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1 **Effects of acute exposure to dispersed oil and burned oil residue on long-term**
2 **survival, growth and reproductive development in polar cod (*Boreogadus saida*)**

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9 **Abstract**

10 The present study investigates the potential long-term physiological effects on maturing
11 polar cod (*Boreogadus saida*), an Arctic key species, after an acute exposure (48 h) to
12 environmentally realistic concentrations of either mechanically dispersed oil (MDO),
13 chemically dispersed oil (CDO) or burned oil residues (BO) (N=58-60 per treatment).
14 Following exposure, fish were monitored in a common tank supplied with clean water for
15 a seven-month period coinciding with the period of reproductive development. Females
16 exposed to BO residues were more frequently found in an earlier phase of gonadal
17 maturation compared to unexposed females while no effects of different oil spill response
18 (OSR) actions were seen in the reproductive development of males. Mechanically and
19 chemically dispersed oil induced a transient short-term reduction in growth in the first week
20 post-exposure. Overall, no significant long-term effects of exposure were seen in growth
21 or mortality. Ultimately, this study provides information for the assessment of population
22 consequences of different OSR actions as part of a net environmental benefit analysis.

23 **Keywords:** Arctic, petroleum hydrocarbons, growth, reproductive cycle, sublethal effects,
24 oil spill response

25 **1. Introduction**

26 Increasing anthropogenic activities related to petroleum in the Arctic elevates the risk of
27 acute and chronic oil spills. Conditions inherent in the Arctic, e.g. weather, sea ice, limited
28 infrastructure, remoteness, and lack of precedent for responses in areas under multinational
29 governance, place specific requirements on operations including eventual oil spill response
30 (OSR) (Harsem et al. 2011). Recent OSR actions have stressed the need for OSR decision
31 making that is site- and situation-specific to adequately consider physical factors and
32 environmental and societal resources at risk (Beyer et al. 2016; Fingas, 2016).
33
34 Polycyclic aromatic hydrocarbons (PAHs) are one group of toxic compounds in petroleum,

35 some of which are known to exhibit carcinogenic, genotoxic, and endocrine disrupting
36 properties, even at low concentrations (Kime, 1995; Horng et al. 2014; Vignet et al. 2014).
37 However, a large portion of the water-soluble fraction of crude oil is made up of poorly
38 characterized mixtures of organic compounds, also classified as unresolved complex
39 mixtures (UCM) (Melbye, 2009), with toxicological effects difficult to discern (Booth et
40 al. 2007, 2008).

41 Mechanical recovery of spilled oil is often the first choice in an OSR and risk mitigation,
42 however, chemical dispersants and *in situ* burning may be better suited in ice infested
43 waters of the Arctic (Fritt-Rasmussen et al. 2015). Use of chemical dispersants as an OSR
44 action is intended to increase the biodegradation potential of petroleum by forming oil-
45 surfactant micelles (Lessard and DeMarco, 2000). The use of chemical dispersants thereby
46 enhances the oil concentration in the water column for a time whilst reducing the surface
47 slick, the risk for encapsulating the oil slick into the sea ice (Brandvik et al. 2006), and the
48 exposure potential for birds and marine mammals (Fingas, 2011). Increased concentrations
49 of small oil droplets in the water column may lead to increased bioavailability of PAHs
50 (Ramachandran et al. 2004; Milinkovitch et al. 2012), which has been linked to adverse
51 effects on behavior, growth, reproduction, and survival in several fish species (Gulec et al.
52 1997; Yamada et al. 2003; Milikinovitch et al. 2011; Wu et al. 2012; Yu et al. 2015;
53 Nwaizuzu et al. 2016).
54

55 *In situ* burning is a countermeasure technology that was first implemented in 1958 along
56 the ice-covered Mackenzie River in Northwest Canada and since then has been used
57 operationally in ice free areas during the 1989 Exxon Valdez oil spill in Alaska and
58 extensively in the 2010 Deepwater Horizon oil spill in the Gulf of Mexico (Beyer et al.
59 2016; Buist, 2004; Hunt, 2009). Burning is a quickly implemented OSR action that requires
60 little infrastructure and, most significantly, is effective, leaving approximately 10% of the
61 original hydrocarbon load (Guenette and Sveum, 1995; Buist, 2004; Buist et al. 2013;
62 Fingas, 2016). Burning creates a burned oil residue (BO) that may sink depending on oil
63 characteristics (Fritt-Rasmussen et al. 2015). A limited number of studies have investigated
64 the potential effects of BO on biota and agreement on the low acute toxicity of BO
65 applications has been reached (for reviews see Buist 2004; Buist et al. 2013; Fingas 2016).

66 Compared to untreated and chemically dispersed oil, burned oil was found to be less
67 acutely toxic in rainbow trout (*Onchoryncus mykiss*), three-spined stickleback
68 (*Gasterosteus aculeatus*) (Blenkinsopp et al. 1996), Australian bass (*Macquaria*
69 *novemaculeata*) (Cohen and Nugegoda, 2000; 2006), amphipods and snails (Gulec and
70 Holdway, 1999), and in copepods (Faksness et al. 2012). However, the long-term effects
71 of acute exposure to burned oil residues has yet to be examined.
72
73 Polar cod (*Boreogadus saida*) is an endemic key species with a pan Arctic distribution
74 (Bradstreet and Cross, 1982) that has been used as a sentinel species in Arctic environments
75 to understand the effects and mechanisms of petroleum exposure (Christiansen and George,
76 1995; Jonsson et al. 2010; Andersen et al. 2015; Nahrgang et al. 2010; 2016; Bender et al.
77 2016). The geographical distribution of polar cod overlaps with oil and gas fields and
78 transport areas in all Arctic seas and fish can therefore encounter petroleum products
79 (Bailly, 2008; Bird et al. 2008; David et al. 2016). Gonadal development in polar cod
80 begins in early autumn and final maturation and spawning takes place from January to
81 March (Nahrgang et al. 2014; Mueter et al. 2016). Gonadal investment in polar cod is high
82 with up to 87% of liver reserves metabolized during reproductive development (Hop et al.
83 1995). Throughout the active gametogenesis period, male fish undergo more rapid
84 development of gonadal tissue and an earlier increase in plasma sex steroid concentrations
85 than female fish (Bender et al. 2016).
86
87 Acute toxicity data (e.g. LC_{50}) using model organisms represents the majority of existing
88 data on the effects of OSR actions while actual oil spills present the challenge of
89 confounding factors (e.g. other sources of pollution or climate and fishing driven pressure),
90 indirect effects, and missing background data when interpreting effects on biota (Beyer et
91 al. 2016; Peterson et al. 2003). Expanding potential effects from short-term responses of
92 biomarkers after acute exposure to a predictive indicator of long-term effects is challenging
93 (Forbes et al. 2006; Claireaux et al. 2013). The objective of this study was to investigate
94 long-term resilience of adult polar cod exposed to mechanically dispersed oil (MDO),
95 chemically dispersed oil (CDO) or BO. To link acute exposure to long-term effects,
96 survival, growth, and reproductive investment in polar cod were monitored for seven

97 months after an acute (48 h) exposure. Simulated wave and current energy in open exposure
98 tanks ensured that acute exposures set up was dynamic whereby organisms were exposed
99 to the whole dispersed oil solution (dissolved fractions and oil droplets) (Milinkovitch et
100 al. 2011; Frantzen et al. 2015, 2016). We hypothesized that addition of chemical
101 dispersants would increase the exposure of polar cod to crude oil resulting in reductions in
102 growth and reduced investment in reproductive development compared to exposure to
103 MDO and BO treatments. Growth and reproductive development are physiological
104 endpoints that can provide an integrative measure of the effects of pollutants on whole
105 organisms and are important to consider when evaluating the potential risks to populations.
106 The aim of this study is to generate sound information on the sensitivity of adult polar cod
107 to support the net environmental benefit analysis (NEBA) of OSR actions in the Arctic
108 marine system.

109 *2. Material and Methods*

110 *2.1 Fish collection and husbandry*

111 Wild polar cod were collected in Svalbard fjords in September 2014 by bottom trawl during
112 a cruise aboard the RV Helmer Hanssen and thereafter transported to the Akvaplan-niva
113 marine laboratory in Tromsø, Norway. The fish were reared in a single common 5000 L
114 tank for an eight-month acclimation and maintenance period and hand fed twice a week on
115 a commercial marine fish feed (ration equal to 4% body weight per feeding; Skretting, 3-4
116 mm dry pellets). The light regime was maintained on a simulated Svalbard light throughout
117 acclimation, exposure and post-exposure periods. The seawater temperature in the tank
118 followed the annual variation of Grøtsundet, the fjord outside the marine laboratory where
119 seawater was collected from 50 m depth, with a high of 8.6 ± 0.1°C in September and low
120 of 3.74 ± 0.02 °C in February and yearly average of 6.2 ± 0.1°C. Oxygen saturation was
121 keep above 90% for acclimation and post-exposure period. On the 19th of May 2015, all
122 fish (n=310) were anesthetized (Metacaine at 0.08 g/L seawater) and received a passive
123 integrated transponder tag (Trovan®) inserted intraperitoneally with no mortality or
124 negative effects observed in the first weeks post tagging.

125 *2.2 Exposure design*

126 Exposure to MDO, CDO or BO commenced in late June 2015, and growth was followed
127 over a seven-month period (i.e. until January 2016) coincident with the active
128 vitellogenesis/spermatogenesis period of polar cod (Bender et al. 2016). Goliat (Kobbe)
129 crude oil, a sweet light crude oil with a density of 0.83 kg /L, an °API gravity of 40.3 and
130 a sulphur content of 0.14% wet weight (Eni Norge, 2015) was used for the MDO and CDO
131 treatments. For preparing BO, 40 L of Goliat (Kobbe) crude oil was added at the surface
132 of a tank containing approximately 200 L sea water. The oil was set on fire and burned for
133 3 minutes until the fire died out, leaving approximately 4 L burned residue in the tank (i.e.
134 ~90 % of the fresh oil volume was burned off). The set-up consisted of four treatments and
135 three replicates per treatment; control (no oil, Ctrl), MDO or CDO (premixed with
136 dispersant FINASOL®; 5% w/w) and BO residue. The BO concentration equaled 10% of
137 the nominal oil concentration used in the mechanically and chemically dispersed oil
138 treatments (i.e. 90% burned off in BO compares to MDO and CDO). To be able to directly
139 compare between dispersed oil toxicity (MDO, CDO) and BO toxicity, the same exposure
140 protocol was used for all treatments.

141

142 The dispersant mixtures were generated according to Frantzen et al. (2015, 2016) following
143 the protocol developed by Cedre, France for the DISCOBIOL project (e.g. Milinkovitch et
144 al. 2011). Briefly, the oil treatments (MDO and CDO; nominal concentration of 67 mg/L)
145 or BO (nominal concentration of 6.7 mg/L) were introduced to individual 120 L exposure
146 tanks through a funnel fixed at the surface. A pump in the bottom of each tank provided
147 continuous mixing energy in all tanks. In order to ensure a homogeneous exposure mixture
148 in the tanks and to allow some weathering of the oil to take place prior to exposure start,
149 water and oil/oil premixed with dispersant/BO were mixed for 24 hours before the
150 introduction of fish to the system. The water system was static and oil exposures were
151 conducted for 48 hours after the introduction of the animals (water temperature 6.4 ± 0.3
152 °C; O_2 saturation was held >80% with aide of aerators). In total, 236 specimens were
153 exposed from the 26th to the 28th of June, 2015 with each replicate exposure tank (n=3 per
154 treatment) containing 18-20 fish.

155 *2.2.1 THC and PAHs in seawater*

156 In order to monitor exposure concentrations, water samples (approximately 1L) were taken
157 from all exposure tanks (n=3 per treatment) at the beginning of the experiment (t 0h), after
158 24 hours (t 24h), and at the end of the 48h exposure (t 48h). Determination of total
159 hydrocarbon content (THC; n-C10 – n-C35) was performed on isooctane extracts by Gas
160 Chromatography-Flame Ionization Detector (GC-FID). Each sample extract was analyzed
161 on the GC simultaneously with control solutions (reference oil EDC95/11; Norwegian
162 Environment Agency M-408/2015) of three known concentrations of THC, covering the
163 concentration range of the sample extracts (Frantzen et al. 2016). Analysis of 26 PAHs (16
164 Environmental Protection Agency [EPA] priority parent PAHs and C1–C3-alkylated
165 naphthalenes, phenanthrenes and dibenzothiophenes) concentrations was performed by
166 GC–Mass Spectrometry (GC–MS) operated in selected ion monitoring mode. Single PAH
167 concentrations were calculated by quantification of altered deuterated standards added
168 prior to extraction, and development of a pre-determined calibration curve of five PAH-
169 standards at different concentrations (Frantzen et al. 2016). The measured THC and PAH
170 concentrations represent dissolved components as well as oil droplets. In the determination
171 of ∑26 PAH concentrations, single components with values below the limit of detection
172 (LOD) were assigned a value of zero. Due to a technical instrument failure, water samples
173 from t0h at the start of the exposure gave unreliable results and were excluded from further
174 analysis.
175

176 *2.3. Post-exposure monitoring and final sampling*

177 The 48h exposure period was followed by a 48h recovery period in 500 L flow-through
178 tanks and subsequent growth registration (T1; see paragraph below) before the fish were
179 transferred back to the common 5000 L rearing flow-through tank. The common tank
180 ensured identical post-exposure rearing conditions for all treatment and replicate groups.
181 The fish were fasted two days prior to exposure start, during the 48h exposure period, and
182 two days prior to every growth measurement. Fish were, however, offered food
183 immediately following exposure in clean water tanks even though it was less than 48 hours
184 before the growth checkpoint.
185

186 Mortality was recorded daily over the entire experiment. Growth was recorded at monthly
187 intervals by first anesthetizing, then measuring the total weight (±0.01 g) and total length
188 (±0.1 cm) at the following time points: T0 (May 19th, pit tagging), T1 (June 30th, 2 days
189 post-exposure), T2 (July 30th), T3 (September 3rd), T4 (October 5th), T5 (November 3rd),
190 T6 (December 9th), and T7 (January 5th). An additional group of "unexposed" polar cod
191 was included in the common rearing tank which consisted of the remaining acclimation
192 fish that fell below (Unexp. 1) and above (Unexp. 2) the desired intermediate size range
193 and were therefore not included the exposure experiment (n=74). These additional
194 unexposed fish provided a control for experimental handling stress related to the exposure
195 with growth measurements undertaken at T0, T2-T7 (excluded from T1 due to logistical
196 limitations).
197
198 On the 5th of January, all remaining experimental fish and the unexposed fish were
199 sacrificed by a sharp blow to the head and the following measurements were collected:
200 total length (±0.1 cm), total weight (±0.01 g wet weight [wwt]), sex, gonad weight (±0.01
201 g wwt), liver weight (±0.01 g wwt) and somatic weight (empty carcass weight, ±0.01 g
202 wwt). The middle section of the testis and ovaries were fixed in a buffered formaldehyde
203 solution (4%) for later histological analysis. Otoliths were collected for age determination
204 and read under a dissection microscope (Leica M205C).

205 Specific growth rate (SGR) for individual fish for the entire experimental period was
206 determined according to the equation:
207 $SGR = [(\ln_t W_2 - \ln_t W_1)t^{-1}]100$
208 where SRG is % increase in body weight per day. $_tW_1$ and $_tW_2$ are the total weights of the
209 fish recorded at times 1 and 2 respectively, and t is the number of days between weighting
210 events.
211
212 Gonadosomatic index (GSI) and hepatosomatic index (HSI) were calculated according to
213 the following equations:
214 GSI = (gonad weight/somatic weight)*100
215 HSI = (liver weight/somatic weight)*100
216

217 Condition factor for the different time points (T0-T7) was calculated:

218 $CF=(W/L^3)*100$

219 where W is total weight in g and L is the total length in cm.

220

221 *2.5. Histological analysis*

222 Briefly, gonad tissues were rinsed of buffered formalin, dehydrated in a series of 70%
223 ethanol baths and embedded in paraffin wax (Aldrich, USA) overnight using Histo-clear®
224 as a clearing agent in a Shandon Citadel 1000 (Micron AS, Moss, Norway). Tissues were
225 then embedded into paraffin and sliced at 5 µm (females) and 3 µm (males) thickness,
226 using a Leica RM 2255 microtome before being stained with haematoxylin and eosin. Two
227 slides were prepared for each fish. Gonad maturity stages in females were classified using
228 the development stage of oocytes within the respective categories of immature, resting, and
229 early and advanced stages of maturation. Immature and resting females had only primary
230 growth (PG) oocytes while maturing females had vitellogenic oocytes present. Resting
231 females were identified by the presence of residual oocytes from previous spawning events
232 with otherwise only PG oocytes. Maturing females exhibited different phases of oocyte
233 development with varying extents of vitellogenin derived oil droplets in the oocyte
234 cytoplasm (Figure 4ab). Oil droplets were present but filling less than ½ of the cytoplasm
235 in early maturing females while advanced maturing females had oocytes completely filled
236 or nearly filled with oil droplets. Abnormal oocyte development was noted with regard to
237 the location of cortical alveolar vesicles and oil droplet within the oocyte. Oocyte diameter
238 ($n \geq 6$ oocytes per female) was counted for oocytes in the most advanced cohort using the
239 image processing software (Leica DFC 295 camera attached to a Leica DM 2000 LED
240 microscope and Leica analysis software) and then averaged for each female. Oocyte stage
241 frequency disruption was determined by classifying all oocytes with a nucleus in an area
242 of 20 mm^2 placed randomly on the tissue slice. Frequency counts were averaged over both
243 replicate slides. Presence of residue oocytes was noted and relative frequencies of atretic
244 oocytes were semi-quantified using a 0-3 scale ranging from 0 (0% of oocytes were atretic);
245 1 (1-2%); 2 (3-20%); and 3 (20-30% of oocytes were atretic) for each female. Male testes
246 were classified into the four different maturity stages of immature, resting, and maturing
247 with either late spermatocytes stage I (Figure S1a) or with late spermatocytes stage II

248 dominating (Figure S1b). Immature males were identified as those with testis containing
249 only early stage spermatocytes (Figure S1c) and a low GSI while resting males had spent
250 testis containing portions of early stage spermatocytes with otherwise empty lumen space
251 (Figure S1d).

252 *2.6 Statistical Analysis*

253 All statistical analyses were performed with R 3.1.1 (R Core Team, 2014). A Levene's test
254 was used to test for normality and homogeneity of variance. When homogeneity criteria
255 were met, a one-way analysis of variance (ANOVA) was run, and when a significant
256 treatment effect was found, the Tukey's HSD post hoc for unequal sample sizes was used
257 to distinguish differences between treatment groups. In cases where homogeneity criteria
258 were not met, a nonparametric Kruskal Wallis ANOVA was used, followed by a multiple
259 comparison of mean rank of all group tests. Difference in SGR variance was tested using
260 an F-test. Maturity stage frequency distributions were tested using a Fishers exact test with
261 the null hypothesis that all treatments have similar maturity stage distributions. With a
262 significant Fishers exact test result, a *chi* squared test was run comparing all treatment
263 groups and control against one another. A probability level of $p \leq 0.05$ was considered
264 significant for all tests. All values are presented as mean ± standard error of the mean (SE).

265 *3. Results*

266 *3.1. Water chemistry*

267 The total hydrocarbon content (THC) in water samples from the control treatment was not
268 detectable throughout the exposure period, and remained relative stabile throughout the
269 last 24 h of exposure with values of 0.9 ± 0.5, 9.2 ± 3.7, 22.5 ± 3.7 mg/L in BO, MDO and
270 CDO, respectively (Fig. 1a). Average ∑26 PAH concentrations were highest after 24 h in
271 all treatments and thereafter decreased by ca. 20 % at 48 h (Fig. 1b, Table S1). Highest
272 ∑26 PAH concentrations were found the CDO treatment (101.5 ± 14.3 μg/L) at 24 h
273 followed by the MDO (62.4 ± 20.7 μg/L), BO (3.5 ± 1.2 μg/L) and Ctrl (1.05 ± 0.0 μg/L)
274 treatments. Dominating PAHs (>98 % of ∑26 PAH) in all treatments (BO, MDO and CDO)
275 were parent and alkylated naphthalenes, phenanthrene/anthracenes and dibenzothiophenes,
276 whereas only parent and C1, C2-naphthalenes were detected in the Control. In BO

277 treatment, the only high molecular weight PAHs measured above detection limits were
278 benzo(b)fluoranthene (0.03 μg/L) and benzo(k)fluoranthene (0.01 μg/L), and these
279 concentrations were comparable to measured concentrations in MDO and CDO (0.01 –
280 0.10 μg/L and 0.01 - 0.013 μg/L, respectively; Table S1). Acenapthylene was the only
281 PAH with a higher concentration in BO (0.01 μg/L) compared to MDO and CDO (<0.004
282 – 0.005 μg/L) (Table S1).
283
284 *3.2. The initial fish population*
285 Fish initially part of the exposure experiment ranged in size from 12.0 – 59.0 g total weight
286 (mean 34.7 ± 0.6 SE), 12.0 – 22.0 cm length (mean 17.3 ± 0.1 SE) and age ranged between
287 2 and 6 years (mean 4.5 ± 0.1 SE) at T0 (Table 1). Fish used for the exposure experiment
288 were all selected from the intermediate size group of the collected fish (size range 24.0 –
289 47.5 g) with no significant difference in size between any of the groups (Ctrl, BO, MDO,
290 CDO). The remaining unexposed polar cod were not included in any of the treatment
291 groups and were classified by size as they exhibited a bimodal size range that was
292 significantly smaller (Unexp1; size range 12-32 g) and bigger (Unexp2; size range 38-59
293 g) than the exposed fish.
294 *3.3. Mortality*
295 Mortality was observed after the first month post collection, and in the period
296 February/March 2015 following the natural spawning period before exposure took place
297 (data not shown), after which mortality subsided. Fish were otherwise in good condition
298 throughout the acclimation, exposure and post-exposure monitoring period. No mortality
299 was registered in any treatments tanks during the 48h exposure period. Mortality was,
300 however, observed during the post exposure period for all treatments independent of
301 exposure. Mortality was most prevalent in the first month post-exposure (T1-T2 [June 30th
302 – July 30th]) with 8-12% mortality occurring in all oil treatments and control. The mortality
303 rate steadied to between 2 and 5 % per month until the final sampling in January for all
304 treatments and control with no statistical difference in cumulative mortality (Fig. 2). The
305 group of larger unexposed fish (Unexp 2.) exhibited the highest cumulative mortality
306 (32%).
307

308 *3.4. Specific growth rate*

309 In general, there was a great variation in SGR within all treatment groups throughout the
310 experiment ranging from −2.5 to 3.5 % change in body weight per day. Overall, growth
311 rates (mean ± SE) were lowest after tagging and during exposure (T0-T1 [May 19th - June
312 30th], (-0.01 – 0.15 % increase in body weight per day) and highest in the consecutive time
313 period (T1-T2 [June 30th –July 30th], 0.47 - 0.73 % increase in body weight per day). In the
314 period from tagging to immediately after exposure (T0-T1), significant treatment effects
315 on growth rates were observed with high rates in the BO compared to lower growth rates
316 in the MDO ($p<0.01$) and CDO treatments ($p<0.01$) (Figure 3). In the following period
317 (T1-T2) growth rates in the BO treatment were significantly reduced only when compared
318 to the CDO treatment ($p<0.01$). No significant differences in SGR were seen between any
319 treatment groups or unexposed fish for the entire period (T0-T7 [May 19th – Jan 5th]) or for
320 any other growth periods beyond the first two periods. Female and male SGR were not
321 significantly different at any time period, therefore both sexes were pooled for statistical
322 analysis.

323 *3.5. Condition factor and hepatosomatic index*

324 At T0 (May 19th), males (exposed and unexposed combined) had a significantly higher
325 condition factor compared to females at 0.68 ± 0.0 and 0.64 ± 0.0, respectively. At T1 (June
326 30th), the condition factor was significantly higher in females in the BO treatment (0.66 ±
327 0.0) compared to females in the other groups (control [0.62 ± 0.0], MDO [0.62 ± 0.0] and
328 CDO [0.60 ± 0.0]). At no other time point were there significant differences found between
329 any of the treatment groups (including control) or sex. Furthermore, no significant
330 difference in age, HSI, or condition factor was seen between any treatment or sex at the
331 end of the experimental period in January (Table 1).

332 *3.6. Reproductive development*

333 *3.6.1. Females*

334 Histological analyses revealed that 56% of female fish had spawned previously as
335 determined by presence of residual oocytes, while 22% exhibited first time maturation with
336 no evidence of previous spawning and the remaining specimens were immature (6%) or
337 resting (16%). From the maturing females, 68% revealed a leading oocyte cohort that had

338 reached the vitellogenic stage II (Vtg II) and were categorized as advanced maturing with
339 mean oocyte diameter of 547 ± 8 µm, a centrally placed nucleus and the cytoplasm filled
340 with vitellogenin derived oil droplets (Fig. 4). In 32 % of maturing females, however, the
341 most advanced oocyte cohort was in an early vitellogenetic stage (Vtg I) and was thus
342 categorized as early maturing with an oocyte diameter of 446 ± 11µm and vitellogenin
343 derived yolk droplets only at the periphery of the cytoplasm and persisting cortical alveolar
344 vesicles, often in combination with atresia (Fig. 4). Abnormal oocyte development,
345 characterized by partial inclusion of cortical alveolar vesicles into the cytoplasm, non-
346 radial yolk globule orientation around nucleus, and few oocytes in the most advanced
347 oocyte cohort, was observed in 35 % of early maturing females with no statistical
348 significance of treatment. Significant differences in gonadal maturity stage was observed
349 in the BO exposed females exhibited by a lower percentage of advanced maturing (35%)
350 and higher percentage of early maturing females (38%) compared to other treatment groups
351 (mean percentage in advanced maturing stage was 61%) ($p=0.042$) and when tested against
352 the control group only, the significance increased (X-squared = 7.99, df = 2, p-value =
353 0.018) (Fig. 5a). No significant differences were found between treatments in mean oocyte
354 diameter, the relative number of oocytes in the leading cohort, presence of residual oocytes
355 or frequency of atresic oocytes. However, significantly greater variation in oocyte diameter
356 was observed in early maturing females in the BO treatment (443.5 ± 42 µm, n=7)
357 compared to the control (409.0 ± 10.7 µm, $p= 0.015$, n=5).
358
359 Gonadosomatic index (GSI) in females ranged between 0.6 and 11.5 with no significant
360 difference between any of the treatments (Fig. 5b). GSI (Mean ± SE) for immature, resting,
361 advanced maturing and early maturing females was (0.9 ± 0.0), (2.6 ± 0.8), (5.7 ± 0.3) and
362 (3.6 ± 0.3), respectively. Advanced maturing female GSI was significantly higher than all
363 other maturity stages ($p<0.001$).
364
365 *3.6.2. Males*
366 Testis development appeared normal for males in all treatments with no significant
367 difference in the frequency of occurrence of different maturity stages among the treatments
368 (Fig. 6a). GSI in males at the end of the experiment (T7) ranged between 0.0 and 33.3 with

369 no significant difference between any of the treatments (Fig. 6b). Immature and resting fish
370 made up 5.9% and 2.9% of the sampled males respectively while 53.9% of males were in
371 an early stage of maturation (late Sc I) and 37.3% of the males were in a later stage of
372 development (late Sc II) (see Fig S1. for maturity stage representations). Immature and
373 resting males had a low mean GSI (2.0 ± 1.6 and 2.0 ± 0.9, respectively). Maturing males
374 with late spermatocytes stage I had a lower GSI (15.8 ± 0.8) compared to those with more
375 developed late spermatocytes stage II (22.6 ± 1.0).
376
377 *4. Discussion*
378 *4.1 Exposure to dispersed oil and burned oil residue*
379 The present study simulates conditions in which dispersant (CDO treatment) or *in situ*
380 burning (BO treatment) might be used to combat an oil spill in Arctic waters in comparison
381 to no action (MDO treatment). THC and PAH water concentrations in both MDO and CDO
382 reflected environmentally realistic concentrations reported from experimental field trials
383 and dispersant operations during actual oil spills (i.e. THC concentrations of 30-50 mg/L
384 below the spill just after treatment before decreasing to <1-10 mg/L, and ∑PAH
385 concentrations of 6-115 mg/L the first days or weeks after accidental oil spills) (Law,
386 1978; Humphrey et al. 1987; Lunel et al. 1995; Short and Harris, 1996; Kingston, 1999;
387 Reddy and Quinn, 1999; Lessard and DeMarco, 2000; Sammarco et al. 2013). Reports of
388 hydrocarbon concentrations in seawater after *in situ* burning operations are scarce. PAH
389 and THC levels in the present study are below seawater concentrations measured after
390 experimentally spilled and burned oil in the Newfoundland Oil Burn Experiment (3.78
391 μg/L ∑16 EPA PAHs) (Daykin et al. 1994), and above THC concentration from an oil spill
392 simulation and test burning experiment in the Barents Sea (13 μg/L) (Brandvik et al. 2010).
393
394 The overall THC and ∑26 PAH concentrations in the Ctrl, MDO and CDO treatments were
395 in agreement with previous experiments using the same nominal oil concentrations and
396 exposure set-up as in the present study (Frantzen et al. 2015, 2016), and confirms that the
397 addition of chemical dispersant increases the efficiency of the dispersion process leading
398 to significantly elevated THC and PAH concentrations in CDO compared to MDO.
399 Measured BO concentrations were 8 ± 2 % of the measured MDO concentrations,

400 indicating that mechanical dispersion of BO into the water column was equally efficient as
401 for oil. In the present study, an identical exposure protocol was used for all treatments to
402 allow for direct comparison of effects between the oil spill response measures investigated.
403 Energy was added to the seawater to simulate a dynamic exposure with wave energy for
404 the period of 4 tidal systems (48 hours) (Merlin, 2005; Milinkovitch et al. 2011), and the
405 measured concentrations of hydrocarbons represented both the water-soluble fraction as
406 well as BO residue particles/dispersed oil droplets. Adding mixing energy to simulate wave
407 action to the BO residue exposure dispersing it in the water column is, however, novel as
408 previous studies have exposed organisms only to the burned oil WSF (Faksness et al. 2012;
409 Gulec and Holdway, 1999), and reported measurements are taken only of seawater
410 hydrocarbon concentrations underneath burned areas (Brandvik et al. 2010).
411
412 Forming of short-term temporary oil slicks, variation in oil adherence to equipment and
413 mixing by fish movements between replicate tanks may be a source of the individual
414 variability in THC and PAHs concentrations between replicate water samples, and the
415 increased PAH/THC concentration at T48h compared to T24h observed in two individual
416 tanks (one MDO and one CDO tank, respectively). Inter- and intra-tank variations did
417 however not influence the overall significant difference in THC/PAH concentrations
418 between the OSR actions investigated. Low concentrations of naphthalene measured in the
419 control water may be considered elevated background levels with no potential toxic effects
420 to biota (Molvær et al. 1997) and are evidence of the ubiquity of PAHs, especially
421 naphthalene, one of the most abundant PAHs in the marine environment (Latimer and
422 Zheng, 2003).
423
424 *4.2 Physiological and reproductive effects*
425 No relationship was found between treatment and mortality. The sustained mortality rate
426 in seen in all groups (both exposed and unexposed) is most likely due to the post spawning
427 physiological state of the mature fish as confirmed by the presence of residual oocytes in
428 56% of females. Handling stress at the beginning of the experiment could have induced
429 higher mortality at this early time point. The mortality rate seen in this experiment (~24%)
430 was lower than the mortality observed (~56%) in a long-term crude oil exposure on adult

431 feral polar cod held in captivity (Bender et al. 2016). Fish were in a good state of health as
432 evidenced by an unanimously high condition factor and HSI in all treatment groups at the
433 final sampling in January, although the HSI values reported for fish in the present study
434 (8.5 - 9.6 %) were lower than for polar cod of a similar size held in captivity at the same
435 time of year (10.9-13.1%) (Bender et al. 2016). Fish in the latter study were fed a natural
436 diet of *Calanus sp.* zooplankton whereas commercial feed was used in the present study
437 and this difference in diet may have influenced the HSI. Higher condition factor in males
438 compared to females at the start of the experiment is most likely due to the difference in
439 the timing of reproductive investment, where males start gonadal investment earlier in the
440 season than females (Hop et al. 1995; Nahrgang et al. 2014).
441
442 Growth rates observed in polar cod of the present study were within reported ranges from
443 previous studies of mature fish (Jensen et al. 1991; Hop et al. 1997). Furthermore, the
444 observed trends in growth rate did not indicate significant long-term effects by any of the
445 OSR actions. The transient decrease in SGR for the MDO and the CDO treatments
446 compared to BO treatment, may however, be due to a transient appetite depression in these
447 two groups in the first days following the exposure. Low feeding activity was visually
448 observed at this time. No effect of crude oil exposure on appetite has been observed in
449 polar cod previously; however, exposure to crude oil contaminated food did lead to reduced
450 growth in exposed fish (Christiansen and George, 1995). The SGR in July (T1-T2) was
451 highest (0.6 ± 0.0 % body weight change per day) in all treatment groups compared to all
452 other periods (0.1-0.2 % change per day) and may reflect some compensatory growth
453 following handling and fasting during exposure (Ali et al. 2003). Reduced feeding and
454 growth was also observed in African catfish (*Claris gariepinus*) for two months following
455 exposure to crude oil and dispersed crude oil (Nwaizuzu et al. 2016).

456 Females likely to spawn in the coming winter season were in the late maturing (Vg II) stage
457 with a GSI around 5.7 ± 0.3 while it is unclear when or if the females in the early maturing
458 stage would spawn. The timing of spawning from other laboratory polar cod populations
459 in an analogous reproductive stage suggests that the late maturing females would be ready
460 to spawn in March (Bender et al. 2016). The high frequency of early maturing females may
461 be an evidence of stress resulting in reduced investment into reproductive development

462 (Rideout et al. 2005; Kime, 1995). However, with only a single histological sampling point
463 it is not possible to resolve if the females in the early maturation stage initiated
464 vitellogenesis at the same time as females in the late maturing phase and then paused
465 further development or if vitellogenesis was ongoing at a reduced pace. Nevertheless,
466 abnormal oocyte development observed in some early maturing females (i.e.
467 nonconforming yolk globule orientation) may suggest that vitellogenesis was interrupted
468 and that these oocytes may soon be reabsorbed through atresia (Rideout et al. 2005).
469 Reabsorbing vitellogenic oocytes result in a lower fecundity and have been observed in
470 Atlantic cod under environmental stressors like low temperature, poor nutritional, and
471 pollution (Rideout et al. 2005). However, no increased incidence of atresia was observed
472 in early maturing females at sampling. The increased frequency of early maturing females
473 in the BO exposure group could indicate a reduced population fecundity compared to the
474 unexposed and control groups. The large variation in oocyte size of early maturing females
475 exposed to BO treatment may be early signs of reabsorption of vitellogenic oocytes or of
476 some other disruption of oogenesis. PAHs have endocrine disrupting properties with
477 potential to impair vitellogenesis in fish (Hylland et al. 2006; Aruwke and Goksøyr, 2003).
478 Despite low tissue PAH concentrations, reproductive impairment was seen in Gulf killifish
479 two months after the Deepwater Horizon oil spill (Whitehead et al. 2012). Similarly,
480 depressed plasma 17β-estradiol concentrations were seen in dolly varden and yellowfin sol
481 after the Exxon Valdez oil spill (Sol et al. 2000).
482
483 Although the overall THC/PAH concentration in BO was an order of magnitude lower than
484 in MDO and CDO, differences in physical characteristics of the BO may have altered the
485 exposure route and time exposed to the BO treatment and thus enhanced the toxicity of the
486 BO residue compared to MDO and CDO. Burned oil residues have increased viscosity and
487 stickiness compared to crude oils (Fritt-Rasmussen et al. 2015; Fingas, 2016). The size of
488 oil droplets and BO particles were not measured in this experiment; however, BO particles
489 were most likely larger than MDO and CDO oil droplets as they could be observed with
490 the naked eye as "black dots" in the water column during the exposure. In contrast to BO
491 particles, mechanically and chemically dispersed oil droplets are generally found to be in
492 the size of ≤ 100 μm and 10-50 μm, respectively (Lessard and DeMarco, 2000; Lewis and

493 Daling, 2001), and could not be observed by eye. The BO residue may have clogged gills,
494 adhered to skin, and/or been ingested and stick to the digestive tract of the fish and thereby
495 increased the exposure time to PAHs and other compounds (including UCM) present in
496 the BO residue compared to MDO and CDO. For instance, burned residues can be enriched
497 in high molecular weight PAHs, pyrogenic PAHs, and metals (Buist 2004; Shigenaka et
498 al. 2012; Fingas, 2016). Indeed, the UCM profiles of burned oil residues from DWH burns
499 have an altered shape compared to unburned fresh oil with enrichment of more volatile *n*-
500 alkanes (Stout and Payne, 2016). These compounds were not quantified in this study
501 despite their potential contribution to adverse effects. Other studies investigating acute
502 toxicity of BO residues have found non-toxic or little effects on snails and amphipods at
503 concentrations below 1.46 mg/L THC or 5.83 µg/L total PAHs when exposed for 24 hours
504 (Gulec & Holdway, 1999). No additional effect of the WSF after burning on *Calanus* spp.
505 when exposed for 96 h at concentrations less than 1 mg/L THC compared to the WSF prior
506 to burning (Faksness et al. 2012). Australian bass exposed to burned oil WAF for four days
507 did express EROD activity levels, a biomarker of PAH exposure, similar to levels in fish
508 exposed to mechanically dispersed oil WAF but significantly lower than fish exposed
509 chemically dispersed WAF (Cohen et al. 2006) However, knowledge on the toxicokinetics
510 of BO compared to other treatments is still lacking and there is a strong uncertainty when
511 it comes to toxicity of UCM.
512
513 Gonadal investment occurred earlier in males compared to females in accordance with
514 other studies investigating polar cod reproductive development (Bender et al. 2016;
515 Nahrgang et al. 2014). No effect of any treatment on the timing, structure, or investment in
516 male reproductive development indicated the relative resilience of this sex. Male polar cod
517 invest less energy in reproductive development compared to females (Hop et al. 1997),
518 which may allow for greater tolerance to xenobiotic exposure during the reproductive
519 development period. Inclusion of the unexposed fish into the experimental design provided
520 additional information on background physiological change due to size differences. The
521 smaller unexposed fish (Unexp. 1) were generally younger and less likely to mature in the
522 current season, with an increased prevalence of immature individuals and lower HSI
523 compared to their larger unexposed counterparts (Unexp. 2). Maturing individuals in

524 Unexp. 1 had generally lower GSI values than maturing fish in larger size categories
525 emphasising the importance of size in reproductive output (Nahrgang et al. 2014). The
526 Unexp. 2 fish were of a similar age and larger size (both length and weight) than fish
527 included in the exposure experiment but exhibited a higher mortality rate and no immature
528 individuals further supporting the hypothesis that mortality is related to previous spawning
529 events.
530
531 *4.4. Conclusion and Outlook*
532 The transient effects observed on growth rate in the present study did not affect overall
533 growth and survival of the polar cod during this 7-month experiment, demonstrating the
534 robustness of adult polar cod. Early life stages of polar cod however have demonstrated
535 increased sensitivity to crude oil (Nahrgang et al., 2016) and therefore are a major source
536 of uncertainty when assessing population level impacts of a potential spill and response
537 actions. The decreased frequency of maturing females exposed to the BO treatment is of
538 importance with regard to potential reductions in population fecundity (Spromberg and
539 Meador, 2006) and may reveal a sensitivity of polar cod when exposed to dispersed BO
540 residues from this OSR countermeasure. This effect observed on the potential reproductive
541 output of female polar cod exposed to BO is not explained by the relatively low THC and
542 PAH levels measured in the BO treatment, therefore other hydrocarbon compounds, the
543 UCM, physical properties and toxicokinetics of the BO residue warrants further
544 investigation.
545
546 With increasing anthropogenic activity in the Arctic, polar cod are at risk for exposure to
547 petroleum and OSR actions through accidental spills. The purpose of a NEBA is to aid in
548 the decision making of an OSR and evaluate the environmental effects of an action or
549 combination of actions *a priori*. However, no long-term effects on polar cod survival and
550 growth were observed under acute dynamic exposure conditions to BO, MDO or CDO.
551 Observed effects were overall limited. The physiological effects of BO need further
552 investigation, including exposure method validation and additional chemical analysis. The
553 reduction of overall oil by ~90% with *in-situ* burning will reduce the oil volume and the
554 potential for organisms to come into contact with the oil and may still be a viable option

555 despite the potential adverse effects observed in this study. The NEBA process will help
556 deciding what response strategy eventually will lead to the least environmental impact and
557 fastest recovery. Overall, this study demonstrates the robustness of the adult life stage of
558 polar cod to a variety of OSR actions. The final endpoints of reproduction, such as
559 fecundity, fertilization success and survival and fitness of offspring of exposed polar cod,
560 were not included in the present study, however these endpoints would provide valuable
561 information on ecosystem sensitive for the NEBA in the Arctic marine system. This study
562 provides new evidence to aid in OSR decision making on the sensitivities of the Arctic key
563 species polar cod.

564 *Ethics Statement*

565 Permission to carry out this experiment was granted by the Norwegian Animal Welfare Authority
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580

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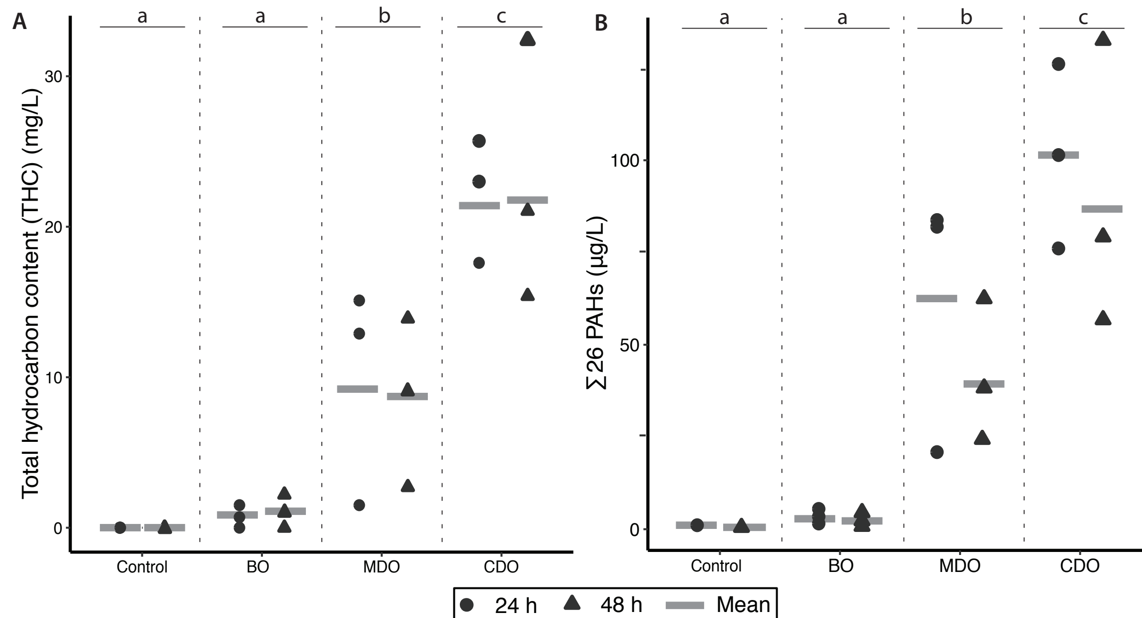

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2 Figure 1. (a) THC and (b) ∑26 PAH concentrations at t24 (circles) and t48 (triangles) for
3 all treatment groups with mean concentrations illustrated by black bars. Treatment mean
4 concentrations that do not share a letter are significantly different ($p<0.01$).

5

6 **Table 1.** Summary of polar cod sampled in January after a 7-month monitoring period
7 following 48 h exposure to *in situ* burned oil residues [BO], mechanically dispersed oil
8 [MDO], and chemically dispersed oil [CDO] treatment, and a control group. Unexposed
9 fish have size distributions which fall outside the intermediate range included in the
10 exposure experiment. Age, as determined by otoliths, total length, total weight,
11 hepatosomatic index (HSI), and condition factor were calculated for all fish. All values
12 are mean ± SE.

	Number of fish sampled							
Treatment	Females	Males	Total	Age	Length (cm)	Total Weight (g)	HSI (%)	Condition factor
Control	27	20	48	4.7 ± 0.1	20.0 ± 0.2	56.5 ± 2.0	9.3 ± 0.3	0.53
BO	26	22	49	4.7 ± 0.1	19.8 ± 0.2	56.4 ± 2.4	9.3 ± 0.3	0.53
MDO	20	19	40	4.4 ± 0.1	19.7 ± 0.2	54.3 ± 2.1	9.5 ± 0.4	0.53
CDO	25	18	46	4.5 ± 0.1	19.9 ± 0.2	56.2 ± 2.1	9.6 ± 0.3	0.54
Unexp. 1	12	18	30	3.8 ± 0.2	17.5 ± 0.2	38.0 ± 1.8	8.5 ± 0.3	0.55
Unexp. 2	17	6	23	4.6 ± 0.2	22.3 ± 0.3	80.0 ± 2.8	9.4 ± 0.4	0.54

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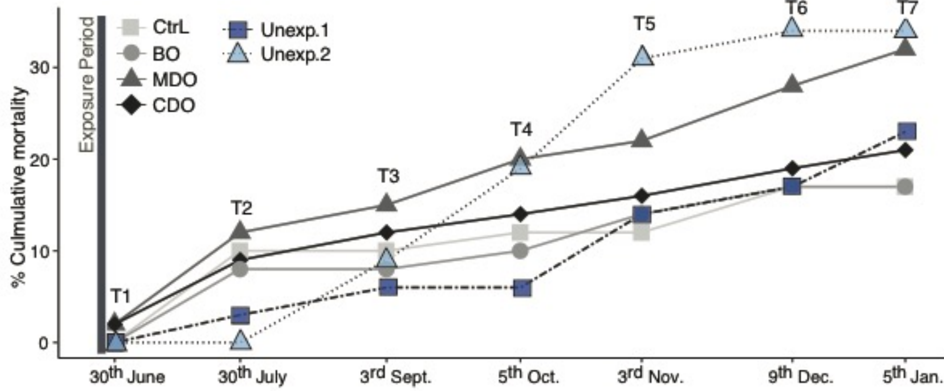

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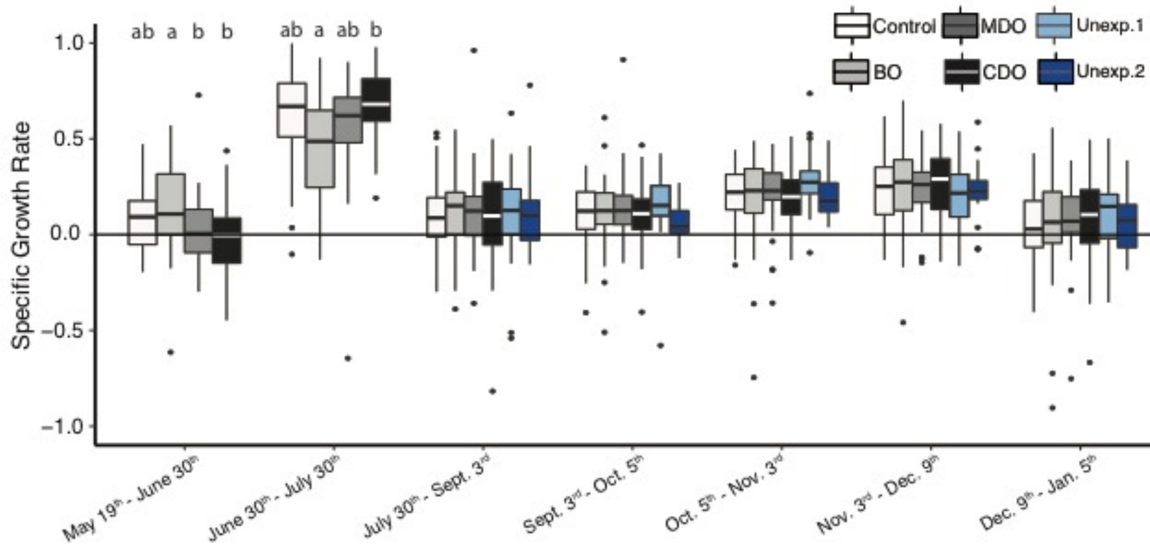
19 Figure 2. Cumulative mortality (% of overall mortality) of polar cod during the course of
20 the exposure and post exposure period (June 2015 – January 2016) for each treatment
21 group. No significant difference in % mortality was found between treatment groups,
22 control or unexposed groups.



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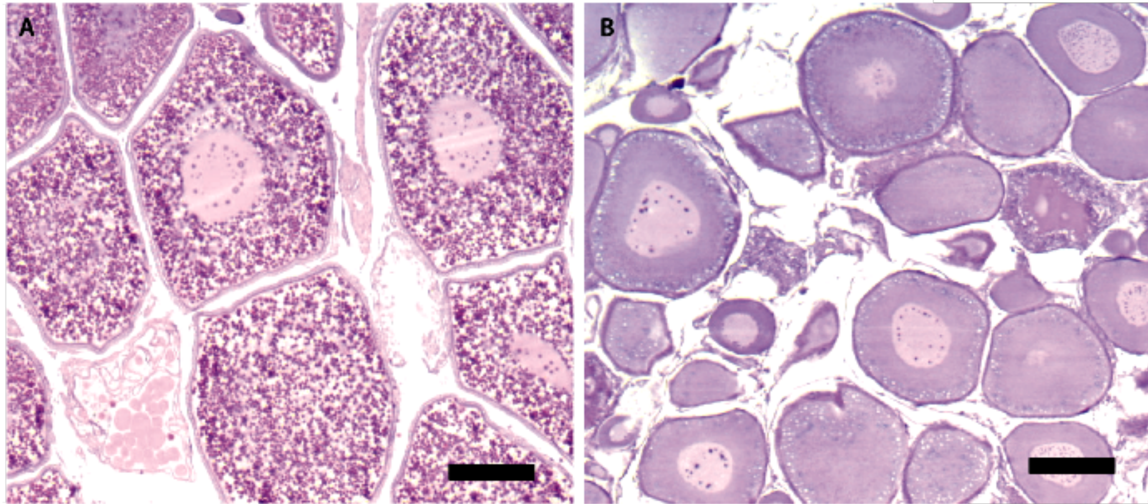
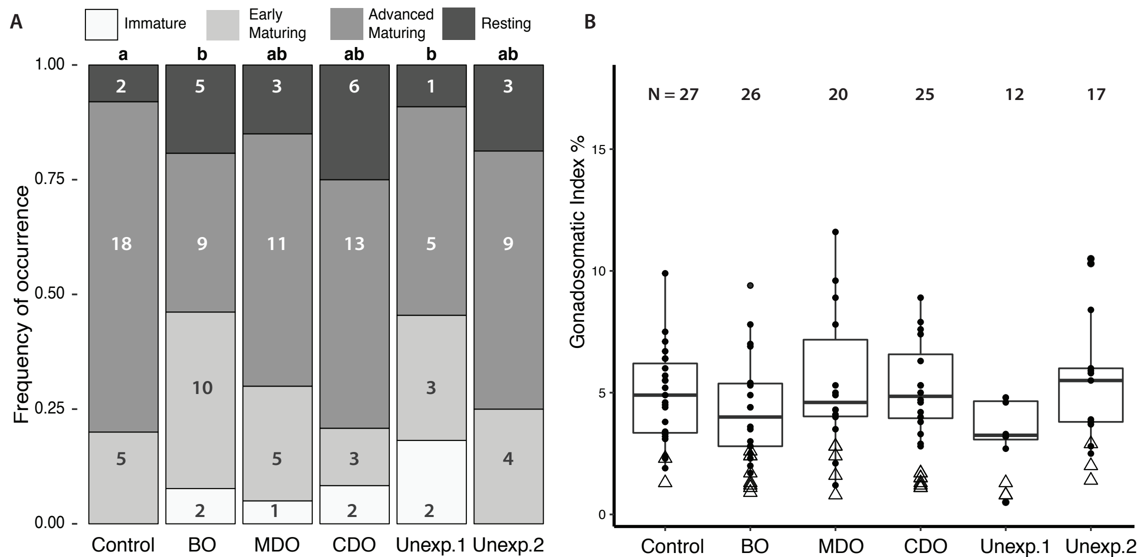
26 Figure 3. Specific growth rate (SGR; % change in body weight per day) of different post-
27 exposure time periods (n=40-49 per treatment [Unexp. n is 23-30 fish]). The box ranges
28 from the first (Q1) to the third quartile (Q3) of the data and represents the interquartile
29 range (IQR). The line across the box indicates the median while the extreme data
30 (outliers) outside Q1 − 1.5 × IQR and Q3 + 1.5 × IQR are displayed as individual points.
31 Limits of displayed data are only for SGR values from 1 to -1 excluding values outside
32 that range. Different letters indicate significant differences between treatment groups.

33



34 Figure 4. Histological representation of an (a) early maturing female with cortical alveoli
35 vesicles and early signs of vitellogenesis with yolk globules present in oocyte periphery;
36 (b) advanced maturing female with vitellogenic oocytes. Scale bare is 200 µm in both
37 pictures.



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40 Figure 5. (A) Maturity stage frequency distribution of females from all treatments; (B)
41 boxplots of GSI of female fish in different treatment groups, maturing females are plotted
42 in the boxplots and immature and resting females are indicated at triangles. Different
43 letters above treatment groups indicate significant differences between treatment groups,
44 those with letters in common are not significantly different from one another. The
45 number in each box refers to the number of fish in that category.
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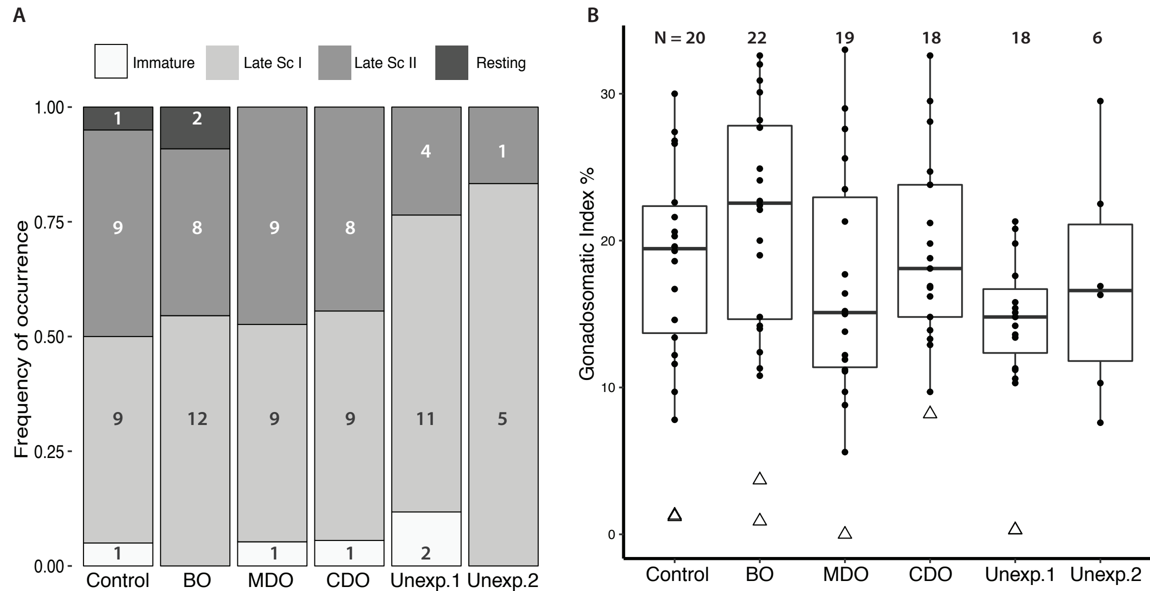

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50 Figure 6. (A) Maturity stage frequency distribution of males from all treatments; (B)
51 boxplot of GSI of male fish in different treatment groups, maturing males are plotted in
52 the boxplots and immature and resting males are indicated at triangles. No significant
53 differences were found between treatments groups. The number in each box refers to the
54 number of fish in that category.