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ORIGINAL ARTICLE

Does Formation of Multicellular Colonies by Choanoflagellates Affect Their Susceptibility to Capture by Passive Protozoan Predators?

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ABSTRACT

Microbial eukaryotes, critical links in aquatic food webs, are unicellular, but some, such as choanoflagellates, form multicellular colonies. Are there consequences to predator avoidance of being unicellular vs. forming larger colonies? Choanoflagellates share a common ancestor with animals and are used as model organisms to study the evolution of multicellularity. Escape in size from protozoan predators is suggested as a selective factor favoring evolution of multicellularity. Heterotrophic protozoans are categorized as suspension feeders, motile raptors, or passive predators that eat swimming prey which bump into them. We focused on passive predation and measured the mechanisms responsible for the susceptibility of unicellular vs. multicellular choanoflagellates, *Salpingoeca helianthica*, to capture by passive heliozoan predators, *Actinosphaerium nucleofilum*, which trap prey on axopodia radiating from the cell body. Microvideography showed that unicellular and colonial choanoflagellates entered the predator's capture zone at similar frequencies, but a greater proportion of colonies contacted axopodia. However, more colonies than single cells were lost during transport by axopodia to the cell body. Thus, feeding efficiency (proportion of prey entering the capture zone that were engulfed in phagosomes) was the same for unicellular and multicellular prey, suggesting that colony formation is not an effective defense against such passive predators.

MICROBIAL eukaryotes that eat bacteria are critical links in aquatic food webs (Azam et al. 1983; Montagnes et al. 2008; Ohtsuka et al. 2015; Weisse et al. 2016). Heterotrophic microbial eukaryotes are unicellular, but some can form multicellular colonies. Many of the organisms that eat microbial eukaryotes are size-selective predators (Montagnes et al. 2008; Strom and Loukos 1998; Verity 1991; Weisse et al. 2016), but the consequences to predator avoidance of being unicellular vs. forming larger multicellular colonies are not yet well understood. Many species of choanoflagellates can be unicellular and can also form multicellular colonies by cell division (King 2004; Leadbeater 2015) (Fig. 1A); thus, they provide research systems that can be used to study the effects of multicellularity on the susceptibility to predation within a single species so that unknown sources of variability involved in comparing unicellular species with different colonial species can be avoided.

Studying the mechanisms that determine the vulnerability to predation of unicellular vs. multicellular choanoflagellates can not only help us understand how forming colonies affects trophic interactions of aquatic protozoans, but may also shed light on the evolutionary origins of animals. Multicellular animals evolved from a unicellular protozoan ancestor more than 600 million years ago (Armstrong and Brasier 2005; Knoll and Lipps 1993; Schopf and Klein 1992), a profound step in the evolution of life on Earth. The development of multicellularity was followed by the Cambrian explosion, when a diversity of complex animals representing most major phyla appeared

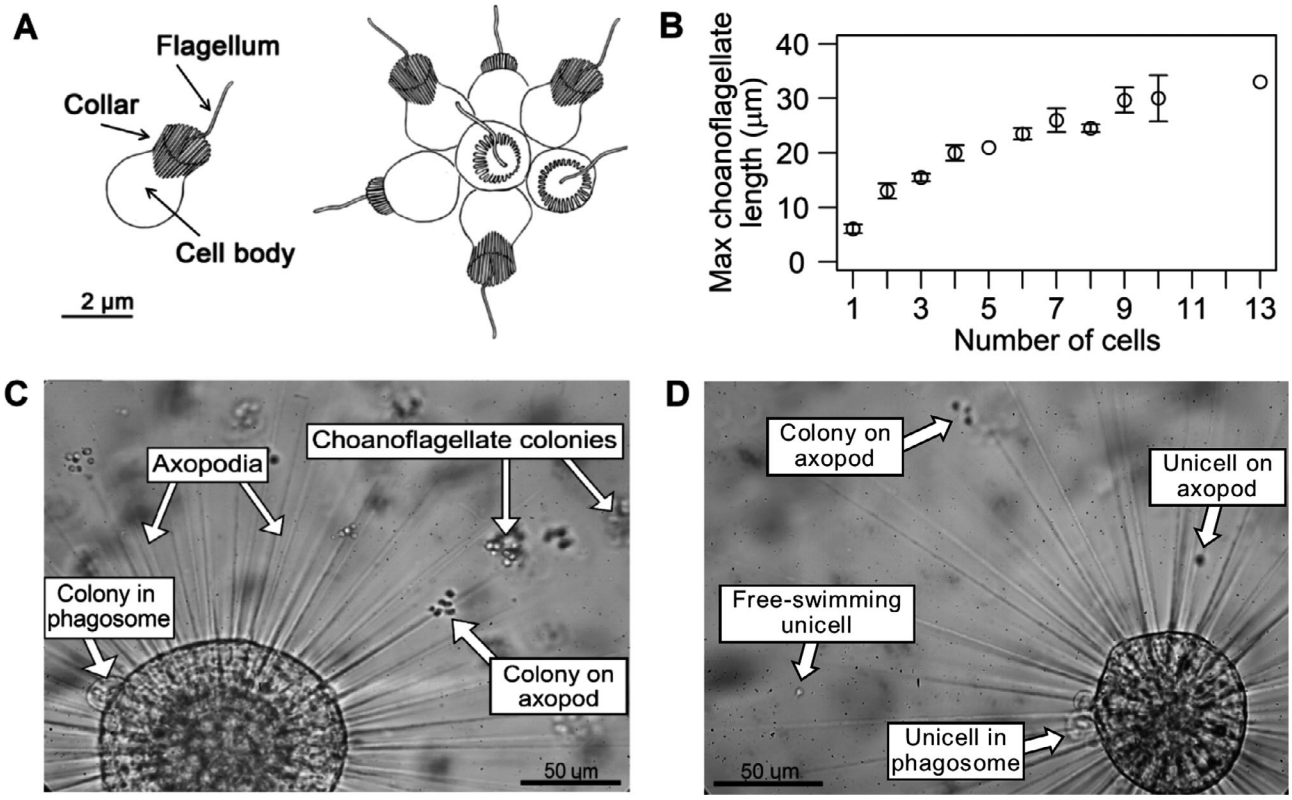


Figure 1 Study organisms. (**A**) Diagram of a unicellular choanoflagellate, *Salpingoeca helianthica,* and a multicellular colony that forms when daughter cells remain attached to each other after mitosis. (**B**) Plot of the maximum length of *S. helianthica* plotted as a function of the number of cells in the choanoflagellate. (**C**) *Actinosphaerium nucleofilum* capturing multicellular colonies of *S. helianthica*. (**D**) *A. nucleofilum* capturing unicellular *S. helianthica.* Prey are captured on the long, slender axopodia, transported to the cell body along an axopod, and engulfed into a phagosome at the cell surface.

in the fossil record (Butterfield 1997; Stanley 1973). Genomic and molecular phylogenetic analyses indicate that animals and choanoflagellate protozoans shared a common ancestor (King et al. 2008). Because colony formation is found in diverse choanoflagellate lineages, it has been suggested that colony formation was present in the last common ancestor of animals and choanoflagellates (Carr et al. 2017). Therefore, studying living choanoflagellates to help us understand how the extinct ancestors of choanoflagellates and animals might have worked enables us to make informed inferences about possible selective pressures at the time of animal origins. Choanoflagellates in the genus *Salpingoeca*, which have both unicellular and multicellular life stages, are used as model organisms to study the evolution of multicellularity in the ancestors of animals (Brunet and King 2017; King 2004; King et al. 2008; Richter and King 2013).

Several selective factors have been proposed that might have favored the evolution of multicellularity in the ancestors of choanoflagellates and animals (reviewed by Koehl 2020). One suggestion is that multicellularity may have enhanced the rates at which cells in colonies captured bacterial prey (Kirkegaard and Goldstein 2016; L'Etoile and King-Smith 2020; Roper et al. 2013; Stanley 1973). It has also been argued that an important selective factor leading to the evolution of multicellularity in the protozoan ancestors of animals was the relative difficulty that protozoan predators may have had in capturing larger multicellular colonies than smaller unicellular prey (Boraas et al. 1998; Richter and King 2013; Stanley 1973). Studies of living heterotrophic protozoans have shown that some have difficulty consuming large prey (Fenchel 1986; Jonsson 1986; Verity 1991).

Fossils, chemical biomarkers, and molecular analyses indicate that diverse heterotrophic protozoans (including ciliates, flagellates, and various amoeboid forms and Rhizaria) evolved before multicellular animals (Armstrong and Brasier 2005; Parfery et al. 2011; Schopf and Klein 1992), so the predators on the ancestors of animals and choanoflagellates were most likely protozoans from these groups. The diverse mechanisms by which swimming, crawling, and sessile protozoan predators of different morphologies capture prey (Arndt et al. 2000; Fenchel 1986; Sleigh 1991) have been categorized by Sleigh (2000) into functional types. Suspension or filter feeders create a feeding current and intercept prey on capture surfaces, motile raptors actively catch prey with pseudopodia or tentacles, and passive predators feed by intercepting prey that swim or drift nearby.

Choanoflagellates and their protozoan predators are so small that inertial forces can be ignored and the viscous resistance of water to being sheared determines the flow around them and hydrodynamic forces on them (reviewed by Koehl 2020). In such viscous flow regimes, it can be difficult for microscopic bodies to approach each other. A mathematical model of a motile sphere approaching a static sphere operating in this viscous regime showed that a sphere pushed by a flagellum behind it (as is the case for choanoflagellates) can approach a nonmotile target (e.g. a passive protozoan predator) that is the same size or bigger than the motile sphere (Jabbarzadeh and Fu 2018). Therefore, basic models of encounter rates between passive predators and motile prey that do not include hydrodynamics are applicable to choanoflagellates and passive protozoan predators. These models predict that the encounter rate of passive protozoan predators with motile prey depends on the swimming speeds and paths of the prey and that large motile prey are more likely than small ones to contact a predator (Fenchel 1982, 1984; Rubenstein and Koehl 1977; Shimeta and Jumars 1991). This suggests that multicellularity might increase rather than reduce the susceptibility to predation by passive protozoan predators. To address these conflicting suggestions, the focus of this study is on the susceptibility of multicellular vs. unicellular choanoflagellates to predation by a passive protozoan.

Research system

The passive protozoan predator *Actinosphaerium nucleofilum* (Fig. 1C) was chosen for this study because it is ecologically important, is easy to maintain in culture, and readily eats choanoflagellates in the laboratory. This heliozoan ("sun animalcule") is found in freshwater habitats worldwide where it consumes a wide variety of other protozoans (Barrett 1958; Leidy 1879). Heliozoans can be found in concentrations of more than 5,000 cells/liter and likely exert a large grazing pressure on protozoans in lakes and ponds (Pierce and Coats 1999). Prey are trapped when they contact the long axopodia of a heliozoan and then are transported along the axopodia to the predator's cell body, where prey items are phagocytosed (Bovee and Cordell 1971; Nikolaev et al. 2004; Suzaki et al. 1980). The Rhizaria (a group of eukaryotes that includes radiolarians, foraminiferans, and heliozoans) are estimated, based on molecular data, to have originated ~620 mya (Cavalier-Smith et al. 2018), and foraminifera 650–920 mya (Cavalier-Smith et al. 2018; Pawlowski et al. 2003), although the oldest fossils of cells are from 545 mya (Groussin et al. 2011). Radiolarians and foraminiferans use the same prey-capturing mechanism as heliozoans, so *A. nucleofilum* is a good model organism for study of a passive amoeboid type of predator that may have been present in premetazoan oceans.

Choanoflagellates in the genus *Salpingoeca* that have both unicellular and colonial life stages are used as model systems to study the functional consequences of and evolution of becoming multicellular (Brunet and King 2017; King 2004; King et al. 2008; Kirkegaard and Goldstein 2016; Richter and King 2013; Roper et al. 2013). We used *S. helianthica* as the prey in our study because it is a freshwater choanoflagellate with both unicellular and colonial forms (Fig. 1A, B) that is readily eaten by *A. nucleofilum* in the laboratory. A choanoflagellate cell (Fig. 1A) bears one flagellum which propels the cell through the water and creates a feeding current past a collar of microvilli, where bacterial prey are caught (Dayel and King 2014; Leadbeater 2015). Rosette colonies are formed when dividing cells of *S. helianthica* remain attached to each other (Fig. 1A). Choanoflagellates such as *S. helianthica* are commonly found in freshwater habitats, where they can be an integral part of aquatic food webs (Leadbeater 2015).

Objectives of this study

The goal of this study was to determine the organismal-level mechanisms responsible for the susceptibility of unicellular vs. multicellular choanoflagellates, *S. helianthica*, to capture by the passive heliozoan predator *A. nucleofilum*. The specific questions addressed were as follows:

1 Is the swimming behavior of unicellular *S. helianthica* different from that of multicellular colonies? Does the behavior of unicellular or multicellular choanoflagellates change when near an *A. nucleofilum*?

2 What are the mechanisms of capture of *S. helianthica* by *A. nucleofilum*, and do they differ between prey of different size (i.e. unicellular choanoflagellates and colonies composed of different numbers of cells)?

3 Do the frequencies of encounters with the capture zone (Fig. 2A) or contacts with the axopodia differ between unicellular and colonial choanoflagellates?

4 Does the size of an *A. nucleofilum* affect its feeding efficiency (number of prey captured per number encountered) on *S. helianthica*?

5 Does the feeding efficiency of *A. nucleofilum* on *S. helianthica* depend on prey size or differ between unicellular and multicellular prey?

MATERIALS AND METHODS

Culture of protozoans

Salpingoeca helianthica were purchased from the American Type Culture Collection (Manassas, Virginia). The original culture was frozen at −70 °C for one week and kept on liquid nitrogen until needed. Three different cultures of *S. helianthica* were revived and cultured at 22 °C using the protocols described in detail by King et al. (2009) and available at http://live-king-lab.pantheon.berkeley.edu/wp-content/uploads/2018/08/King-Lab-Choanoflagellate-Protocol-Handbook-April-2015.pdf. After a culture was revived, it was passaged every 3–4 d by pipetting 1 ml of culture into 9 ml of new media (25% cereal germ; King et al. 2009). The proportion of colonies in a culture decreased as the number of passages increased. Therefore, one culture was passaged 180 times before the culture was used in the experiment to encourage the formation of single

cells. The second and third cultures were revived later, were passaged every 3–4 d, and were used after 75 passages when the cultures were still rich in colonies. Aliquots of each of the three cultures were used during the first two weeks following passaging.

Cultures of *Actinosphaerium nucleofilum* were purchased from Carolina Biological Supply Company (Burlington, NC) and were kept in their original culture jars at room temperature (20 °C). No passaging or sub-culturing of these organisms was necessary, as all were used within four weeks of delivery and the original containers included wheat media that provided sustenance to the predators. Exposure to light was minimized by keeping the cultures in an opaque box when not actively being used.

Videomicrography

Predator–prey interactions were observed at room temperature (20 °C) in the flat-bottomed well (0.7 mm depth; 15 mm diameter) of a depression slide. For each experiment, one individual *A. nucleofilum* was pipetted directly from the culture into the well, followed by the addition of enough choanoflagellate culture to fill the well, which was then covered by a coverslip (total volume in well = 0.124 ml). After 30 min, the protozoans were observed using a Leica DMLS microscope with fiber-optic lighting so that illumination did not affect stage temperature. Videos were taken at a magnification of 10X to measure the swimming speeds of the choanoflagellates, and at 40X to record the process of prey capture and ingestion. To minimize wall effects on the motions of the protozoans, the microscope objective lenses had long working distances so that the plane of a video was at least mid-depth (150 μm below the cover slip) in the well for the 10X videos, and at least 120 μm below the coverslip for the 40X videos. Videos were made using a Fastec HiSpec 1 camera. The videos at 40X of predator–prey interactions were taken at 5 frames/s for a duration of 120–300 s and the videos of choanoflagellate swimming at 10X were taken at 5 frames/s for a duration of 327 s.

The concentrations of choanoflagellates in the well was determined by counting the number of colonies and of single cells in sharp focus in the region of water outside the

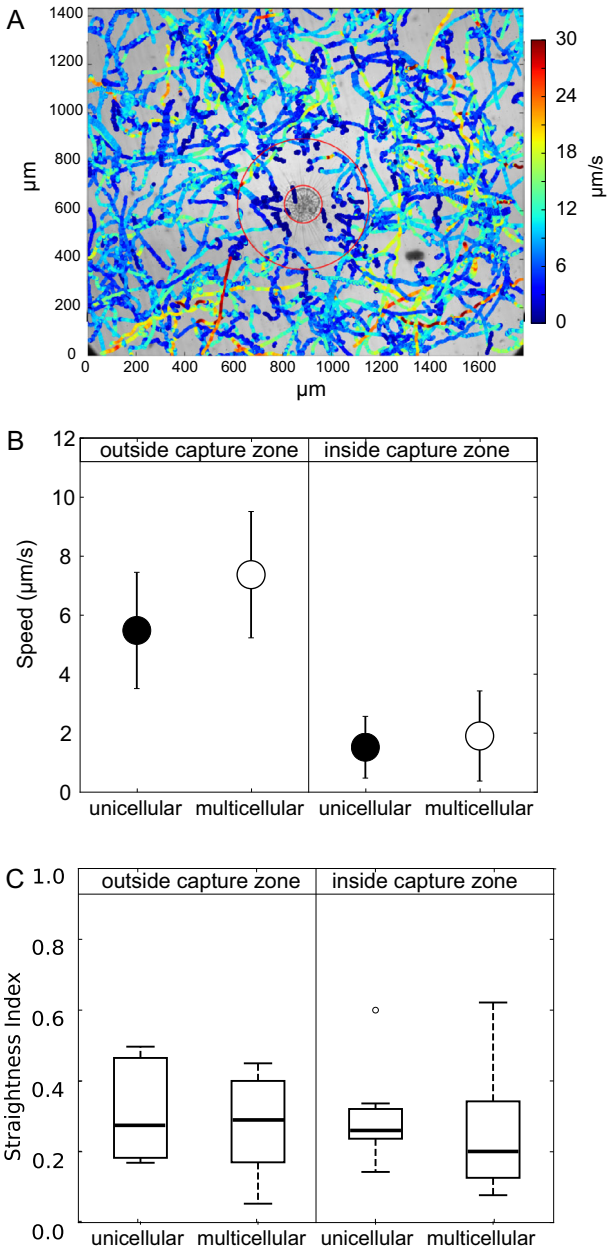


Figure 2 *Salpingoeca helianthica* movement patterns near an *Actinosphaerium nucleofilum*. (**A**) Swimming trajectories of *S. helianthica*, with higher speeds denoted by warmer colors. The capture zone (CZ) is indicated by the bright red circle. (**B**) Instantaneous speed of unicellular (black circles) and multicellular (white circles) *S. helianthica* outside and inside the CZ. For each replicate predator individual, we calculated the mean velocity of each choanoflagellate and then calculated the mean velocity outside and mean velocity within the capture zone of all unicellular choanoflagellates and of all colonial choanoflagellates. The mean of those means for all replicate predators is plotted here ($n = 6$ predators). Error bars indicate one standard deviation. (**C**) Straightness index (ratio of the distance between the position of the choanoflagellate at the start and the end of the trajectory, to the length of the path that the choanoflagellate followed along its trajectory) of unicellular and multicellular *S. helianthica* outside and inside the capture zone. The median of the straightness indices outside and the median within the capture zone were calculated for all the unicellular choanoflagellates or for all the colonies for an individual predator, and then, the median of those median straightness indices was calculated for each case for all the predators ($n = 6$ predators for all cases except unicellular choanoflagellates outside the CZ, for which $n = 5$ predators). Black bars indicate medians, boxes the first quartiles about the median, and error bars the range. For B and C, 12 videos of unicellular *S. helianthica* and 11 videos of colonies were used for this analysis, with 1–266 choanoflagellates per video; 6 predators fed on unicellular prey and 6 fed on colonies, and data from multiple videos of a single predator were pooled for that predator.

perimeter of the *A. nucleofilum* in the first frame of videos taken at 10X. The area of water in the frame in which the choanoflagellates were counted was measured using ImageJ (Rasband 2016) and was multiplied by the depth of the focal plane (11.4 μm) to determine volume. The mean concentration of single cells was 3.3×10^7 cells/ml (SD = 4.3×10^7, $n = 5$) and of colonies was 2.9×10^7 colonies/ml (SD = 3.9×10^7, $n = 5$). Although concentrations of protozoans in natural waters vary greatly (Fenchel 1987), we chose to use high concentrations of choanoflagellate prey in our brief experiments to assure that encounters would occur between the predator and prey in our small field of view (e.g. Fig. 1C, D).

Video analysis of choanoflagellate swimming

Choanoflagellate motions near *A. nucleofilum* were recorded in videos made at a magnification of 10X and were analyzed using in-house software written to use Python (version 2.7) bindings to the OpenCV (version 2.4) Computer Vision Library (https://opencv.org/) (Bradski and Kaehler 2008). The capture zone (CZ, a circle with a radius that is the distance between the cell center and the tip of the longest axopod of the heliozoan; Fig. 2A) was determined for each *A. nucleofilum*. The positions of an *A. nucleofilum* in each video were tracked using a combination of pyramid smoothing and blob detection filters that were thresholded by pixel brightness (Bradski and Kaehler 2008). Thus, the CZ of each predator was defined relative to the moving predator. The smaller choanoflagellates were identified using the cvGoodFeaturesToTrack function of the OpenCV library (Shi and Tomasi 1994), and their positions were followed through time using the Lucas–Kanade optical flow methods of the cvCalcOpticalFlowPyrLK function (Bradski and Kaehler 2008; Lucas and Kanade 1981). Tracking of an individual was terminated when the feature being tracked was no longer discernable by the algorithm, either due to swimming out of field or out of the focal plane. After tracking, only those tracked features that were clearly choanoflagellates (not suspended detritus) were manually chosen and used for further analysis. Each choanoflagellate was also identified as either unicellular or colonial at this stage in the analysis.

These choanoflagellate trajectories were used to determine the encounters of prey with the CZ of the predators. The proportion of unicellular and of colonial choanoflagellates that were swimming outside the CZ near an *A. nucleofilum* (within a radius of 400 μm from the center of the predator) that subsequently entered the CZ was determined for each *A. nucleofilum*.

The trajectories of the choanoflagellates were split into two categories for analysis of swimming behavior: "outside" the CZ of the predator or "within" the CZ. Central differences were used to calculate instantaneous swimming speeds from the positions of a choanoflagellate in successive frames of the video. Then, for each choanoflagellate, the mean of its instantaneous velocities when "outside" and the mean when "within" the CZ were calculated. We also determined a straightness index for the entire trajectory for prey "outside" vs. "within" the CZ, where straightness index (also called "net-to-gross-displacement ratio") is the ratio of the distance between the position of the choanoflagellate at the start of the trajectory and the end of the trajectory, to the length of the path that the choanoflagellate followed during its trajectory (Hadfield and Koehl 2004). Because the straightness index can differ between short trajectories and longer ones, we only determined straightness index for trajectories lasting ≥ 50 s. Straightness indices close to one denote nearly linear swim paths, while lower indices indicate paths characterized by turns or circling. For each individual predator, we calculated the mean of velocities outside and the mean of velocities within the capture zone of all unicellular choanoflagellates and of all colonial choanoflagellates, and then, the mean of those mean velocities was calculated for all predators. Similarly, for each individual predator, the median of the straightness indices outside and the median within the capture zone was calculated for all the unicellular choanoflagellates and for all the colonies, and then, the median of those median straightness indices was calculated for all the predators.

Video analysis of prey contact, transport, and capture

Each video made at a magnification of 40X was saved into digital.avi format and imported into ImageJ software (Rasband 2016) for analysis. For each video, the diameter of the cell body of each *A. nucleofilum* was measured on a single frame of a video to the nearest 10 μm and the CZ was determined.

All unicellular and colonial choanoflagellates that were in sharp focus and that entered the CZ were tracked by hand. Colonies and single cells were easily distinguished from each other while they were swimming freely (Fig. 1C, D), and the number of cells in each *S. helianthica* colony was counted while it was swimming freely. The longest dimension of freely swimming single-celled choanoflagellates and of freely swimming colonies were measured to the nearest 1 μm on single frames of videos. The edges of cell bodies were clearly visible, while collars and flagella were not always in sharp focus, so only cell bodies were used to measure choanoflagellate lengths.

Contacts with the axopodia of a predator by choanoflagellates that were in the CZ were determined. Because the depth of field was thicker than an axopod, a contact was only counted if the choanoflagellate near an axopod stopped when it overlapped with the axopod and then was transported along the axopod. The proportions of unicellular and colonial *S. helianthica* in the CZ that contacted an axopod were determined for individual *A. nucleofilum*. The instantaneous speed at which each prey on an axopod was transported toward the cell body of the predator was measured to the nearest 0.1 μm/s, and the mean speed for each prey item was calculated.

The feeding efficiency (number of prey caught per number encountered) of each predator at capturing choanoflagellates was determined. Encountered prey were all those that entered the CZ of the predator, and captured prey were only those that were eventually engulfed into a

phagosome (Fig. 1C). Cells that were already contained within a phagosome at the start of the video were not counted because single cells and colonies could not be distinguished when in phagosomes. These data were used to calculate the feeding efficiency of an *A. nucleofilum* for each size category (number of cells) of choanoflagellate prey:

$$\text{Feeding efficiency} = \frac{n_{\text{consumed}}}{n_{\text{encountered}}},$$

where n is the number of individual prey (single-celled choanoflagellates or multicellular colonies) in a size category. If several videos of the same individual *A. nucleofilum* were made, the data from those videos were pooled to give a single feeding efficiency for each size of choanoflagellate prey for that individual predator.

Statistical analyses

Swimming speeds of *S. helianthica* of different sizes were compared using ANOVA–Bonferroni tests performed using Python (version 2.7) and packages from the SciPy software stack (https://www.scipy.org/). Nonparametric tests performed using R software (R Core Team 2017) (Mann–Whitney *U*, Kruskal–Wallis, Kendall's tau-b, and Kolmogorov–Smirnov tests) were used to analyze data that were ratios (encounter with CZ, contact with axopodia, feeding efficiency, straightness index) or percentages, and data for which the sample sizes of some cases were small (e.g. speeds of axopodial flow).

RESULTS

Choanoflagellate behavior near *Actinosphaerium nucleofilum*

The swimming trajectories of *Salpingoeca helianthica* near *Actinosphaerium nucleofilum* predators are shown in Fig. 2A. The instantaneous swimming speeds of unicellular *S. helianthica* were not significantly different from those of multicellular colonies, both when outside and when inside the capture zone (CZ) (ANOVA–Bonferroni, $P > 0.05$, $n = 6$ *A. nucleofilum* predators) (Fig. 2B). Both colonies and single cells moved significantly more slowly when inside than when outside the CZ (ANOVA–Bonferroni, for colonies $P = 0.012$; for single cells, $P = 0.0024$, $n = 6$ predators). The straightness indices for the trajectories of unicellular and multicellular *S. helianthica* were not significantly different from each other, nor did they change when the choanoflagellates entered the CZ (Kruskal–Wallis, $P = 0.81$, $n = 6$ predators) (Fig. 2C).

Mechanisms of prey capture by *Actinosphaerium nucleofilum*

The process of capturing *S. helianthica* prey by *A. nucleofilum* involved several steps. First, a choanoflagellate swam into the CZ. Then, a prey item physically contacted an axopod and became adhered to it. We observed two different mechanisms used by *A. nucleofilum* to transport adhered prey to the cell body, named "axopodial flow" and "rapid axopodial contraction" by Suzaki et al. (1980) when reporting on heliozoans eating other types of prey. When prey reached the cell body of the predator, they were engulfed in phagosomes that formed outwards from the surface of the cell body (Fig. 1C). These phagosomes were occasionally observed to combine with other phagosomes to form a single, large phagosome. The phagosome and the prey item were then moved into the cell body of the predator.

Axopodial flow (indicated by red in Fig. 3A) is the slow movement (Fig. 3C) of the axopodial plasma membrane, which draws a prey item toward the cell body (Suzaki et al. 1980). We observed axopodial flow velocities ranging from 1.1 to 21.5 μm/s (median = 2.4 μm/s, $n = 7$ *A. nucleofilum* individuals). This speed was not significantly different between unicellular choanoflagellate prey and colonies (Mann–Whitney *U*, $P = 0.63$, $n = 7$ predators), and no trend was found between axopodial flow velocity and number of cells in a choanoflagellate colony (Kendall's tau-b, $P = 0.53$, $n = 7$ predators).

Rapid axopodial contraction (indicated by red in Fig. 3B) occurs when the microtubule skeleton of the axopod breaks down at high speed, triggered by the contact of a prey item with the axopod (Suzaki et al. 1994). When *A. nucleofilum* transported *S. helianthica* prey by rapid axopodial contraction, the entire motion occurred between two successive frames of the video (i.e. the motion was completed in a period of ≤ 0.2 s), so speeds were measured in excess of 1,500 μm/s (Fig. 3D).

The rapid axopodial contraction occurred more frequently than did axopodial flow (median percent of axopodial transports done by rapid contraction = 75%, $n = 8$ predators). The distribution of the percent of transports by rapid contraction was significantly different from a random normal distribution (two-sided Kolmogorov–Smirnov test, $P = 0.0335$). Large choanoflagellate colonies were more likely to be transported via rapid axopodial contraction than were small ones or single cells (there was a significant positive association between the number of cells in a choanoflagellate and the percent transported by rapid contraction, Kendall's tau-b, $P = 0.015$, $n = 30$ choanoflagellates). Large heliozoans were also more likely to use rapid axopodial contraction than were smaller predators, as there was a significant positive association between the percent of prey transported by rapid axopodial contraction and *A. nucleofilum* cell diameter (Kendall's tau-b, $P = 0.0495$, $n = 10$ predators).

Encounters with capture zone and contacts with axopodia

There are two steps in the feeding process of a heliozoan for which encounter rates are important: (i) entry of the prey into the CZ and (ii) contact of prey item with an axopod. Because the capture zone of a passive heliozoan predator is a sphere with the length of the longest axopod

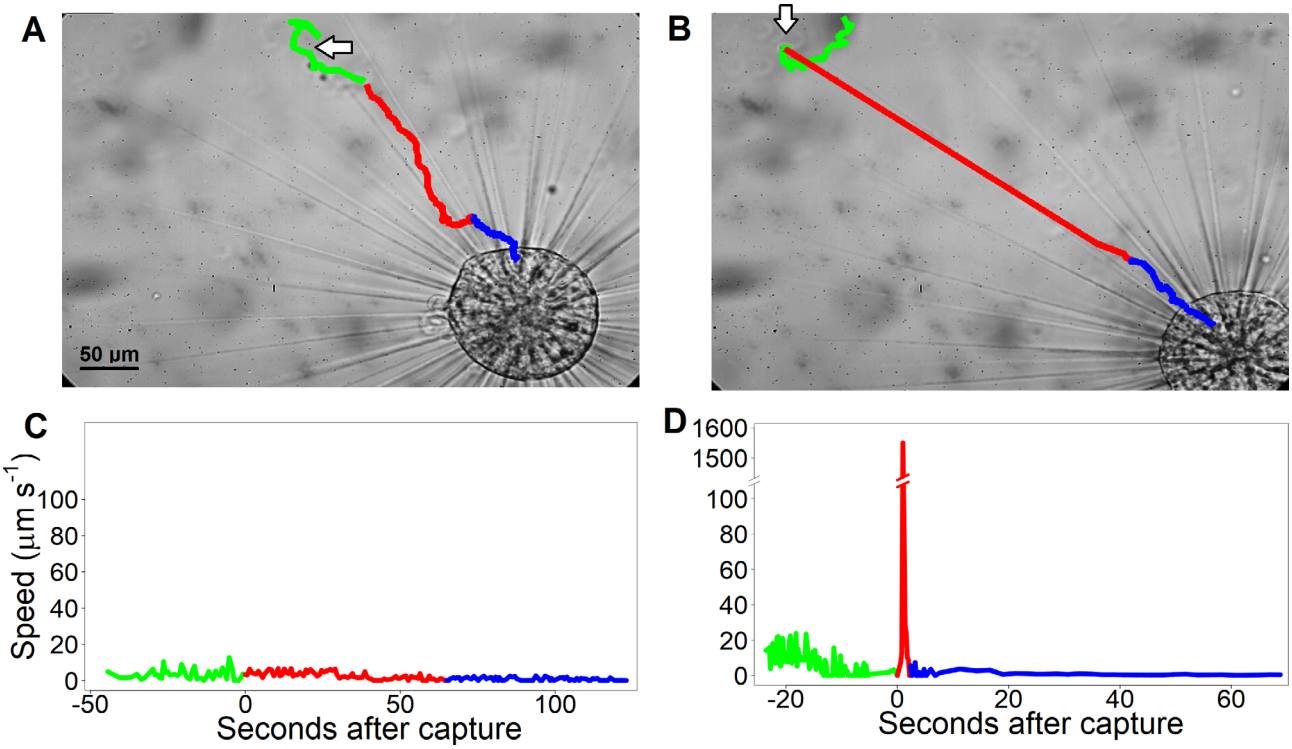


Figure 3 Examples of the two types of axopodial transport of prey by a single *Actinosphaerium nucleofilum*. (**A**) and (**B**) show the paths of prey (arrow indicates the location of the tip of the axopod before prey contact) and (**C**) and (**D**) show prey speeds plotted as a function of time before contact with an axopod (green), during axopodial transport (red), and during phagocytosis (blue). (A) and (C) present data for an example of axopodial flow, during which a prey item that touches an axopod is transported along the axopod toward the cell body. (B) and (D) show data for rapid axopodial contraction, in which a captured prey is rapidly moved toward the predator by the retraction of an axopod.

as the radius, we can use the empirical relationship provided by Fenchel (1982) and Shimeta and Jumars (1991) for passive predators to estimate the rate (F_{CZ}) at which motile choanoflagellates (radius r_p) in still water encounter the spherical CZ (radius r_s) of an *A. nucleofilum*:

$$F_{CZ} = 4\pi CD(r_s + r_p)$$

where C is the concentration of the prey (number/volume) and D is the diffusivity of the prey when outside the CZ. Diffusivity, D, is used in models to account for prey swimming when there is no quantification of the actual swimming trajectories, and so a random walk is assumed. Instead, we have measured the velocities and the straightness indices of the prey trajectories and found that they do not differ between single cells and colonies when outside the CZ. Therefore, we assume that D is the same for colonies and single cells. Thus, when we calculate the ratio of the F_{CZ} of colonies to the F_{CZ} of single cells for a CZ of a given r_s, D is a constant that is canceled out.

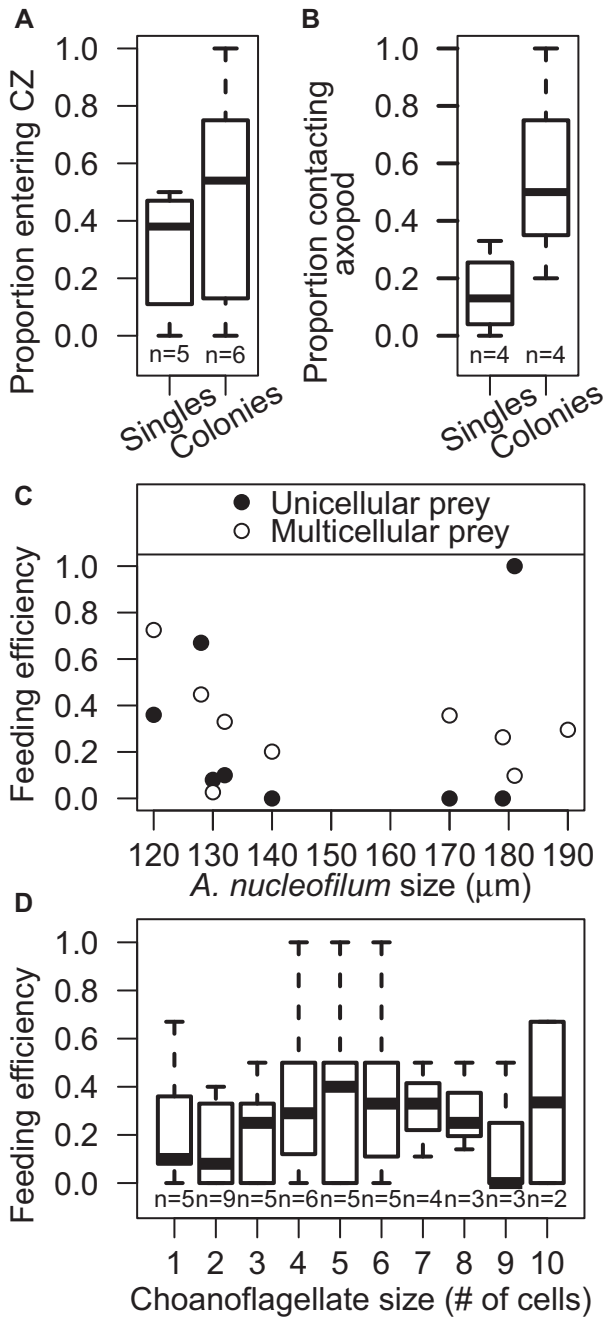
Once the prey are within the spherical CZ, they must also contact an axopod to be captured. We modeled an axopod as a cylindrical collector and calculated the contact rate (F_A) of choanoflagellates in the CZ with an axopod of length l and radius r_c using the relationship given by Shimeta and Jumars (1991):

$$F_A = 2\pi(r_c + r_p)D_A\frac{\delta C}{\delta r}l$$

where r_p is choanoflagellate radius, $2\pi(r_c + r_p)$ is the circumference of the site of encounter between the prey and axopod, D_A is the diffusivity of the prey within the CZ, and $\delta C/\delta r$ is the concentration gradient of prey around the cylinder. Prey swimming in the water within the CZ can approach an axopod from any direction, and our data meet the assumptions of this model because when an axopod is in the focal plane, we can see prey approaching it from all directions. Since both the swimming velocities and straightness indices of unicellular and multicellular *S. helianthica* are the same when within the CZ of an *A. nucleofilum*, we assume that D_A is the same for colonies and single cells. Thus, when we calculate the ratio of the F_A of colonies to the F_A of single cells at a given concentration gradient around an axopod of length l, then D_A, $\delta C/\delta r$, and l are constants that are canceled out.

Colonies are more likely to encounter the CZ than are single cells. For example, if an *A. nucleofilum* has a CZ radius of 300 µm and an axopod radius of 3 µm, the ratio of the F_{CZ} for a colony of 10 cells (r_p = 15.00 µm, Fig. 1B) to the F_{CZ} for a single cell (r_p = 3.02 µm, Fig. 1B) is 1.04, indicating that unicellular choanoflagellates are just as likely to enter the CZ as are colonies. This prediction is

consistent with our data showing no difference between the proportion of single cells or of colonies near an *A. nucleofilum* that enter the CZ (Fig. 4A). In contrast, the ratio of F_A for the 10-celled colony to the F_A of a unicellular *S. helianthica* is 3.0, indicating that contact rates with axopodia are higher for large multicellular prey than for small unicellular prey. This prediction is also consistent with our data showing that a significantly greater proportion of colonies in the CZ contact axopodia than do single cells (Fig. 4B).



The measured encounter frequencies of unicellular and colonial choanoflagellates with the CZ are shown in Fig. 4A, and the measured contact frequencies with axopodia are shown in Fig. 4B. The proportion of single cells encountering the CZ (median = 0.38, $n = 5$ predators), was not significantly different from that of colonies (median = 0.54, $n = 6$ predators) (Mann–Whitney U, $P = 0.463$). In contrast, the proportion of colonies contacting axopodia (median = 0.50, $n = 4$ predators) appeared to be greater than that of single cells (median = 0.13, $n = 4$ predators) (Mann–Whitney U, $P = 0.059$).

Feeding efficiency of *Actinosphaerium nucleofilum*

Feeding efficiency (number of choanoflagellates consumed divided by the number of choanoflagellates that entered the CZ) was not affected by predator or prey size. There was no significant correlation between the size (cell diameter) of *A. nucleofilum* and feeding efficiency on unicellular *S. helianthica* (Kendall's tau-b, $P = 0.36$, $n = 8$ predators) or on multicellular colonies (Kendall's tau-b, $P = 0.28$, $n = 9$ predators) (Fig. 4B). There also was no significant difference between the feeding efficiency on colonies vs. single cells (Mann–Whitney U, $P = 0.59$, $n = 9$ predators), nor was there any trend in feeding efficiency as a function of the number of cells in a prey colony (Kendall's tau-b, $P = 0.41$, $n = 9$ predators) (Fig. 4C).

DISCUSSION

Steps in the capture of *Salpingoeca helianthica* by *Actinosphaerium nucleofilum*

The process of capture of choanoflagellate prey by *A. nucleofilum* occurs in four distinct steps: (i) entry of prey into the capture zone (CZ), (ii) contact of the prey with an axopod, (iii) transport of the prey toward the cell body of the

Figure 4 *Salpingoeca helianthica* interactions with *Actinosphaerium nucleofilum*. (**A**) Proportion of the single-celled choanoflagellates and of colonies swimming near an *A. nucleofilum* that enter the CZ. A total of 41 single cells and 30 colonies of *S. helianthica* were tracked for 9 *A. nucleofilum* predator individuals. (**B**) Proportion of the single-celled choanoflagellates and of colonies swimming in the CZ of *A. nucleofilum* that contact axopodia. A total of 22 *S. helianthica* colonies and a total of 35 unicellular *S. helianthica* were tracked for 4 individual predators. (**C**) Feeding efficiency (number of prey engulfed in a phagosome per number of prey in the CZ) of *A. nucleofilum*, plotted as a function of the size of the *A. nucleofilum* for unicellular choanoflagellates (black circles) and for colonies (white circles). Each circle shows the efficiency for an individual predator, eight of which fed on single cells and nine of which fed on colonies. (**D**) Feeding efficiency plotted as a function of the size (number of cells) of *S. helianthica* prey. The number of individual predators (n) for which efficiency data could be determined for a given prey size is listed on the graph. In (A), (B), and (D), black bars indicate medians of the values from all the individual predators, boxes show the first quartiles about the median, and whiskers the range.

predator, either by axopodial flow or by rapid axopodial contraction, and (iv) phagosome formation around prey and movement of the phagosome into the cell body of the predator. Steps 2, 3, and 4 have also been documented for *A. nucleofilum* feeding on protozoan prey other than choanoflagellates (Suzaki et al. 1980).

The swimming speeds and the straightness indices of the trajectories of unicellular and multicellular *S. helianthica* are the same, both outside and within the CZ (Fig. 2). The choanoflagellates swim more slowly when in the CZ (Fig. 2B). Viscous forces determine the hydrodynamic performance of microscopic swimmers, so the viscous resistance of the water to being sheared between swimming choanoflagellates and nearby axopod surfaces is most likely the mechanism responsible for the reduction in swimming speed in the CZ (Vogel 1994).

We found that *S. helianthica* that contacted axopodia were transported by *A. nucleofilum* using either rapid axopodial contraction, (~75% of the transports) or axopodial flow. In contrast, Suzaki et al. (1980) found that *A. nucleofilum* used only axopodial flow to transport the flagellated cryptophyte *Chilomonas* and the ciliates *Paramecium* and *Stentor*, while both rapid contraction and axopodial flow were used to transport the flagellated unicellular alga *Chlamydomonas* and the ciliate *Tetrahymena.*

Susceptibility of unicellular vs. multicellular choanoflagellates to predation by passive protozoan predators

Models of passive protozoan predators indicate that large, motile prey are more likely than small ones to contact a predator (Fenchel 1982; Shimeta and Jumars 1991), as long as the prey item is not significantly larger than the predator (Jabbarzadeh and Fu 2018). This suggests that multicellular choanoflagellates might be more susceptible than unicellular choanoflagellates to predation. In contrast, it has been suggested that the larger size of choanoflagellate colonies makes them less vulnerable than unicellular choanoflagellates to protozoan predators and hence that predation might have been an important selective factor in the evolution of multicellularity in the ancestors of animals (Boraas et al. 1998; Fenchel 1986; Jonsson 1986; Richter and King 2013; Stanley 1973). Our study addresses these contradictory suggestions.

We found that the feeding efficiency (ratio of the number of prey taken into a phagosome in the cell body of the predator to the number of prey in the CZ) of *A. nucleofilum* is independent of their size (Fig. 4C) and of the size (number of cells) of *S. helianthica* prey (Fig. 4D). Because colonies are more likely to encounter an axopod after they have entered the CZ than are the smaller unicellular choanoflagellates, this indicates that more colonies than single cells are lost during transport along the axopodia. We conducted experiments in which *A. nucleofilum* were exposed to dead *S. helianthica* to determine whether colony loss during axopodial transport was due to predator choice or prey escape, but the nonmotile choanoflagellates did not enter the CZ of the passive heliozoan predator, and thus no axopodial transport could be tracked. Prior studies of various species of *Actinosphaerium* have noted both successful (Greenwood 1886; Suzaki et al. 1980) and failed (Bovee and Cordell 1971; Greenwood 1886; Tilney and Porter 1967) prey capture, but did not report the number of successful captures relative to the number of prey encountered.

We found that multicellularity does not affect the susceptibility of *S. helianthica* to capture by a heliozoan passive predator, *A. nucleofilum.* Our result suggests that colony formation is not an effective defense against such heliozoan predators, but other passive predators such as foraminiferans and radiolarians should also be tested. If the mechanisms we documented in this study also operate for other passive predators, this would suggest that predation on the protozoan ancestors of animals by such passive protozoan predators might not have been an important selective factor in the evolution of multicellularity. However, colony formation by choanoflagellates increases susceptibility to capture by raptorial predators and decreases vulnerability to ingestion by suspension-feeding ciliates (reviewed by Koehl 2020). Many active heterotrophic eukaryotes show size-selective feeding, with preferences for large prey in some cases and smaller prey in others (e.g. Fenchel 1980, 1986; Hansen et al. 1996; Jonsson 1986; Koehl 2020; Montagnes et al. 2008; Strom and Loukos 1998; Verity 1991; Weisse et al. 2016), so colony formation may well increase or decrease susceptibility to being eaten in ecosystems where such predators are abundant.

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