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Size-dependent Catalysis of *Chlorovirus* Population Growth by A Messy Feeding Predator

John P. DeLong[1] · **Zeina Al-Ameeli**[2] · **Shelby Lyon**[1] · **James L. Van Etten**[3] · **David D. Dunigan**[3]

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Abstract Many chloroviruses replicate in endosymbiotic zoochlorellae that are protected from infection by their symbiotic host. To reach the high virus concentrations that often occur in natural systems, a mechanism is needed to release zoochlorellae from their hosts. We demonstrate that the ciliate predator *Didinium nasutum* foraging on zoochlorellae-bearing *Paramecium bursaria* can release live zoochlorellae from the ruptured prey cell that can then be infected by chloroviruses. The catalysis process is very effective, yielding roughly 95% of the theoretical infectious virus yield as determined by sonication of *P. bursaria*. *Chlorovirus* activation is more effective with smaller *Didinia*, as larger *Didinia* typically consume entire *P. bursaria* cells without rupturing them, precluding the release of zoochlorellae. We also show that the timing of *Chlorovirus* growth is tightly linked to the predator-prey cycle between *Didinium* and *Paramecium*, with the most rapid increase in chloroviruses temporally linked to the peak foraging rate of *Didinium*, supporting the idea that predator-prey cycles can drive cycles of *Chlorovirus* abundance.

Keywords Chloroviruses · Predator-prey interaction · Virus dynamics · *Didinium* · *Paramecium bursaria* · Trait mediated

Introduction

Viruses are the most diverse and abundant biological organisms in nature [1, 2]. Although many viruses (and other parasites) are considered in the context of their interactions with specific hosts, it is becoming clear that parasites in general are integral parts of food webs [3–8]. This is because food webs influence the abundance and distribution of hosts and because foraging interactions can influence the transmission, dispersal, and host encounter rates of parasites [5, 9, 10]. Thus, a variety of species interactions—including those not directly involved in a virus-host interaction—can influence virus activation and play a role in determining the structure of virus communities.

Chloroviruses are large DNA viruses that infect chlorella-like green algae including the zoochlorellae endosymbionts of a range of organisms such as protists and hydra [11]. Zoochlorellae contained within intact symbionts are refractory to infection by chloroviruses. Some mechanism is required to disrupt the holobionts to release the zoochlorellae and expose them to encounters with virus particles. In the case of *Paramecium bursaria*, chloroviruses can be found attached to the outer membrane of the cell, where they would be in good position to encounter zoochlorellae if the *Paramecium* is ruptured [12]. Such rupture may occur upon cell death, potentially from freeze-thaw events, certain chemical exposures, or through the messy feeding of predators. Chloroviruses also may be activated by predators that pass viable zoochlorellae through their digestive systems intact. This latter ecological catalyst mechanism operates within the copepod-*P. bursaria* system, rapidly amplifying *Chlorovirus* populations to levels similar to that found in

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✉ John P. DeLong
jpdelong@unl.edu

✉ David D. Dunigan
ddunigan2@unl.edu

[1] School of Biological Sciences, University of Nebraska-Lincoln, Lincoln, NE 68588-0118, USA

[2] School of Natural Resources, University of Nebraska-Lincoln, Lincoln, NE 68583-0961, USA

[3] Department of Plant Pathology/Nebraska Center for Virology, University of Nebraska-Lincoln, Lincoln, NE 68583-0900, USA

natural systems [10, 13]. This catalyst mechanism also indicates that the abundance of chloroviruses depends on predator-prey interactions of species that are not their hosts.

P. bursaria (hereafter just *Paramecium*) is a widespread freshwater protist. It is consumed by a wide variety of protists and metazoan zooplankton [14], including the *Paramecium* specialist *Didinium nasutum* (hereafter just *Didinium*) [15]. *Didinium* may consume *Paramecium* whole, without rupture, or by rupturing the *Paramecium* cell and consuming parts of the cell while some of the cell contents leak into the water (messy feeding) (Fig. 1, online movies 1 and 2). Thus, *Didinium* has the potential to catalyze *Chlorovirus* infection of *Paramecium* zoochlorellae if its feeding is messy, releasing potentially hundreds of zoochlorellae hosts into the water [16], but it is unknown whether this occurs and whether there are cell traits that influence foraging behavior that could also influence the effectiveness of the catalyst mechanism. One hypothesis is that larger *Didinium* cells would be more likely to consume entire *Paramecia* and thus be less likely to catalyze *Chlorovirus* population growth. Here, we assess *Chlorovirus* amplification by *Didinium* feeding on *Paramecium*. Using a short-term foraging experiment, we investigate whether *Didinium* can catalyze *Chlorovirus* amplification and whether this effect depends on *Didinium* cell size. In a longer-term foraging experiment, we also ask whether *Chlorovirus* production is temporally connected to the predator-prey dynamics between *Didinium* and *Paramecium*.

Methods

We acquired *Didinium* from Carolina Biological Supply (Burlington, North Carolina), and we isolated *Paramecium* from a pond at the Spring Creek Prairie Audubon Center southwest of Lincoln, NE, USA [17]. Stock cultures of both species were maintained in the laboratory at 23 °C in medium made from protozoan concentrate (Carolina Biological Supply) mixed with filtered and autoclaved pond water acquired from the source pond for *Paramecium* (1:9 ratio of concentrate to water). Naturally associated *Chlorovirus* is present in the *Paramecium* stock cultures.

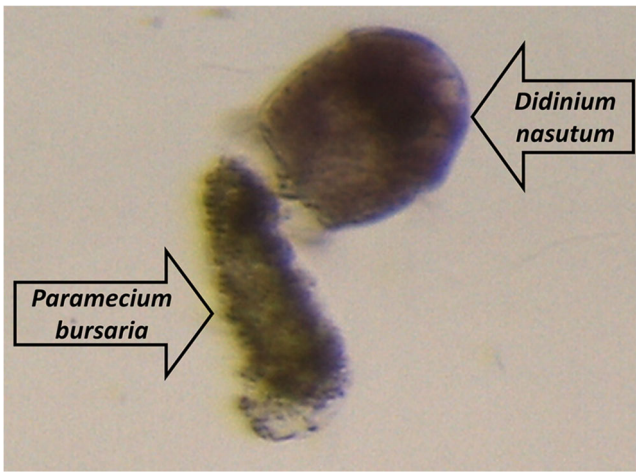

Fig. 1 *Didinium nasutum* (above right) consuming *P. bursaria* (below left). The predator was unable to get the entire *Paramecium* cell into its own cell. After trying for several minutes, it regurgitated part of the cell, leaving unconsumed and exposed zoochlorellae available as targets for *Chlorovirus* infection. See online resources 1 and 2 for videos of foraging

In the short-term foraging trial, we assembled 1.75 mL microcosms in 35-mm diameter plastic Petri dishes with lids. Replicate microcosms were randomly assigned to be control dishes (six replicates), foraging dishes (30 replicates), and sonication dishes (six replicates). We first transferred 30 *Paramecia* in 0.2 mL medium to each dish. We then rinsed *Didinium* three times in sterile pond water and added one *Didinium* in 0.05 mL of rinse water to the foraging dishes. We also added 0.05 mL of the rinse water (without a *Didinium*) to the control and sonication dishes. We then added 1.5 mL of the 1:9 protozoan medium to complete the microcosms. We sonicated microcosms used in the sonication treatments for 15 s at output level 5 (Heat Systems), which achieved near-complete disruption of the *Paramecia* and release of zoochlorellae, essentially as previously described [10]. We pooled the sonicated samples to standardize the sonication procedure and zoochlorellae density across replicates.

Before the trials began, we photographed each *Didinium* with a Leica M165C microscope and digital camera, measured cell length and width, and calculated cell volume using the formula for a prolate spheroid. *Didinium* foraged overnight (~ 19 h) at 26 °C, after which we counted the number of *Didinium* and *Paramecia* remaining in the microcosm. Because the foraging trial lasted longer than the generation time for *Paramecia* (~ 12–24 h; [18]), the *Paramecia* underwent about one cell division in most cultures, so the number of remaining *Paramecia* provided an estimate of the minimum number consumed rather than the exact number. In two foraging dishes, the *Paramecium* population grew to sizes greater than the initial population of 30, so we assigned those dishes a value of zero for a minimum number of *Paramecia* consumed. Overnight foraging trials also were long enough for about one cell division in *Didinium* to occur. At the end of the foraging period, *Didinia* were removed, and the microcosms were left for one more day to allow the *Chlorovirus* population to grow before they were filtered for *Chlorovirus* plaque assays (see below).

We analyzed data from the short-term foraging experiment using linear models with *Chlorovirus* plaque-forming units (PFUs) per milliliter as the dependent variable and *Didinium* volume, number of *Paramecia* consumed, and final *Didinium* number as explanatory variables. We began with a full model including all interactions and removed non-significant terms until we arrived at a final model, which we compared to the other models using Akaike's information criteria corrected for

small samples (AIC_c; Table 1). We then used partial regression analysis to visualize the effect of *Didinium* volume and number of *Paramecia* consumed on *Chlorovirus* PFU density, while holding other significant effects constant.

In the longer-term (population dynamics) experiment, we assembled 7.4 mL microcosms in 60-mm diameter plastic Petri dishes with lids. Microcosms were randomly assigned to four control dishes, five foraging dishes, and four sonication dishes. To create microcosms, we transferred 7.3 mL of *Paramecia* stock culture (sampled ahead of time to estimate initial density) to each dish. We then rinsed *Didinium* three times in sterile pond water and added one *Didinium* in 0.1 mL rinse water to the foraging dishes and added 0.1 mL of the rinse water (without a *Didinium*) to the control and sonication dishes to control for potential rinse water effects. We then used a sonicator to disrupt the membranes of *Paramecia* in the sonication dishes [10].

We counted *Paramecia* and *Didinium* daily in the longer-term experiment. Each day, we removed 0.1 mL of culture and replaced it with 0.1 mL of autoclaved pond water plus 0.05 mL of 0.1 μm filtered pond water to account for evaporation. When *Paramecia* were abundant, we counted cells in the 0.1 mL sample, but when rare, we counted cells in the entire microcosm (i.e., a scaled sampling regime; [19]). We also estimated the per capita daily foraging rate (f_{pc}) of *Didinium* on *Paramecium* through time using an estimate of the functional response:

$$f_{pc} = \frac{aRC^m}{1 + ahRC^m} \quad (1)$$

Equation 1 is the standard Holling disc equation for predators [20] modified for mutual interference competition among predators, which is known to be important for *Didinium* [21–23]. In this model, a is the space clearance rate (how much of the occupied prey space is completely cleared of prey per unit time), h is the handling time (the time cost of consuming prey), m is the "mutual" interference, R is prey density, and C is predator density. To get the total foraging rate, f, which would reflect the total amount of potential *Chlorovirus* catalysis in the system [10], we multiply Eq. 1 by predator density to get:

$$f = \frac{aRC^{m+1}}{1 + ahRC^m} \quad (2)$$

We used parameters from [24] for *Didinium* foraging on *P. aurelia* (a similar-sized *Paramecium*) and observed mean daily values of R and C to estimate the total foraging rate through time in the longer-term foraging experiments. Functional response parameters for *Didinium* foraging on a variety of *Paramecium* species are quantitatively similar [25], so the use of these parameters will give us a robust indication of the timing of maximum *Paramecium* consumption.

We assessed *Chlorovirus* abundance daily starting on the second day of the trial. For each sample, we vigorously mixed each microcosm longitudinally as opposed to centripetally so as to avoid uneven organism distribution within the dish and extracted 0.3 mL of the culture for assay. We used plaque assays to detect infectious chloroviruses as described previously [16], with *Chlorella variabilis* Syngen 2–3 (product no. 30562; American Type Culture Collection) cells as the lawn.

Results

In the short-term foraging experiment, *Chlorovirus* density was boosted above controls by *Didinium* foraging, nearly to the maximum level as determined by sonication (ANOVA: $F_{2,41} = 72.42$, $p < 0.001$, all treatment differences were significant based on Tukey's honest significant difference (HSD) post hoc comparisons; Fig. 2). *Chlorovirus* abundance was positively associated with the number of *Paramecia* killed (Table 2; Fig. 3a) and negatively associated with *Didinium* cell volume (Table 2; Fig. 3b). Although the final number of *Didinium* did not affect *Chlorovirus* density as a main effect, it did interact with the number of *Paramecia* killed (Table 2), indicating that the positive effect of *Didinium* foraging got weaker the more *Didinium* divided during the experiment. To isolate and visualize the effects of the number of *Paramecia* killed and *Didinium* cell volume on *Chlorovirus* PFUs, we plotted the partial regression between these

Table 1 Comparison of linear models evaluating the effects of *Didinium* cell volume (DCV), number of *Paramecia* killed (NPK), and *Didinium* cell division (DCD) on the production of *Chlorovirus* plaque-forming units (PFUs). Models ranked by Akaike's information criterion corrected for small samples (AICc)

Model	AICc	ΔAIC
PFUs ~ DCV + NPK + DCD + NPK × DCD	825.16	0
PFUs ~ DCV + NPK + DCD + NPK × DCD + NPK × DCV + DCD × DCV	828.34	3.18
PFUs ~ DCV + NPK + DCD + NPK × DCD + NPK × DCV + DCD × DCV + NPK × DCD × DCV	829.40	4.24

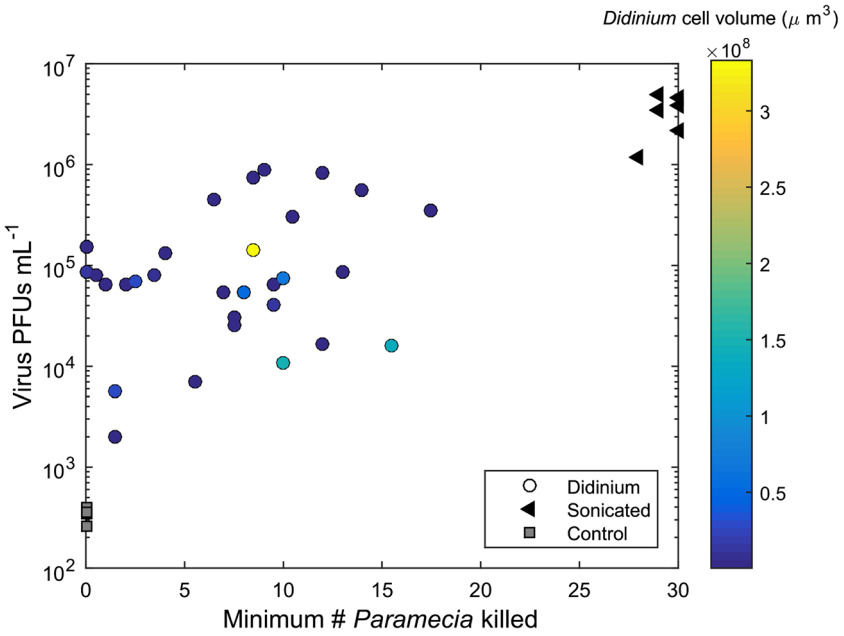

Fig. 2 Chlorovirus concentrations (PFUs per mL) as a function of the minimum number of *Paramecia* killed and treatment in the short-term foraging experiment. Each point is a replicate microcosm. Color refers to *Didinium* cell volume (μm^3)

variables, which shows the residual of the expected *Chlorovirus* density (i.e., the observed *Chlorovirus* PFU minus model-predicted *Chlorovirus* PFU) against the residual of the predictor variables (Fig. 3).

Didinium foraging generated 1.6×10^5 infectious chloroviruses per *Paramecia* killed, estimated by the slope of the regression between final *Chlorovirus* density and the density (not number) of *Paramecia* killed (i.e., the linear model slope estimate of 9.1×10^4 PFUs per *Paramecium* in Table 2 times the microcosm volume of 1.75 mL). In the sonication treatment, the yield of chloroviruses was 5×10^6 per 30 *Paramecia* (i.e., the initial *Paramecium* density), which gives 1.7×10^5 chloroviruses per *Paramecium* (theoretical yield). Thus, *Didinium* foraging was roughly 95% efficient (i.e., 1.6×10^5 PFUs generated by *Didinia*/1.7×10^5 PFUs generated by sonication) in generating *Chlorovirus* production.

In the longer-term experiment, *Chlorovirus* concentrations remained low in the controls, increased by three orders of magnitude in the sonication treatments, and were intermediate in the foraging dishes (Fig. 4a). This pattern was consistent with changes in *Paramecia* populations; they were nearly eliminated in the sonication treatment, they were stable initially and then increased in the controls, and they were eliminated after 3–5 days in the foraging treatments (Fig. 4b). Together, *Didinium* and *Paramecium* showed typical predator-prey dynamics for this system, with *Didinium* increasing in abundance in the first few days, consuming all of the *Paramecia*, and then undergoing a population crash (Fig. 4c) [24, 26]. Chlorovirus abundance increased alongside the increase in *Didinium* and decrease in *Paramecia*, indicating a strong temporal relationship between *Chlorovirus* production and foraging interactions between *Didinium* and *Paramecium*. The total foraging rate (*f*; Eq. 2) peaked on day two, immediately preceding the most rapid increase in virus concentration.

Discussion

Our results illustrate how the abundance of chloroviruses depends upon foraging interactions among species that are not actually hosts for the viruses. As *Chlorovirus* hosts (zoochlorellae) may be ensconced in protective symbiotic relationships, access to hosts depends in part on the

Table 2 Final model evaluating the effects of *Didinium* cell volume, number of *Paramecia* killed, and *Didinium* cell division on the production of *Chlorovirus* plaque-forming units (PFUs)

Term	Estimate	SE	*t*	*p* value
Intercept	-9.74×10^4	1.44×10^5	−0.68	0.5
number of *Paramecia* killed	9.09×10^4	24,907	3.65	0.001
Didinium cell division	9.89×10^4	1.09×10^5	0.91	0.37
Didinium cell volume	-1.41×10^{-3}	6.02×10^{-4}	−2.34	0.027
number of *Paramecia* killed: *Didinium* cell division	-6.91×10^4	2.57×10^4	−2.68	0.012

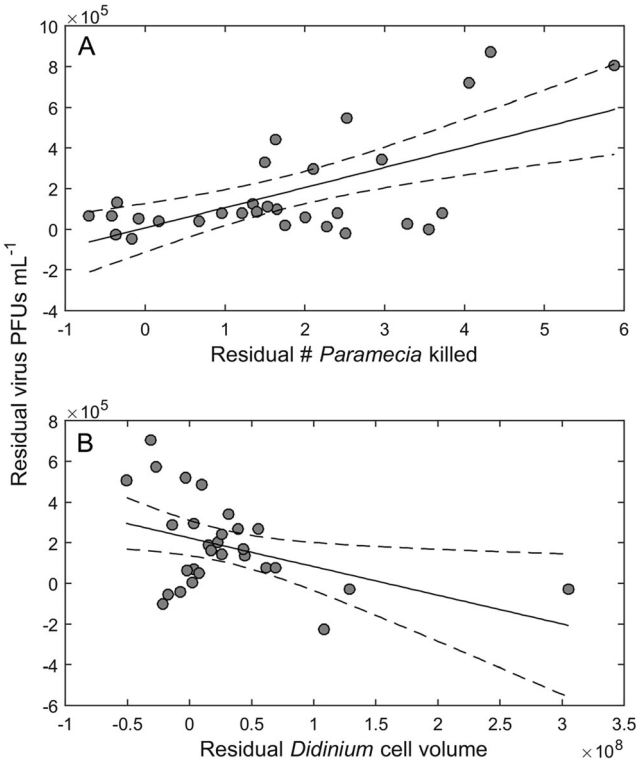

Fig. 3 Partial regression plots for the best linear model (Table 1) relating the effect of **a** minimum number of *Paramecia* killed (controlling for *Didinium* cell volume) and **b** *Didinium* cell volume on *Chlorovirus* plaque-forming unit (PFU) density (controlling for number of *Paramecia* killed) in the short-term foraging experiment. Residual refers to variation in PFUs not accounted for by other terms in the model

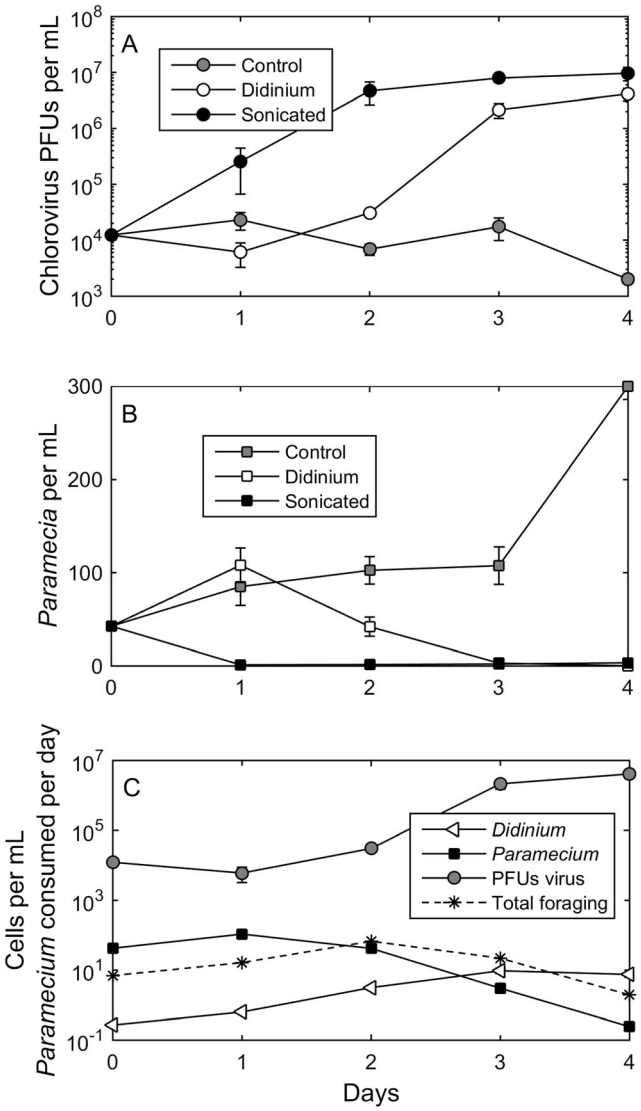

Fig. 4 Time series of **a** *Chlorovirus* PFU density and **b** *Paramecia* density in the longer-term experiment by treatment type. **c** The density of virus, *Paramecia*, and *Didinium* are shown for the foraging (with *Didinium*) dishes. The estimated number of *Paramecia* consumed per day for the whole *Didinium* population is also shown (asterisks)

presence of specific predator-prey interactions as well as the strength of these interactions. This type of catalysis is distinct from other predator effects on parasites and disease transmission, such as the promotion of epidemics in *Daphnia* when predators release parasitic fungal spores from infected *Daphnia* prey into the environment [27] or when predators defecate virus particles into new areas after they consume infected prey [9]. Together, these results indicate that food web context can play a substantial role in driving virus dynamics.

P. bursaria is a widespread protist that may be consumed by several types of aquatic predators, including protists, copepods, nematodes, and planarians [10, 14, 15, 28, 29]. We previously showed that interactions between copepods (*Eucyclops gracilis*) and *P. bursaria* can generate *Chlorovirus* blooms though the passage of fecal pellets containing viable zoochlorellae [10]. In the current study, we show that messy feeding also can catalyze *Chlorovirus* population growth by the simpler process of releasing (or regurgitating) zoochlorellae into the water when *Paramecium* cells were ruptured by ciliate predators. This activation gave rise to 2–3 orders of magnitude increases in *Chlorovirus* density, similar to the magnitude of *Chlorovirus* spikes seen in natural systems [13].

This predator catalyst process is different than the interactions where predators release spores through messy feeding (e.g., [27]); here, the predators are releasing hosts (zoochlorellae) rather than actual infectious agents such as spores. Furthermore, *Didinia* appear to be more efficient at spurring *Chlorovirus* growth than copepods, here generating ~ 95% of the theoretical yield (generated through sonication) compared with ~ 17% for copepods [10]. Thus, there are multiple ways that predators may catalyze *Chlorovirus* activation, and given that both copepods and *Didinium* may be present together in freshwater ponds along with *Paramecium*, it is likely that both mechanisms are operating, perhaps simultaneously or at different times. Copepods also may consume *Didinium*, indicating that the

predator catalyst mechanism may operate in a real food web through the net effect of multiple food web interactions. Furthermore, predators that consume copepods or *Didinium* (e.g., larger zooplankton or fish) may have a trophic-cascade-like effect on virus activation and dispersal, with *Chlorovirus* activation dependent on the length of the food chain leading to the catalyzing predators [30].

Catalyzing *Chlorovirus* population growth in the *Didinium-Paramecium* system depends on rupture of the *Paramecium* cell and release of zoochlorellae. We hypothesized that this rupturing is less likely with larger *Didinium* cells that can engulf entire *Paramecium* cells (Fig. 1; online movie 2). This conclusion is consistent with our results that indicate that larger *Didinium* cells were in fact less likely to stimulate *Chlorovirus* activation than smaller cells (Fig. 3b), which implies several relevant things about the process of *Chlorovirus* activation in nature. First, because older cells are likely to be bigger, most of the activation is likely to occur during periods of rapid cell division in *Didinium* populations, when many cells are smaller. Second, because *Didinium* cells are likely to be larger at the top of their population cycles [24], *Chlorovirus* activation may have a temporal component wherein more activation occurs when *Didinium* populations are in the growth phase of their cycles. And finally, because cold temperatures tend to lead to larger cells [14, 31], *Chlorovirus* activation potentially may be more effective in warmer environments. This effect would operate in addition to the potential effects of temperature on aquatic virus replication or host cell physiology [32, 33]. Thus, more than just being connected to the food web, *Chlorovirus* activation through predator catalysis depends on predator traits and also may depend on abiotic factors such as temperature.

In conclusion, we have shown that a messy feeding microbial predator can catalyze *Chlorovirus* population growth by releasing zoochlorellae hosts into the water where they can be infected. This process can be nearly as effective as simply rupturing cells mechanically. The process is also trait dependent, since larger *Didinium* cells appear to have a greater capacity to ingest *Paramecium* cells without rupturing them. Our results contribute to the growing realization that virus dynamics are inextricably linked to the structure and dynamics of the food webs in which the viruses reside.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing of interests.

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