscopy**

223 Copepods (N=6) from each treatment as well as eggs from females from each treatment group were
224 subjected to fluorescence microscope imaging as described in Hansen et al. (Hansen et al., 2012)
225 (2012). To achieve simultaneous identification of oil droplets either ingested or adhered to the
226 copepod surface, ingested algae as well as accumulated fluoranthene, the reported B-2A filter cube
227 (Nikon Corp., Tokyo, Japan) was replaced with a triband filter cube (D/F/Tr, Nikon Corp., Tokyo, Japan)
228 producing a better resolution of the fluorescence-signatures of interest.

229

230 **2.9. Transcriptional profiling**

231 Eggs from exposed females were collected over the following 3-6 days in recovery after the exposure
232 period, hatched and reared in clean seawater through to the late naupliar stages (N5-6) before they
233 were harvested and stored in RNAlater (Invitrogen) at -20°C. For RNA extraction, nauplii were
234 recovered onto filter paper (Whatman Qualitative Grade 2) using a vacuum pump. The collected nauplii
235 were then rinsed into a glass Potter Elvehjem tissue grinder with 1 ml of Purezol Reagent (Bio-Rad) and
236 homogenized using a PFTE pestle. Total RNA was extracted from the homogenate using the Aurum
237 Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad). RNA samples were sent to the Genomic Services
238 Laboratory at HudsonAlpha (Huntsville AL, US), where RNA quality was evaluated using a Bioanalyzer
239 and libraries were synthesized for high-throughput sequencing using Illumina TruSeq Reagents. Three

240 replicate libraries were prepared from each treatment. For the control and dispersion treatments
241 these were derived from independently-reared batches of nauplii. For the WSF treatment, one group
242 of nauplii was lost due to a technical error during the grow-out phase; therefore, the two of the
243 libraries were synthesized from subsamples of the same RNA sample and serve as technical replicates.
244 The libraries were sequenced as 100 basepair paired-end reads across two lanes of an Illumina
245 HiSeq2000. All raw sequences are archived within the NCBI database as BioProject PRJNA358233.

246 Adapter sequences and low-quality portions of reads (< phred 20) were trimmed using Trimmomatic
247 (Lohse et al., 2012). In exploratory data analysis, reads were assembled into a transcriptome *de novo*
248 using Trinity 2.0.3 with default parameters (Grabherr et al., 2011). Differential gene expression
249 analysis was conducted using scripts bundled with Trinity. Sequences were mapped to the new
250 transcriptome as well as two previously published transcriptomes (Lenz et al., 2014; Tarrant et al.,
251 2014) using RSEM (Li&Dewey, 2011). The highest mapping success was obtained by using a published
252 transcriptome derived from multiple copepod life stages (Lenz et al., 2014), so these results were used
253 in subsequent analyses, except where noted. Differentially expressed genes were identified using
254 EdgeR software (Robinson et al., 2010) and annotated through Blastx-based queries of the Swiss-Prot
255 database. The distribution of gene ontology (GO) terms in differentially expressed genes was assessed
256 using WEGO (Ye et al., 2006). Within WEGO, Pearson Chi-Square tests were conducted to compare GO
257 terms specifically associated with a particular oil exposure (dispersion vs. WSF) or direction of
258 regulation (up-regulated vs. down-regulated within a treatment).

259

260 3. Results and Discussion

261

262 3.1. Exposure concentrations – description, partitioning and relevance

263 Based on the chemical analyses of water samples, the experimental conditions appear to have caused
264 PAH loading as anticipated (

265). The concentrations of volatile organic components (VOC) were comparable between dispersions and
266 WSFs reflecting their solubility in water, i.e. they were not removed by filtration of the dispersion to
267 generate the WSF. The PAH concentrations were higher in the dispersion compared to the
268 corresponding WSF due to the presence of oil droplets in the dispersions. The filtration of dispersion
269 caused a decrease in PAH concentration in WSFs depending on the water solubility of the PAH, i.e. the
270 fraction of heavier 4-6-ring PAHs associated with droplets were higher than for the lighter
271 naphthalenes. The naphthalenes were found at comparable concentrations in the dispersions and
272 corresponding WSFs displaying their higher water solubility. In terms of environmental relevance, the
273 T-PAH concentrations in the experiment (approximately 20 µg/L) are in the high range of the
274 concentrations observed in the field following oil spills. The same applies for the total oil
275 concentrations (approx. 1 mg oil/L) (Li et al., 2011). After the Deepwater Horizon incident in the Gulf
276 of Mexico in 2010, most water samples taken displayed T-PAH concentrations below 10 µg/L (Sandoval
277 et al., 2017). Keeping a steady exposure concentration over 4-days is also not environmentally
278 relevant, however, our design was chosen primarily to isolate the contribution of oil droplets to chosen
279 effect endpoints by comparing observed effects after treatments with and without oil droplets present
280 (Nordtug et al., 2011a; Nordtug et al., 2011b).

281

282 **3.2. Uptake of polycyclic aromatic hydrocarbons in adult copepods**

283 The exposure was well below the LC_{50} recorded for *C. finmarchicus* for the current oil and weathering
284 (Hansen et al., 2012), and very low mortality was observed in the experiment with only three dead (of
285 250) in dispersion, one dead in WSF and none in the negative controls. The concentrations of PAHs
286 associated with the copepods clearly was higher in animals exposed to dispersion than those exposed
287 to the corresponding WSF (Fig. 2). The difference between the two exposures is related to the size of
288 the PAHs with differences increasing from approximately 20% in naphthalenes to 80 % in 4-6 ring PAHs.
289 Previous studies have shown that *C. finmarchicus* may accumulate oil micro-droplets through active

290 filtration of and through adhesion onto carapace surface (Nordtug et al., 2015). Thus, it is expected
291 that PAHs contained in oil associated with the copepods contribute to the measured body residue
292 copepods exposed to dispersions.

293

294 In Figure 3 the fraction between body residue (Cb) and water concentration (Cw) of individual PAHs
295 represented by their octanol water partitioning coefficient (Kow). The Figure show that below
296 approximately Log Kow = 6, the fraction between water and tissue is similar for the two exposures.
297 Based on the assumption that octanol is a suitable proxy for lipids in organisms the bioconcentration
298 factor (BCF) for exposure to dissolved lipophilic chemicals is generally assumed to be related to the
299 octanol water partitioning coeffiecient (Kow). Thus, the linear relationship observed between the bio-
300 concentration and Kow of the dissolved components in the WSF is expected (Figure 3). Components
301 with Log Kow > 6 were below the analytical detection limit for the animals exposed to WSF. In the
302 individuals exposed to oil dispersions, components with Log Kow up to above 7 could be detected, and
303 the relation with Kow was best fitted by a bilinear model (Kubinyi, 1977) where the initial part up to
304 curve is similar to the WSF exposure. This indicate that these components are largely dissolved in the
305 water phase of the dispersion. The fall-off from a linear correlation at higher Kow values is consistent
306 with studies on fish and several other aquatic organisms (Chiou, 1985; Banerjee&Baughman, 1991)
307 exposed to dissolved components. However, in the current experiment the components with high Log
308 Kow (> 6) could also be contained in oil droplets in the digestive system and on the surface of the
309 copepods. If droplets were sole source of components above log Kow = 6, the bioaccumulation for
310 these components should be equal and represented by a straight horizontal line (slope = 0) in Figure
311 3. The fact that the slope is negative above log Kow = 5.5 strongly indicates that a portion of the larger
312 PAHs is also accumulated in the tissue of the animals.

313

314 **3.3. Effects of exposure on adult female copepods**

315 Biometric analyses of female copepods after exposure revealed that there were no differences in
316 prosome length and projected 2D lipid sac area on the images, however, the area of the lipid sac was
317 significantly lower in copepods treated with oil dispersions compared to control ($p<0.0001$) and WSF-
318 treated copepods ($p<0.001$) (Supplemental 1 Figure S1). The underlying reason for this is unknown.
319 Exposure to oil dispersions may be more costly to recover from, and reduced lipid content may be a
320 function of increased demand for energy to recover and uphold maintenance during exposure. It is
321 unknown whether a relatively short (4-day) exposure could cause a measurable decrease in lipid sac
322 size. These copepods are adapted to withstand long periods of starvation during diapause (Hirche,
323 1996), but diapause is associated with a substantial metabolic depression. While the metabolic rate of
324 copepods within the experiment was not known, exposure to oil dispersions could have resulted in a
325 sustained or elevated metabolic rate, and/or a complete stop in filtration and a subsequent depletion
326 of lipid reserves. Lower mortality of lipid-poor copepods than lipid-rich copepods is also unlikely as
327 previous studies have shown the opposite; that lipid-poor copepods are more susceptible to oil
328 exposure than lipid-rich copepods (Hansen et al., 2011; Hansen et al., 2016; Hansen et al., 2013).
329 Whatever the mechanistic basis for the dispersion-induced low lipid content is, it may be expected
330 that low lipid content in adult females will result in reduced fecundity and/or reduced quality of
331 offspring.

332

333 **3.4. PAH transfer between adults and offspring**

334 Due to the small biomass of copepod eggs, we did not attempt to measure PAH concentrations in eggs
335 of reproducing females after exposure. Instead, fluorescence microscopy was used to visualize PAHs
336 (tuned to fluoranthene) in both eggs and adult females. In adult females, when comparing a negative
337 control (**Error! Reference source not found.**A) against a dispersion-treated copepod (**Error! Reference
338 source not found.**B), a clear blue area is apparent in the exposed specimen. This represents the lipid
339 sac of the copepod, and strongly suggests lipid-accumulation of PAHs. The dispersion-treated copepod

340 also displays a greenish stain in the filtration apparatus, which is produced by oil droplets stuck there.
341 In addition, a red-orange color is evident for both treatments representing the digestive system with
342 partly degraded algae (Hansen et al., accepted). The eggs from the negative control, WSF- and
343 dispersion-exposed copepods are displayed in **Error! Reference source not found.**C, D and E,
344 respectively. The eggs from females exposed with oil dispersion are blue in color (E) compared to both
345 the controls (C) and the WSF-treated eggs (D), suggesting accumulation of heavy PAHs. Although this
346 is not a quantitative measure of PAH content in eggs, it strongly indicates that maternal transfer of
347 PAHs to eggs occurs, and that this process is more pronounced in copepods exposed to oil droplets. To
348 the authors' knowledge, PAH transfer has not been reported previously between mother and offspring
349 in copepods. In vertebrates, transfer of PAHs between parent and offspring has been shown. For
350 example, maternal PAH exposure has been shown to affect endocrine function and behavioral traits in
351 both F1 and F2 generations in zebrafish (*Danio rerio*) (Vignet et al., 2015). Other organic contaminants
352 have been shown to transfer between crustacean parents and offspring. Notably, in the cladoceran
353 *Daphnia magna*, mercury (ionic as well as methyl-mercury) is transferred between mothers and
354 neonates. In fact, as much as 41% of accumulated methyl-mercury in adult daphnids was transferred
355 to their neonates (Tsui&Wang, 2004).

356

357 **3.5. Implications of PAH-transfer on hatching success and development**

358 After transfer to clean seawater for recovery the female copepods started producing eggs. Hatching
359 success was monitored in eggs collected after 48 hours of recovery, and was monitored at 48, 72 and
360 96 hours of incubation of the test. (**Error! Reference source not found.**). Significantly, lower hatching
361 was observed in the eggs after 48 and 72 hours in eggs from the females exposed to dispersion
362 compared to the negative control, whereas no significant differences between treatments were
363 observed after 96 hours of incubation. No significant differences in hatching were observed between
364 the groups exposed for dispersion or WSF, or between the negative control and the WSF-exposure.

365 Thus, if maternal exposure to oil droplets has an effect on the hatching success of the offspring, the
366 effect is subtle. Several studies have reported reduced hatching rates or hatching success of copepods
367 following oil exposure. Han et al. (2014) reported reduced hatching rate in response to a 10-day WSF
368 exposure in the harpacticoid copepod *Tigriopus japonicus*. Jensen & Carroll (2010) found reduced
369 hatching success in *Calanus glacialis* females treated with WSF (10.4 µg 16 EPA PAH/L), which was
370 argued by the authors to have resulted from passive uptake by the eggs or transfer from the mothers.
371 They did not observe differences in the cumulative egg production. For *C. finmarchicus* lower hatching
372 success has also been shown after female exposure to pyrene, however, interestingly direct exposure
373 of eggs to pyrene did not cause reduced hatching success (Jensen et al., 2008), suggesting the
374 importance of maternal exposure and transfer on this endpoint.

375 Delay in initiation of reproduction after treatment with dispersed oil (5.5 mg oil/L) was observed in *C.*
376 *finmarchicus* in a previous study (Hansen et al., 2015), but the copepods were able to fully recover and
377 produce offspring at the rate of (and even above) untreated copepods over a period of 25 days.
378 Similarly, in a study by Olsen et al., (2013), *C. finmarchicus* were exposed for 120 hours to
379 concentrations ranging 0.02 to 16.5 mg oil/L followed by recovery in clean seawater. 13 days post
380 exposure, no significant differences were observed in egg production rates or hatching success,
381 however, a significantly lower portion of the surviving females from the highest exposure participated
382 in the egg production.

383

384 **3.6. Maternal exposure causes altered nauplii gene expression**

385 Differentially expressed genes were identified through pairwise comparisons between treatments
386 (**Error! Reference source not found.**). Using a significance cut-off of 4-fold change in expression and
387 1% false discovery rate, expression of 173 genes was altered in response to the dispersion, 184 in
388 response to the WSF, and 35 genes were affected by both treatments. In each group, a larger number
389 of genes was downregulated following exposure compared to the number of genes upregulated.

390 Approximately 2/3 of the differentially identified genes could be annotated according to their similarity
391 to known genes in the SwissProt database. The complete list of differentially expressed genes is
392 contained in Supplemental File 2.

393 Overall, the differentially expressed genes were associated with a total of 339 level 2 and level 3 GO
394 terms. Given the relatively small number of differentially expressed genes, many terms were not
395 sufficiently represented to enable statistical comparisons. Of the categories that could be compared,
396 several GO terms were enriched in one of the two exposure treatments or according to the direction
397 of regulation within an exposure (Table 2). For example, following exposure to the dispersion, several
398 GO terms associated with cellular metabolism were more frequently associated with up-regulated
399 genes than with down-regulated genes (e.g., cellular metabolic process, primary metabolic process,
400 cellular process, negative regulation of cellular process). Of particular interest, within the WSF
401 treatment the catalytic activity GO term was more frequently found among the up-regulated genes.

402 These included several genes with known roles in responses to chemical stressors (Table 3 and
403 Supplemental File 2), such as Phase I oxidizing enzymes (Cyp2J-like), Phase 3 exporting enzymes
404 (multidrug resistance proteins), oxidoreductases (thioredoxin reductase, xanthine dehydrogenase,
405 quinone oxidoreductase, and 2-oxoglutarate dehydrogenase-like), and apoptosis regulators (mitogen-
406 activated protein kinase). While the specific role of Cyp2J-like enzymes in copepods is not known, many
407 enzymes in cytochrome P450 family 2 are involved in oxidative detoxification of contaminants and
408 metabolism of endogenous lipids; upregulation of this gene in nauplii from exposed mothers supports
409 the assumption that PAH transfer to nauplii occurs. Thioredoxin reductase and xanthine
410 dehydrogenase were up-regulated by both the WSF and dispersion treatments suggesting that the
411 nauplii are subjected to oxidative stress as crustacean thioredoxin is regarded a potent antioxidant
412 protein (Aispuro-Hernandez et al., 2008). It may also be suggested that the nauplii are sensitive to
413 pathogen challenge as well, as expression of thioredoxin has been observed following exposure to

414 pathogens *Vibrio alginolyticus*, *Micrococcus luteus* and *Pichia pastoris* in swimming crab (*Portunus*
415 *triberculatus*) (Song et al., 2012).

416 Based on previous studies demonstrating robust up-regulation of glutathione-S-transferase (GST, a
417 Phase 2 conjugating enzyme) in response to oil exposure in copepodites and adult *C. finmarchicus* and
418 *C. glacialis* (Hansen et al., 2009; Hansen et al., 2011), we specifically investigated expression of this
419 gene within our data set. No GST sequences were included in the set of differentially expressed genes,
420 and we did not detect this GST sequence through blast-based searches of the transcriptome used for
421 these analyses (i.e., published by Lenz et al (2014)). However, this sequence is present in the
422 transcriptomes we assembled within this study (c163217_g1 within Supplemental File 3) and
423 previously ((Tarrant et al., 2014), comp257357_c0 within PRJNA231164). When differential expression
424 analysis was conducted on reads mapped to the transcriptome assembled within this study, the GST
425 showed a trend toward higher expression with both exposures, but this difference was modest in
426 magnitude (<2-fold) and not statistically significant (Supplemental File 1 Figure S2). Overall the gene
427 expression analyses indicate that exposure of females to both the dispersion and the WSF produced
428 mild changes in gene expression in the offspring consistent with responses to oil exposure. While most
429 of the differentially expressed genes were unique to each treatment, we did not see evidence for
430 increased toxicity of the dispersion relative to the WSF. This is somewhat surprising and contrasts with
431 the visual observations (Fig. 4) that suggest increased PAH exposure in eggs produced by females
432 exposed to oil-dispersion. One possible explanation is that the effects on gene expression primarily
433 result from exposure to the smaller more water soluble PAHs.

434

435 **4. Conclusions and suggestions for future work**

436 Oil droplet exposure contributes to PAH uptake in dispersion-treated copepods as displayed by PAH
437 body residue analyses and visualized in copepods using fluorescence microscopy. Utilizing the latter
438 methodology, transfer of heavy PAH from copepod mothers to offspring was strongly supported. Only

439 subtle effects were observed in offspring, namely a temporary reduction in hatching success, which
440 appears to be relevant only when mothers were exposed to the oil dispersion that included droplets.
441 Dispersion-treated adult copepods also displayed lower lipid levels than WSF-treated copepods and
442 controls, suggesting droplets caused alterations in copepod energy budgets. Gene expression analyses
443 of offspring during the last naupliar stage suggest affected expression of genes that were
444 toxicologically relevant. However, the small number of differentially expressed genes suggest a
445 relatively mild long-term effect of maternal exposure on the offspring.

446

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449

450 **References**

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581

582

FIGURE CAPTIONS

Figure 1: Schematic of experimental design. Female copepods were exposed to clean sea water (negative control), oil dispersion or WSF for 4 days, after which they were allowed to recover in clean seawater where eggs were harvested for assessment of hatching success and development of nauplii until the first copepodites appeared. For each treatment, there were four replicates.

Figure 2: PAH body residue in the copepod *Calanus finmarchicus* exposed to seawater (negative control), oil dispersion and WSF. Data are given as average ± STDEV (N=3).

Figure 3: Bioaccumulation in *Calanus finmarchicus* plotted as a function of Kow of individual PAH components. Data are given as average ± STDEV (N=3). Both datasets fitted by a bilinear equation (y=A*log(x)-B*log(C*x+1)+D)

Figure 4: Fluorescence microscopy. Example of female copepod from the negative control group (A, seawater) and dispersed oil (B) as well as eggs from females from the negative control group (C, seawater), WSF (D) and dispersion (E). In the top images the red fluorescence represents partly digested algae in their intestine. The dispersion treated copepod (B) displays a blue lipid sac indicating uptake of heavy PAHs in the lipid sac, whereas the control copepod (A) does not display the same coloration. The eggs) from females treated with oil dispersion are blue in color (E) compared to both the negative controls (C) and the eggs from WSF-exposed copepods (D), suggesting transfer of heavier PAHs from the dispersion. Photo: Dag Altin, BioTrix.

Figure 5: Hatching success of eggs from female copepods treated with clean seawater (negative control), dispersion and WSF. Bars display average ± STDEV (N=4 throughout). Asterisks * denote significant differences ($p<0.05$) between treatments determined using 2-way ANOVA with Tukey's multiple comparison test.

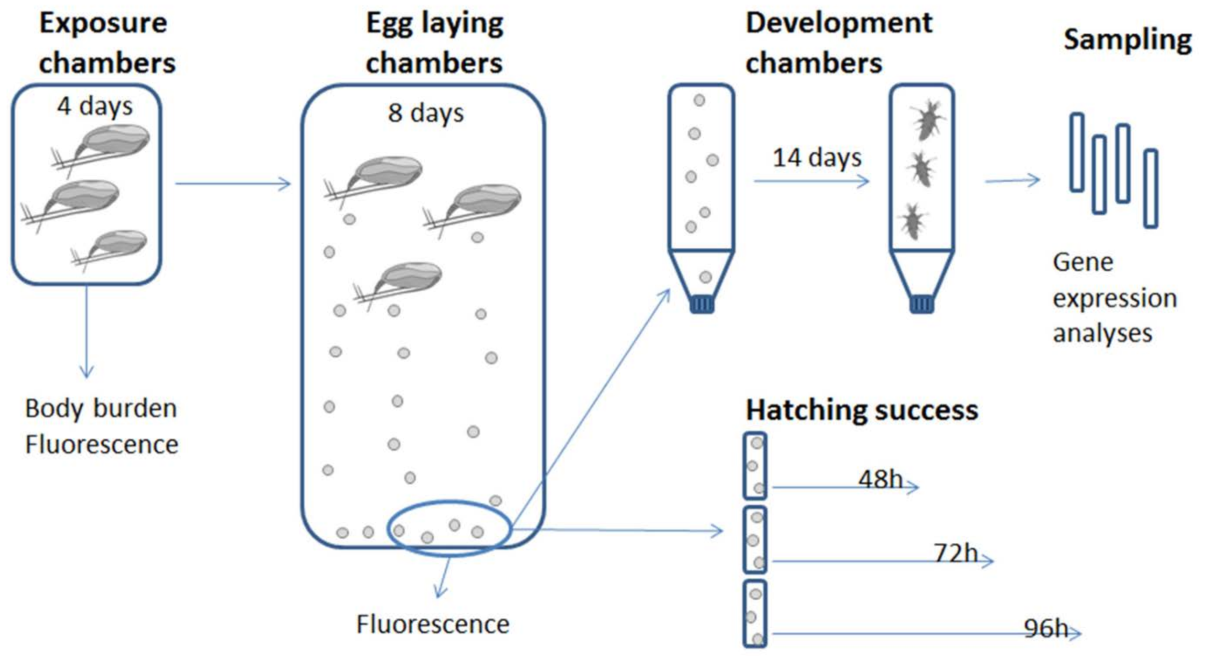
Figure 6: Summary of gene expression results for the offspring (nauplii) of mothers exposed to oil dispersion. (A) Heat map indicating normalized expression patterns of genes differing in expression among treatments. Columns indicate replicate samples clustered into treatments (colored bars at top). Rows represent individual differentially expressed genes. (B) Venn diagram indicating the number of differentially expressed genes in the WSF and dispersion treatments relative to control. Initial numbers indicate results using a significance threshold of 4-fold change in expression and 1% false discovery rate (FDR). Numbers indicated parenthetically indicate a 0.1% false discovery rate.

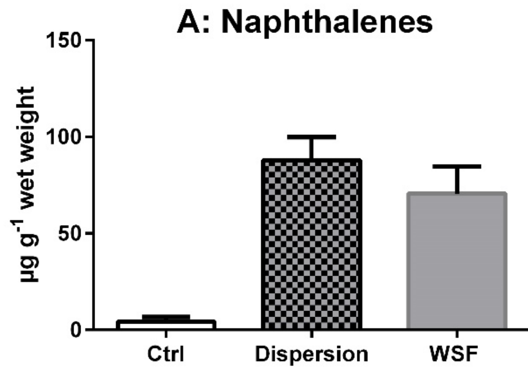
Table 1: Concentrations of oil components in the water samples taken during exposure. For each treatment, three replicates were used, so results are presented as mean ± STDEV (N=3).

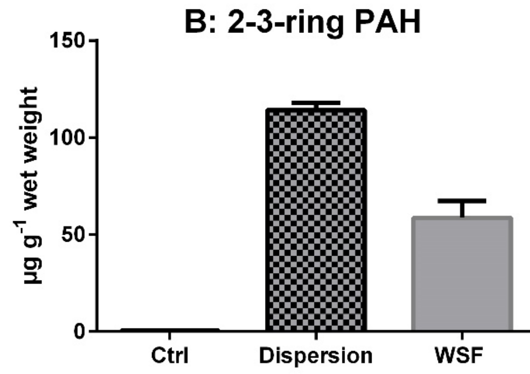
	Control (µg/L)	WSF (µg/L)	Dispersion (µg/L)
Sum VOC	0,42 ± 0.10	16,53 ± 1.10	16,89 ± 0.51
Sum BTEX	0,36 ± 0.05	4,72 ± 0.25	5,15 ± 0.13
Sum SVOC	0,11 ±0.00	16,44 ± 0.84	24,22 ± 0.42
Sum Naphthalenes	0,07 ± 0.01	11,85 ± 0.72	12,62 ± 0.55
Sum 2-3-ring PAH	0,04 ± 0.00	3,28 ± 0.15	6,51 ± 0.15
Sum 4-6-ring PAH	ND	ND	1,26 ± 0.06
Sum Phenols	ND	0,93 ± 0.17	1,13 ± 0.06
Hopane	ND	ND	0,21 ± 0.01

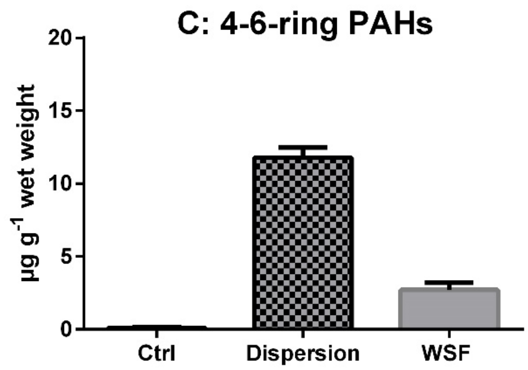
Table 2: GO terms exhibiting significant enrichment in pairwise comparisons between groups of differentially expressed genes (Chi square, $p < 0.05$).

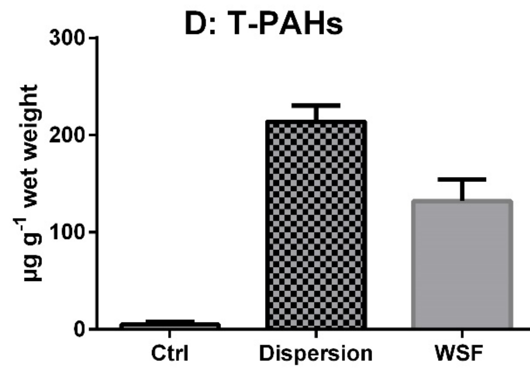
Dispersion vs. WSF	
Up-regulated in dispersion	GO:0006996 organelle organization; GO:0007017 microtubule-based process; GO:0043473 pigmentation; GO:0048519 negative regulation of biological process; GO:0050794 regulation of cellular process; GO:0065007 biological regulation; GO:0050789 regulation of biological process
Up-regulated in WSF	None
Down-regulated in dispersion	None
Down-regulated in WSF	None
Dispersion: Up vs. Down	
Primarily up-regulated	GO:0044237 cellular metabolic process; GO:0044238 primary metabolic process; GO:0009987 cellular process; GO:0006996; GO:0007017 microtubule-based process; GO:0006996 organelle organization; GO:0048523 negative regulation of cellular process; GO:0016740 transferase activity; GO:0043167 ion binding
Primarily down-regulated	None
WSF: Up vs. Down	
Primarily up-regulated	GO:0003824 catalytic activity; GO:0001882 nucleoside binding
Primarily down-regulated	GO:0006996 organelle organization; GO:0048646 anatomical structure formation involved in morphogenesis; GO:0009605 response to external stimulus; GO:0005515 protein binding

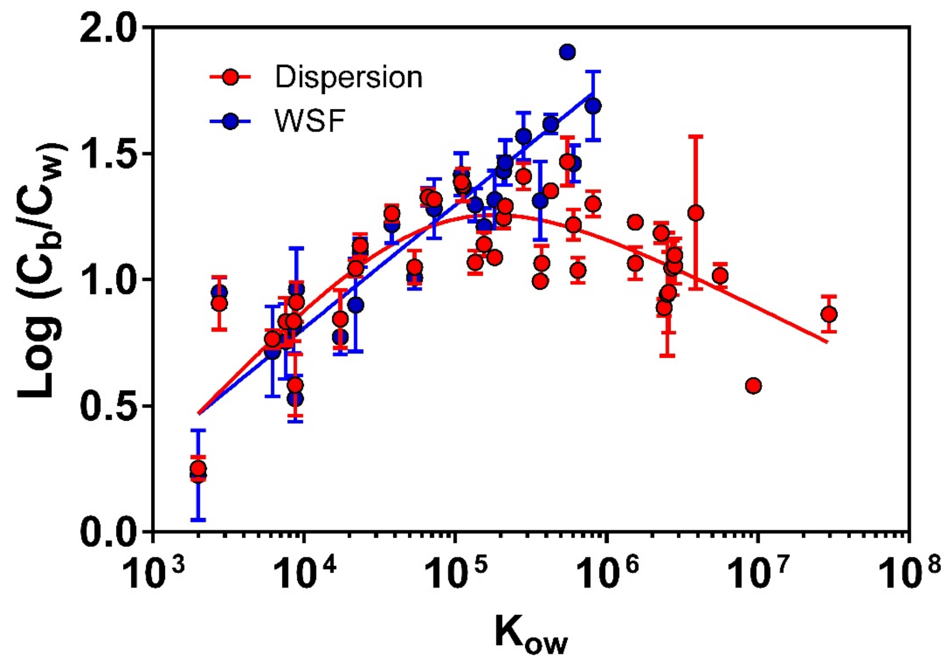

Exposure chambers
4 days
Body burden
Fluorescence
Egg laying chambers
8 days
Fluorescence
Development chambers
14 days
Sampling
Gene expression analyses
Hatching success
48h
72h
96h


A: Naphthalenes
150
100
50
0
μg g-1 wet weight
Ctrl
Dispersion
WSF

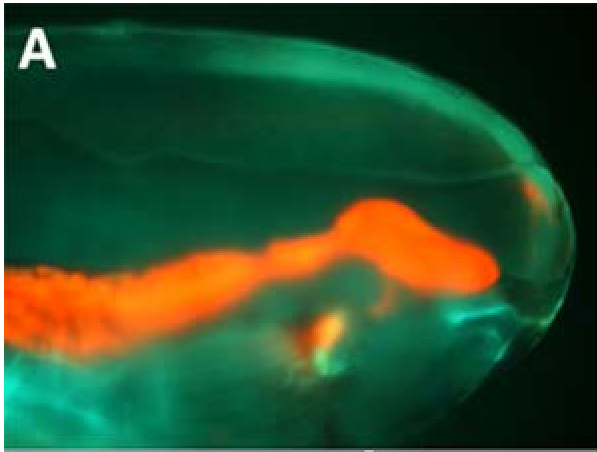
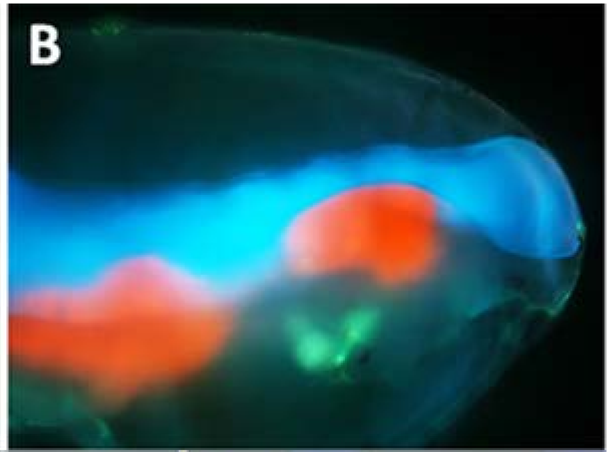

B: 2-3-ring PAH
150
100
50
0
μg g-1 wet weight
Ctrl
Dispersion
WSF

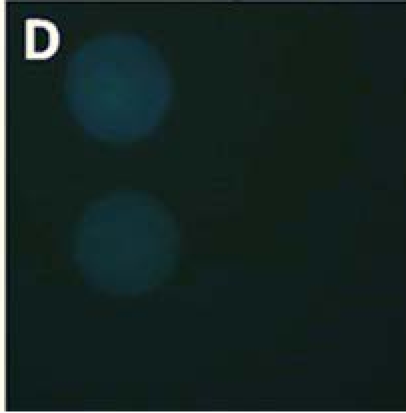
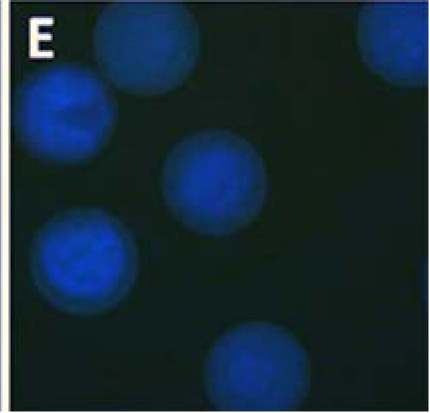

C: 4-6-ring PAHs
20
15
10
5
0
μg g-1 wet weight
Ctrl
Dispersion
WSF


D: T-PAHs
300
200
100
0
μg g-1 wet weight
Ctrl
Dispersion
WSF



Dispersion
WSF
Log (C_b/C_w)
K_{ow}
2.0
1.5
1.0
0.5
0.0
10^3
10^4
10^5
10^6
10^7
10^8

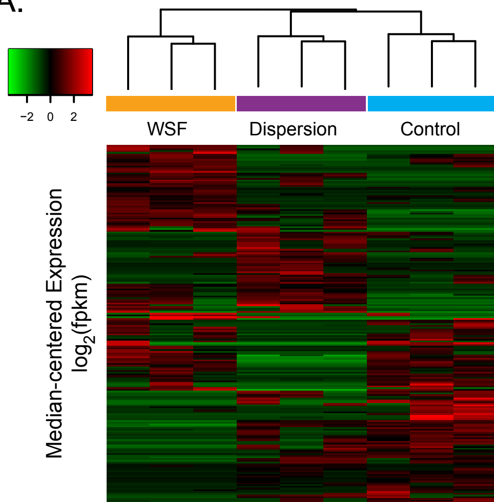

A

B

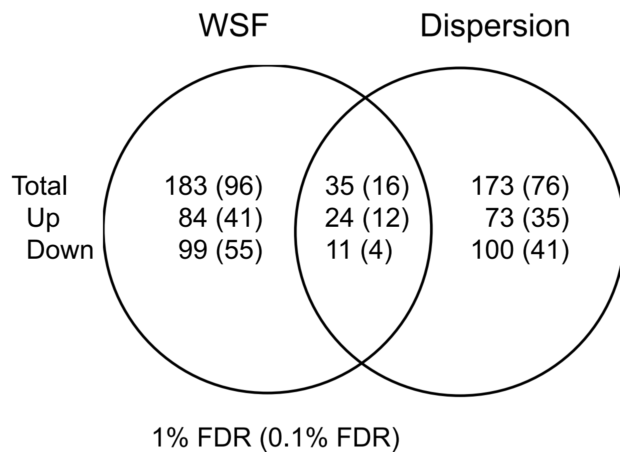
C

D

E

100
95
90
85
80
% hatching
*
*
48
72
96
Hrs after egg being laid
Control
Dispersion
WSF

Table 3: Examples of differentially expressed genes with known roles in responses to chemical stressors.

Gene Name	Transcript	Expression relative to Control	Description
Hypoxia upregulated protein 1	GAXK01175378.1	Up WSF	Hsp70 family member (endoplasmic reticulum)
Cyp2J	GAXK01019268.1	Up WSF	Phase 1 metabolism, eicosanoid metabolism
Thioredoxin 3	GAXK01014080	Up Dispersion and WSF	Antioxidant response
Xanthine dehydrogenase	GAXK01025498.1	Up Dispersion and WSF	Antioxidant response
Phospholipase A2 activating protein	GAXK01017887	Down Dispersion and WSF	Inflammatory response, involved in cell death pathways


A.
−2
0
2
WSF
Dispersion
Control
Median-centered Expression
log2(fpkm)


B.
WSF
Dispersion
Total
Up
Down
183 (96)
84 (41)
99 (55)
35 (16)
24 (12)
11 (4)
173 (76)
73 (35)
100 (41)
1% FDR (0.1% FDR)