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Virus infection of *Emiliania huxleyi* deters grazing by the copepod *Acartia tonsa*

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Copepods are an important link in marine food webs bridging primary and secondary production with higher trophic levels. Viral infections of phytoplankton can short circuit this traditional food web and release significant quantities of carbon from phytoplankton into the dissolved organic pool. The magnitude of the carbon released is in part dependent on the direct and indirect effects of viral infections on the grazing rates of copepods. During viral infection, phytoplankton undergo significant physiological and biochemical changes which could influence their susceptibility to herbivorous zooplankton. In this study, grazing experiments were conducted to investigate the impact of *Emiliania huxleyi* infection by the coccolithovirus EhV-86 on the ingestion rates of the calanoid copepod *Acartia tonsa*. Our results showed significantly lower ingestion of infected cells as compared to uninfected cells. High-resolution grazing experiments suggest that the lower ingestion rates are initiated within hours of infection. These results suggest that viral infections can alter food web structure and may result in lower food web efficiency. Hence, the overall impact of coccolithovirus infection on ecosystem function and carbon transfer during the progression of an *E. huxleyi* bloom should be considered in the context of copepod grazers.

KEYWORDS: copepod grazing; EhV-86; *Acartia*; *Emiliania huxleyi*; virus

INTRODUCTION

Copepods play important roles in oceanic biogeochemical cycling and the transfer of organic matter through the food web. Ingested phytoplankton cells that are not assimilated are packaged into fecal pellets (FPs) and transported to deeper waters and sediments. This transfer of surface derived organic matter provides food for deep sea organisms and is a mechanism by which the ocean sequesters atmospheric CO_2 (Turner, 2015).

Viral infections of algal cells can divert the traditional food web by releasing organic carbon within phytoplankton cells into the dissolved phase, fueling an active bacterial population. This process, known as the viral

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shunt, potentially releases 6–26% of carbon from photosynthetic plankton to the dissolved organic pool (Wilhelm and Suttle, 1999; Suttle, 2005). The magnitude of the viral shunt depends in part on the indirect effects of the viral infection on the grazing rates of both micro- and macrozooplankton. During viral infection, phytoplankton undergo significant physiological and biochemical changes (Evans, 2005; Evans *et al.*, 2006; Rosenwasser *et al.*, 2014) which could influence their susceptibility to predation. For example, in laboratory cultures, the single-celled microzooplankton *Oxyrrhis marina* selectively grazed on virus-infected cells over uninfected cells of the phytoplankton *Emiliania huxleyi* (Evans and Wilson, 2008). In a natural community selective grazing behavior could slow down the progression of viral infection by reducing the release of newly produced infectious *E. huxleyi* viruses (coccolithoviruses). This would subsequently result in less *E. huxleyi*-derived organic matter released into the dissolved phase, and potentially diminish the magnitude of the viral shunt. In contrast, avoidance of virus-infected prey cells would lead to higher *E. huxleyi* mortality via viral lysis and lead to the accumulation of viruses in the water column. Under this scenario, more dissolved organic carbon (DOC) would be released and the carbon flux through the microbial loop would be enhanced. The selection process could essentially act as a switch. If virally infected cells are avoided, more DOC passes through the microbial loop and is remineralized. If virus-infected cells are preferentially ingested organic carbon and virally-derived genetic vectors would be channeled through the traditional food web. However, the question remains, do copepods graze or avoid virally infected cells? Copepods are known to selectively feed, distinguishing between prey based on size, motility, nutritional value and palatability of the cell (Demott and Watson, 1991; Kleppel, 1993; Isari *et al.*, 2013). Furthermore, the degree of selectivity can be modified by chemical compounds released from ruptured algal cells such as amino acids (Poulet and Ouellet, 1982; Gill and Poulet, 1988; Demott and Watson, 1991) and dimethyl sulfide (DMS; Steinke *et al.*, 2006) and possibly due to changes in cell surface characteristics that occur during viral infection (Mackinder *et al.*, 2009; Rosenwasser *et al.*, 2014). Despite its potential biogeochemical significance, to our knowledge positive or negative selection of virus infected algae by copepods has not been shown.

In this study, the ingestion rates of the calanoid copepod *Acartia tonsa* were measured when fed virus infected or uninfected *E. huxleyi* cells. *Acartia tonsa* is an important copepod species in many temperate and subtropical coastal marine environments and also widely used in aquaculture (Knuckey *et al.*, 2005). Its ecological and potential economic importance has fueled numerous grazing studies (e.g. Houde and Roman, 1987; Durbin *et al.*, 1990; Chinnery and Williams, 2004). *Emiliania huxleyi* is a widely distributed, biogeochemically significant coccolithophorid species that forms large-scale oceanic blooms (Townsend *et al.*, 1994) that provide food for secondary trophic levels such as micro- and meso-zooplankton and may be decimated by viral infection (Wilson *et al.*, 2002). Grazing experiments with high temporal resolution were conducted to investigate copepod ingestion rates during different periods of the infection cycle.

METHOD

Host culture maintenance

Emiliania huxleyi (strain CCMP374 - non-axenic, non-calcifying; 3–5 μm) was obtained from the Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA-Bigelow Laboratory, Maine, USA). Viral infection is known to cause *E. huxleyi* cells to lose their liths (Brussaard *et al.*, 1996; Frada *et al.*, 2008). *Acartia tonsa* have been shown to have higher ingestion rates when feeding on non-calcified cells (Fields unpubl; ASLO-Abs 2013). We chose to use a non-calcifying strain to minimize the indirect effects of changes in calcification on ingestion rates. Cultures were maintained in exponential growth phase at 16°C in f/2-Si seawater medium (Guillard, 1975). Light (250 μmol photons m^{-2} s^{-1}) was supplied by fluorescent tubes under a light-dark cycle of 14:10 h. Cell concentrations were measured using a FACScan flow cytometer (Beckton Dickinson, Franklin Lakes, NJ), equipped with an air-cooled laser providing 50 mW at 488 nm and with standard filter setup.

Virus pathogen stocks

Emiliania huxleyi-virus (EhV) isolate EhV-86 (Schroeder *et al.*, 2002) was used throughout. Fresh working stocks of EhV-86 lysate were produced prior to performing an experiment. Briefly, 5–10% (v/v) lysate was added to an exponentially growing culture of *E. huxleyi* strain CCMP374. Once clearing of the host culture was observed, the lysate was passed through a 0.45 μm syringe filter (Sartorius AG, Germany) to remove cellular debris and most bacteria, and the filtrate containing virus was stored at 4°C for a maximum of 48 h prior to being used in an experiment. Virus concentration was calculated by flow cytometry methods (FCM) using SYBR Green I as described by Brussaard (2004).

Copepod culture maintenance

A multi-generational laboratory culture of the copepod *A. tonsa* was maintained at 16°C on a mixed diet of *Thalassiosira weissflogii* (CCMP1336; 10–12 μm), *Rhodomonas salina* (CCMP1319; 6–8 μm) and *Pleurochrysis carterae* (CCMP645; 8–12 μm) at a combined concentration of 10^5 cells mL^{-1}. All food cultures were maintained in log phase growth in L1 media.

E. huxleyi growth curves and infection dynamics

Cell surface characteristics were used as a proxy to track the infection progress. *Emiliania huxleyi* culture aliquots were collected at different time points throughout the infection and during grazing experiments (see details below). Cells were labeled with lipid-specific fluorescent dye N-(3-Triethylammoniumpropyl)-4-[4-(dibutylamino) styryl] pyridinium dibromide (FM 1–43, Invitrogen Co., Carlsbad, CA, USA) to allow for the discrimination between infected cells that display membrane blebbing and non-infected, or cells at early stages of infection prior to obvious membrane blebbing, by FCM as described by Martínez Martínez *et al.*, (2011). Labeled *E. huxleyi* cells were discriminated by FCM on the basis of their red, chlorophyll, autofluorescence at 610 nm versus the orange fluorescence of the FM 1–43 dye at 488 nm (Fig. 1B). Infection dynamics of EhV-86 on *E. huxleyi* are well characterized (Mackinder *et al.*, 2009) and highly repeatable among biological replicates within a given experiment (Fig. 1) and between experiments (Martínez Martínez *et al.*, 2011).

Grazing experiments

Flow cytometry analysis of *E. huxleyi* growth curves indicated that cell division occurs from ~1 h before the light came on until 4 h into the light phase. After this period cultures showed a slight decrease in abundance until the next division phase (Fig. 1). Also, preliminary experiments showed optimum infection with EhV-86 during cell division, independent of the light phase. Based on those results, all virus infections were done 2 h into the light phase and incubated under normal culturing conditions for at least two additional hour post infection (h PI), until the end of the cell division period, prior to being used in a grazing experiment. Virus inoculations were done at a 30:1 virus:host ratio. Four grazing experiments were conducted (Table I) to determine the ingestion rates of *A. tonsa* during different stages of the infection cycle of *E. huxleyi* (Fig. 1A). A fifth grazing experiment was conducted to determine if copepods that had ingested EhV-infected cells experienced persistent changes in grazing rates once removed from infected cultures.

Prior to each grazing experiment, adult female *A. tonsa* were removed from the stock culture and held in 0.2 μm filtered seawater for 2 h to evacuate their gut contents. All *E. huxleyi* grazing experiments were conducted at 16°C in algal concentrations of 2×10^4 cells mL^{-1}. Experiments were run in the dark and grazing jars were placed on a plankton wheel (0.25 rpm) to maintain algae in suspension. All grazing experiments included control flasks containing algae (infected or uninfected) but no copepods and grazing flasks containing algae (infected or uninfected) and copepods. Changes in cell concentration over time were measured in triplicate using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The fluid volume removed for cell counts was replaced with the same quantity of fluid at approximately the same algal concentration, to maintain a constant grazing volume and the absence of bubbles in the control and grazing flasks. The combination of incubation flask volume and the number of copepods used were chosen such that changes in phytoplankton cell numbers due to grazing would be detectable (>5%) yet not deplete cell concentrations by >20% compared to the controls flasks (no grazers). Cell diameter was determined by a Beckman Coulter Multisizer 3 Coulter Counter. Carbon content per cell was calculated using the equation for "other phytoplankton organisms" given by Strathmann (1967).

At the selected times during Experiment #2 and at the end of all the experiments, each jar was removed from the plankton wheel, gently mixed by inverting it several times, and subsampled for immediate cell counts. At the end of all the experiments, the contents of the grazing jars were gently poured through an 80 μm Nitex screen to recover the grazers. The copepods were counted and checked for mortality. Only live copepods were used in the final ingestion rate calculations. Grazing experiments were conducted on different days and the results combined into a linear time series. Individual copepods were only used once.

Experiment 1: 24-hour Grazing (starting 2 h PI). The aim of this experiment was to compare ingestion rates of *A. tonsa* on uninfected and infected *E. huxleyi* cells over a 24 h period. This experiment was conducted on three different days each using four grazing flasks and three control flasks for infected and uninfected treatments, respectively. The grazing flasks contained 0.12 *A. tonsa* mL^{-1}. Experiments were run in the dark to maintain low algal growth rates during the experiment. After the 24 grazing experiment, the copepods and FPs were

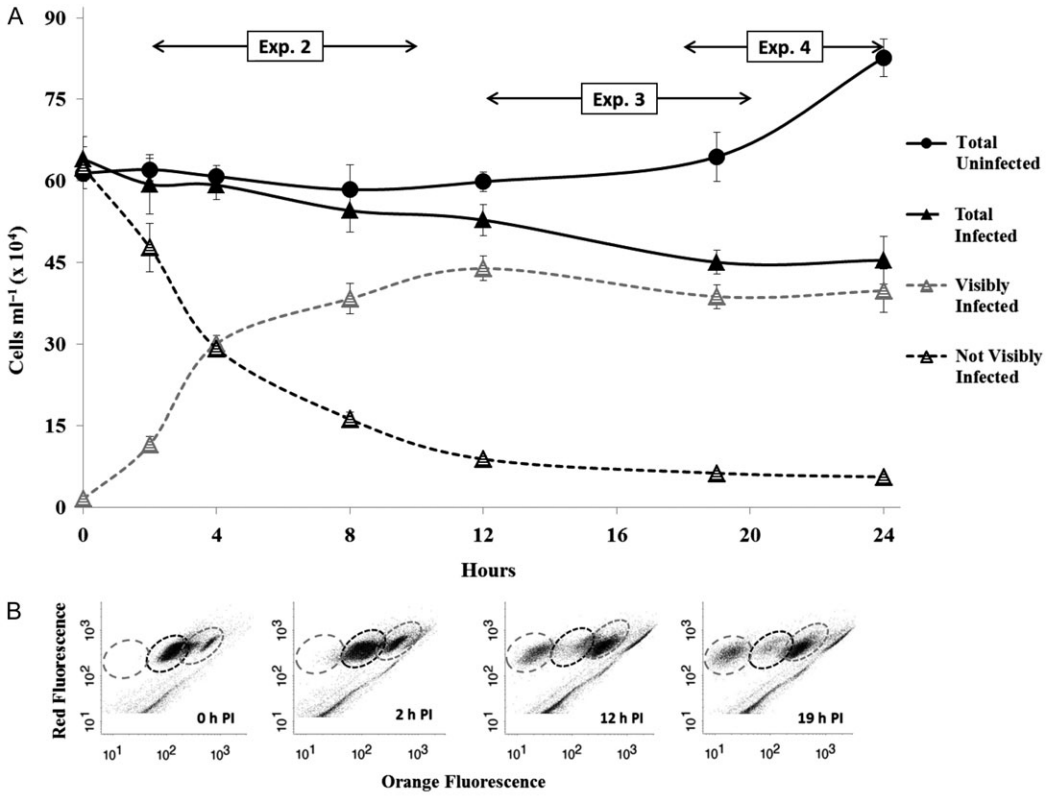

Fig. 1. Representative *Emiliania huxleyi* CCMP374 cell dynamics within uninfected (virus-free, circles) and virus-infected (with EhV-86, triangles) cultures over a 24 h period. Cultures were maintained on a 14:10 L:D cycle. Virus lysate was added to treatment cultures at T0 (2 h after the start of the light cycle), while uninfected cultures received a virus-free (0.1 μm filtered) EhV-86 lysate addition. (**A**) *E. huxleyi* cell abundance ± SE as enumerated by FCM. Total cell counts in virus-infected cultures were further divided into "visibly infected" and "not visibly infected" subpopulations based on changes in orange fluorescence of cells stained with lipid stain FM1-43 (blebbing). (**B**) FCM dotplots of virus-infected *E. huxleyi* at approximately the starting times post-inoculation (PI) chosen for the grazing experiments. As the infection progressed, changes in lipid staining were revealed by the appearance of subpopulations with relatively higher and lower orange fluorescence signal (gray dashed gates). The black dashed gate corresponds to the subpopulation of the cells not visibly infected. The dotplot at T_0 is also representative of the uninfected cultures throughout the experiment. Note that the high orange fluorescence population was also observed in the uninfected cultures, yet, unlike in virus-infected cultures, it remained a small (mean 3.6%) and relatively constant subpopulation over the course of the experiment.

Table I: Experimental design for each grazing experiment. Grazing rates in exp 2 were 628 calculated at 2 h intervals.

Grazing experiment	Hours post infection (h PI)	Flask volume (mL)	Copepods (mL^{-1})	Experiment duration (h)
Exp. 1	2–26	250	0.12	24
Exp. 2[a]	2–10	170	0.59	8
Exp. 3	12–20	160	0.42	8
Exp. 4	18–24	250	0.12	6
Exp. 5	N/A	250	0.12	24

[a]Algal concentrations measured every 2 h.

collected on an 80 μm mesh and counted in both virally infected and uninfected chambers under a dissecting microscope. We recognize that copepods may break or re-ingest produced pellets which will underestimate the total pellets produced in both treatments. However in combination with measured grazing rates the number of pellets collected in each treatment provides an independent measure of differential feeding on infected and uninfected cultures.

Experiment 2: 8-hour Grazing (starting 2 h PI). This experiment was designed to provide greater temporal resolution of the ingestion rates of *A. tonsa* on uninfected and infected *E. huxleyi* cells during the early stages of infection (first 10 h PI). Experimental design included a total of

eight grazing flasks (0.59 *A. tonsa* mL^{-1}) and four control flasks (no grazers). Of the grazing flasks, four flasks contained virus infected *E. huxleyi* while the other four contained uninfected cells. Two flasks for each algal condition were run as controls. Cell concentrations were measured in all flasks at 0, 2, 4 and 8 h after adding *A. tonsa* to the grazing flasks. To facilitate accurate measurements of grazing rates over short, 2 h intervals, we employed relatively lower volume flasks and higher numbers of copepods to achieve a higher predator:prey encounter rate.

Experiment 3: 8-hour Grazing (starting 12 h PI). The aim of this experiment was to measure the ingestion rates of *A. tonsa* on infected *E. huxleyi* cells during the period when the majority of the cells in the infected culture had undergone a major lipid membrane change, as revealed by a significant variation in FCM orange fluorescence signal from FM 1–43 dye labeling (i.e. from 12 h PI to 20 h PI). Each treatment, uninfected or infected *E. huxleyi* cells, consisted of five grazing (0.42 *A. tonsa* mL^{-1}) and three control flasks.

Experiment 4: 6-hour Grazing (starting 18 h PI). The aim of this experiment was to compare ingestion rates of *A. tonsa* on uninfected and infected *E. huxleyi* cells from 18 h PI to 24 h PI, a period where the majority of the cells in the culture were visibly infected (low FM 1–43 orange fluorescence signal) but before high cell lysis was measured. Again, each treatment consisted of five grazing flasks (0.12 *A. tonsa* mL^{-1}) and three control flasks for each algal treatment.

Experiment 5: Copepod Recovery. This experiment was designed to determine if feeding on *E. huxleyi* infected cells had a prolonged effect on *A. tonsa* grazing rates when presented a non-infected food source. Adult *A. tonsa* females were maintained on a diet of either infected or uninfected *E. huxleyi* cells for 96 h. During this time, copepods were transferred to newly infected (2 h PI) or uninfected *E. huxleyi* cells at concentrations of 5×10^4 cells mL^{-1} (430 μg C L^{-1}) every 24 h. After 96 h, copepods were transferred to 250 mL grazing flasks containing *R. salina* at concentrations of 2×10^4 cells mL^{-1} and placed on the grazing wheel for 24 h. *Rhodomonas salina* was chosen instead of uninfected *E. huxleyi* in order to avoid the possibility of algal re-infection from viral particles potentially carried over by the copepods (Frada *et al.*, 2014).

Data analysis

Algal growth rates for the infected and uninfected *E. huxleyi* were calculated (Frost, 1972) from control flasks within each time interval and tested using a two-way ANOVA with a *post hoc* pairwise multiple comparison (Holm-Sidak method). Ingestion rates were calculated based on the equations provided by Frost (1972). Ingestion rates in Experiments 1 & 3–5 and FP production rates were analyzed with a two-tailed *t*-test assuming equal variance. The high-resolution grazing experiments were analyzed using a two-way ANOVA with a *post hoc* pairwise multiple comparison (Holm-Sidak method). Statistical significance levels were $P < 0.05$ throughout all experiments.

RESULTS

Changes in size and growth rates of *E. huxleyi* during infection

Cell size did not vary over the course of the infection. The average estimated spherical diameter for both infected and uninfected *E. huxleyi* was 3.82 (+/− 0.02; SE) μm. In contrast, algal growth rates differed significantly over time and as a function of infection status. When measured over an entire 24 h period (Exp. 1), the growth coefficient of *E. huxleyi* maintained in the dark showed negative values in both the infected and uninfected cultures. However, the infected cultures showed significantly higher mortality than uninfected cultures (two tail *t*-test; $P = 0.047$; df = 12). The shorter experiments (Exp. 2–4) provided an opportunity to investigate changes in the growth characteristics of *E. huxleyi* over the 24 h period. A comparison of algal growth rates measured within the first 10 h PI (Exp. 2), 12–20 h PI (Exp. 3) and 18–24 h PI (Exp. 4) shows significant changes as a function of time and infection status and no interaction (two-way ANOVA; Table II). In all cases the growth rates were significantly lower in the infected cultures compared to the uninfected cultures (*post hoc* pairwise comparison Holm-Sidak method; Table IIB). A comparison of the growth rates of the infected cultures over time showed no significant difference in the mortality rates of infected *E. huxleyi* between 2–10 h PI (Exp. 2) and 12–20 h PI (Exp. 3) but both experiments had significantly lower growth rates than measured between 18 and 24 h PI (Exp. 4; *post hoc* pairwise comparison—Holm-Sidak method; Table IIC). Interestingly, both the infected and uninfected culture showed positive growth between 18 and 24 h despite being held in the dark. The later results suggest that both infected and uninfected cultures maintained an endogenous rhythm and began to divide despite the lack of light (Fig. 1; Table IIB). These results also suggest that a small fraction of the cells in the infected cultures either remained uninfected or are capable of dividing despite the infection. The progression of the virus infection was monitored using changes in the membrane characteristics. Cell membrane blebbing provides an excellent proxy for the infection status of individual cells. Martinez

Table II: Coefficient of growth (calculated as in Frost, 1972) in the control jars (no copepod grazers) of infected (coccolithovirus EhV-86) and uninfected Emiliania huxelyi *during three time periods (Experiments 2–4). All cultures were maintained in the dark on a rotating wheel*

(A)

Source of variation	DF	SS	MS	*F*	*P*
Time period (T)	2	0.0414	0.0207	33.446	<0.001
Infection status (IS)	1	0.0172	0.0172	27.825	<0.001
T × IS	2	0.0010	0.0005	0.809	0.458
Residual	22	0.0136	0.0006		
Total	27	0.0746	0.0027		

(B)

Grazing experiment	Hours post inoculation (h PI)	UC h^{-1} (SD)	IC h^{-1}(SD)	th	*P*
Exp. 2[a]	2–10	–9.44E-03 (2.77E-02)	–5.43E–02 (2.42E–02)	3.61	0.002
Exp. 3	12–20	–3.54E-02 (2.16E-02)	–8.14E–02 (2.28E–02)	2.26	0.034
Exp. 4	18–24	8.83E-02 (3.32E-02)	1.40E–02 (2.79E–03)	3.66	0.001

(C)

Experiment	*t*	*P*
Exp. 2 vs 3	1.60	0.123
Exp. 2 vs 4	4.05	0.001
Exp. 3 vs 4	4.69	<0.001

[a]Growth coefficient averaged over entire duration.
A) Results from a two-way ANOVA for time and infection status. B) *Post hoc* comparison of the growth coefficient (+/– SD) as a function of infection status within each time period using pairwise multiple comparison procedures (Holm-Sidak method). C) *Post hoc* comparison between the coefficients of growth (+/– SD - calculated as in Frost, 1972) between time periods (Experiments 2, 3 and 4) for the virally infected cultures of *E. huxleyi*.

Martinez *et al.* (2011) found that only a small percentage of single sorted cells from the "not-visibly infected" population (as in Fig. 1) were proven by PCR to be infected or to have attached viruses (amplification with virus-specific primers). Within 4 h PI 50% of the cells showed signs of infection and by 12 h PI ~80% of the culture was infected.

Copepod grazing rates

During the 24 h grazing experiment (Exp. 1), *A. tonsa* females (F) fed on infected *E. huxleyi* at less than half the ingestion rate of copepods fed on uninfected cells (Fig. 2A; Table III). *Acartia tonsa* consumed on average 378 (+/− 88 SE) infected cells $F^{-1}\,h^{-1}$ (2.4×10^{-3} µg C h^{-1}) with average clearance rates of ~0.45 mL $F^{-1}\,d^{-1}$. The ingestion rate on uninfected cultures was 1048 (+/− 138 SE) cells $F^{-1}\,h^{-1}$ (4.8×10^{-3} µg C h^{-1}) with average clearance rates of ~1.26 mL $F^{-1}\,d^{-1}$ over the 24 h period. Less than 1% of the copepods in either treatment suffered mortality and all the animals contained pigment within the gut at the end of the experiment. It is possible that the measured ingestion rates underestimated the ingestion rates of copepods in the infected treatment if the copepods consumed the algae before the infected cells lysed. To constrain this potential bias in ingestion rates, FP production rates during the 24 h experiment were measured as an independent proxy of grazing rates within the flasks. The data show that copepods produced ~80% more pellets when feeding on uninfected *E. huxleyi* cells over infected cells (Fig. 3; *t*-test; $t = 2.78$; df = 4; $P = 0.05$).

Subsequent experiments that investigated grazing rates over shorter time periods and at different stages of infection (Exp. 2–4), revealed that ingestion rates varied significantly throughout the first 24 h of the infection cycle (Fig. 2A; Table III). During the early phase of the infection cycle (Exp. 2, 2–10 h PI) when the number of algal cells showing cell surface blebbing was rapidly increasing (Fig. 1B), copepods grazed less than half the number of infected cells than uninfected cells (Fig. 2B; Tables IV and V). At 12–20 h PI (Exp. 3; Fig. 2B) copepods ingested nearly an order of magnitude fewer infected cells h^{-1} (144 +/− 56 SE) than uninfected cells (1288 +/− 30 SE). In addition, the rate of ingestion on the infected cells was less than half the ingestion rates measured during the first 10 h post infection. After 18 h PI (18–24 h PI; Exp. 4; Fig. 4), the average ingestion rates of *A. tonsa* on infected cells was only 36% of the uninfected cells although the difference was not statistically significant (Table III).

High-resolution grazing rates (2 h resolution level) during the first 10 h PI (Exp. 2; Fig. 2C) show that the ingestion rates changed significantly over time and as a function of the infection status (Tables IV and V). In both the control and the infected treatment, females ingested more cells per hour during the first 2 h of feeding than during the remainder of the 8 h experiment. Additionally, during the initial two time periods (2–4 h PI and 4–6 h PI) there was no significant difference in the ingestion rates of uninfected cells versus infected cells ($t = 2.15$; $P = 0.06$ & $t = 1.98$; $P = 0.08$, respectively). These results suggest that during 2–6 h PI, *A. tonsa* did not detect and/or discriminate between infected and uninfected cells and ate both cells equally. However as the infection progressed, after 6 h PI there was no measureable ingestion of infected cells and copepods did not resume feeding for the remainder of the experiment (6–10 h PI). In contrast, copepods feeding on uninfected

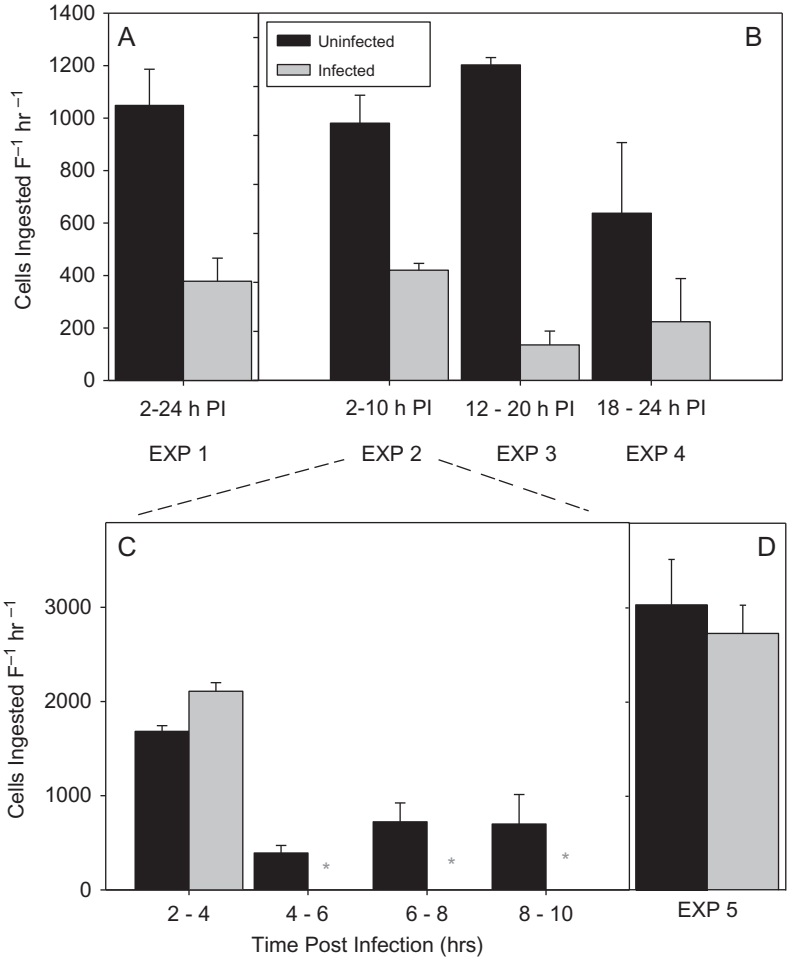

Fig. 2. The ingestion rates of adult female (F) *Acartia tonsa* on EhV-86 virus infected or uninfected *Emiliania huxleyi* over (**A**) a 24 h period (Exp. 1); (**B**) early (Exp. 2), mid (Exp. 3) and late (Exp. 4) time periods during the infection cycle; (**C**) every 2 h (Exp. 2) during the first 10 h PI. Copepods were introduced to the algae 2 h post inoculation. The asterisk (*) indicates no measurable grazing; (**D**) The ingestion rates of female *A. tonsa* on *Rhodomonas salina* after prolonged exposure to EhV-86 virus infected or uninfected *E. huxleyi* (Exp. 5).

Table III: Ingestion rates (+/− SEM) of Acartia tonsa *females (F) on uninfected (UC) and infected (IC) cultures of* Emiliania huxleyi *(calculated as in Frost, 1972) during different periods of the infection cycle*

Grazing experiment	Hours post inoculation (h PI)	Ing-UC cells F^{-1} h^{-1} (SEM)	Ing-IC cells F^{-1} h^{-1} (SEM)	df	*t*	*P*
Exp. 1	2–24	1048 (138)	378 (88)	18	4.10	<0.001
Exp. 2[a]	2–10	1050 (114)	450 (28)	1	12.31[b]	0.008
Exp. 3	12–20	1287 (17)	144 (56)	4	19.33	<0.001
Exp. 4	18–24	682 (288)	239 (176)	8	1.31	0.230
Exp. 5[c]	NA	3032 (179)	2729 (173)	4	0.92	0.409

Statistical results compare the ingestion rates for infected vs uninfected within a given time periods (Experiments 1–4; Fig. 2A and B). Data within each experiment were tested with a two-tailed *t*-test with the exception of Experiment 2, which was tested using a two-way ANOVA (see Tables IV and V).

[a]Analyzed in a two-way ANOVA see Table IV.

[b]*F* statistic from Table IV.

[c]Copepods were maintained on a diet of either infected or uninfected *E. huxylei* cells for 96 h and then transferred to uninfected cultures of *Rhodomonas salina*.

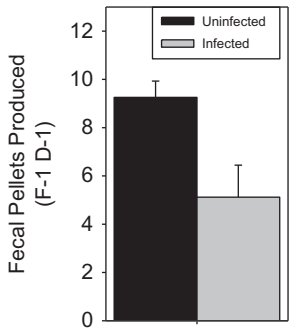

Fig. 3. FPs produced per female *Acartia tonsa* per day on a diet of uninfected or EhV-86 virus infected *Emiliania huxleyi* cells.

Table IV: Statistical results for Exp. 2 (2–10 h PI) during three time periods (Fig. 2C). Results from a two-way ANOVA for the ingestion rates of Acartia tonsa *feeding on infected and uninfected* Emiliania huxelyi *within Experiment 2*

Source of variation	DF	SS	MS	*F*	*P*
Time period (*T*)	**3**	7687721.483	2562573.828	65.475	<0.001
Infection status (IS)	**1**	481782.518	481782.518	12.310	0.008
T × IS	**3**	862690.294	287563.431	7.347	0.011
Residual	**8**	313107.488	39138.436		
Total	**15**	9345301.783	623020.119		

Table V: Post hoc *comparison of the grazing rates in Experiment 2 (2–10 h PI) as a function of infection status using a pairwise multiple comparison procedure (Holm-Sidak method)*

Hours post infection (h PI)	*t*	*P*
2–4	2.15	0.064
4–6	1.98	0.083
6–8	3.65	0.006
8–10	3.53	0.008

cells continued to feed, albeit at a significantly slower rate (Fig. 2C). Visual inspection of the copepod at the end of Exps. 2–4 showed full guts in both treatments although there were substantially fewer FPs produced in the infected treatment. These results suggest that gut passage time increased in *A. tonsa* when feeding on virally infected *E. huxleyi* cells within the first 24 h PI.

Copepods that were maintained on a diet of freshly infected *E. huxleyi* cells for 96 h and then transferred to cultures of *R. salina* showed no difference in grazing rates from copepods maintained on a diet of healthy

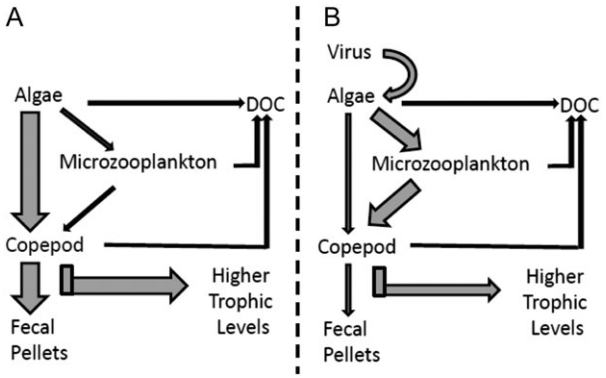

Fig. 4. The dominant trophic pathway during the progression from (**A**) uninfected (**B**) to late stage infection of an *Emiliania huxleyi* bloom by the EhV-86 virus. During an uninfected bloom of *E. huxelyi*, copepods have ingest and package organic carbon into rapidly sinking FPs which transport a fraction of the organic matter out of the surface waters. When *E. huxleyi* become infected, the ingestion/growth rates of microzooplankton on *E. huxleyi* increase. Copepods decrease feeding rates on the infected *E. huxleyi* and increase grazing pressure on microzooplankton. The increased trophic level of the copepod results in a lower abundance of animals and a decrease in the flux of organic matter via FPs out of the surface ocean.

E. huxleyi cells and then transferred to cultures of *R. salina* (*t*-test; $t = 0.92$; df = 4; $P = 0.41$) (Fig. 2D, Table III). These results suggest that there are no long-term effects of ingesting virus infected cells on the ingestion rates of *A. tonsa*.

DISCUSSION

Acartia tonsa ingests significantly fewer *E. huxleyi* cells infected with the EhV-86 coccolithovirus than uninfected cells. Ingestion rates are similar to literature values with the same copepod and algal combination (Nuester *et al.*, 2014) but are not as high as those reported for *A. tonsa* ingesting other algal species (Kleppel and Hazzard, 2000; Ismar *et al.* 2008). Because of its small size *E. huxleyi* is not considered a primary food source for adult copepods but it is likely to be important for the naupliar stages (Smith *et al.*, 2008) and during bloom periods when their cell concentrations are dominant (Frada *et al.* 2014). *Acartia tonsa* consumed ~2.5 × 10^4 cells F^{-1} d^{-1} when grazing on uninfected cells over a 24 h period. When cells were virally infected, consumption fell to 0.9 × 10^3 cells F^{-1} d^{-1}, or a 64% decrease in ingestion. To our knowledge these are the first data which quantify the ingestion rates of copepods on virally infected cells and the first experiments to measure changes in grazing rates as viral infection progresses.

Our results show that grazing pressure on infected cultures decreased as the infection within the cultures

advanced. During the early stages of the infection cycle (4 h PI; Exp. 2; Fig. 2C) copepods readily ingest cells from both the infected and uninfected cultures. At this early stage it is unclear if the copepods in the infected cultures were feeding only on the fraction of uninfected cells. The high grazing rates on cells 2–4 h PI could be either the result of copepods indiscriminately consuming both infected and uninfected cells or selectively choosing only the uninfected cells. However, given that only 50% of the cells within the infected cultures showed visible signs of infection it is unlikely that copepods would consume only uninfected cells in the infected cultures at the same rate as cells in the uninfected cultures since the uninfected cells would be at half the concentration. After 4 h PI (2 h of feeding), the same copepods stopped grazing on the cells in the infected cultures while grazing continued in the cultures with uninfected cells. Finally, it is unlikely that the high feeding rates during the initial 2 h of Exp. 2 are due to a starvation effect as a result of being held for 2 h without food. When fresh copepods were given *E. huxleyi* cells that were well into the infection cycle (12 h PI and 18 h PI, Exps. 3 and 4), the copepods ingested the infected cells at a very low rate (10% and 30%, respectively of the ingestion rate on uninfected cells) despite being held under the same conditions. Combined, these results suggest that copepods can differentiate infected cells after 4 h PI. Determining when the copepods can detect the infected cells is critical to link changes in the phytoplankton biochemistry with the ingestion rates of the copepods. This aspect certainly warrants future studies.

The underlying mechanism causing the decreased feeding rates on infected cells remains unclear. It is well documented that copepods feed at different rates on different prey types and can choose specific prey based on both mechanical and chemical properties of the cells (Paffenhöfer and Lewis, 1990; Fields and Yen, 1997; Moore *et al.*, 1999). Viral infections cause physical, biochemical and physiological changes within infected *E. huxleyi* cells (Brussaard *et al.*, 2001; Evans *et al.*, 2007; Rosenwasser *et al.*, 2014, Gilg *et al.*, 2016) that can influence the feeding rates. Cell characteristics that vary between healthy and virus infected *E. huxleyi* include cell size, the release of dissolved infochemicals (DeMott, 1989; Brussaard *et al.*, 2001; Steinke *et al.*, 2006; Evans *et al.*, 2007) and cell lipid membrane properties (Mackinder *et al.*, 2009, Martinez Martinez *et al.*, 2011). Viral infection causes *E. huxleyi* to lose its coccoliths, and consequently, as viral infection progresses the percentage of non-lithed cell can exceed that of calcified cells during natural bloom conditions (Jacquet *et al.*, 2002). Cell size changes as a result of decalcification may alter ingestion rates (Frost, 1972). In an effort to uncouple the effects of calcification and size on feeding rates we use the non-calcifying *E. huxleyi* strain CCMP374. Our data show no significant change in size during the infection process or between infected and uninfected cells, suggesting that differences in ingestion rates observed in this study were not being driven by differences in cell size. Among the indicated chemical cues it is worth mentioning, for example, the release of DOC by stressed phytoplankton cells (Strom *et al.*, 1997; Muller-Kerger *et al.* 2005). DOC has been shown to stimulate the feeding activity in copepods (Gill and Poulet, 1988) which could provide a positive feedback that intensified further grazing pressure on the algal culture (Poulet and Ouellet, 1982). Thus, one might suspect that as *E. huxleyi* progresses through the lytic cycle, the increase in released DOC would fuel intensified copepod feeding (Poulet and Ouellet, 1982). However, our data indicate that grazing rates are not enhanced during the infection process. In contrast, certain phytoplankton species also produce inhibitory or toxic compounds under stress conditions (Verity *et al.* 1988; Malej and Harris, 1993; Muller and Schlegel, 1999) including DMS (Dacey and Wakeham, 1986), a putative deterrent to copepod grazing (Steinke *et al.*, 2006). Virally infected *E. huxleyi* cells produce and release significantly more DMS and dimethylsulfoniopropionate (DMSP) than uninfected cells (Evans *et al.*, 2007; Floge, 2014). Other studies have proposed that the rapid enzymatic conversion of DMSP to DMS and the subsequent production of acrylate, rather than DMS itself, may act as an activated defense system to deter copepod grazing (Wolfe and Steinke 1996; Steinke *et al.*, 2002). This is supported by the results from Exp. 5 that showed how the ingestion rates of *A. tonsa* returned to normal levels immediately when transferred to jars containing uninfected *R. salina* after having fed solely on infected *E. huxleyi* cells for 96 h.

Acartia spp. respond to lower food quantity and quality by decreasing ingestion rates (Fields *et al.*, 2011) and the rate of gut passage (Tirelli and Mayzaud, 2005). While food concentrations in this study were constant, food quality has been shown to change considerably due to viral infection (Rosenwasser *et al.*, 2014). Transcriptome and gene expression analyses show viral infection alters numerous metabolic pathways, including glycolysis, fatty acid synthesis, nucleotide biosynthesis and enzymes responsible for photo damage repair (Rosenwasser *et al.*, 2014; Gilg *et al.*, 2016). Some changes occurred early on in the infection cycle (<6 h PI) while other were initiated later (<24 h PI). For example, within 1 h PI Rosenwasser *et al.*, (2014) reported increased gene expression for FA biosynthesis which manifests later in the accumulation of C8–C16 saturated fatty acids within the cells (Evans *et al.*, 2009).

Viral infection also caused cell-specific decreases in the production of essential fatty acids such as DHA and other polyunsaturated FA (Floge, 2014; Rosenwasser *et al.*, 2014) that are necessary for zooplankton growth and reproduction. Similarly, early in the infection cycle virus sphingolipid genes are upregulated while the host's sphingolipid genes are down regulated (Rosenwasser *et al.*, 2014). Sphingolipids are key lipids within the cell membrane and are particularly important for normal development of neural systems. Differences in protein (Tirelli and Mayzaud, 2005) and fatty acid content (Jones and Flynn, 2005) of the copepod prey have been directly correlated with the ingestion rates of copepods.

Coccolithovirus infection impacts *E. huxleyi* bloom dynamics and termination, community succession and biochemistry in coastal and open ocean ecosystems (Bratbak *et al.*, 1993; Wilson *et al.*, 2002; Stefels *et al.*, 2007; Martínez Martínez *et al.*, 2012; Lehahn *et al.*, 2014). *Emiliania huxleyi* is a commonly available algal species for copepods in temperate waters (McIntyre *et al.*, 1972) and maybe expanding into polar water. Studies (including this study) have shown that copepods actively graze *E. huxleyi* in the laboratory (Harris, 1994; Jones *et al.*, 2002) and during large oceanic *E. huxleyi* blooms (Frada *et al.*, 2014) although the ingestion rates of *A. tonsa* on uninfected *E. huxleyi* are low (this study) compared to other phytoplankton species (Kleppel and Hazzard, 2000; Ismar *et al.*, 2008). Low ingestion rates are probably the result of the small size of *E. huxleyi* rather than poor nutritional qualities since *E. huxleyi* can support rapid naupliar development in *A. tonsa* (Ismar *et al.*, 2008) and high egg production (67–81 eggs copepod^{-1} d^{-1}) in *Calanus finmarchicus* (Nejstgaard *et al.*, 1994; 1997).

When *E. huxleyi* is virally infected, the ingestion rates by *A. tonsa* decrease dramatically. *Acartia tonsa* consumed less than half the number of infected cells compared to uninfected cells. During the first 24 h the decreased ingestion rate of copepods lead to a decreased FP production. Lower FP production could lead to a decrease in the flux of organic matter out of the surface waters and also decrease the potential for storage and redistribution of active viral particles via FPs (Frada *et al.*, 2014). While our data show that copepods ingest substantially less virally infected cells, previous work reported enhanced microzooplankton feeding rates on virally infected *E. huxleyi* cells (Evans and Wilson, 2008). When taken together, this suggests that the viral infection of *E. huxleyi* can shift the flow of organic matter that would normally go directly to copepods (Fig. 4A) to the microzooplankton community (Fig. 4B). This shift in carbon flow may be pronounced given the measured consumption rates of *A. tonsa* on *E. huxleyi* cells. In response to the lower availability of algal food, the copepod population could switch from an omnivorous diet to a carnivorous diet focusing their grazing pressure on the microzooplankton (Tiselius and Jonsson, 1990; Kleppel, 1993). The increased trophic level of the copepod would decrease the transfer efficiency of organic matter to higher trophic levels (Joassin *et al.*, 2011) and diminish the export of carbon out of the photic zone. Given the scale of the infections in the ocean and the rapid dynamics of the infection cycle incorporating these processes into current ecosystem models are essential.

CONCLUSIONS

A growing body of literature has shown that viral infections alter the metabolic capacity and biochemical composition of their algal host. Reprogramming of host metabolism can alter the transfer of carbon and key nutrients through trophic levels if primary consumers modify their grazing rates during the infection cycle. This study shows changes in the ingestion rates of a coastal copepod (*A. tonsa*) during the course of the infection cycle of a marine coccolithophore (*E. huxleyi*). Copepods consumed significantly fewer infected cells as compared to uninfected cells. High-resolution grazing experiments indicate that the copepods showed a marked decrease in the ingestion rate of infected cells within 2–4 h post infection. Future studies which combine these grazing results with detailed analysis of the changes in algal biochemistry during the infection cycle will offer important insight into the underlying modes of discrimination of copepods that help drive selective feeding.

These results are in strong contrast to previous work reporting higher grazing rates of microzooplankton on infected cells using the same host virus system used in our study. Together they suggest that during an infected *E. huxleyi* bloom, copepods may alter the grazing pressure from *E. huxleyi* to microzooplankton. This shift in diet will increase the trophic level of copepods and alter the flow of carbon to higher trophic levels.

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