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Effects of Dispersant and Oil on Survival and Swimming Activity in a Marine Copepod

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Abstract Knowledge of lethal and sublethal effects of crude oil and dispersants on mesozooplankton are important to understanding ecosystem impacts of oil spills in marine environments. Here we (1) establish median lethal concentrations for water accommodated fractions of Corexit EC9500A dispersant, MC-252 crude oil (WAF), and dispersed crude oil (CEWAF) for the coastal copepod *Labidocera aestiva*, and (2) assess acute effects on *L. aestiva* swimming activity. Mortality assays with *L. aestiva* support that copepods are more sensitive than other zooplankton taxa to dispersant toxicity, while WAF and CEWAF are generally similar in their toxicity to this copepod species and other zooplankton. Acute effects on *L. aestiva* activity included impaired swimming upon WAF and CEWAF exposure. These results highlight that copepods are particularly sensitive to dispersant exposure, with acute effects on survival most evident with dispersant alone, and on swimming behavior when dispersant is mixed with crude oil.

Keywords Corexit EC9500A · Water accommodated fraction · Chemically-enhanced water accommodated fraction · *Labidocera aestiva*

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Zooplankton play a key role in marine pelagic ecosystems as grazers on phytoplankton and microzooplankton, exporters of carbon and nutrients from surface waters, and vectors for carbon and toxins to higher trophic levels (Banse 1995). As such, zooplankton responses to oil and the consequent impacts on ecosystem function during and after prolonged oil releases are of interest to understanding ecosystem effects of such pollution events. Some sustained oil spills have caused declines in zooplankton biomass during subsequent years, along with changes in species composition and ecosystem function, while other spills have resulted in negligible changes in zooplankton biomass and community structure (Penela-Arenaz et al. 2009). Mesozooplankton in the northern Gulf of Mexico accumulated polycyclic aromatic hydrocarbons (PAHs) from Macondo well oil during the 2010 Deepwater Horizon spill (Mitra et al. 2012), but long-term effects on zooplankton in the Gulf of Mexico resulting from such exposure remain to be determined. A decline in mesozooplankton abundance following oil spills is predicted from field and mesocosm studies, but this negative response appears to depend on dispersant additions rather than oil alone, and is mediated by interactions between lower trophic levels and oil/dispersants (Almeda et al. 2013). This highlights that knowledge of both direct and indirect toxicity, as well as sublethal effects that in turn influence trophic interactions, are critical to understanding population and community level responses to spills. Such data are necessary for informed application of chemical dispersants in the future. However, studies on both oil and dispersant toxicity in zooplankton have primarily focused on a few model taxa, which limit application of these results to highly diverse zooplankton populations affected by spills (e.g., Mitra et al. 2012). Accordingly, in this study we examined both acute lethal and behavioral effects of water accommodated

fractions of the dispersant Corexit® EC9500A (Corexit), crude oil alone (WAF), and crude oil with dispersant (CEWAF) on a common coastal copepod, *Labidocera aestiva*. Mortality assays were conducted to establish lethal levels of exposure, with additional experiments testing effects of acute exposure on *L. aestiva* swimming activity.

Materials and Methods

Water accommodated fractions of oil and dispersant solutions were prepared in 2 L aspirator flasks in darkness according to standard CROSERF methods (Aurand and Coelho 2005). All solutions used 0.4 μm-filtered ambient seawater (FSW) from Boca Ciega Bay (FL, USA; salinity = 34). Oil was Macondo (MC-252) crude oil sampled in May 2010 by British Petroleum from a riser insertion tube during the *Deepwater Horizon* oil spill, and maintained in a sealed vial at 4°C until use. Oil WAF was prepared by variable loading (Aurand and Coelho 2005) at nominal concentrations of 0.1–125 mg oil L^{-1}. CEWAF was prepared at nominal concentrations of 1–50 mg oil L^{-1}, with oil loading immediately followed by the dispersant Corexit EC9500A (Nalco Company) at an oil:dispersant ratio of 10:1. Total petroleum hydrocarbons (TPHs) were measured in WAF and CEWAF test solutions at Mote Marine Laboratory (Sarasota, FL USA) following extraction using modified EPA Method 3510C, prior to which samples were spiked with 5-α androstane and *o*-terphenyl standards. TPHs were analyzed by GC–FID (Agilent 7890A) on a Phenomenex ZB-5MSi capillary column (30 m × 0.25 μm film thickness × 0.25 mm i.d.) with ultrahigh-purity helium as the carrier gas using modified EPA Method 8260. The injector (splitless mode) and FID were set to 250 and 340°C, respectively. The oven temperature program was as follows: 40°C (2 min hold), then 6°C min^{-1}–320°C (16 min hold) for a total run time of 64.67 min. The results are reported based on integration of the FID signal over the hydrocarbon range from *n*-C9 to *n*-C42 and quantified using an internal standard, with reported concentrations corrected for the background TPHs concentration in filtered seawater used to prepare all test solutions. A performance-based quality-assurance and quality-control program, which included the parallel analysis of procedural blanks, matrix spikes, and standard reference materials was implemented to ensure data of the highest quality. The GC response was monitored every 10–12 samples with product check standards. Procedural blanks were checked to confirm they were clear of targeted analytes. Test solutions containing only Corexit were prepared as for CEWAF, but lacking oil loadings, at nominal concentrations of 2.73–22.63 mg dispersant L^{-1}. Corexit concentrations in these solutions were measured by absorbance (Beckman 610 UV/VIS spectrophotometer) at 360 nm in a 100 mm pathlength quartz cuvette against a Corexit standard curve prepared immediately prior to use. The method's detection limit was based on the lowest detectable calibration standard of 1.55 mg L^{-1}. Control solutions were prepared with FSW added at the largest volume of oil or dispersant for the corresponding experiment.

Copepods were collected from Boca Ciega Bay by plankton net (333 μm mesh) between 08:00 and 10:00. Within 1 h of collection, adult male and female *L. aestiva* that were active and swimming were sorted following Gibson and Grice (1977) into mixed-sex groups of five individuals in 5 mL FSW for immediate use in experiments. Following CROSERF static-renewal protocols for mortality experiments (Aurand and Coelho 2005), glass bottles (125 mL) with Teflon-lined caps were partially filled with test solution, and a group of five *L. aestiva* in 5 mL FSW was gently added, along with 160–180 newly hatched *Artemia* nauplii as food. Replicate bottles (n = 8 per concentration of each test solution, but see Fig. 1) were filled completely, capped to exclude all air, and placed in darkness in an incubator at 23°C (t_0). After 24 h (t_{24}), 100 mL of test solution was gently pipetted away. Copepods were scored under a stereomicroscope as *alive* (active swimming and/or regular appendage movement), *incapacitated* (on bottom of bottle with occasional twitching movements and/or digestive tract pulsation over 2 min), or *dead* (on bottom of bottle with no appendage or digestive tract movement over 2 min). Dead copepods were removed and the bottles refreshed with newly prepared solutions and food, then sealed and returned to the incubator. After another 24 h (t_{48}), copepod survival was again scored. Salinity, pH, and dissolved oxygen were measured in freshly prepared test solutions (t_0), and in pooled samples from three experimental bottles at each concentration of the test solutions (t_{24} and t_{48}). LC_{50}s were calculated for both t_{24} mortality and total mortality at t_{48} by the Trimmed Spearman–Karber method (Hamilton et al. 1977). Additional WAF and CEWAF solutions were prepared at approximate LC_{50}s for *L. aestiva* (8.8 mg L^{-1} WAF, and 8.0 mg L^{-1} CEWAF) and analyzed for PAHs. Approximately 50 PAHs, including parent compounds and their homologues, were analyzed in a 1 μL injection volume of extract (as described above for TPHs) by GC/MS (Agilent 7890A/5975C). Analyte separation was achieved on an Agilent HP-5MS capillary column (30 m × 0.25 μm film thickness × 0.25 mm i.d.) with ultrahigh-purity helium as the carrier gas. PAHs were determined in electron impact selective ion-monitoring mode (EI-SIM) with helium as the carrier gas at 1 mL min^{-1}. The injector (splitless mode) and transfer line temperatures were set to 250 and 280°C, respectively. The oven temperature program was as follows: 35°C (1 min hold), then 6°C min^{-1} to 275°C

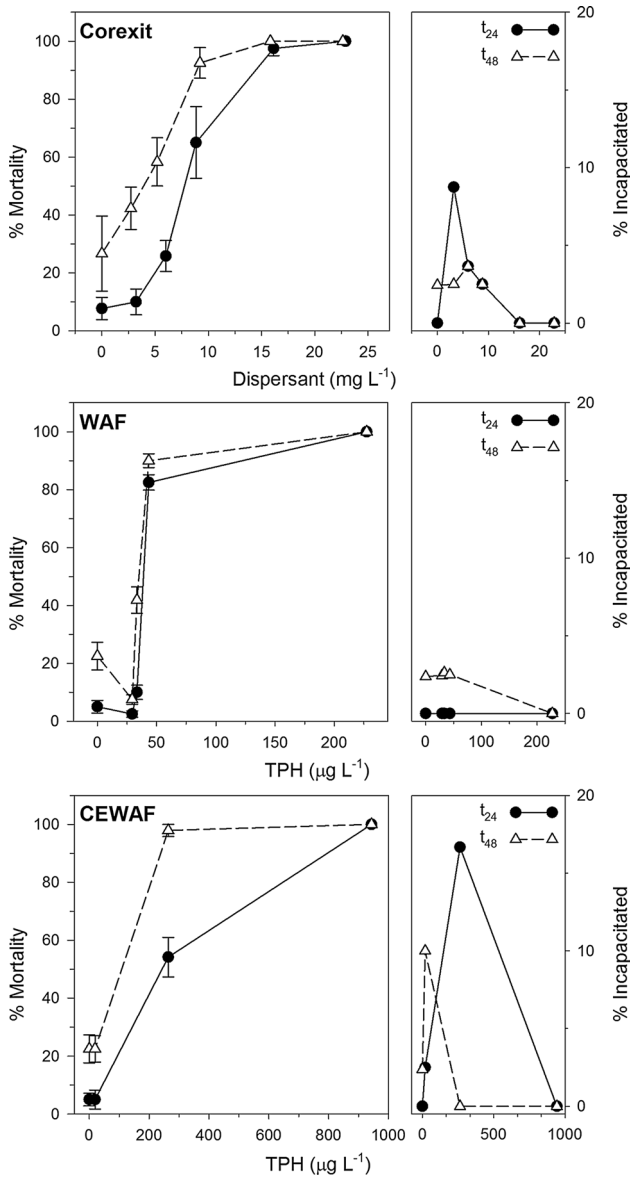

Fig. 1 Mortality in the copepod *L. aestiva* exposed to dispersant, oil, and dispersed oil. *Left panels* are mortality *curves*, where data are mean (±SE, n = 8 bottles of five copepods for all treatments except Corexit controls where n = 16) percentage mortality of copepods after 24 h (t_{24}, *solid circles*) and 48 h (t_{48}, *open triangles*) exposures as a function of measured dispersant or TPH concentration. *Right panels* are the percentages of tested copepods in each treatment (40–82 copepods treatment^{-1}) that were scored as "incapacitated" plotted as a function of measured concentration

(10 min hold) then 3°C min^{-1} to 300°C for 3 min for a total run time of 62.33 min. The source and quadrapole temperatures were set to 230 and 150°C, respectively. Individual and standard mixtures of PAHs were purchased from AccuStandard (New Haven, CT, USA). Contaminant levels were quantified from a 5-point calibration curve. The method's detection limit was based on the lowest detectable calibration standard of 20 μg L^{-1}. All mass spectral data were compared to spectra produced by authentic standards and to previously published library spectra.

A time course for acute sublethal effects of oil and dispersant on swimming activity of *L. aestiva* was measured. A subset of test solutions was prepared as above: FSW, 1 and 5 mg L^{-1} Corexit, 10 and 50 mg L^{-1} WAF, and 10 and 50 mg L^{-1} CEWAF. For WAF and CEWAF, these approximated the nominal concentration of the 24-h LC_{50} (10 mg oil L^{-1}) for *L. aestiva*, and 5-times that value (50 mg oil L^{-1}), while for dispersant they were the loadings used in these CEWAF preparations. The intent of these select concentrations was to examine swimming behavior upon immediate exposure to treatment solutions at concentrations showing copepod incapacitation in mortality studies and thus not to create a dose–response curve (see Results and Discussion). Such a scenario would mimic copepods exposed to a patch of oil/dispersant in the environment, and represent an ecologically relevant time scale at which to observe behavioral effects of pollutants (e.g., Michalec et al. 2013). Replicate groups (n = 5–6) of 30 copepods, collected as described above, were tested for each solution. For a given replicate, 30 copepods were added to an acrylic cuvette (5 × 5 × 5 cm) containing a test solution that was then capped to exclude all air. The cuvette was placed in darkness, backlit with far-red light (>740 nm), and copepod swimming activity was recorded to digital video as described in Smith and Cohen (2012). The entire cuvette was in the camera's field of view, and the camera's depth of field was sufficient to resolve copepods at all points between the front and back walls. Swimming activity was analyzed in video records at 0, 15, and 30 min following placement of a cuvette into darkness. The number of copepods actively swimming in the cuvette and number on the cuvette bottom were counted, serving as one endpoint for toxicity. CellTrak software (Motion Analysis Corp.) was then used to track the simultaneous positions of individual copepods swimming in the X–Y plane of the video during a single 3 s period at each time point for calculation of another endpoint, swimming speed. To minimize wall effects, only copepods in the center of the cuvette (i.e., away from the cuvette sides, top, and bottom) were tracked. On average, 46 copepods (±9, SD) were tracked for each time point of each test solution. Swimming speed was calculated for each individual based on the change in X–Y position between successive video frames, and averaged for the individual's track. Number swimming and swimming speed were compared across exposure time within a test solution using a 1-way repeated measures (RM) ANOVA and Holm–Sidak post hoc tests. Swimming activity within 0, 15, and 30 min time points was compared across test solutions by 1-way ANOVAs and Holm–Sidak post hoc tests.

Table 1 Median lethal concentrations of Corexit EC9500A dispersant, crude oil WAF, and CEWAF for the copepod *L. aestiva*

Treatment	Time	LC_{50} (95 % CI)		
		TPH (μg L^{-1})	Dispersant (mg L^{-1})	Nominal loading (mg L^{-1})
Corexit	t_{24}	–	7.8 (7.0–8.7)	12.9 (11.7–14.4)
	t_{48}	–	4.5 (3.7–5.5)	8.5 (7.0–10.3)
WAF	t_{24}	40.5 (36.2-45.2)	–	9.8 (6.7–14.5)
	t_{48}	37.5 (34.8-40.4)	–	5.0 (2.8–9.1)
CEWAF	t_{24}	190.4 (130.2–278.3)	–	8.2 (5.6–12.0)
	t_{48}	74.3 (68.5–80.6)	–	3.3 (3.1–3.5)

Results and Discussion

Mean mortality of *L. aestiva* increased above FSW control levels with increasing Corexit, WAF, and CEWAF solution concentration, saturating at 100 % mortality after 48 h (Fig. 1). Apart from oil/dispersant additions, water quality was sufficient (e.g., Stalder and Marcus 1997) for copepod survival throughout the 48 h exposures with salinity of 34, dissolved oxygen ranging between 3.4 and 6.6 mg L^{-1} (median = 5.0 mg L^{-1}), and pH between 7.56 and 8.23 (median = 8.07). Corexit and CEWAF LC_{50}s calculated from either nominal or measured concentrations were higher at t_{24} than t_{48} as expected, whereas no difference was observed between t_{24} and t_{48} LC_{50}s for WAF (Table 1). At each time point, LC_{50}s for measured dispersant concentrations were lower than for nominal dispersant loadings (Table 1). Few studies exist for Corexit EC9500A toxicity in copepods; available data suggest LC_{50}s in the copepods *Acartia tonsa* (48-h LC_{50} = 2.2 μL L^{-1}) and *Eurytemora affinis* (96-h LC_{50} = 5.9 μL L^{-1}) (rev. in George-Ares and Clark 2000; Wise and Wise 2011) are similar to *L. aestiva*. Studies are needed for a broader range of zooplankton taxa (e.g., Goodbody-Gringley et al. 2013), but available data (e.g., Wise and Wise 2011) suggest other zooplankton are less sensitive to Corexit EC9500A than copepods. In contrast, toxicity of crude oil to *L. aestiva* appears similar to both the smaller copepod *A. tonsa* (Avila et al. 2010) and to other zooplankton (e.g., Neff et al. 2000; Almeda et al. 2013), but these comparisons are difficult given variability in oil types and experimental methods across studies.

A common scenario during pelagic oil spills is exposure to dispersed oil rather than either oil or dispersant alone, and uncertainty remains regarding lethal effects of dispersed oil on zooplankton relative to oil alone (Wise and Wise 2011). When chemical dispersants are applied it is generally thought that the toxicity of oil itself does not change, but rather exposure of planktonic organisms to dissolved hydrocarbons increases as floating oil is degraded. This ultimately leads to greater hydrocarbon exposure for a planktonic organism with a given amount of oil in the water column when dispersant is present (George-Ares and Clark 2000). This was observed in the present study where CEWAF solutions had higher TPH concentrations for given nominal oil loadings. Interestingly, in mortality assays with *L. aestiva*, LC_{50}s for CEWAF solutions calculated as TPHs concentration were significantly greater than for WAF solutions at each time point, but no differences were observed between CEWAF and WAF when LC_{50}s were calculated based on nominal oil loadings. If CEWAF was more toxic to this copepod, then the WAF/CEWAF ratio for 48 h LC_{50}s calculated as TPH should be several-fold less than 1 due to the elevated TPH in CEWAF, while that ratio calculated from nominal loadings would be several-fold greater than 1 given that far less oil would be needed in CEWAF to achieve the same level of mortality (e.g., Wu et al. 2012). Given the calculated LC_{50}s (Table 1), our WAF/CEWAF ratios for TPH and nominal loading were 0.5 and 1.5, respectively. These suggest WAF and CEWAF have similar toxicities to *L. aestiva*, and dispersant addition did not meaningfully increase hydrocarbon toxicity. Wu et al. (2012) reported a similar ratio for TPH-based LC_{50}s of trout embryos, leading them to similarly conclude that nominal LC_{50}s alone may overestimate CEWAF toxicity.

TPHs themself do not accurately reflect toxicity of oil, and two- to four-ring PAHs are considered more representative of bioavailable toxicity in hydrocarbon solutions prepared from crude oil (Neff et al. 2000). TPHs concentrations in WAF and CEWAF test solutions approximating *L. aestiva* LC_{50}s were 61 and 255 μg L^{-1} respectively, which are within the range reported for epipelagic waters during oil spills (e.g., Haddad and Murawski 2010). In these solutions, total PAHs were similar between WAF (70.8 μg L^{-1}) and CEWAF (74.3 μg L^{-1}), and alkylated naphthalenes were the dominant PAHs (67 %–69 %; Fig. 2) as reported in other studies (e.g., Neff et al. 2000; Wu et al. 2012). Concentrations of alkylated PAHs in both WAF and CEWAF (6.4 and 7.6 mg PAH g^{-1} nominal oil) were higher than non-alkylated PAHs. However, CEWAF was enriched relative to WAF in C3-naphthalene and all greater molecular weight alkylated PAHs (Fig. 2). Naphthalenes have been shown to accumulate in mesozooplankton tissues during oil spills (Mitra et al. 2012), and alkylated naphthalenes are more toxic to copepods than their parent compound (Barata et al. 2005). CEWAF did not appear to be acutely more toxic than WAF in mortality experiments with *L. aestiva*, but PAH measurements are consistent with the potential for increased toxicity in

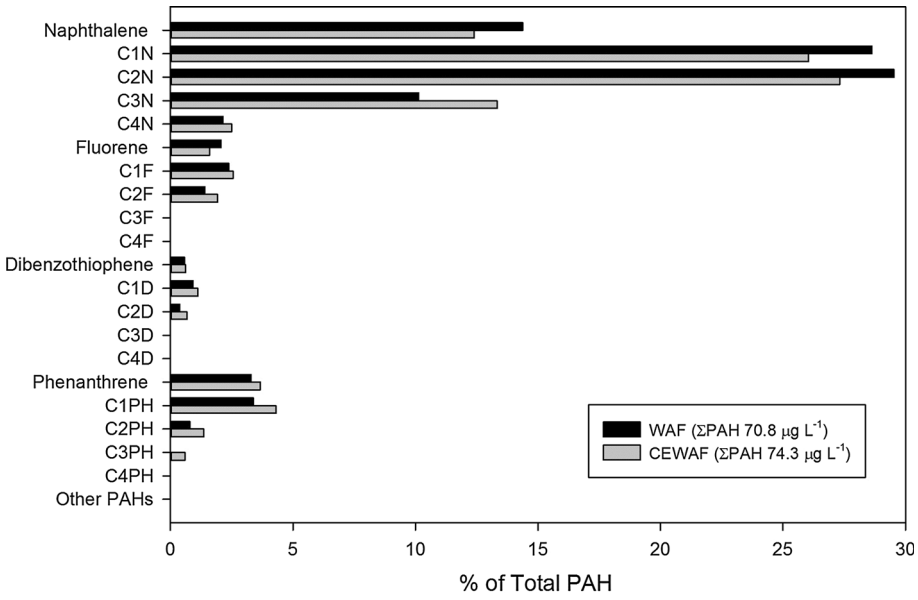

Fig. 2 PAHs in WAF and CEWAF. Classes of PAH, represented as parent compounds and their alkylated homologues (C1–C4), measured in freshly prepared 8.8 mg L^{-1} WAF (*black bars*) and 8.0 mg L^{-1} CEWAF (*grey bars*). 'Other PAHs' refers to the sum of the remaining PAHs that are EPA priority pollutants and their alkylated derivatives

CEWAF relative to WAF. CEWAF had proportionately more alkylated naphthalenes and a higher concentration of three-ringed alkylated PAHs (fluorene, dibenzothiophene, phenanthrene) (Fig. 2) which are more toxic to copepods than either two-ringed naphthalenes or in most cases their three-ringed parent compounds (Barata et al. 2005). It may be that these less soluble compounds were contained in droplets too large for ingestion by *L. aestiva*; oil droplet ingestion has been suggested as a potential route of exposure for PAH accumulation in zooplankton (Almeda et al. 2013). This question of bioavailability of PAHs in dispersed oil for uptake by zooplankton warrants further study.

In *L. aestiva* mortality assays, test solutions differed in the magnitude of incapacitation evident upon microscopic examination of copepods at t_{24} and t_{48} (Fig. 1). Incapacitation occurred at or below the calculated LC_{50}s in Corexit and CEWAF solutions, with a greater percentage of tested copepods incapacitated at t_{24} as compared to t_{48}. In contrast, individuals were either actively swimming or dead in all WAF solutions at t_{24}, with incapacitation in sublethal concentrations comparable to control levels at t_{48} (Fig. 1). Concentration-dependent narcosis, which has been reported in zooplankton during the initial hours to days of continuous crude oil and hydrocarbon exposure (e.g., Barata et al. 2005; Almeda et al. 2013), likely explains the incapacitation observed in 24-h Corexit and CEWAF exposures, where inactivity at the 24 h observation was associated with subsequent death during the remainder of the 48 h exposure period.

Additional support for narcosis during mortality assays comes from swimming activity experiments where narcosis was evident as an increased number of copepods on the cuvette bottom (Fig. 3). In these experiments, more copepods were observed swimming in the cuvettes over time, with the strongest effect seen in FSW controls, and little or no difference across time in dispersant or oil solutions (Fig. 3). The number of individuals swimming at each of the 0, 15, and 30 min time points varied depending on the experimental solution ($p = 0.005$, $p < 0.001$, and $p = 0.003$, respectively, 1-way ANOVAs). After 15 min, fewer individuals were swimming in the 50 mg L^{-1} WAF as well as the 10 and 50 mg L^{-1} CEWAF solutions than in FSW, with reduced numbers swimming relative to FSW continuing at 30 min in the highest concentrations ($p < 0.05$, Holm–Sidak tests). It was apparent from video records that many non-swimming copepods in these three solutions were alive but moving with reduced activity on the bottom of the cuvette, suggesting narcotization.

Speeds of swimming copepods are consistent with this interpretation. Individual *L. aestiva* swam fastest upon introduction to darkness (i.e., 0 min) and decreased with time in FSW controls and also in the 10 mg L^{-1} WAF and 1 mg L^{-1} Corexit solutions ($p < 0.05$, 1-way RM ANOVAs; $p < 0.05$, Holm–Sidak tests) (Fig. 3). The elevation in swimming speed upon addition to darkness could have resulted either from hyperactivity upon introduction to a novel chemical stimulus such as hydrocarbons or surfactants (e.g., Michalec et al. 2013), or from a stereotypic startle response upon sudden light decreases that is common to estuarine/coastal copepods for predator avoidance (Buskey et al. 1987) and would result from placing light-acclimated copepods in darkness. The former is not likely as copepods were in FSW prior to being added to cuvettes, and swimming speed was also elevated in FSW controls at the 0 min time point. Rather, the lack of elevated

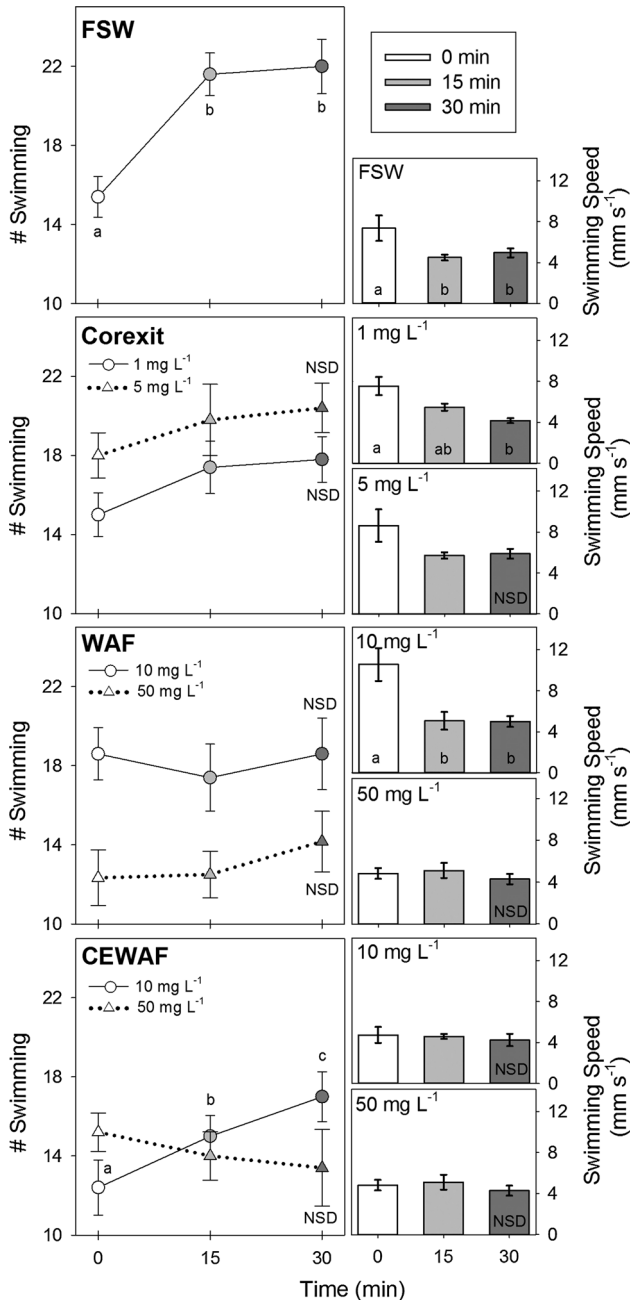

Fig. 3 Effects on swimming activity in the copepod *L. aestiva* exposed to dispersant, oil, and dispersed oil. *Left panels* are *L. aestiva* actively swimming 0, 15, and 30 min after addition to test solutions. Means (±SE, n = 5–6 replicate groups of 30 copepods) are plotted for both low (*circles*, *solid line*) and high (*triangles*, *dotted line*) nominal concentrations. WAF loading of 50 mg L^{-1} corresponds to 43.4 µg TPHs L^{-1}; TPHs data are not available for 10 mg L^{-1}. CEWAF loadings of 10 and 50 mg L^{-1} correspond to 263.9 and 943.3 µg TPHs L^{-1}. *Right panels* are mean speeds (±SE) of actively swimming copepods. Differences within each treatment across time identified by 1-way RM ANOVA and Holm–Sidak post hoc tests are shown by letters; *NSD* no significant difference, $\alpha = 0.05$

swimming speeds at the 0 min time point seen in copepods in the 50 mg L^{-1} WAF as well as in both CEWAF solutions ($p > 0.05$, 1-way RM ANOVAs) (Fig. 3) suggests narcotization in these solutions, and rapid disruptive effects from WAF and CEWAF exposure to photoreception and/or motor behavior in otherwise active copepods.

In conclusion, these collective results suggest copepods may be particularly vulnerable to dispersant toxicity. Further, given that behavioral endpoints also demonstrate toxicity of WAF and to a greater extent CEWAF, such approaches are useful for assessing more subtle effects of oil and dispersant toxicity in zooplankton.

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