2019

Chloroviruses lure hosts through long-distance chemical signaling

David Dunigan
University of Nebraska - Lincoln, ddunigan2@unl.edu

Maitham Ahmed Al-Sammak
University of Nebraska - Lincoln, s-malsamm1@unl.edu

Zeina Al-Ameeli
University of Nebraska-Lincoln, zeina@huskers.unl.edu

Irina Agarkova
University of Nebraska-Lincoln, iagarkova2@unl.edu

John DeLong
University of Nebraska - Lincoln, jpdelong@unl.edu

See next page for additional authors

Title

Chloroviruses lure hosts through long-distance chemical signaling

Authors

David D. Dunigan[1,2]*, Maitham Al-Sammak[2,3], Zeina Al-Ameeli[4,5], Irina V. Agarkova[1,2], John P. DeLong[6], James L. Van Etten[1,2]

Affiliation

1. Department of Plant Pathology, University of Nebraska—Lincoln, Lincoln, Nebraska, USA
2. Nebraska Center for Virology, University of Nebraska—Lincoln, Lincoln, Nebraska, USA
3. College of Science, University of Bagdad, Bagdad, Iraq
4. School of Natural Resources, University of Nebraska—Lincoln, Lincoln, Nebraska, USA
5. Medical Technical Institutes, Middle Technical University, Bagdad, Iraq
6. School of Biological Sciences, University of Nebraska—Lincoln, Lincoln, Nebraska, USA

Contact information

*Corresponding author - David D. Dunigan (Telephone: 402-472-5776; E-mail: ddunigan2@unl.edu; ORCID ID: https://orcid.org/0000-0001-9496-5396)

Maitham Al-Sammak (Telephone: 402-472-5776; E-mail: maitham@huskers.unl.edu; ORCID ID: https://orcid.org/0000-0001-6819-7143)

Zeina Al-Ameeli (Telephone: 402-472-5776; E-mail: zeina@huskers.unl.edu; ORCID ID: https://orcid.org/0000-0002-9444-5347)

Iriina V. Agarkova (Telephone: 402-472-5776; E-mail: irina@unl.edu; ORCID ID: https://orcid.org/0000-0002-0930-8541)

John P. DeLong (Telephone: 402-472-7752; E-mail: jpdelong@unl.edu; ORCID ID: https://orcid.org/0000-0003-0558-8213)

James L. Van Etten (Telephone: 402-472-3168; E-mail: jvanetten1@unl.edu; ORCID ID: https://orcid.org/0000-0002-5063-0049)

Keywords

Chlorovirus, symbiosis, chemotaxis, population dynamics, giant virus

Abstract

Chloroviruses exist in aquatic systems around the planet where they infect certain eukaryotic green algae that are mutualistic endosymbionts in a variety of protists and metazoans. Natural chlorovirus populations are seasonally dynamic but the precise temporal changes in these populations and the mechanisms that underlie them have, heretofore, been unclear. We recently reported the novel concept that predator/prey-mediated virus activation regulates chlorovirus population dynamics, and in the current manuscript demonstrate virus packaged chemotactic modulation of prey behavior.

Importance

Viruses have not previously been reported to act as chemotactic/chemo-attractive agents. Rather, viruses as extracellular entities are generally viewed as non-metabolically active spore-like agents that await further infection events upon collisions with appropriate host cells. That a virus might actively contribute to its fate via chemotaxis and change the behavior of an organism independent of infection is unprecedented.

Introduction

Virus particles (virions) are generally considered to be inanimate, influencing cells only upon contact. Virions typically contact appropriate host cells through biological, mechanical, or other physical processes but virions are not known to have their own mechanisms for attracting motile cells from a distance. Here, we report that chloroviruses can attract *Paramecium bursaria* from a distance by altering their movements. The action of a virus as a chemotactic agent has significant implications in biological systems from immune functions to predator-prey interactions.

Chloroviruses (Family *Phycodnaviridae*) are large icosahedral (190 nm in diameter) dsDNA viruses (genomes of 290 to 370 kb) containing an internal, single bi-layered lipid membrane (1, 2). Chloroviruses infect certain eukaryotic green algae that are mutualistic endosymbionts (referred to as zoochlorellae) of organisms such as the protozoan *Paramecium bursaria* (Cilophora) (3, 4). However, the zoochlorellae as endosymbionts are resistant to virus infection because the viruses have no way of reaching their hosts. For virus expansion to occur the protective barrier provided by *P. bursaria* must be disrupted. We have determined that one mechanism for increasing the chlorovirus population is due to an ecological catalytic event driven by predators, including a cyclopoid copepod predator (*Eucyclops agilis*) that engulfs the entire *P. bursaria* (prey) during feeding (5) or the ciliate *Didinium nasutum* that disrupts the *P. bursaria* during feeding (referred to as messy feeding) (6) In the case of copepod consumption, when a fecal pellet is released into the water column, the virus replicates in the released zoochlorellae and nascent virions subsequently diffuse from the fecal pellet, resulting in a localized high concentration of virus (5). Messy feeding by *Didinium* releases algal cells into the water column where they are infected (6). These catalytic processes can contribute to cycles of chlorovirus expansion in temperate lakes (e.g., 7). The efficiency of this process is enhanced because the chloroviruses reside on the outer surface of the paramecia, often at the base of the ciliary pits that can number in the thousands per cell (8, 9, 10).

Previous estimates suggest that hundreds of infectious chloroviruses can be attached to the surface of a single cell (5). How so many virions accumulate on paramecia cells is unknown.

The accumulation of virions on the cell surface could occur through random contacts between the paramecia and virus particles as the paramecia moves through the water. This process would be consistent with the view that virions cannot attract cells from a distance. If this is true, there should be no observable shifts in paramecium movement in response to gradients in virus density. In contrast, if chloroviruses are able to signal to paramecia from a distance through some chemical means, then the paramecia should show detectable behavioral shifts as they orient toward the virus particles (chemotaxis). Here, we describe choice/no-choice experiments that reveal strong directional movement of *P. bursaria* toward concentrations of chloroviruses relative to alternative targets, demonstrating the chemotactic influence of a virus on cells from a distance.

Results

We used a simple three-way system wherein *P. bursaria* placed in the center of a Petri dish could move out into one of three arms toward different targets (Fig. 1). Targets were paper disks loaded with target agents. Each experiment was run with one of two possible sets of three choices (Fig. 1A, B) paired with a no-choice experiment as a negative control (Fig. 1C), and replicated 4-6 times. Each replicate experiment was analyzed with *chi*-square tests to assess differences in frequency of *P. bursaria* cells moving towards the three different targets, and outcomes were summarized across all replicates. We ran a series of trials that show that *P. bursaria* are not attracted to algal host cells but are attracted to infected cells, that *P. bursaria* respond more intentionally to higher densities of virions, and that *P. bursaria* respond to a variety of chlorovirus strains. *P. bursaria* showed no directional movements in control dishes.

First, we determined that chlorovirus-infected cells could influence the movement behavior of *P. bursaria*. In these experiments, we used the paramecium-free zoochlorellae that are

susceptible to the *Chlorovirus* Osy-NE-ZA1 (5, 6). We estimated that Osy-NE-ZA1 infection kinetics are similar to the type member of the genus *Chlorovirus* PBCV-1 (1), indicating a burst size of ~1,000 virus particles per cell, of which 20-30% are infectious (11). Thus, cell-free extracts were prepared from 4 hr-infected zoochlorellae and used as the choice target in microcosm chambers, because intact, infectious virus particles are inside host cells by 4 to 6 hr post infection (11, 12) as described in the Methods. Cell-free extracts from mock-infected zoochlorellae and sterile pond water (hereafter, pond water) were used as choice controls. The paramecium movement-behavior was assessed by counting the population distribution after 12 hr of free ranging movement within the microcosm. We observed that paramecia were attracted to the virus-infected cell-free extract relative to the mock-infected cell-free extract or the pond water control ($p = 10^{-17} – 10^{-5}$, $n = 6$; Table 1); whereas, in microcosm chambers with no choices (all targets were pond water), the paramecium were equally distributed ($p = 0.08 – 0.67$, $n = 5$) (Fig. 2).

Pilot experiments with purified virions as the target showed that these movements of paramecia were directed toward the virus itself. We then varied the amount of virions in the target and showed that the movements were concentration dependent. Even at the lowest concentration tested [10^1 plaque-forming units (PFU)], *P. bursaria* showed orientation toward the viruses (Fig.3). As virion concentrations increased, the number of paramecia located at the virus target increased. This increase was confirmed by an increasing chi-square value showing that an increase in virion numbers resulted in higher chi-square values and decreased *p*-values; these results indicate that there is increasing confidence in the orientation toward the virus target as virion concentrations increase (Table 2). A dose-response curve revealed an apparent two-phase response with a lower limit of efficacy between 10^1 - 10^3 PFU per disk, and a secondary response above 10^5 PFU per disk.

We used an Osy virus (Osy-NE-ZA1) as the target in the initial experiments because the *P. bursaria* cultures containing zoochlorellae were infected by Osy viruses (5, 6). Currently our laboratory has four such algal/virus systems; they are *Chlorella variabilis* NC64A and its viruses

(referred to as NC64A viruses), *Chlorella variabilis* Syngen 2-3 and its viruses (referred to as Osy viruses), *Chlorella heliozoae* SAG 3.83 and its viruses (referred to as SAG viruses) and *Micractinium conductrix* Pbi and its viruses (referred to as Pbi viruses) (1, 13 14 15). To determine if the behavior of *P. bursaria* was chlorovirus specific, we conducted identical experiments with other chlorovirus strains (PBCV-1 an NC64A virus, TN603 an SAG virus and CVM-1 a Pbi virus), and each chlorovirus attracted the paramecia (Fig. 4; Table 3). Therefore, the chemotaxis was not chlorovirus type specific.

The observed chemotaxis was not due to reagents used in preparing the virus stocks. The chloroviruses were isolated using either sucrose or Iodixanol density gradients. We evaluated the density gradient materials to determine if residuals of these chemicals influenced the ability of the virus to attract *P. bursaria*. No significant differences were detected using virions purified by either sucrose or Iodixanol gradients to attract *P. bursaria* (Fig. 5; Table 4). We also substituted disks soaked in 40 % of either sucrose or Iodixanol in place of virus for one of the three arms in the choice microcosms. *P. bursaria* did not show any preferential movement toward sucrose or Iodixanol targets. Therefore, we conclude that the virus alone was responsible for attracting *P. bursaria*.

To begin to evaluate the soluble nature of the chemoattractive agent, virions were separated from the aqueous phase by centrifugation, so that we could recover and evaluate both the pellet and supernatant fractions (Fig. 6; Table 5). The majority of the attractive "signal" was retained in the "wash" fraction that is essentially free of virus, indicating that there was a soluble agent(s) "leaking" from the particles. The virions (pellet fraction) remain intact as indicated by plaque assay, where essentially all of the initial PFUs were recovered.

Discussion

Collectively, our results indicate that *P. bursaria* could detect and move toward chloroviruses, and this intentional behavior was virus concentration-dependent. These results stand in contrast to the paradigm that viruses do not signal to other cells from a distance and are dependent on biological, mechanical, or other physical processes to facilitate contacts with hosts and other intermediary cells. We infer that chloroviruses have associated chemical signals that are detected in low concentrations at a great distance by *P. bursaria* (roughly 400 cell lengths for cells ~70-80 μm in length in the microcosms used in these studies). Although *P. bursaria* is not the host of the chloroviruses, attracting *P. bursaria* cells increases the possibility that chloroviruses can adhere to their outer membrane. These contacts would otherwise depend on substantial movement of *P. bursaria* cells through the water column to facilitate random encounters to lead to effective accumulation of chloroviruses. This adherence in turn places the chloroviruses in the correct location to take advantage of ecological catalysts by predation that release zoochlorellae through either messy feeding or by passing fecal pellets. These processes may not be isolated to just this *P. bursaria* system, as zoochlorellae are also associated with other symbiotic hosts such as the coelenterate *Hydra viridis* (e.g., 16 and the heliozoan *Acanthocystis turfacea* (e.g., 17).

Although paramecia moved towards the chloroviruses at potentially high ratios (up to ~5:1 virus to alternative targets), not all individual paramecia chose to move toward the virus target. There was always a subset of the *P. bursaria* population that did not track to the virus target, independent of the virus amount. This suggests that individuals may differ in either their ability to detect the chemical cue or the motivation to respond to it. It is likely that individuals either vary in the number of viruses already on the cell surface at the time of the experiment, in their stage of the cell cycle, or in their physiology. Individual variation in propensity to seek out concentrations of chloroviruses could have significant implications for the evolution of chloroviruses and *P. bursaria*, as there may be fitness benefits to either carrying or shunning chlorovirus surface loads.

We do not know what the chemical nature of the chemotactic/chemo-attractive signal is. Virus particle complexity tends to increase with the size of the genome, including virion size and shape, protein composition and diversity, membrane content and composition, small molecule content, and charge neutralizing agents such as cations (18). All of these factors contribute to particle stability and infection potential as an extracellular agent that is animated upon contact with the host. Chloroviruses are large dsDNA-containing icosahedral particles composed of a glycoprotein outer capsid, which surrounds an inner lipid membrane. This shell encapsulates the 300+ kb genome, along with many proteins that are mostly virus encoded (19). Additionally, the chlorovirus PBCV-1 contains several small molecules associated with polyamine biosynthesis, including putrescine, spermidine, homospermidine and cadaverine (20 21). These molecules were evaluated for their potential to neutralize the large negative charge associated with the virion DNA; however, the abundances measured indicate the mole ratio was insufficient for this purpose. The best evidence for charge neutralization of the virion DNA is via divalent cations including calcium and magnesium, as well as certain cationic proteins (22). Thus, chloroviruses consist of both large and small molecules, some of which may contribute to the chemotactic properties of the virions reported here.

To begin to understand the chemical nature of chemotactic agent(s), virions were allowed to incubate in the VSB, then the supernatant fraction was collected after centrifugation and the pellet fraction was re-suspended in an equivalent volume. These fractions were evaluated in the standard 3-chamber microcosm, as shown in Fig. 6. The paramecia migrated to both fractions, but more so to the soluble fraction. The data support the hypothesis that there is a soluble agent that "leaks" from the virus particles. However, this is apparently not due to virus particle degradation, as indicated by a full recovery of PFUs in the pellet fraction.

Virions have not previously been reported to act as chemotactic/chemo-attractive agents. Rather, viruses as extracellular entities are generally viewed as non-metabolically active spore-like

agents that await further infection events upon collisions with appropriate host cells. That a virus might actively contribute to its fate via chemotaxis and change the behavior of an organism independent of infection is unprecedented. However, a recent report indicates that at least some bacteriophage can communicate with one another by producing and sensing small peptides as chemical messages (23). It has not escaped our attention that other viruses may attract motile cells; e.g., macrophages, but this has not been demonstrated to date to our knowledge. We must now re-think how virus particles can play an active role in directing their own fate. If one virus can do this, it is likely that others do as well.

Materials and Methods

1.1. Preparing the paramecia

Paramecia bursaria, a zoochlorellae-bearing holobiont, was provided by Dr. John DeLong's Lab at the University of Nebraska—Lincoln. They were originally collected from Spring Creek Prairie Audubon center pond water, Denton, NE, USA (*GPS* Coordinates: 40° 41' 37.6764'' N, 96° 51' 12.2544'' W). The paramecia were grown on protozoan medium (Carolina Biological Supply, Burlington, NC, USA) under constant light (light flux: 38-42 μmol/m^2×s) at room temperature (~25 °C). Before each experiment, paramecia were transferred to pond water from the Spring Creek Prairie pond that had been filtered through Whatman No.1 filter paper, aspirated through a 0.45 μm bottle-top filter (Sarstedt, Newton, NC, USA), autoclaved and stored at room temperature. The paramecia were washed three times with pond water to remove unattached viruses and residue such as culture medium and algae (5).

1.2. Virus isolation

The primary strain of virus tested in this project was *Chlorella variabilis* Syngen 2-3-infecting *Chlorovirus* OSY-NE-ZA1 (5). Other chlorovirus strains used were *C. variabilis* NC64A-infecting *Chlorovirus* PBCV-1, *C. variabilis* Syngen 2-3-infecting *Chlorovirus* OSY-NE-5, *C. heliozoae*

SAG 3.83-infecting *Chlorovirus* TN603 virus. All of these viruses were propagated using algal cells grown in MBBM medium (Modified Bold's Basal Medium) as described (11, 24) except for *Micractinium conductrix* Pbi-infecting chlorovirus CVM-1, which was propagated in FES medium (25).

Cell-free extracts

Chlorella variabilis Syngen 2-3 cells (0.6-1.0 × 10^8 cells/ml) in Basal Bold's Medium (BBM) were infected with *Chlorovirus* OSY-NE-ZA1 suspended in virus stabilization buffer [50mM Tris HCl, 10 mM $MgCl_2$, pH 7.8 (VSB)] at a multiplicity of infection (moi) of 3 PFU per cell, or mock infected with VSB as a control. After 30 min of infection, unattached viruses were removed by low speed centrifugation (1,900 × g for 3 min) of the virus- and mock-infected cells, and the pellet fractions containing the treated cells were re-suspended in BBM. The infection was then allowed to proceed to 4 hr under normal incubation conditions of light, temperature and shaking. After this 4 hr incubation the cells were harvested by centrifugation at 3,000 × g for 5 min, washed one time (by re-suspending and centrifugation) with pond water and re-suspended in pond water at a concentration of 0.9-1.3 × 10^9 cell/ml. Aliquots of 0.5 ml of infected cells were mixed with ~0.5 gm of glass beads [equal mix of 0.25-0.30 mm and 0.45-0.5 mm (Glasperlen, B. Braun Melsungen AG, Pennsylvania, USA), extensively washed with deionized distilled water and then with sterilized pond water] and placed in a bead beater (Disruptor Genie; Scientific Industries, Bohemia, NY) for 15 min at 4 °C. Disrupted cells were centrifuged for 1 min at 1,000 × g (to remove glass beads), and the supernatant fraction was collected and frozen in liquid nitrogen.

1.3. *Chlorovirus* purification

All the algal cells were maintained at 25 °C with continuous light and shaking (200 rpm) (26). The algal cell concentration was 1.5 × 10^7 cells/ml in 1600 ml of culture with tetracycline (10 µg/ml). The cells were inoculated with 0.45 µm filter sterilized virus (stock concentrations at ~8 × 10^{10} pfu/ml) at a moi of ~ 0.005 pfu/cell and incubated for 2 days with continuous light at 25 °C

and shaking until complete cell lysis. The lysates were adjusted with Triton X-100 to a final concentration of 1% (stock concentration 20%, w/v) to dissolve membranes, pigments and lipids that trap virions. The suspensions were stirred at room temperature for approximately one hr.

The detergent treated lysates were filtered through Whatman No.1 filter paper to remove cell debris, and the filtrates were collected. The filtered lysates were centrifuged in a Beckman Type 19 ultracentrifuge rotor at 53,000 × g for 50 min at 4 °C. The supernatant fractions were discarded, and the virion-enriched pellet fractions were re-suspended in 1 ml of VSB. The re-suspended virion fractions were further purified by density gradient fractionation. NC64A and Pbi viruses were separated on sucrose density gradients; whereas, SAG and Osy-NE viruses were separated on Iodixanol density gradients (OptiPrep; Axis-Shield, Oslo, Norway). The gradients were pre-established 10-40% linear sucrose or Iodixanol equilibrated with VSB for the Beckman SW32 rotor. Three to 4 ml of virion suspensions were layered on the pre-formed gradients, then centrifuged at 72,000 × g for 20 min at 4 °C. The virion bands were approximately 1/2 to 2/3 deep in the gradients, which were aspirated using a sterile bent cannula to remove the particles from the top. The suspended virions were transferred to a Beckman Type 19 tube, diluted with VSB and pelleted at 53,000 × g as above. The supernatant fractions were discarded, and pellet fractions were re-suspended overnight at 4 °C with 2 ml of VSB. After fully re-suspending the pelleted virions, the material was treated with proteinase K (final concentration of 0.02 mg/ml) and incubated at 45 °C for one hr to degrade non-virion associated proteins (26). The proteinase K-treated virions were applied to another density gradient fractionation, as described above, then collected and fully suspended in VSB and pelleted with the Beckman Type 19 rotor. This process was carried out two times, then the viral pellets were suspended in 1 ml of VSB. The final gradient purified virus particles were aspirated through a 0.45 μm bottle-top filter, and the virions were enumerated by plaque assay to determine the virus concentrations of plaque-forming units per ml.

1.4 Soluble agent from virions

Chlorovirus Osy-NE-ZA1 was freshly prepared as described in above, evaluated for virus concentration by plaque assay, and stored at 4 °C for 1 week. The 1 week old virus prep (100 µl at a concentration of 2.5×10^{11} PFU/ml) was incubated on the bench top at room temperature overnight, then was centrifuged for 1 hr at 20,000 × g [S_{20} = 2,300 for the type virus PBCV-1 (27)]. The supernatant fraction was removed, and the pellet fraction (containing the vast majority of virions) was re-suspended in 100 µl of fresh virus stabilization buffer (VSB). The pellet and supernatant fractions were serially diluted to an equivalent of virus concentration of 1×10^8 PFU/ml, then 10 µl of the diluted pellet and supernatant fractions were applied to filter disks. Untreated virus at the equal concentration served as a positive control. The preparations were evaluated with our standard 3-chamber choice microcosm experimental design (described below); each condition was replicated four times and each microcosm was read by four individuals. The infectivity of the virus in the re-suspended pellet fraction was not affected by these manipulations, as measured by plaque assay.

1.5 Microcosm setup

Paramecia movement behavior was evaluated by observing populations placed in a microcosm environment formed in Petri dishes with molded sterile agar media made in pond water (1.5 %, w/v, ThermoFisher). We used a locally fabricated three-channel mold (Fig. 7A) that allowed us to provide a three-way choice for the paramecia while moving freely in an aqueous condition. To set up the microcosm chamber, a thin layer of agar was first added and allowed to cool in a level Petri dish (92 × 16 mm Petri dish, Sarstedt, Germany), and the mold was put in the middle of the dish. Then hot liquid agar medium was added slowly (25-30 ml agar/dish) and left to solidify at room temperature. The mold was a three-channel triangle like shape (Plastic tubing connector-Y, tapered: 9.9-11.5 mm) (Fig. 7A, B); the end of each channel's width was 7 mm, and the entrance to the channel was 15 mm; the length of each channel was 30 mm. Once solidified, the mold was carefully removed from the Petri dish, and a small amount of hot liquid agar was added to smooth

the channel and make them equal in all directions. The depth of each channel was 10 mm, and the agar thickness was 16 mm.

Using three grade AA Whatman paper discs (6 mm from GE Healthcare, UK), 20 µl of each treatment condition was spotted onto the filter paper. Each disc was then placed at the end of each of the three channels (Fig. 7C). The paramecia (110-125 paramecia/ml in pond water) were counted and 1 ml was added to the middle of the microcosm so that they could move freely in all directions. Each experimental treatment had five replicas. The choice-type microcosm included a *Chlorovirus*, VSB and pond water, whereas, the non-choice-type microcosm contained three treatments of pond water, as a control. The treatment conditions were blind-coded.

The microcosm chambers with ranging paramecia were left overnight, then a micro cover glass (22 mm × 22 mm VWR Scientific products, US) was used to block each channel from the middle distance (15 mm between the filter paper disc to the Petri dish center) (Fig. 7C) before we started counting. Counting the paramecia was done using a dissection microscope (Leica Wild W3Z). At least four individuals counted the paramecia in each blind-coded microcosm chamber, and the mean values of these counts were used to represent the paramecia distributions within the microcosm chambers with respect to the treatment conditions.

1.6 Statistical analysis

We used chi-square tests to assess differences in the movement of paramecia toward the three targets within each replicate experimental microcosm. We replicated each treatment and control 3-5 times. The frequencies were the counts of paramecia located at the three targets at the end of the experimental period. We then compiled results across replicates to show the consistency of chemotactic movement toward each target type.

Acknowledgments

323 This material is based upon work supported by the National Science Foundation under Grant No.
324 1736030 (JLVE, DDD, JPD); the University of Nebraska—Lincoln Agricultural Research Division
325 and the Office of Research and Economic Development (DDD); and was partially supported by
326 the Ministry of Higher Education & Scientific Research, Republic of Iraq, and the Iraqi Cultural
327 Office in Washington, DC (ZA-A).
328
329 DDD was responsible for the project concept, contributed to the experimental design, analyses
330 and manuscript writing; MA-S contributed to the experimental design and data collection; ZA-A
331 contributed to the experimental data collection and critical materials development; IVA
332 contributed to the initial pilot experiments, experiment materials, and data analyses; JPD
333 contributed to the experimental design, data reduction and statistical analysis, as well as the
334 manuscript writing; JLVE contributed to the experimental design and manuscript development.
335
336 The authors declare no competing interests.
337
338 Correspondence and material requests should be addressed to David D. Dunigan (mail: 203
339 MOLR, University of Nebraska—Lincoln, Lincoln, NE, 68583-0900, USA; email:
340 ddunigan2@unl.edu)
341
342 **References**

343 1. Van Etten JL, Dunigan DD. 2012. Chloroviruses: not your every day plant virus. *Trends Plant*
344 *Sci* 17: 1-8. https://doi.org/10.1016/j.tplants.2011.10.005
345 2. Van Etten JL, Dunigan DD. 2016. Giant chloroviruses - five easy questions. *PLOS Pathogens*
346 12: e1005751. https://doi.org/10.1371/journal.ppat.1005751

3. Karakashian SJ, Karakashian MW. 1965. Evolution and symbiosis in the genus *Chlorella* and related algae. *Evolution* 19: 368-377. https://doi.org/10.1111/j.1558-5646.1965.tb01728.x

4. Kodama Y, Suzuki H, Dohra H, Sugii M, Kitazume T, Yamaguchi K, Shigenobu S, Fujishima M. 2014. Comparison of gene expression of *Paramecium bursaria* with and without *Chlorella variabilis* symbionts. *BMC Genom* 15: 183. doi: 10.1186/1471-2164-15-183. https://doi.org/10.1186/1471-2164-15-183

5. DeLong JP, Al-Ameeli Z, Duncan GA, Van Etten JL, Dunigan DD. 2016. Predators catalyze an increase in chloroviruses by foraging on the symbiotic hosts of zoochlorellae. *Proc Natl Acad Sci USA* 113: 13780-13784. https://doi.org/10.1073/pnas.1613843113

6. DeLong JP, Al-Ameeli Z, Lyon S, Van Etten JL, Dunigan DD. 2018. Size-dependent catalysis of chlorovirus population growth by a messy feeding predator. *Microbial Ecol* 75: 847-853. https://doi.org/10.1007/s00248-017-1106-8

7. Quispe CF, Sonderman O, Seng A, Rasmussen B, Weber G, Mueller C, Dunigan DD, Van Etten JL. 2016. Three-year survey of abundance, prevalence, and genetic diversity of Chlorovirus populations in a small urban lake. *Arch Virology* 161: 1839-1847. https://doi.org/10.1007/s00705-016-2853-4

8. Kleene SJ, Van Houten JL. 2014. Electrical signaling in motile and primary cilia. *Biosci* 64: 1092 – 1102. https://doi.org/10.1093/biosci/biu181

9. Kawakami H, Kawakami N. 1978. Behavior of a virus in a symbiotic system *Paramecium bursaria* – zoochlorella. *J Protozool* 25: 217–225. https://doi.org/10.1111/j.1550-7408.1978.tb04399.x

10. Yashchenko VV, Gavrilova OV, Rautian MS, Jakobsen KS. 2012. Association of *Paramecium bursaria* chlorella viruses with *Paramecium bursaria* cells: Ultrastructural studies. *Eur J Protistol* 48: 149-159. https://doi.org/10.1016/j.ejop.2011.05.002

11. Meints RH, Lee K, Van Etten JL. 1986. Assembly site of the virus PBCV-1 in a chlorella-like green alga: Ultrastructural studies. *Virology* 154: 240-245. http://doi: 10.1016/0042-6822(86)90448-4

12. Van Etten JL, Burbank DE, Xia Y, Meints RH. 1983. Growth cycle of a virus, PBCV-1, that infects chlorella-like algae. *Virology* 126: 117-125. https://doi.org/10.1016/0042-6822(83)90466-X

13. Yamada T, Onimatsu H, Van Etten JL. 2006. Chlorella viruses. In: "Advances in Virus Research" (K. Maramorosch and A.J. Shatkin, eds). Vol 66, pp 293-336. Elsevier Inc. https://doi.org/10.1016/S0065-3527(06)66006-5

14. Dunigan DD, Fitzgerald LA, Van Etten JL. 2006. Phycodnaviruses: a peek at genetic diversity. *Virus Res* 117: 119-132. https://doi.org/10.1016/j.virusres.2006.01.024

15. Quispe CF, Esmael A, Sonderman O, McQuinn M, Agarkova I, Battah M, Duncan GA, Dunigan DD, Smith TPL, De Castro C, Speciale I, Ma F, Van Etten JL. 2017. Characterization of a new Chlorovirus type with permissive and nonpermissive features on phylogenetically related strains. *Virology* 500: 103-113. https://doi.org/10.1016/j.virol.2016.10.013

16. Bosch TC. 2012. What hydra has to say about the role and origin of symbiotic interactions. *Biol. Bull.* 223: 78-84. https://doi.org/10.1086/BBLv223n1p78

17. Proschold T, Darienko T, Silva PC, Reisser W, Krienitz L. 2011. The systematics of zoochlorella revisited employing an integrative approach. *Environ. Microbiol* 13: 350-364. https://doi.org/10.1111/j.1462-2920.2010.02333.x

18. Glasgow J, Tullman-Ercek D. 2014. Production and applications of engineered viral capsids. Appl Microbiol Biotechnol 98: 5847-5858. doi: 10.1007/s00253-014-5787-3.

19. Dunigan DD, Cerny RL, Bauman AT, Roach JC, Lane LC, Agarkova IV, Wulser K, Yanai-Balser GM, Gurnon JR, Vitek JC, Kronschnabel BJ, Jeanniard A, Blanc G, Upton C, Duncan GA,

McClung OW, Ma F, Van Etten JL. 2012. *Paramecium bursaria* chlorella virus 1 proteome reveals novel architectural and regulatory features of a giant virus. *J Virol* 86: 8821-8834. http://doi: 10.1128/JVI.00907-12

20. Kaiser A, Vollmert M, Tholl D, Graves MV, Gurnon JR, Xing W, Lisec AD, Nickerson KW, Van Etten JL. 1999. *Chlorella* virus PBCV-1 encodes a functional homospermidine synthase. *Virology* 263: 254-262. https://doi.org/10.1006/viro.1999.9972

21. Baumann S, Sander A, Gurnon JR, Yanai-Balser GM, Van Etten JL, Piotrowski M. 2007. *Chlorella* viruses contain genes encoding a complete polyamine biosynthetic pathway. *Virology* 360: 209-217. https://doi.org/10.1016/j.virol.2006.10.010

22. Wulfmeyer T, Polzer C, Hiepler G, Hamacher K, Shoeman R, Dunigan DD, Van Etten JL, Lolicato M, Moroni A, Thiel G, Meckel T. 2012. Structural organization of DNA in *Chlorella* viruses. *PLoS One* 7: e30133. doi: 10.1371/journal.pone.0030133. Epub 2012 Feb 16.

23. Erez Z, Steinberger-Levy I, Shamir M, Doron S, Stokar-Avihail A, Peleg Y, Melamed S, Leavitt A, Savidor A, Albeck S, Amitai G, Sorek R. 2017. Communication between viruses guides lysis-lysogeny decisions. *Nature* 541: 488-493. https://doi.org/10.1038/nature21049

24. Nicols HW, Bold HC. 1965. *Trichosarchina polymorpha* gen.et. sp. nov. *J Phycol* 1: 34–38. https://doi.org/10.1111/j.1529-8817.1965.tb04552.x

25. Reisser W, Becker B, Klein T. 1986. Studies on ultrastructure and host range of a *Chlorella* attacking virus. *Protoplasma* 135: 162-165. https://doi.org/10.1007/BF01277009

26. Agarkova IV, Dunigan DD, Van Etten JL. 2006. Virion-associated restriction endonucleases of chloroviruses. J Virol 80: 8114-8123. http://doi: 10.1128/JVI.00486-06

27. Van Etten JL, Meints RH, Kuczmarski D, Burbank DE, Kit Lee K. 1982. Viruses of symbiotic *Chlorella*-like algae isolated from *Paramecium bursaria* and *Hydra viridis*. *PNAS* 79: 3867-3871. https://doi.org/10.1073/pnas.79.12.3867

Figure legends

Fig. 1. Experimental scheme for evaluating potential of chemotaxis agents associated with chloroviruses.

The microcosms are described in the Methods section. A) In the first experiment, *P. bursaria* are provided with a choice of cell-free extracts of mock-infected cells, chlorovirus-infected cells, and pond water. B) In later experiments, *P. bursaria* were provided a choice of purified virions suspended in virus stabilization buffer, virus stabilization buffer, or pond water. C) All experiments were paired with a negative control where all targets were the same (pond water).

Fig. 2. *Paramecium bursaria* showed significant movement toward cell-free extracts of virus-infected zoochlorellae relative to mock-infected cells and pond water.

A) Box-plots showing numbers of *P. bursaria* found at target sources in the counting zone. Boxes are central 50% of observations and points are outliers, defined as greater than a box distance away from the box. B) Box-plots summarizing chi-square values across replicate choice and no-choice experiments. C) Box-plots summarizing *p*-values across replicate experiments (all $p < 0.00001$), showing that all choice experiments revealed significant shifts in *P. bursaria* behavior and that none of the no-choice experiments showed significant orientation toward any target (all $p > 0.08$).

Fig. 3. Dose-response curve showing an increasing response of *P. bursaria* cells to chlorovirus targets as the amount of virus loaded on the target increased.

Blue circles represent the virus treatment, red boxes represent the virus stabilization buffer, and orange triangles represent pond water. Values are the mean of n = 4 for each concentration. Error bars are the standard deviation.

Fig. 4. *P. bursaria* showed chemotactic movements toward all tested chloroviruses.

Panels as in Figure 3. Virus types: C (black, pond water no-choice), M (red, Pbi-CVM-1), N (orange, SAG-TN603), P (purple, NC64A-PBCV-1), NE (green, Osy-NE5), Z1 (light blue, Osy-NE-ZA1), and Z2 (dark red, Osy-NE-ZA2). Each virus was evaluated at 10^4 PFU per target disk.

Fig. 5. Sucrose versus Iodixanol.

Paramecium bursaria showed no significant movement toward virions prepared with sucrose density gradients compared to virions prepared with Iodixanol density gradients. A) Box-plots showing numbers of *P. bursaria* found at target sources in the counting zone for preparations using either sucrose or Iodixanol prepared virus, sucrose or Iodixanol spiked VSB, or spiked pond water. Boxes are central 50% of observations and points are outliers, defined as greater than a box distance away from the box. B) Box-plots summarizing chi-square values across replicate choice experiments. C) Box-plots summarizing *p*-values across replicate experiments (all $p < 0.001$). The data indicate that all choice experiments revealed no significant shifts in *P. bursaria* behavior as a result of virus preparation.

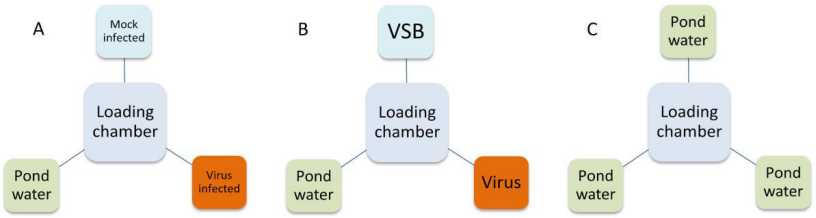
Fig. 6. Distribution of *Paramecium bursaria* in the presence of a soluble fraction of *Chlorovirus* Osy-NE-ZA1 virions to evaluate particle "leakage" of a chemotactic agent.

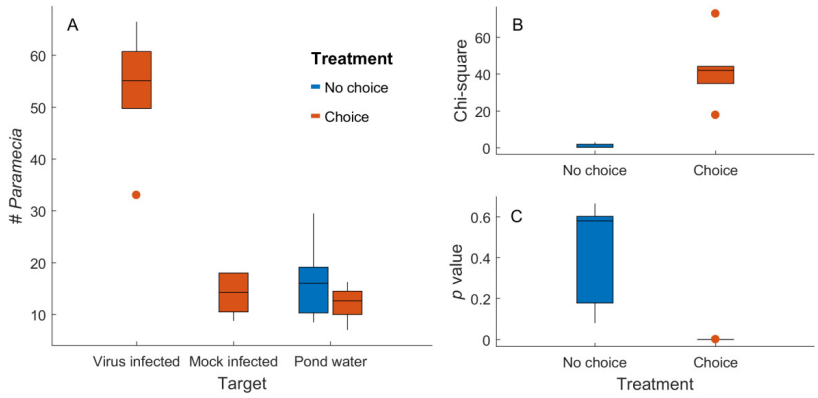
Panel A represents the distribution of the paramecia in the 3-chamber microcosms after an overnight incubation that allows the paramecia to roam throughout the microcosm space. The negative control (C) and the positive control (VC) were as observed previously (e.g., Figure 4). Both the pellet fraction (VP) and the supernatant fraction (VS) attract paramecia relative to the VSB and pond water choices; however, the VS attracted more paramecia than did the VP. Panels B and C provide the statistical support for these observations. Sample codes: "C" is the no-choice negative control where all targets are pond water (blue); "VC" is the non-treated virus suspended in virus stabilization buffer (VSB) after 24 hours incubation at room temperature (yellow); "VP" is the virus

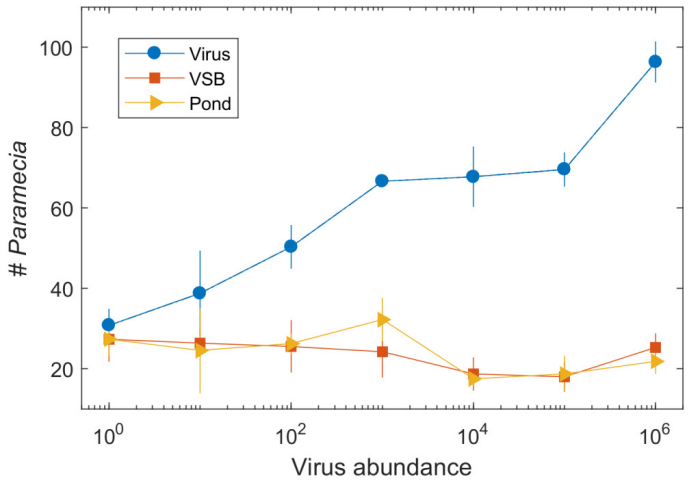
after 24 hours incubation at room temperature, then centrifuged at 20,000 x g for 1 hour and re-suspended in VSB as the pellet fraction (which is intended to contain the vast majority of virions) (purple); "VS" is the virus after 24 hours incubation at room temperature, then centrifuged at 20,000 x g for 1 hour separated as the supernatant fraction (which is intended to be essentially free of virus) (orange). VC, VP and VS were compared to VSB and pond water as choices. The data and statistical analyses for this experiment are found in Table 5.

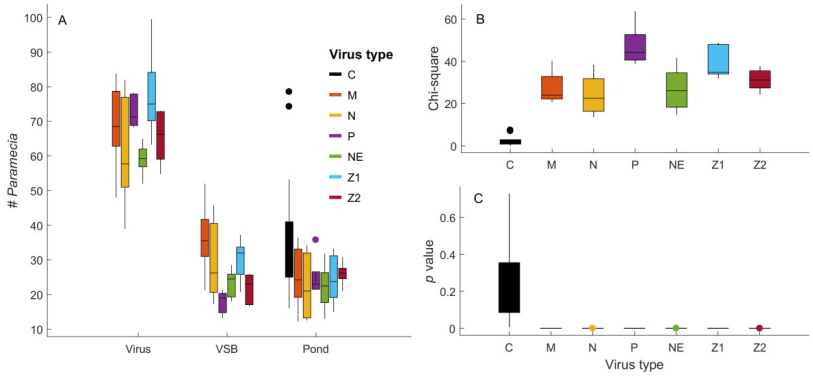
Fig. 7. Microcosm configuration.

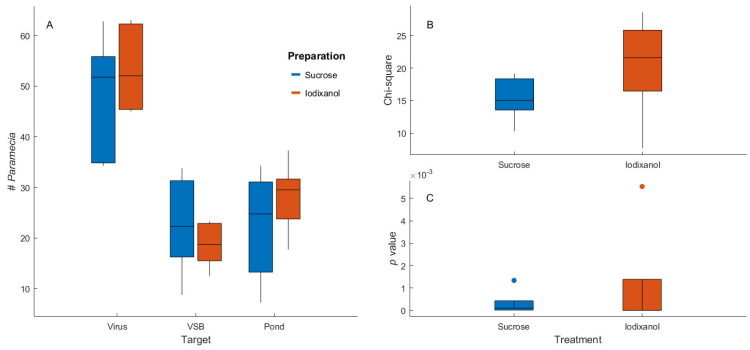
Microcosms were molded into pond water agar using plastic tubing connectors fashioned with a pipet tip as a handle (A); 100 mm plastic Petri dishes were used to contain the agar (B, C). When the three-channel mold (A) was removed from the agar, the microcosm consisted of three connected channels. Chemotactic agents (experimental and control) were impregnated into Whatman paper discs, and these were placed at the terminal points of the open channels (D). At the time of incubation, paramecia were transferred to the open channels and allowed to swim freely.

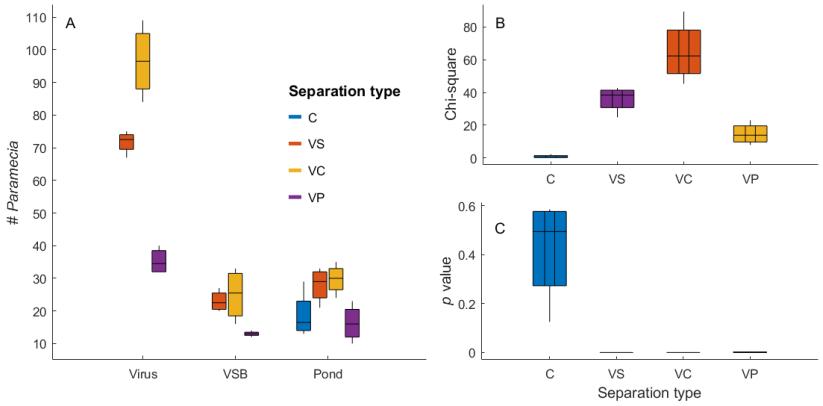












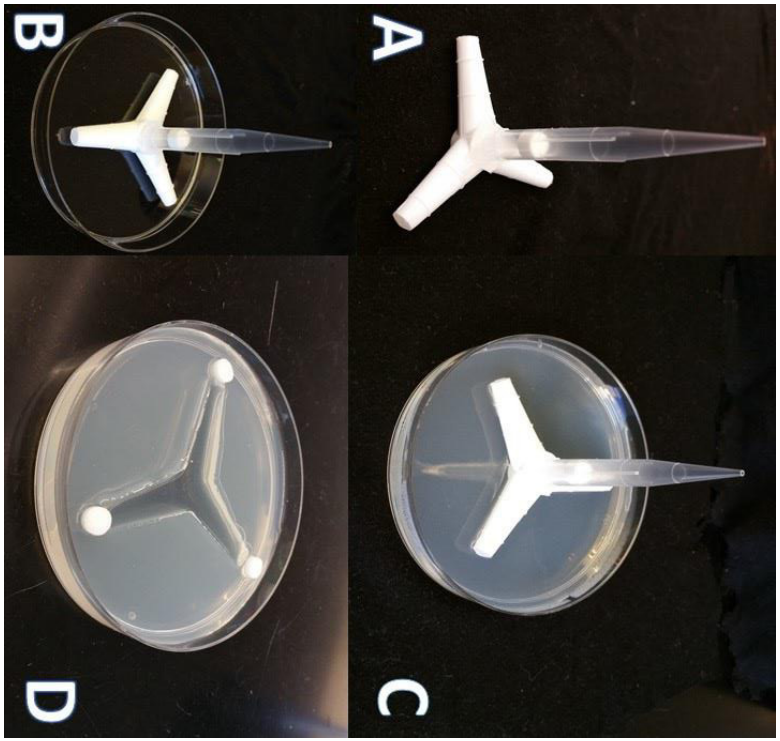

B
A
D
C

Table 1. Paramecium distribution in the presence of cell-free extracts

Treatment	Target/stats	Replicate experiment averages					
Cell free isolate	**Virus-infected**	33	57	49.75	60.75	53.25	66.5
	Mock-infected	8.75	18	14.25	18	14.25	10.5
	Pond Water	13.75	16.25	7	14.5	11.5	10
	Chi square	17.72	34.90	44.23	42.67	41.41	72.74
	p	2.56E-05	3.47E-09	2.92E-11	6.48E-11	1.23E-10	1.48E-17
Mock-infected	**Pond Water**	10.25	11	12.25	27.75	19.25	
	Pond Water	10	16	9.75	18	22	
	Pond Water	8.5	17.5	10.5	29.5	18.75	
	Chi square	0.19	1.56	0.30	3.06	0.31	
	p	0.67	0.21	0.58	0.08	0.58	

Columns are the measured values and associated statistical data from each of six microcosms for the virus-infected cell-free extracts, and five for the mock-infected; the values represent the average of the blind-coded readings by at least four individuals. The data are represented in Figure 2.

Table 2. Paramecium distribution in the presence of varying amounts of virions

Treatment (PFU/disk)	Target/stats	Replicate experiment averages			
	Virus	88.75	97.75	99.75	99
	VSB	23.25	23.75	30.5	23.75
1 × 10^6	**Pond Water**	19.25	26	22.25	19.75
	Chi square	69.61	72.06	71.28	83.92
	p	7.22E-17	2.09E-17	3.10E-17	5.14E-20
	Virus	64.25	68.25	74	71.75
	VSB	13.75	18.25	22.75	17
1 × 10^5	**Pond Water**	19.75	24.5	16.25	14.25
	Chi square	46.72	40.12	53.13	61.28
	p	8.21E-12	2.39E-10	3.12E-13	4.96E-15
	Virus	58.75	68.75	66.5	77
	VSB	14.25	24.25	18.25	18
1 × 10^4	**Pond Water**	17.75	18	15	19
	Chi square	40.48	41.40	50.03	60.05
	p	1.99E-10	1.24E-10	1.51E-12	9.24E-15
	Virus	67.25	65	67.75	66.5
	VSB	21.25	33.75	22.5	19.5
1 × 10^3	**Pond Water**	31.75	35.25	37	24.75
	Chi square	28.99	13.91	25.17	35.93
	p	7.26E-08	1.92E-04	5.24E-07	2.04E-09
	Virus	52	46.5	57.25	45.5
	VSB	22.25	19.5	34.5	26
1 × 10^2	**Pond Water**	26.5	27.75	28	22.5
	Chi square	15.42	12.25	11.82	9.80
	p	8.62E-05	4.65E-04	5.86E-04	1.74E-03
1 × 10^1	**Virus**	45.25	23	45	41.75

	VSB	30	23.5	28.5	23.5
	Pond Water	28.75	8.75	28.5	32
	Chi square	4.87	7.62	5.34	5.15
	p	0.027	0.006	0.021	0.023
Control - non choice test	**Pond Water**	25.25	30	34.5	33.25
	Pond Water	24.25	23	26.5	35.25
	Pond Water	22	28.75	26.25	32.25
	Chi square	0.23	1.02	1.51	0.14
	p	0.63	0.31	0.22	0.71

Columns are the measured values and associated statistical data from each of four microcosms; the values represent the average of the blind-coded readings by at least four individuals. The data are represented in Figure 3.

Table 3. Paramecium distribution in the presence of varying chloroviruses

Treatment [virus name (type)]	Target/stats	Replicate experiment averages				
CVM-1 (Pbi)	Virus	48	68.5	67.75	77	83.75
	VSB	35.5	52	38.25	21.25	34.25
	Pond Water	12.25	21.5	24.25	32	36.5
	Chi square	20.63	24.02	22.71	40.30	30.34
	p	5.59E-06	9.51E-07	1.88E-06	2.18E-10	3.62E-08
TN603 (SAG 3.83)	Virus	75.25	57.75	82	55	39
	VSB	45.75	26.25	38.75	17.25	21.75
	Pond Water	34.25	21	31.25	12.5	13.5
	Chi square	17.29	22.58	29.62	38.39	13.68
	p	3.22E-05	2.02E-06	5.25E-08	5.78E-10	2.17E-04
Osy-NE-ZA1(Syngen 2-3)	Virus	63.25	72.5	75	99.5	79
	VSB	20.75	27.5	32	37.25	32.5
	Pond Water	20.5	15	23.75	33.25	30.5
	Chi square	34.77	47.72	34.75	48.71	31.82
	p	3.70E-09	4.92E-12	3.75E-09	2.97E-12	1.69E-08
Control - non choice test	Pond Water	40.25	39	42.5	41	78.5
	Pond Water	49.25	53.25	52.5	40.5	74.25
	Pond Water	33.75	29.5	39.75	33.75	49.25
	Chi square	2.95	7.04	2.00	0.85	7.42
	p	0.086	0.008	0.157	0.356	0.006
Osy-NE5 (Syngen 2-3)	Virus	58.5	52	61	59.25	65
	VSB	18	25	24.5	19.75	28.5
	Pond Water	13	24.5	22.5	19.25	31.75
	Chi square	41.73	14.63	26.09	32.16	19.54
	p	1.04E-10	1.30E-04	3.25E-07	1.41E-08	9.81E-06
PBCV-1 (NC64A)	Virus	68.25	78.25	71.25	69	77.75
	VSB	20	21.25	19	15.25	13.25

	Pond Water	21.5	35.75	23.5	21.5	23
	Chi square	41.15	38.93	44.22	49.02	63.62
	p	1.41E-10	4.39E-10	2.93E-11	2.53E-12	1.51E-15
Control - non choice test	**Pond Water**	25	16	33	25.75	31
	Pond Water	28.75	17.25	31.25	16.75	19.25
	Pond Water	30	18	24.75	18.25	25.25
	Chi square	0.49	0.12	1.27	2.30	2.74
	p	0.49	0.73	0.26	0.13	0.10

Columns are the measured values and associated statistical data from each of five microcosms; the values represent the average of the blind-coded readings by at least four individuals. All virus concentrations were 1×10^4 PFU/disk. The data are represented in Figure 4.

Table 4. Paramecium distribution in the presence of density gradient materials used in virion preparations.

Treatment	Target/stats	Replicate experiment averages				
Sucrose	**Virus**	83.5	119	95.5	72.25	52.75
	VSB	30.75	76	45.5	41.5	21.25
	Pond Water	45.25	81.5	59	38.25	22.75
	Chi square	19.12	10.29	15.02	18.09	14.64
	p	1.23E-05	1.34E-03	1.06E-04	2.11E-05	1.30E-04
Iodixanol	**Virus**	81.25	61.75	86.75	82.75	86
	VBS	33.5	26.5	18.25	33.25	14.25
	Pond water	44	37.25	48.75	52.75	26.75
	Chi square	19.37	21.59	28.56	7.70	24.91
	p	1.08E-05	3.38E-06	9.08E-08	5.53E-03	6.02E-07

Columns are the measured values and associated statistical data from each of five microcosms; the values represent the average of the blind-coded readings by at least four individuals. The data are represented in Figure 5.

Table 5. Paramecium distribution in the presence of a soluble fraction from Osy-NE-ZA1 virions

Treatment	Target/stats	Replicate experiment averages			
Virus supernatant	Virus	67	73	72	75
	VSB	24	27	20	21
	Pond Water	33	21	31	27
	Chi square	24.89	40.13	36.63	42.73
	p	6.08E-07	2.37E-10	1.43E-09	6.28E-11
Virus control	Virus	84	92	101	109
	VSB	16	30	33	21
	Pond water	24	35	31	29
	Chi square	66.84	45.34	57.75	89.36
	p	2.95E-16	1.66E-11	2.98E-14	3.29E-21
Virus pellet	Virus	37	32	32	40
	VSB	12	13	13	14
	Pond water	10	23	14	18
	Chi square	23.02	7.97	11.63	16.33
	p	1.61E-06	4.75E-03	6.50E-04	5.31E-05
Control - non choice test	Pond Water	17	24	16	14
	Pond Water	13	24	13	17
	Pond Water	22	29	14	16
	Chi square	2.35	0.65	0.33	0.30
	p	0.13	0.42	0.57	0.59

Columns are the measured values and associated statistical data from each of four treatments in four replicate microcosms that are evaluating untreated virions (Virus control), and the soluble (Virus supernatant) and pellet (Virus pellet) fractions of the corresponding virus preparation, as described in the Materials and Methods. The values represent the average of the blind-coded readings by four individuals. The data are represented in Figure 6.