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## Physiological responses of Oxyrrhis marina to a diet of virally infected Emiliania huxleyi

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The coccolithophore *Emiliania huxleyi* forms some of the largest phytoplankton blooms in the ocean. The rapid demise of these blooms has been linked to viral infections. *E. huxleyi* abundance, distribution, and nutritional status make them an important food source for the heterotrophic protists which are classified as microzooplankton in marine food webs. In this study we investigated the fate of *E. huxleyi* (CCMP 374) infected with virus strain EhV-86 in a simple predator-prey interaction. The ingestion rates of *Oxyrrhis marina* were significantly lower (between 26.9 and 50.4%) when fed virus-infected *E. huxleyi* cells compared to non-infected cells. Despite the lower ingestion rates, *O. marina* showed significantly higher growth rates (between 30 and 91.3%) when fed infected *E. huxleyi* cells, suggesting higher nutritional value and/or greater assimilation of infected *E. huxleyi* cells. These results show that virally infected *E. huxleyi* support higher growth rates of single celled heterotrophs and in addition to the "viral shunt" hypothesis, viral infections may also divert more carbon to mesozooplankton grazers.

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14	Oxyrrhis marina, virus, carbon, grazing, growth, fatty acids, food web
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24 Abstract:

The coccolithophore *Emiliania huxleyi* forms some of the largest phytoplankton blooms 25 in the ocean. The rapid demise of these blooms has been linked to viral infections. E. huxlevi 26 abundance, distribution, and nutritional status make them an important food source for the 27 heterotrophic protists which are classified as microzooplankton in marine food webs. In this 28 study we investigated the fate of E. huxleyi (CCMP 374) infected with virus strain EhV-86 in a 29 simple predator-prey interaction. The ingestion rates of Oxyrrhis marina were significantly lower 30 (between 26.9 and 50.4%) when fed virus-infected *E. huxleyi* cells compared to non-infected 31 cells. Despite the lower ingestion rates, O. marina showed significantly higher growth rates 32 (between 30 and 91.3%) when fed infected *E. huxleyi* cells, suggesting higher nutritional value 33 and/or greater assimilation of infected E. huxleyi cells. No significant differences were found in 34 35 O. marina cell volumes or fatty acids profiles. These results show that virally infected E. huxleyi support higher growth rates of single celled heterotrophs and in addition to the "viral shunt" 36 hypothesis, viral infections may also divert more carbon to mesozooplankton grazers. 37

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#### 39 INTRODUCTION

Cell lysis, due to viral infection, accounts for up to 30% of daily mortality rates of marine 40 microorganisms (Suttle 1994; van Hannen et al. 1999), influences shifts in microbial community 41 structure (Martínez Martínez et al. 2006; Thingstad 2000), and is hypothesized to result in the 42 reduction of net primary productivity (Suttle 1994). Conventional dogma holds that virus-43 induced cell lysis can divert energy away from the traditional food web by releasing the organic 44 carbon (C), nitrogen (N), and phosphorus (P) in phytoplankton cells to the dissolved phase, 45 fueling an active bacterial population. This process, known as the "viral shunt", is hypothesized 46 to transfer 6 - 26% of C (estimated 150 gigatons of C per year) from photosynthetic plankton to 47 the dissolved organic pool (Suttle 2005; Wilhelm & Suttle 1999). However, to the best of our 48 knowledge, the magnitude of the C "shunt" during viral infection has not been directly measured. 49 A quantitative understanding of the pathways and factors that affect the flow of organic C in 50 marine systems is key to understanding community structure and for predicting resource 51 availability to support important commercial species. Although it is known that viral infection of 52 algal cells alters crucial cellular and biogeochemical processes (Evans et al. 2009; Gilg et al. 53 2016; Malitsky et al. 2016; Rosenwasser et al. 2014; Suzuki & Suzuki 2006), the impacts of 54 these changes on the nutritional value of cells and on the grazing and growth rates of both micro-55 and macrozooplankton are largely unexplored (Evans & Wilson 2008; Vermont et al. 2016). 56

57 The coccolithophore *Emiliania huxleyi* is a globally distributed and abundant oceanic 58 phytoplankton species whose blooms can cover thousands of square kilometers (Holligan et al. 59 1993). They are a key component in pelagic food webs contributing essential amino acids and 60 fatty acids (FA) to the base of the food chain, which are crucial for supporting multiple cellular 61 functions and growth in higher trophic level organisms. The collapse of *E. huxleyi* blooms have

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62	been linked to infection by double-stranded (ds) DNA viruses (EhVs) (Bratbak et al. 1993;
63	Brussaard et al. 1996; Wilson et al. 2002). Infection with EhV causes rapid physiological
64	changes in <i>E. huxleyi</i> that divert host resources toward virus replication and assembly; e.g.,
65	decreased photochemical efficiency (Gilg et al. 2016) and altered metabolic pathways such as
66	glycolysis, FA, and nucleotide biosynthesis (Evans et al. 2009; Malitsky et al. 2016;
67	Rosenwasser et al. 2014). Within three hours post inoculation with EhV, infected cultures shift
68	from producing polyunsaturated (PUFA) to monounsaturated (MUFA) and saturated (SFA) fatty
69	acids relative to non-infected cultures (Floge 2014). Additionally, viral infection can increase the
70	uptake capacity of N and P by expressing viral genes which code for nutrient transporters not
71	found in the host's genome and increase access to diverse nutrient sources unavailable to
72	uninfected cells (Monier et al. 2017; Monier et al. 2012; Wilson et al. 1996). High P and/or N
73	resources are critical for optimal viral proliferation in phytoplankton hosts (Maat & Brussaard
74	2016; Maat et al. 2016; Mojica & Brussaard 2014; Monier et al. 2017), including E. huxleyi
75	(Bratbak et al. 1993; Martínez Martínez 2006) At the scale of large oceanic E. huxleyi blooms it
76	remains unclear if the sum of viral alterations enhance or diminish the overall amount of C and
77	essential nutrients that are passed to higher trophic levels.
78	Predation by heterotrophic and mixotrophic protists (microzooplankton) dominates
79	grazing on phytoplankton in aquatic microbial food webs, and plays a key role in C cycling and
80	nutrient regeneration (Sherr & Sherr 2002; Sherr & Sherr 2009). The heterotrophic

*huxleyi* cells, relative to uninfected cells (Evans & Wilson 2008). Although the mechanism

driving this preferential grazing is unclear, Evans and Wilson (2008) proposed possible changes

dinoflagellate Oxyrrhis marina has been shown to preferentially graze on EhV-infected E.

84 in prey size, motility, nutritional value, palatability, and chemical cues as potential causes.

However, to the best of our knowledge, those results have not yet been reproduced in any
independent studies. Also, one aspect that was not investigated is *if* and *how* preferential grazing
on infected *E. huxleyi* might modify the transfer of C and essential nutrients up the food web. In
this study we investigated the effect of viral infection on the growth and ingestion rates, cell
volume, and FA composition of *O. marina* cells to better understand how the grazing behavior
and physiology of microzooplankton is influenced by viral infection of abundant and important
phytoplankton prey.

#### 92 MATERIALS AND METHODS

#### 93 <u>Culture maintenance:</u>

Clonal Emiliania huxleyi strain CCMP 374 (non-axenic, non-calcifying; 3-5µm) and non-94 axenic clonal O. marina strain CCMP 1795 were obtained from the Provasoli-Guillard National 95 96 Center for Marine Algae and Microbiota (NCMA-Bigelow Laboratory, Maine, USA). A nonaxenic clonal Dunaliella sp. strain was sourced from the University of South Carolina. E. 97 huxleyi, O. marina, and Dunaliella cultures were maintained at 16 °C under a light:dark cycle 98 (14:10 h; 250  $\mu$ moL photons m<sup>-2</sup> s<sup>-1</sup>). *E. huxlevi* and *Dunaliella* sp. cultures were kept in 99 exponential growth phase by periodically transferring 10% (v/v) culture into fresh f/2-Si 100 seawater medium (Guillard 1975). O. marina stock cultures were fed weekly with fresh 101 Dunaliella sp. cultures (5% (v/v)). Fresh EhV-86 (Wilson et al. 2002) lysates were obtained by 102 inoculating E. huxleyi cultures in exponential growth phase. Once culture clearance was 103 observed (typically 3 – 5 days post inoculation (p.i.)), cell debris was removed by filtration (0.45 104  $\mu$ m PES filter) and the EhV-86 lysates were then stored at 4°C in the dark for up to two weeks 105 prior to being used in an experiment. The same EhV-86 lysate stock was used to determine 106 107 infection dynamics and for grazing experiments 1 - 3 (see experimental details in the sections

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108	below). Cell and virus concentrations were measured using a FACScan flow cytometer (Beckton
109	Dickinson, Franklin Lakes, NJ), equipped with an air-cooled laser providing 50 mW at 488 nm
110	with standard filter set-up, as previously described (Brussaard 2004; Marie et al. 1999).
111	Emiliania huxleyi virus infection dynamics:
112	Fifty milliliter aliquots of exponentially growing E. huxleyi culture were inoculated at
113	four EhV-86 to host ratios of 5:1, 20:1, 50:1, and 100:1, in triplicate. Fresh f/2-Si media was
114	added to each flask in order to achieve the same <i>E. huxleyi</i> cell concentration in all flasks.
115	Aliquots were taken from each culture at 2, 4, 6, and 20 h p.i. for cell enumeration using flow
116	cytometry (FCM). Cells were stained with the orange fluorescent lipid-specific dye N-(3-
117	Triethylammoniumpropyl)-4-[4-(dibutylamino)styryl] pyridinium dibromide (FM 1-43,
118	Invitrogen Co., Carlsbad, CA, USA) to allow discrimination between visibly infected and non-
119	infected E. huxleyi cells (Martínez Martínez et al. 2011) (Fig. S1). Progression of the viral
120	infection was quantified by tracking the percentage of visibly infected E. huxleyi over time.
121	Emiliania huxleyi C and N content:
122	A culture of <i>E. huxleyi</i> at exponential growth phase was divided in two equal volumes.
123	One of the aliquots received EhV-86 to achieve a 50:1 virus:host ratio; the second one received
124	an equal volume of fresh f/2-Si medium to achieve similar cell concentration in both cultures.

125 *Emiliania huxleyi* concentration and percentage of visibly infected cells in each flask was

determined immediately after the addition of EhV-86 and f/2-Si medium at 5 h and at 24 h p.i.

127 Six 5 ml samples were taken from each flask immediately after the addition of EhV-86 and f/2-Si

medium and at 24 h p.i. and were gravity filtered through a combusted glass fiber filter

129 (Whatman GF/F; GE Healthcare Life Sciences, Pittsburg, PA) to collect particulate matter. The

130 filtrates were then passed through fresh combusted GF/F filters to serve as C and N background

controls (residual dissolved C and N retained by the filters). Filters were stored at -80° C until
analysis. Prior to testing, the filters were dried at 45 °C for 24 h before being placed in 9 × 10
mm Costech tin capsules using clean forceps and sample preparation block. Calibration
standards were prepared from acetanilide. The samples, standards, and filter blanks were
analyzed using a Costech ECS 4010 elemental analyzer (980°C combustion).

#### 136 *Oxyrrhis marina specific growth and grazing rates:*

Four independent experiments (experiments 1-4) were performed. Oxyrrhis marina was 137 not fed for 3 days prior to each experiment to ensure their feeding vacuoles were empty. FCM 138 139 was employed to check for the absence of prey-derived chlorophyll red autofluorescence signal within *O. marina* vacuoles after the 3-day period. Stock *E. huxleyi* cultures ( $\sim 1 \times 10^6$  cells ml<sup>-1</sup>) 140 were split into two equal volumes. One of the flasks was inoculated with fresh EhV-86 lysates to 141 142 achieve the virus:host ratios specified for each experiment in Table 1. The second flask received f/2-Si media equal to the virus stock volume to match the dilution of cells. The flasks were 143 incubated without shaking under the standard culture conditions indicated above. Incubations 144 were carried out for 6 h to allow sufficient viral infection levels (see results from virus infection 145 dynamics below, Fig.1). Equal volume aliquots of either infected or non-infected E. huxleyi 146 cultures were fed to triplicate O. marina cultures. Additional aliquots of the E. huxleyi cultures 147 (infected and non-infected) were maintained separately as non-grazing controls. It should be 148 noted that non-grazing control cultures were not maintained during experiment 3, instead, 149 average E. huxleyi growth rates from experiment 2 were used to normalize for E. huxleyi cell 150 growth and lysis. Both these experiments employed the same EhV stock, virus:host ratio, and 151 culture volume, and only differed in the length of time of the experiment (Table 1). We have 152 153 shown in this study and elsewhere (Gilg et al. 2016; Vermont et al. 2016) that under comparable

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154	conditions infection dynamics and virus production are highly reproducible. E. huxleyi and O.
155	marina cell concentrations were monitored in each flask by FCM. Prey and predator cell
156	concentrations were measured immediately after the initial feeding and every 30 min for the first
157	2 h and then every hour up to 6 h for experiment 1 (Fig. S2) and every 24 h for experiments $2 -$
158	4. During experiments 2 – 4 <i>O. marina</i> cultures were fed, either infected (6 h after virus addition)
159	or non-infected E. huxleyi cultures, immediately after determining cell concentrations at the end
160	of each 24 h incubation period for a total of $3 - 7$ days (Table 1). The time length of our
161	experiments is ecologically relevant and representative of high rates of viral infection during
162	induced blooms of mixed assemblies of <i>E. huxleyi</i> (Castberg et al. 2001; Martínez Martínez et al.
163	2007). Fresh prey cell additions were calculated to bring the prey:predator ratio to the same level
164	as at the beginning of the experiment. Sterile f/2-Si medium was added, as needed, to the O.
165	marina cultures to maintain comparable cell concentration between treatments. Additional
166	experimental design information can be found in Table 1. Oxyrrhis marina specific growth and
167	grazing rates were determined by the equations of Frost (1972) and used to calculate O. marina
168	growth per E. huxleyi cell consumed.

#### *Projected Oxyrrhis marina's abundance:* 169

The removal of cells within samples collected at each time point together with the 170 subsequent additions of fresh prey cells and f/2-Si medium to maintain prey and predator cell 171 concentrations at near to initial concentrations hindered direct measurement of total O. marina 172 production and prey cells consumed. Consequently, we performed a mathematical projection to 173 estimate both consumption and production of O. marina. Average growth and grazing rates of 174 individual cultures from experiments 2 - 4 (n=9) were combined to calculate the overall average 175 ± 1 standard error (SE) growth and grazing rates of *O. marina* fed either infected or non-infected 176

177 *E. huxleyi* cells. We postulated a starting population size of 6,000 *O. marina* cells and assumed 178 *E. huxleyi* prey saturation and no mortality for *O. marina* over a 7-day period. We applied the 179 overall *O. marina*'s average  $\pm 1$  SE growth rate over the 7-day period to calculate a reasonable 180 abundance range for each diet. We then used the equations of Frost (1972) with the overall *O.* 181 *marina*'s average  $\pm 1$  SE grazing rates and the hypothetical population size calculated above to 182 predict the potential total ingestion of *E. huxleyi* cells under such scenario.

#### 183 *Oxyrrhis marina and E. huxleyi fatty acid (FA) analysis:*

The effect of feeding on virally infected or non-infected E. huxleyi on the FA 184 composition of O. marina was investigated during experiment 2. Aliquots of 5 ml for FA 185 analysis were taken from non-infected E. huxleyi cultures and from cultures 6 h after inoculation 186 with EhV-86 (in duplicate), as well as from O. marina cultures (in triplicate) before feeding them 187 188 with E. huxleyi cells (Day 0) and after three days being fed E. huxleyi (Day 3). Samples were vacuum filtered through a combusted glass fiber filter (Whatman GF/F; GE Healthcare Life 189 Sciences, Pittsburg, PA), and stored at -80° C until analysis. FAs were converted to FA methyl 190 191 esters (FAMEs) in a one-step extraction direct methanolysis process (Meier et al. 2006) following the procedures detailed in Jacobsen et al. (2012). FAMEs were analyzed on a gas 192 chromatograph with mass spectrometric detector (Shimadzu GCMS-QP2010 Ultra, Shimadzu 193 Scientific Instruments, Columbia, MD). FAME samples were reconstituted in 200 µl of hexane 194 and 1 µl was injected into the GC/MS injector which was kept at 250°C. FAMEs were separated 195 on a SGE BPX-70 column, in a helium mobile phase at a flow rate of 1.17 ml min<sup>-1</sup>. A Supelco 196 37 Component FAME Mix (47885-U; Supelco Analytical, Bellefonte, PA) standard solution was 197 used for instrument calibration. Individual FAMEs were identified via comparison to standard 198 199 mixture peak retention times and fragmentation patterns using the NIST-library of compound

- 200 mass spectra. FAME concentrations were calculated from peak area relative to that of a C19:0 201 internal standard that was added to each sample prior to extraction. FA type concentrations were converted to percentages of the combined total FA concentration. 202
- 203 Oxyrrhis marina cell volume:

Aliquots of 500 µl were taken from a prey-depleted O. marina culture (i.e., 3 days 204 without being fed *Dunaliella* sp. cells or any other prey type) and once a day for three days from 205 each O. marina culture during experiment 4. Fixation is required to immobilize the dinoflagellate 206 cells to facilitate light microscopy examination. Common fixatives such as Lugol's or 207 glutaraldehyde alter cell volume (Menden-Deuer et al. 2001). Alternatively, live cells can be 208 immobilized by adding nickel sulfate (0.003% final concentration), which appears to have no 209 effect on cell shape and size (Menden-Deuer et al. 2001). We chose to fix culture O. marina 210 211 culture aliquots by transferring 50% (v/v) into 70% ethanol and storing at  $4^{\circ}$ C for 30 minutes prior to analysis. Ethanol did not appear to alter cell size since our results were very similar to 212 the measurements with added nickel sulfate from Menden-Deuer et al. (2001); however, no 213 direct comparison of these two methods was carried out. Ten randomly selected individual O. 214 *marina* cells from each aliquot were photographed on a hemocytometer. The hemocytometer 215 gridding served as scale for cell sizing. Images were and measured for cell width and length 216 using ImageJ (Schneider et al. 2012). Volume was calculated for each cell using the equation for 217 the volume of a rotational ellipsoid;  $V = \frac{\pi}{6} x d^2 x h$  (Edler 1979; Menden-Deuer & Lessard 2000). 218 In this calculation we assume that the width and depth of *O. marina* were equal. Differences in 219 cell volume between treatments were evaluated using a standard t-test. Total C per O. marina 220 cell was estimated based on the average cell volume using the equation  $\log pg C \operatorname{cell}^{-1} = -0.665 +$ 221 222  $\log vol \times 0.939$  (Menden-Deuer & Lessard 2000).

#### 223 <u>Statistical analyses:</u>

Temporal differences within the same diet treatment for E. huxleyi C and N content and 224 for O. marina specific growth and ingestion rates, and cell volume were analyzed with a two-225 226 tailed, paired t-test, Alpha level 0.01. When comparing parameters between treatments and for FA composition, the differences were analyzed with a two-tailed, unpaired t-test assuming equal 227 variance, Alpha level 0.01. P-values (P) < 0.05 were significant and P < 0.01 were considered 228 highly significant. 229 RESULTS 230 231 *Emiliania huxleyi virus infection dynamics:* 

The percentage of visibly infected cells (as revealed by FCM) increased at higher 232 virus:host inoculation ratios over a 20 h period. During this same period cell abundance did not 233 234 change significantly in virally-infected cultures compared to non-infected cultures (Fig. S1C). The highest virus:host ratio (100:1) yielded ~19% visibly infected *E. huxleyi* cells by 6 h p.i., and 235 ~57% by 20 h p.i. (Fig. 1); consequently we chose this ratio for experiments 1 - 3, which were 236 237 carried out with the same EhV-86 stock and under the same environmental conditions employed to determine the infection dynamics (Fig. 1). The infection dynamics of *E. huxleyi* (CCMP374) 238 and viral (EhV-86) production are highly consistent and reproducible when using the same host 239 and virus strains and conditions, in particular when using the same virus lysate stock for a series 240 of experiments within 2-4 weeks (Gilg et al. 2016; Vermont et al. 2016). Infected cells begin to 241 release virus progeny at around 4.5 h p.i. (Mackinder et al. 2009) and any cells not infected by 242 the initial EhV inoculum can become infected during successive infection rounds by the new 243 EhV progeny. At the high virus:host ratios in our study, close to 100% of the E. huxleyi cells 244 245 become infected by 24 h p.i. (Gilg et al. 2016; Vermont et al. 2016), even if not evident by FCM

(Martínez Martínez et al. 2011). In experiment 4, in which we used a fresh EhV-86 lysate stock
and carried out the inoculations at a 50:1 virus:host ratio, 36% of *E. huxleyi* cells were visibly
infected by 6 h p.i.

249 *Emiliania huxleyi C and N content:* 

Over a 24h incubation, both infected and non-infected cultures of *E. huxleyi* exhibited a 250 slight but significant increase ( $P = 4.94 \times 10^{-4}$  and P = 0.018, respectively) in C content of 9.27 ± 251 0.19 to 10.86  $\pm$  0.43 pg C cell<sup>-1</sup> ( $\pm$  SD) for infected cells and 8.94  $\pm$  0.94 to 10.42  $\pm$  0.26 pg C cell<sup>-1</sup> 252 <sup>1</sup> for non-infected cells, respectively. Carbon content was not statistically different between 253 treatments (P = 0.42 at t0 and P = 0.057 at t24) (Fig. 2A, Table S1). *Emiliania huxleyi* N content 254 was not statistically different between samples at the beginning of the experiment (P = 0.989), 255 but it increased significantly over the 24 h incubation; from  $1.51 \pm 0.08$  to  $1.89 \pm 0.13$  pg N cell<sup>-1</sup> 256 (± SD) ( $P = 6.36 \times 10^{-4}$ ) for infected cells and  $1.51 \pm 0.05$  to  $2.09 \pm 0.07$  pg N cell<sup>-1</sup> (P = 1.80 x 257 10<sup>-5</sup>) for non-infected cells, respectively. The N content of non-infected cells was significantly 258 higher than in non-infected cells after the 24 h incubations (P = 0.008) (Fig. 2B). 259

260 *Oxyrrhis marina specific growth rate:* 

The 6 h duration of experiment 1 was too short to measure *O. marina* growth rates. In the longer experiments, the growth rates (experiments 2-4) of *O. marina* ranged from 0.28 to 0.43 day<sup>-1</sup> (average  $0.35 \pm 0.08$  day<sup>-1</sup> ( $\pm$  SD)) when fed non-infected prey and from 0.47 to 0.56 day<sup>-1</sup> (average  $0.52 \pm 0.05$  day<sup>-1</sup>) when fed infected prey (Fig. 3A, Table S2). Specifically, *O. marina* specific growth rates were 30% (P = 0.002), 43.4% ( $P = 5.29 \times 10^{-6}$ ), and 91.3% (P = 0.006) higher when fed infected *E. huxleyi* during experiments 2, 3, and 4, respectively (Fig. 3 A). Based on the average growth rates, and assuming no loss term for *O. marina* cells, we calculated

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an increase of *O. marina* cells over a 7-day period that is 233% higher with a diet of infected
than with a diet of non-infected *E. huxleyi* cells (Fig. 3D).

270 *Oxyrrhis marina ingestion rates:* 

During the initial 1.9 h in experiment 1, *O. marina* ingestions rates were not significantly 271 different (P = 0.68) when feeding on infected (14.48 ± 0.18 cells Om<sup>-1</sup> h<sup>-1</sup> (± SD)) vs uninfected 272 cells  $(14.88 \pm 1.57 \text{ cells h}^{-1})$ . Between 1.9 and 6 h no additional ingestion was measurable (Fig. 273 3B, Fig. S2, Table S2). Initial pulse-feeding following a period of starvation is commonly 274 observed in grazing experiments and it is likely the reason for the equal ingestion rates we 275 measured in experiment 1. The combined results from grazing experiments 2-4 yielded 276 ingestion rates that were on average 35.4% lower (P = 0.001) for O. marina fed infected (39.74 ± 277 12.14 cells  $Om^{-1} day^{-1}$ ) versus non-infected *E. huxleyi* cells (60.34 ± 10.13 cells  $Om^{-1} day^{-1}$ ) (Fig. 278 3B, Table S3). Compared to experiment 1, the higher total number of ingested E. huxlevi cells, 279 both infected and non-infected, measured in experiments 2 - 4 indicated that O. marina resumed 280 ingestion after 6 h, as prey cells were digested. Also, the relatively low standard deviation values 281 indicated daily ingestion rates were fairly constant from day to day in experiments 2-4. 282 Normalizing O. marina growth rate to the number of cells ingested renders the highly significant 283  $(P = 1.60 \times 10^{-5})$  differences between diets even more striking (i.e., 86.30%, 238.62%, and 284 154.44% higher when fed infected *E. huxleyi* cells, for experiments 2, 3, and 4 respectively) (Fig. 285 3C, Table S2).Combining the higher growth rate of *O. marina* (i.e., higher end abundance, Fig. 286 3D) and the average ingestion rates (Fig. 3B), we estimated that the total consumption of virus-287 infected E. huxleyi cells would exceed that of non-infected cells after 4 - 5 days and would be on 288 average 63.2% higher for virus-infected E. huxleyi over a 7-day period (Fig. 3E). 289 Oxyrrhis marina and E. huxleyi fatty acid analysis: 290

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291 Minor differences were detected in the proportions of individual FAs between non-

infected and recently-infected *E. huxleyi* cultures (Table 2). Similarly, minor differences in the

FA profile were observed in *O. marina* that had consumed infected versus non-infected cells.

294 The cultures containing O. marina fed infected E. huxleyi contained slightly higher proportions

of C17:0 and 2-fold higher proportions of C20:2 (Table 2).

296 *Oxyrrhis marina cell volume:* 

The average volume of *O. marina* cells was not significantly different (P = 0.21) between individuals fed a diet of infected (5226 ± 1267 µm<sup>3</sup> (± SD)) versus non-infected (4706 ± 1259

299  $\mu$ m<sup>3</sup>) *E. huxleyi* cells (Table 3, Fig. S3).

#### 300 DISCUSSION

The results presented here show compelling evidence that feeding on virus-infected E. 301 302 huxleyi fuels O. marina specific growth, but it does not significantly alter their FA profile and cell size. Furthermore, we show that the higher growth rates of O. marina were not due to higher 303 ingestion rates of infected cells or to a tradeoff of O. marina cells becoming smaller (i.e., 304 305 containing less C and nutrients per cell). Consequently, the higher growth efficiencies of O. marina feeding on virally infected E. huxleyi cells suggest that viral infection of prey cells 306 increases the production of microzooplankton. These results suggest a shift in the "viral shunt" 307 paradigm by pointing toward the flow of organic matter to higher trophic levels being enhanced 308 by viral infection of algae rather than just being short-circuited. 309

310 *Oxyrrhris marina* feed and grow on a wide range of prey types, some prey enhance

311 growth rates more than others (Montagnes et al. 2011). Indeed this study shows that virus-

infected *E. huxleyi* cells supported higher *O. marina* growth than non-infected *E. huxleyi* cells,

despite lower ingestion rates, suggesting higher nutritional value or higher assimilation

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314 efficiency of infected prev cells. It is worth noting that O. marina ingestion rates on virally infected *E. huxleyi* cultures might have been overestimated. A reduction in prey abundance due 315 to viral lysis over each 24 h interval, prior to fresh-prey replenishment, during the experiments 316 might have led to temporarily reduced grazer-prev encounter and ingestion rates. Under such 317 scenario O. marina's growth per ingested infected cell would have be even larger than we 318 estimated, adding further significance to our results. Consequently, our study would represent a 319 conservative estimate of C transfer efficiency. 320

Based on our measurements, the mechanisms underlying the lower ingestion rates and 321 higher growth efficiency remain unknown. Calcification reduces digestion efficiency and 322 predator growth (Harvey et al. 2015). In the environment, E. huxleyi cells lose their liths during 323 an active viral infection (Brussaard et al. 1996; Frada et al. 2008; Jacquet et al. 2002). In this 324 325 study we chose a non-calcifying E. huxleyi strain to uncouple the effects of calcification and prey size, on feeding and growth rates. Furthermore, we found no differences between C content in 326 infected and non-infected E. huxleyi cells, while N content was only slightly higher in non-327 328 infected cells. Nitrogen depletion in some prey cells causes O. marina to cease grazing, possibly due to the buildup of an inhibitor or a change in prey recognition (Flynn et al. 1996; Martel 329 2009). However, C:N ratios in all of our E. huxleyi cultures (virus-infected or not) were lower 330 than 6.6, indicative that N was replete (Davidson et al. 2005; Flynn et al. 1994). Large dsDNA 331 viruses of eukaryotic algae, such as EhVs, have a high demand of C, N and P for the production 332 of lipids, proteins, and nucleotides to support typical high burst sizes. Viral infection can 333 modulate host metabolic pathways and nutrient uptake to fulfill the metabolic requirements of 334 viral production (Malitsky et al. 2016; Monier et al. 2017; Monier et al. 2012; Rosenwasser et al. 335 336 2014; Wilson et al. 1996). The production of intermediary biomolecules and changes in E.

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337 *huxleyi*'s lipidome induced by infection with EhV-86 (Evans et al. 2007; Evans et al. 2006; Evans et al. 2009; Malitsky et al. 2016; Rosenwasser et al. 2014; Suzuki & Suzuki 2006) could 338 potentially lead to increasing the nutritional value of infected cells. During EhV infection, 339 changes in biosynthesis pathways result in the production of more highly saturated FAs (Evans et 340 al. 2009; Floge 2014; Malitsky et al. 2016) and the enhanced production of sphingolipids 341 (Pagarete et al. 2009; Rosenwasser et al. 2014). It should be noted that the majority of these 342 virus-induced alterations in lipid composition have been detected after prolonged infection (> 24 343 h) of *E. huxleyi* cultures. At the relatively coarse level of detail in lipid profile carried out in the 344 345 present study, only minor differences in FA composition were observed between non-infected E. huxleyi cultures and cultures that had been infected for 6 h (Table 2). This suggests that 346 differences in FA composition between recently-infected and non-infected E. huxleyi, were not 347 responsible for the differences in growth rates of *O. marina*. However, it is possible that the 348 relatively small sample volume collected for FA analysis of *E. huxleyi* cells limited the 349 resolution and detection of differences in FA between infected and non-infected E. huxleyi cells 350 351 (Evans et al. 2009; Floge 2014; Malitsky et al. 2016). Alterations in lipid profile between infected and non-infected cells that were not apparent in our analysis, may contribute to the 352 higher growth efficiencies of O. marina fed virally infected prey cells. An additional factor that 353 may influence the nutritional value of the phytoplankton prey is P. In addition to providing a 354 much needed resource for viral replication, P-rich phytoplankton cells increase grazing 355 efficiency and secondary production in cladocerans (Elser et al. 2001; Sterner 1993; Urabe & 356 Sterner 1996; Urabe & Watanabe 1992). Low P availability reduces viral replication in E. 357 huxleyi (Bratbak et al. 1993) and other eukaryotic algae (Maat et al. 2016; Wilson et al. 1996), 358 359 possibly by limiting the production of nucleic acids. It has been hypothesized that virally

360 encoded putative phosphate transporters increase accumulation of P in host cells (Monier et al. 2012; Wilson et al. 1996). While to the best of our knowledge this has not been tested during the 361 infection of E. huxleyi cells, most available EhV isolates, including EhV-86, carry an E. huxleyi-362 homolog putative phosphate repressible phosphate permease (PPRPP) gene (Martínez Martínez 363 2006; Nissimov et al. 2011; Nissimov et al. 2012; Wilson et al. 2005), which we hypothesize led 364 to higher P uptake in virally infected cells in our experiments. Additionally, the stoichiometric 365 "light:nutrient hypothesis" poses that low supply of light relative to P yields more P-rich 366 producers (i.e., low tissue C:P ratios) (Sterner et al. 1997); possibly due to the algae allocating 367 high levels of P to light-harvesting cellular machinery and storing excess P intracellularly 368 (Hessen et al. 2002). In our study, E. huxleyi cells were grown in P-rich f/2-Si culture medium 369 and both EhV-infected and non-infected cultures were kept under the same light conditions. In 370 371 addition to the role of the PPRPP gene in P uptake, we hypothesize that virus-induced reduction in E. huxleyi's photochemical efficiency from the early stages of EhV infection (Gilg et al. 2016) 372 might also induce an increased P uptake and intracellular accumulation. While a reduction in 373 374 photochemical efficiency might translate into lower C fixation rates, our results show that C content was not affected in infected compared to non-infected cells. Phosphorus content in 375 infected and non-infected E. huxleyi cells and its impact on grazing warrants investigation in 376 future studies. 377

*E. huxleyi* is an important food source at the base of the food chain and grazing pressure influences population and bloom dynamics (Fileman et al. 2002; Olson & Strom 2002). Virusinduced mortality also plays a prominent role in bloom demise (Bratbak et al. 1993; Brussaard et al. 1996; Castberg et al. 2001; Lehahn et al. 2014; Martínez Martínez et al. 2007) and diverts organic C away from upper trophic levels to the dissolved phase, which fuels the microbial

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383 loop—"viral shunt" (Wilhelm & Suttle 1999). Our results suggest that viral infection also boosts microzooplankton production. High rates of viral infection can last from a few days (as in this 384 study) to a few weeks during a natural *E. huxleyi* bloom progression (Brussaard et al. 1996; 385 Castberg et al. 2001; Martínez Martínez et al. 2007), which could result in large differences in C 386 flow through the food web. Extrapolating the results in our study, the enhanced growth rates of 387 microzooplankton populations that feed on virally infected phytoplankton cells would lead to 388 more organic C available for higher trophic levels. Thus, contrary to the idea that viral infection 389 leads only to the production of dissolved organic matter (Wilhelm & Suttle 1999), viral 390 391 infections at the base of the food chain may augment the flow of C to higher trophic levels as well as toward the microbial loop. Although, to the best of our knowledge, the specific functional 392 response of copepods ingestion of O. marina fed infected and non-infected E. huxleyi has yet to 393 394 be investigated, nutrition and reproduction rates are enhanced in copepods fed O. marina (grown on other phytoplankton diets) compared to copepods that feed directly on small phytoplankton 395 cells (Broglio et al. 2003; Chu et al. 2008; Parrish et al. 2012; Veloza et al. 2006). Phytoplankton 396 397 are considered the primary producers of long chain n-3 (LCn-3) PUFAs; however, heterotrophic protists such as O. marina are also able to produce sterols and essential FAs (e.g., EPA (C20:5 n-398 3) and DHA (C22:6 n-3)) from lipid precursors (Chu et al. 2008; Klein Breteler et al. 1999; Lund 399 et al. 2008; Veloza et al. 2006), which emphasizes the important role of microzooplankton in 400 trophic upgrading and C transfer and highlights the need for a better quantitative understanding 401 402 of the factors that influence microzooplankton grazing behavior and secondary production rates. Incorporating quantitative data for viral lysis and the effect of viral infection in grazing behavior 403 and transfer efficiency into ecosystem models is essential for accurate budgeting of C flow 404 405 throughout the food web in the global marine ecosystem. As a cautionary reminder, when

406 interpreting these results it is important to note that O. marina is not typically found in open waters (Yang et al. 2011) and is not likely to be a common natural predator of *E. huxleyi* cells. 407 However O. marina is frequently used as a model predator in laboratory-based experiments 408 because of its morphological similarity to a wide variety of heterotrophic and mixotrophic 409 dinoflagellates and its plasticity in feeding behavior allow it to represent a broad range of marine 410 dominant microzooplankton (Lowe et al. 2011; Roberts et al. 2011). Furthermore, several studies 411 have shown that O. marina responds in a similar way to other microzooplankton taxa to various 412 experimental stimuli (Strom et al. 2003a; Strom et al. 2003b; Tillmann 2004). E. huxleyi's true 413 414 protozoan predators in nature have yet to be precisely identified (Wolfe 2000). A final consideration is that the lower ingestion rates of O. marina on E. huxleyi (strain 415 CCMP374) cells infected with coccolithovirus EhV-86, compared to non-infected cells, are in 416 417 contrast with an earlier study that used the same virus strain but a different E. huxleyi strain (CCMP 1516—non-calcifying) (Evans & Wilson 2008). Strain-specific differences in the 418 ingestion and clearance rates of O. marina feeding on E. huxleyi (Harvey et al. 2015) might have 419 420 played a role in our findings. However, in light of our findings, the results from Evans and Wilson (2008) need to be revisited and revalidated and future studies should include multiple 421 strains within a species (predator, prey, and/or virus) to test differences driven by intraspecific 422 diversity. Importantly, future research is needed that focuses on a range of abundant and 423

424 ecologically meaningful predator-prey-virus systems.

#### 425 CONCLUSIONS

426 Viruses cause biochemical alterations to their *E. huxleyi* host cells to facilitate viral assembly

427 (Gilg et al. 2016; Malitsky et al. 2016; Rosenwasser et al. 2014; Suzuki & Suzuki 2006). The

428 data presented in this study show that changes due to viral infection of *E. huxleyi* cause higher

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429 growth efficiency and an increase in heterotrophic protist production. Despite the faster growth 430 rates, we found no major difference in cell size, total FA content or FA profile of O. marina maintained on a diet of virally infected E. huxleyi cells during 3 days as compared with O. 431 *marina* individuals reared on non-infected cells for the same period of time. These results 432 suggest that after a feeding period of time of 3 days the nutritional value of *O. marina* is 433 unaffected by the infection status of their E. huxelyi diet despite known changes in the FA 434 profiles and biochemical pathways in EhV-86 infected E. huxleyi cells. Combined, these results 435 suggest that during viral infection of *E. huxleyi*, there may be a proportionally increased flow of 436 C to higher trophic levels. Thus, in addition to the "viral shunt" hypothesis, these results suggest 437 that food webs with virally infected E. huxleyi cells may shunt proportionally more C to higher 438 trophic levels than non-infected systems. In order to gain a more comprehensive understanding 439 440 of ocean ecosystem function in relation to C flow, it is crucial that we get quantitative knowledge of each of those processes and the factors that determine the relative magnitude of each pathway. 441 The significance of our work is that, given the global scale and rapid dynamics of viral infections 442 in the ocean, infection of primary producers is likely to be one of the compounding factors that 443 influences the qualitative and quantitative flow of C in oceanic systems and determines overall 444 efficiency of transfer to higher trophic levels. 445

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O. MARINA RESPONSE TO INFECTED PREY

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O. MARINA	<b>RESPONSE TO</b>	INFECTED PREY
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## Figure 1

Infection progression of E. huxleyi

Fig. 1. Infection progression of *E. huxleyi* at four different virus:host ratios; 5:1, 20:1, 50:1, and 100:1. Values are mean percentage (%) of cells visibly infected over time (hours)  $\pm$  one standard deviation.



## Figure 2

### E. huxleyi C and N concentration

Fig. 2. *E. huxleyi* C (**A**) and N (**B**) concentration (pg cell<sup>-1</sup>) at 0 and 24 hours p.i. Values are mean  $\pm$  one standard deviation. Letters indicate statistical similarity. Same letters indicate no statistical significance between compared treatments and different letters denote statistical significance.



### Figure 3

### Growth and grazing rates

Fig. 3. Differential growth and grazing rates of *O. marina* fed non-infected versus infected *E.* huxleyi. **A**: *O. marina* growth rates (day <sup>-1</sup>). **B**: *O. marina* grazing rates (Cells Om<sup>-1</sup> h<sup>-1</sup> or day<sup>-1</sup>). **C**: *O. marina* growth rate divided by grazing rate (*O. marina* divided per *E. huxleyi* consumed). Values mean  $\pm$  one standard deviation (Experiments 2, 3, and 4) and standard deviation (Experiment 1). **D**: Projected abundance of *O. marina*. **E**: Projected total consumption of *E. huxleyi*. Dashed lines are average values and shaded regions are one standard error from Experiments 2, 3, and 4. Asterisks indicate statistical significance: \*\*\* p < 0.001, \*\* p < 0.01, \* p < 0.05.



### Table 1(on next page)

Table 1. Details of experiments performed.

*Emiliania huxleyi* (Eh); *Oxyrrhis marina* (Om); o indicates that the grazing rates were calculated using *E. huxleyi* k-values from non-grazing controls in experiment 2.

#### 1 Table 1. Details of experiments performed.

	Experimental Conditions					Parameters Measured (Om)				
	EhV: Eh ratio	% infected Eh cells 6 h p.i.	Eh:Om ratio	Initial Om (cells/ml)	Vol (ml)	Duration (days)	Growth Rate	Grazing Rate	Fatty Acids	Cell Vol
<b>Experiment 1</b>	100:1	30	30:1	6000	50	0.25		Х		
<b>Experiment 2</b>	100:1	30	100:1	4500	150	3	х	х	Х	
<b>Experiment 3</b>	100:1	30	100:1	4000	150	7	х	0		
<b>Experiment 4</b>	50:1	36	100:1	6000	150	5	х	х		х

*Emiliania huxleyi* (Eh); *Oxyrrhis marina* (Om); o indicates that the grazing rates were calculated using *E. huxleyi* k values from non-grazing controls in experiment 2.

### Table 2(on next page)

Table 2. Percentage (%) of individual fatty acids to total FA concentration

Percentage (%) of individual fatty acids to total FA concentration of cultures in which: i) *O. marina* was depleted of prey at the start of the experiments (Day 0 Om); ii) the *E. huxleyi* cultures fed to *O. marina*; iii) after three days fed non-infected *E. huxleyi* (Om + Eh non-inf); and iv) after 3 days fed infected *E. huxleyi* (Om +Eh inf). Values are mean  $\pm$  one standard deviation, n = 4, n = 4, n = 3 and n = 3, respectively. Note the values for *E. huxleyi* are the average of duplicate non-infected and duplicate infected cultures. Significant differences in the proportions of individual compounds are shown as: <sup>a</sup> Day 0 Om vs. *E.huxleyi*; <sup>b</sup> Day 0 Om vs. Day 3 Om + Eh non-inf; <sup>c</sup> Day 0 Om vs. Day 3 Om + Eh inf; and <sup>d</sup> Day 0 Om vs. Day 3 Om + Eh non-inf vs. Day 0 Om vs. Day 3 Om + Eh inf.

1	Table 2. Percentage (%) of individual fatty acids to total FA concentration of cultures in which:
2	i) O. marina was depleted of prey at the start of the experiments (Day 0 Om); ii) the E. huxleyi
3	cultures fed to O. marina; iii) after three days fed non-infected E. huxleyi (Om + Eh non-inf);
4	and iv) after 3 days fed infected <i>E. huxleyi</i> (Om +Eh inf). Values are mean ± one standard
5	deviation, $n = 4$ , $n = 4$ , $n = 3$ and $n = 3$ , respectively. Note the values for <i>E. huxleyi</i> are the
6	average of duplicate non-infected and duplicate infected cultures. Significant differences in the
7	proportions of individual compounds are shown as: <sup>a</sup> Day 0 Om vs. <i>E.huxleyi</i> ; <sup>b</sup> Day 0 Om vs.
8	Day 3 Om + Eh non-inf; <sup>c</sup> Day 0 Om vs. Day 3 Om + Eh inf; and <sup>d</sup> Day 0 Om vs. Day 3 Om + Eh
9	non-inf vs. Day 0 Om vs. Day 3 Om + Eh inf.

	FA Class	Day 0 Om	E. huxleyi	Day 3 Om + Eh non-inf	Day 3 Om + Eh inf
	C14:0	$0.2\pm0.2$	$0.2\pm0.1$	$0.4 \pm 0.1$	$1.2 \pm 1.3$
	C15:0	$0.4\pm0.3$	$0.3\pm0.2$	$0.5\pm0.0$	$0.8\pm0.4$
	C16:0	$30.9\pm4.5$	$27.6\pm3.6$	$29.4\pm7.6$	$24.6\pm2.6$
SFA	C17:0	$2.7\pm0.1$	$2.7\pm0.7$	$2.7\pm0.3$	$3.2\pm0.1$ c, d
	C18:0	$36.2\pm11.1$	$60.9\pm7.6$ $^{\rm a}$	$46.9\pm6.4$	56.4 ± 7.1 °
	C20:0	$1.1 \pm 0.1$	$1.3\pm0.3$	$1.5\pm0.8$	$1.2\pm0.4$
	C22:0	$0.8\pm0.2$	$0.7\pm0.6$	$3.3\pm2.3$	$1.8\pm0.9$
	C24:0	$0.7\pm0.5$	$1.1 \pm 0.1$	$0.6\pm0.4$	$0.4\pm0.3$
	C16:1	0.8 ± 1.6	0.0	0.0	0.0
MUFA	C18:1(n-9cis)	$5.0\ \pm 4.0$	0.0 <sup>a</sup>	1.6 ±1.4 <sup>b</sup>	$1.6\pm1.6$ °
WOIA	C18:1(n-9trans)	$4.6\pm3.3$	0.0 <sup>a</sup>	$1.7 \pm 1.5$	$1.3 \pm 1.2$
	C22:1	$1.1\pm0.9$	$0.1\pm0.1$	0.0	0.0
	C18:2	$2.7 \pm 1.4$	0.0	0.0 <sup>b</sup>	$0.2\pm0.3$ c
PUFA	C20:2	$2.9\pm2.3$	$5.0 \pm 3.7$	$7.1\pm1.9^{\text{ b}}$	$3.2\pm1.3$ <sup>c, d</sup>
	C20:5 (n-3)	$1.2 \pm 1.2$	$0.1\pm0.1$	$0.2\pm0.3$	$0.3\pm0.3$
	C22:6 (n-3)	$8.6\pm7.7$	0.0	$4.0\pm4.1$	$3.9\pm3.3$
	$\sum$ SFA	73 ± 15	$95\pm4^{a}$	$86\pm9$	89 ± 6
	∑ MUFA	$12 \pm 8$	$0.1\pm0.1$ a	$3\pm3$	$3\pm3$
	∑ PUFA	$15\pm 8$	$5\pm4$	$11 \pm 6$	$8\pm3$

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### Table 3(on next page)

Table 3. Cell volumes ( $\mu$ m<sup>3</sup>) of *O. marina* fed non-infected and infected *E. huxleyi* over three days during experiment 4.

*Oxyrrhis marina* (Om); prey-depleted (prey-depl), i.e., not fed for three days; *Emiliania huxleyi* non-infected (Eh non-inf); *Emiliania huxleyi* infected with EhV-86 (Eh inf). Values are mean  $\pm$  one standard deviation.

1 Table 3. Cell volumes (µm<sup>3</sup>) of *O. marina* fed non-infected and infected *E. huxleyi* over three

Treatment	Day	Replicate	Volume (µm³)
Om prey-depl	0		$5586 \pm 917$
		А	$4723 \pm 1535$
	1	В	$5759 \pm 1123$
		С	$5696 \pm 1842$
Om + Eh non-inf	2	А	$4107 \pm 1689$
	2	В	$3801\pm 660$
		С	$5602 \pm 1045$
-	3	А	$4675 \pm 1141$
		В	$4038 \pm 1319$
		С	$3949\pm977$
	_	А	$5004 \pm 1245$
	1	В	$4286 \pm 1053$
		С	$4829 \pm 1435$
Om + Eh inf	_	А	$6105 \pm 462$
	2	В	$5561 \pm 1483$
		С	$6977 \pm 1371$
-	_	А	$4267 \pm 1218$
	3	В	$4478 \pm 1184$
		С	5527±1956

2 days during experiment 4.

3 Oxyrrhis marina (Om); prey-depleted (prey-depl), i.e., not fed for three days; Emiliania huxleyi non-infected (Eh

4 non-inf); *Emiliania huxleyi* infected with EhV-86 (Eh inf). Values are mean  $\pm$  one standard deviation.

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