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ORIGINAL ARTICLE

Formation of multicellular colonies by choanoflagellates increases susceptibility to capture by amoeboid predators

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Abstract

Many heterotrophic microbial eukaryotes are size-selective feeders. Some microorganisms increase their size by forming multicellular colonies. We used choanoflagellates, *Salpingoeca helianthica*, which can be unicellular or form multicellular colonies, to study the effects of multicellularity on vulnerability to predation by the raptorial protozoan predator, *Amoeba proteus*, which captures prey with pseudopodia. Videomicrography used to measure the behavior of interacting *S. helianthica* and *A. proteus* revealed that large choanoflagellate colonies were more susceptible to capture than were small colonies or single cells. Swimming colonies produced larger flow fields than did swimming unicellular choanoflagellates, and the distance of *S. helianthica* from *A. proteus* when pseudopod formation started was greater for colonies than for single cells. Prey size did not affect the number of pseudopodia formed and the time between their formation, pulsatile kinematics and speed of extension by pseudopodia, or percent of prey lost by the predator. *S. helianthica* did not change swimming speed or execute escape maneuvers in response to being pursued by pseudopodia, so size-selective feeding by *A. proteus* was due to predator behavior rather than prey escape. Our results do not support the theory that the selective advantage of becoming multicellular by choanoflagellate-like ancestors of animals was reduced susceptibility to protozoan predation.

KEYWORDS

Amoeba proteus, choanoflagellate, feeding behavior, hydrodynamic signal, multicellularity, phagocytosis, predation, protozoa, pseudopodia, *Salpingoeca helianthica*

INTRODUCTION

HETEROTROPHIC microbial eukaryotes that eat bacteria, phytoplankton, or other protozoans are important links in marine and freshwater food webs (e.g. Azam et al., 1983; Boenigk & Arndt, 2002; Fenchel, 1987; Jürgens & Matz, 2002; Lagenheder & Jurgens, 2001; Laybourn-Parry & Parry, 2000; Montagnes et al., 2008; Ohtsuka et al., 2015; Schekenbach et al., 2010; Tillman, 2004; Weisse et al., 2016; Worden et al., 2015). Many of the predators on microbial eukaryotes are size-selective feeders (e.g. Fenchel, 1980; Montagnes et al., 2008; Strom & Loukos, 1998; Verity, 1991; Weisse et al., 2016). One of the ways in which microbial eukaryotes can change their size is by forming multicellular colonies, but the effects of being multicellular versus unicellular on vulnerability to predation by other microbial eukaryotes is not yet well understood. Choanoflagellate species that have unicellular life stages and that can also form multicellular colonies via cell division (e.g. King, 2004; Leadbeater, 2015) (Figure 1) provide useful research systems for studying the effects of multicellularity on predator avoidance.

Understanding mechanisms that affect the susceptibility of unicellular versus multicellular choanoflagellates to being captured by diverse protozoan predators can also provide insights into the evolutionary origins

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of animals. Multicellular animals evolved about 600 million years ago (Armstrong & Brasier, 2005; Knoll & Lipps, 1993; Schopf & Klein, 1992). Molecular phylogenetic and genomic analyses indicate that animals and choanoflagellate protozoans shared a common ancestor (Carr et al., 2017; King et al., 2008; López-Escardó et al., 2019; Richter et al., 2017). It is thought that the ability to form multicellular colonies was present in the last common ancestor of animals and choanoflagellates because colony formation is found in a number of different choanoflagellate lineages (e.g. Carr et al., 2017). Thus, by studying the performance of colonial versus unicellular choanoflagellates at swimming, feeding, and avoiding capture by predators, we can make informed inferences about selective pressures that might have affected the evolution of multicellularity in the ancestors of animals and choanoflagellates (reviewed in Koehl, 2020). Before animals evolved, the predators on the ancestors of animals and choanoflagellates were most likely heterotrophic eukaryotes, so it has been suggested that multicellular colonies might have been too big for those microbial predators to capture and consume (Boraas et al., 1998; Fenchel, 2019; Richter & King, 2013; Stanley, 1973).

Evidence from molecular phylogenetic analyses, fossils, and chemical biomarkers shows that heterotrophic forms of eukaryotes such as ciliates, flagellates, and various amoeboid protozoans evolved before multicellular animals (e.g. Armstrong & Brasier, 2005; Parfery et al., 2011; Schopf & Klein, 1992). Therefore, studying how living examples of these groups interact with unicellular and multicellular choanoflagellates can help us evaluate the idea that a selective advantage of forming colonies by animal ancestors was an escape in size from predation.

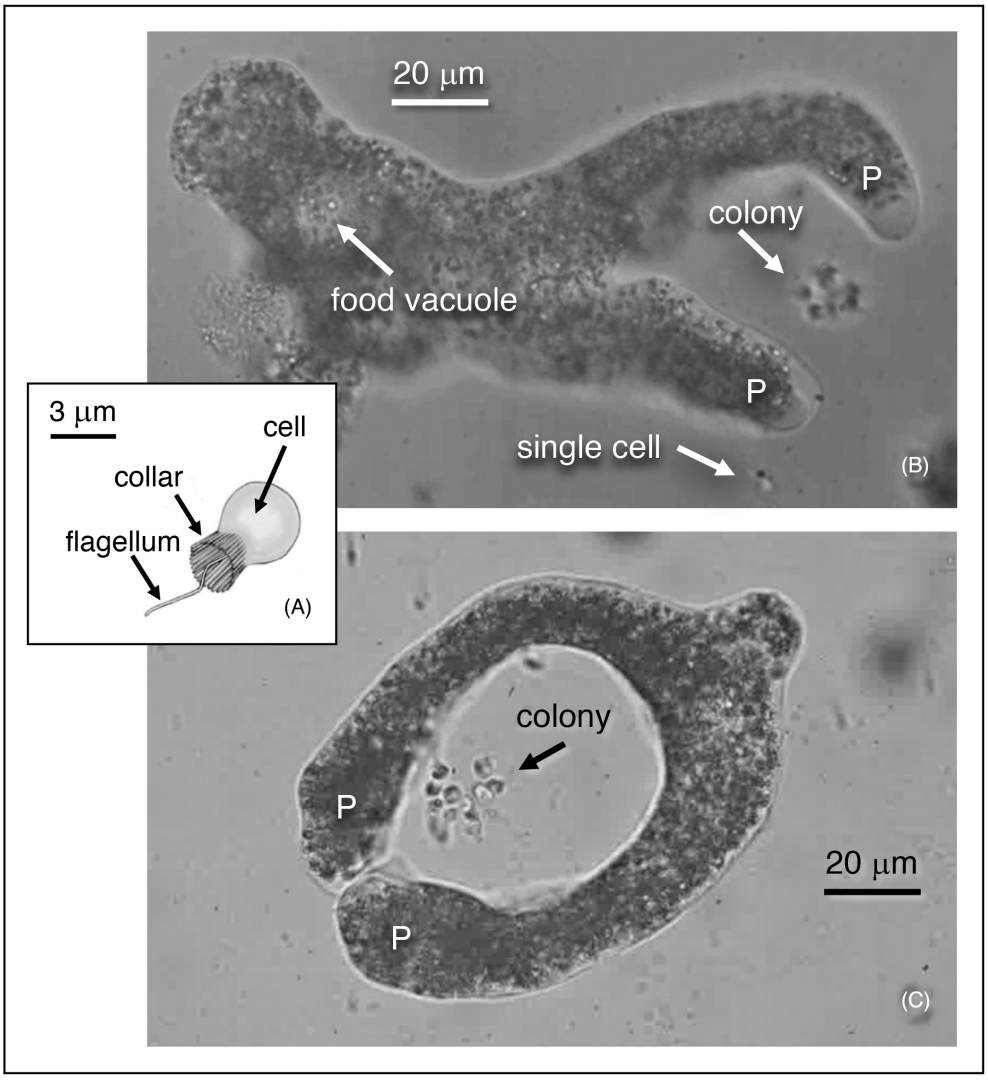

FIGURE 1 *Salpingoeca helianthica* prey and *Amoeba proteus* predators. (A) Diagram of an *S. helianthica* cell showing the ovoid cell body and the single flagellum surrounded by a collar of microvilli. (B) Frame of a video of an *A. proteus* extending two pseudopodia (P) around an *S. helianthica* colony. A food vacuole in the *A. proteus* contains a captured colony of *S. helianthica*. A unicellular *S. helianthica* (single cell) is ignored by the *A. proteus*. (C) Frame of a video of a different *A. proteus* that has completely encircled a colony of *S. helianthica* by two pseudopodia that are fusing to create a food vacuole.

Feeding modes and size-selective feeding by heterotrophic microbial eukaryotes

Although diverse sessile, crawling, and swimming protozoans use a variety of mechanisms to capture prey (reviewed by Arndt et al., 2000; Fenchel, 1986, 1987; Jürgens & Massana, 2008; Montagnes et al., 2008; Sleigh, 1991), their feeding modes have been categorized into functional types (Montagnes et al., 2008; Sleigh, 2000): (1) a "suspension feeder" or "filter feeder" produces a water current that carries prey into a capture area, (2) a "passive predator" or "diffusion feeder" traps swimming or drifting prey that bump into it, and (3) a "motile raptor" or "raptorial interception feeder" uses structures such as pseudopodia or tentacles to actively capture prey. The effects of increasing size via colony formation on the susceptibility of choanoflagellates to being eaten might differ depending on predator feeding mode.

It has long been known that different types of heterotrophic microbial eukaryotes are size-selective feeders (e.g. Fenchel, 1980, 1987; Hansen, 1992; Montagnes et al., 2008; Verity, 1991). For example, diverse types of protozoans are unable to consume large prey (e.g. suspension feeders: Fenchel, 1986; Jonsson, 1986) or preferentially feed on smaller prey (e.g. dinoflagellates: Jakobsen & Tang, 2002; nanoflagellates: Callieri et al., 2002). In contrast, diverse bacteria-eating protozoans preferentially feed on large cells (ciliates: Epstein & Shiaris, 1992; Gonzalez et al., 1993; Jonsson, 1986; Sanders, 1988; flagellates: Chrzanowski & Šimek, 1990; Epstein & Shiaris, 1992; and nanoflagellates: Boenigk & Arndt, 2000; Šimek & Chrzanowski, 1992). However, some protozoans eat small and large cells at lower rates than mid-sized prey (reviewed by Jürgens & Güde, 1994; ciliates: Jakobsen & Hansen, 1997; flagellates: Jakobsen & Tang, 2002; Pfandl et al., 2004; dinoflagellates: Jakobsen & Hansen, 1997; and nanoflagellates: Güde, 1979). Herbivorous amoeba preferentially engulf larger diatoms (Van Wichelen et al., 2006), whereas a variety of amoebae feeding on benthic bacteria consume smaller cells at higher rates than large ones (Dillon & Parry, 2009). The mechanisms underlying size-selective feeding can be an active choice by predators, the inability of predators to engulf large prey, or the higher rates of encountering predators by large motile prey than by small prey (e.g. Fenchel, 1982, 1987; Kumler et al., 2020; Rubenstein & Koehl, 1977; Shimeta & Jumars, 1991; Verity, 1991). Some protozoans use different feeding mechanisms to capture large prey than they employ for smaller prey (Berge et al., 2008; Jeong et al., 2005). Heterotrophic microbial eukaryotes also distinguish among prey using chemical signals (e.g. Montagnes et al., 2008; Stoecker, 1988; Verity, 1991). These studies suggest that an increase in prey size via colony formation might only reduce the danger of capture by some types of microbial predators.

Features of prey that affect susceptibility to predation by microbial eukaryotes

Microorganisms use a variety of mechanisms that can reduce their susceptibility to predation, including chemical defenses and changes in cell surface properties, increase in size, and motile escape maneuvers (reviewed in Lancaster et al., 2019; Montagnes et al., 2008).

Various prey of heterotrophic microbial eukaryotes respond to predators by increasing their size. Some ciliates do so by increasing cell size (Kusch, 1993b; Wicklow, 1988) or by producing wings, spines, and protuberances (Kuhlmann & Heckmann, 1994; Kusch, 1993a; Kusch & Heckmann, 1992; Wicklow, 1988) in response to predatory ciliates and amoebae. Other microorganisms increase their size in response to predators by becoming multicellular via colony formation or aggregation. For example, some unicellular algae form multicellular filamentous colonies or increase colony size in response to predation by flagellates (Boraas et al., 1998; Jakobsen & Tang, 2002; Kapsetaki & West, 2019). Similarly, in response to heterotrophic microbial eukaryotes, some bacteria and cyanobacteria form colonies or aggregate into multicellular clusters, filaments, or biofilms that are resistant to predation (Corno & Jürgens, 2006; Deleo & Baveye, 1997; Güde, 1979; Hahn et al., 1999; Jürgens et al., 1994; Lancaster et al., 2019; Matz & Kjelleberg, 2005; Pajdak-Stós et al., 2001; Pernthaler et al., 1997; Posch et al., 2001; Sommaruga & Psenner, 1995). Although the formation of spherical rosette colonies (Figure 1) by choanoflagellates does not affect their capture by passive heliozoan predators (Kumler et al., 2020), the consequences of forming rosette colonies to capture by protozoans using other feeding modes are not known.

The motility of prey can affect their susceptibility to predation. Mathematical models indicate that planktonic prey have more frequent encounters with predators as prey swimming speed increases (e.g. Andersen & Dölger, 2019; Crawford, 1992; Visser, 2007), and some experiments show that motile or faster microorganisms are captured at higher rates (bacteria captured by flagellates: Gonzalez et al., 1993; dinoflagellates and ciliates captured by zooplankton: Jakobsen et al., 2005) than are nonmotile or slowly-swimming microorganisms. In contrast, faster swimming by bacteria reduces the rates at which nanoflagellate (Matz & Jürgens, 2005) and ameboid (Lancaster et al., 2019) predators capture them. Some motile protozoans show escape behaviors. For example, ciliate and flagellate prey may initiate evasive behaviors when they detect fluid motions produced by predators (Jakobsen, 2001, 2002). Furthermore, some ciliates are induced by a chemical signal to move away from ameboid predators (Kusch, 1993b). Modeling also predicts that the risk of predation is greater for prey that swim along straight paths than for prey that have

meandering or spiral trajectories (Visser, 2007). Rosette colonies of the choanoflagellate, *Salpingoeca rosetta*, swim more slowly and along more circuitous paths than do unicellular *S. rosetta* (Koehl, 2020), but the effects of such differences in swimming on susceptibility to predation are not known. Furthermore, whether unicellular or multicellular choanoflagellates have escape behaviors elicited by predators has not been documented.

If microbial eukaryote predators can sense nearby prey via hydrodynamic signals produced by the prey, a mechanism by which prey can be cryptic is to minimize the disturbance of the water around them as they swim. Microbial eukaryotes are so small that inertial forces can be ignored and the viscous resistance of water to being sheared determines the flow around them and the hydrodynamic forces they experience (e.g. reviewed by Koehl, 2020; Vogel, 1994). In such viscous flow regimes, the water is sheared (e.g. one layer of water moves faster than the layer next to it) by moving bodies, and the layer of sheared water around a microscopic swimmer is large relative to the size of the organism (e.g. Vogel, 1994). Raptorial ciliates detect hydrodynamic shear produced by motile prey (Jakobsen et al., 2006). Further evidence that microbial eukaryotes respond to hydrodynamic disturbances is that some flagellates and ciliates are stimulated to execute escape maneuvers by predator-feeding currents or by siphon flow that mimics the water shear in predator-produced currents (Jakobsen, 2001, 2002; Jakobsen et al., 2006). Rosette colonies of the choanoflagellate, *S. rosetta*, create flow fields that are much larger than those produced by unicellular *S. rosetta* (Koehl, 2020), suggesting that raptorial predators might be more likely to detect colonies than single cells, and might be able to sense multicellular colonies at greater distances than single cells.

Research system

We studied the effects of rosette colony formation by choanoflagellates on their susceptibility to capture by raptorial protozoan predators using *Salpingoeca helianthica* as the prey and *Amoeba proteus* as the predators.

Salpingoeca helianthica

Choanoflagellates in the genus *Salpingoeca* are used as model organisms to study the evolution of multicellularity in the ancestors of animals because they have unicellular life stages and can also form multicellular colonies by cell division (e.g. Brunet & King, 2017; King, 2004; King et al., 2008; Kirkegaard & Goldstein, 2016; Koehl, 2020; Kumler et al., 2020; Richter & King, 2013; Roper et al., 2013). *S. helianthica* is a freshwater choanoflagellate that has a number of life stages, including benthic thecate cells, unicellular swimmers, and multicellular swimming rosette colonies (Figure 1; Carr et al., 2017; Richter et al., 2017). Choanoflagellates such as *S. helianthica*, which eat bacteria and are the prey to other protists, can be important links in freshwater food webs (Leadbeater, 2015).

Amoeba proteus

The raptorial predator, *A. proteus*, was chosen for this study because it is ecologically important (reviewed in Anderson, 2018; Rodríguez-Zaragoza, 1994), is easily maintained in culture, and readily eats both multicellular and unicellular swimming *S. helianthica*. *A. proteus* are found worldwide on surfaces (e.g. sediment, vegetation, and particulate floc) in freshwater environments (e.g. puddles, ponds, lakes, and streams), and in wet soil and moist detritus (e.g. Anderson, 2018; Nishibe et al., 2004; Rodríguez-Zaragoza, 1994; Rogerson et al., 2003; Waite et al., 2000). *A. proteus* range in size from ~250 to 600 μm (Levy, 1924; Rogerson, 1980; Schaeffer, 1916a) and prey on a wide variety of smaller organisms that move slowly enough to be captured, such as bacteria, desmids, diatoms, flagellates, ciliates, rotifers, and other amoebae (e.g. Anderson, 2018; Dillon & Parry, 2009; Gibbs & Dellinger, 1908; Jeon & Bell, 1962; Kepner & Taliaferro, 1913; Lancaster et al., 2019; Mast & Hahnert, 1935; Van Wichelen et al., 2006). Amoebae play a vital role in the dynamics of nutrient cycling and energy flow in microbial communities and thus are essential components of both terrestrial and aquatic ecosystems (e.g. Anderson, 2018; Shi et al., 2021).

It has long been known that amoebae crawl and capture prey using pseudopodia, which are temporary arm-like extensions of the cell (e.g. Cameron et al., 2007; Dellinger, 1906; Gibbs & Dellinger, 1908; Kepner & Taliaferro, 1913; Mast, 1926; Schaeffer, 1916b, 1917). The cellular mechanisms and biophysics of pseudopod extension and amoeboid crawling have received much attention (e.g. reviewed in Álvarez-González et al., 2014; Barry & Corson, 2005; Lämmermann & Sixt, 2009; Swanson & Baer, 1995), as have the locomotory and food-searching strategies of amoebae (e.g. Miyoshi et al., 2001; Van Haastert & Bosgraaf, 2009). *A. proteus* capture prey by phagocytosis, encircling prey with one or more pseudopodia and digesting them in food vacuoles (e.g. described in Jeon & Bell, 1962; Jeon & Jeon, 1976; Kepner & Taliaferro, 1913; Kepner & Whitlock, 1921; Lancaster et al., 2019; Prusch & Britton, 1987; Salt, 1968; Sobczak et al., 2008; Swanson & Baer, 1995). Both mechanical and chemical stimuli produced by prey can induce *A. proteus* to extend pseudopodia (e.g. Kepner & Taliaferro, 1913; Weisman & Korn, 1967), and amoeboid protozoans have been shown to respond to hydrodynamic shear (Décavé et al., 2003). *A. proteus* can capture more than one prey

at a time (Kepner & Whitlock, 1921; Salt, 1961), but they do not phagocytose all the prey that they encounter (Kepner & Taliaferro, 1913). How the behavior and kinematics of *A. proteus* pseudopodia might change in response to prey of different sizes has not yet been quantified.

Objectives of this study

The goal of this study was to determine the organismal-level mechanisms responsible for the susceptibility of unicellular versus multicellular choanoflagellates, *S. helianthica*, to capture by the raptorial predator *A. proteus*. We measured swimming by the choanoflagellate prey and behavior of the pseudopodia of the predators, and used these data to address specific questions:

1. Does the susceptibility of *S. helianthica* to capture by *A. proteus* depend on prey size (i.e. number of cells)?
2. Do the responses and kinematics of pseudopodia of *A. proteus* vary with prey size?
3. Do *S. helianthica* colonies or single cells change their behavior in response to the pseudopodia of *A. proteus*?

MATERIALS AND METHODS

Culture of protozoans

Salpingoeca helianthica cultures (from the American Type Culture Collection, Manassas, VA) were frozen at −70°C for 1 week and then kept on liquid nitrogen until needed. Frozen cultures of *S. helianthica* were revived and cultured at 22°C using the protocols described in detail by King et al. (2009; available at http://live-king-lab.pantheon.berkeley.edu/wp-content/uploads/2018/08/King-Lab-Choanoflagellate-Protocol-Handbook-April-2015.pdf).

Each culture was passaged every 3–4 days by pipetting 1 ml culture into a measured volume of fresh medium (25% cereal germ; King et al., 2009). Cultures were a mix of unicellular and multicellular choanoflagellates, and the proportion of single cells in culture was higher if the volume of culture was low relative to the volume of medium when cultures were passaged. To have cultures of colonies and cultures of single cells to study, we used a range of ratios of culture volume to medium volume when we passaged the cultures (culture:medium ratios of 1:9, 1:2, and 1:50). Furthermore, the proportion of colonies in a culture decreased as the number of passages increased. Cultures were still rich in colonies after 75 passages, so we did >180 passages to get cultures with high proportions of single cells. Aliquots of cultures were used in our experiments during the first 2 weeks following passaging.

Cultures of *A. proteus* (from Carolina Biological Supply Company) were kept in their original culture jars at room temperature (20°C). All cultures were used within 4 weeks of delivery. The original containers included wheat media that provided sustenance to the *A. proteus*, so no passaging of these organisms was necessary. Exposure to light was minimized by keeping the cultures in an opaque box.

Videomicrography

Video recordings were made of *A. proteus* predators interacting with *S. helianthica* prey in the flat-bottomed well (0.7 mm depth; 15 mm diameter) of a depression slide at room temperature (20°C). For each experiment, one *A. proteus* was pipetted into the well, after which enough choanoflagellate culture was added 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 40×, and the depth of field was 1.84 µm. To minimize wall effects on the swimming of the choanoflagellates, we used a microscope objective lens that had a long working distance so that the plane of a video was >120 µm below the coverslip. Videos were made at various framing rates (40, 50, or 100 frames s^{-1}) using a Fastek Hi-Spec 1 camera system.

Video analysis of choanoflagellate swimming

Video records of choanoflagellate motions near *A. proteus* were analyzed with in-house software written to use Python (version 3.5) bindings to the OpenCV (version 3.4) Computer Vision Library (https://opencv.org/; Bradski & Kaehler, 2008). Choanoflagellates in a video were identified as either unicellular or colonial, and their positions in successive frames of the video were determined using the blob-tracking function for all individuals above a threshold pixel brightness. Tracking of an individual was terminated when it was no longer discernable by the algorithm, either due to swimming out of the field or out of the focal plane.

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 was calculated. For all trajectories lasting ≥50 s, a straightness index for the entire trajectory was also determined, where the straightness index 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 & Koehl, 2004).

Straightness indices close to one denote nearly linear swim paths, while lower indices indicate paths characterized by turns or circling.

The capture zone (CZ) for each *A. proteus* predator was defined as 100 μm from the edge of the cell (the distance at which *A. proteus* can sense food, Schaeffer, 1917). To determine if a choanoflagellate came within the CZ, the distance between the closest edge of the choanoflagellate and the closest edge of the *A. proteus* was measured using a straight line. For each individual *A. proteus*, we calculated the mean of the velocities of (1) all the unicellular choanoflagellates outside the CZ, (2) all the choanoflagellate colonies outside the CZ, (3) all the unicellular choanoflagellates within the CZ, and (4) all the choanoflagellate colonies within the CZ. Then the grand means of each of those mean velocities were calculated for all the *A. proteus* that we videoed. Similarly, for each individual *A. proteus*, the median of the straightness indices was calculated for all the unicellular choanoflagellates and for all the colonies outside the CZ, and for all the unicellular choanoflagellates and for all the colonies within the CZ, and then the median of each of those median straightness indices was calculated for all the *A. proteus*.

Video analysis of flow fields produced by choanoflagellates

Some videos of *S. helianthica* swimming outside the CZ of predators were made with marker particles in the water so that the hydrodynamic disturbances produced by the choanoflagellates could be visualized. ImageJ (version 1.53f51) software with MTrackJ plugin (version 1.5.1; Meijering et al., 2012) was used to determine the position of the centroid of each choanoflagellate at 0.2 s intervals, and to calculate the instantaneous velocities (to the nearest 0.1 μm/s) of particles in the surrounding water.

Video analysis of speeds of pseudopodia

The extension speeds of the pseudopodia of the *A. proteus* were analyzed using the OpenCV Computer Vision Library (version 3.4) described above. The edges of an *A. proteus* were highlighted using a combination of Laplacian filtering and thresholding of pixel brightness. The pseudopodia that formed in response to choanoflagellate prey were identified. The path of each pseudopodium was traced manually, and that path was used to identify and mask a small region on the pseudopod leading edge that was followed frame-by-frame using the blob-tracking algorithm described above. Central differences were used to calculate instantaneous pseudopodium speeds from the position on successive frames of the tracked spot on the leading edge. Instantaneous pseudopodium velocity data was passed through either a 2 or a 0.5 Hz lowpass filter prior to further analysis.

Video analysis of predator–prey interactions

Each video was saved into digital .avi format and imported into ImageJ version 1.52 software for frame-by-frame analysis. All measurements of distance and size were made to the nearest 1 μm. A response by an *A. proteus* to a choanoflagellate was defined as the formation of a pseudopodium. To determine how close prey had to come to the predator to elicit a response, we measured the distance between the closest edge of the *A. proteus* to the prey (Kepner & Taliaferro, 1913) and the closest edge of the choanoflagellate in the frame when the first pseudopod began to form in response to that choanoflagellate.

Only choanoflagellates that were in focus and whose fates (captured, not captured, and ignored) were clear by the end of the video were analyzed. A choanoflagellate was considered "captured" if the *A. proteus* completed the formation of a food vacuole around the prey (i.e. the pseudopodia completely encircled the prey and fused with each other or the cell body of the *A. proteus*). A choanoflagellate was considered "not captured" if the *A. proteus* responded to the choanoflagellate, but the prey moved away from the predator and ended up outside of the volume of water encircled by the pseudopodia. A choanoflagellate was considered to be "ignored" if it entered the CZ but elicited no response from the *A. proteus*. In these cases, the shortest distance between the ignored prey and the surface of the *A. proteus* was measured.

For every choanoflagellate that entered the CZ (100 μm from the edge of the *A. proteus*) and whose fate was clear, we used ImageJ to measure the diameter of the choanoflagellate, and we recorded the number of cells in the choanoflagellate and its fate. *S. helianthica* colonies rotate as they swim, so cells in colonies were counted in a video frame when most of the cells were clearly visible. If some of the cells were unclear within one frame, the colony was followed for ~16 frames to assure that all the cells in the colony were counted. During analysis, we categorized the choanoflagellate into three size categories: single cells, small colonies (2–5 cells), and large colonies (≥6 cells).

A. proteus responded to choanoflagellate prey by forming one or more pseudopodia. We recorded the frame at which each pseudopodium was initiated to calculate the timing of pseudopod formation. In some cases, a pseudopodium is split into two pseudopodia (i.e. the new pseudopodium formed along the surface of an already formed pseudopodium). We recorded the frame number when such a split was initiated.

Statistical analyses

Data used in parametric statistical tests met the assumption of normality (Shapiro–Wilke test) and homogeneity of variance (Levene's test). Shapiro–Wilke tests, Levene's tests, Kruskall–Wallis tests, and paired-sample *T*-tests were done using Statistics Kingdom Online Calculators (Statistics Kingdom 2017; https://www.statskingdom.com). One-way ANOVA with post hoc Tukey HSD analyses, and Kendall's tau rank correlation tests were done using the Astasa Online Statistical Calculator (Navendu Vasavada, 2016; https://astatsa.com).

RESULTS

Effect of *S. helianthica* size on their susceptibility to capture by *A. proteus*

Only some of the *S. helianthica* that swam into the CZ of an *A. proteus* (100 µm from the predator's surface) were captured. We found that the percent of the prey in the CZ that were caught by *A. proteus* correlated with the size (number of cells) of the choanoflagellate prey (Figure 2). Therefore, the hypothesis that the larger size of multicellular choanoflagellates makes them less vulnerable to predation was rejected for the amoeboid raptorial predator, *A. proteus*. To determine the mechanism(s) responsible for the greater susceptibility of large *S. helianthica* than of small ones to capture by *A. proteus*, we measured various aspects of the behavior of both the predators and the prey.

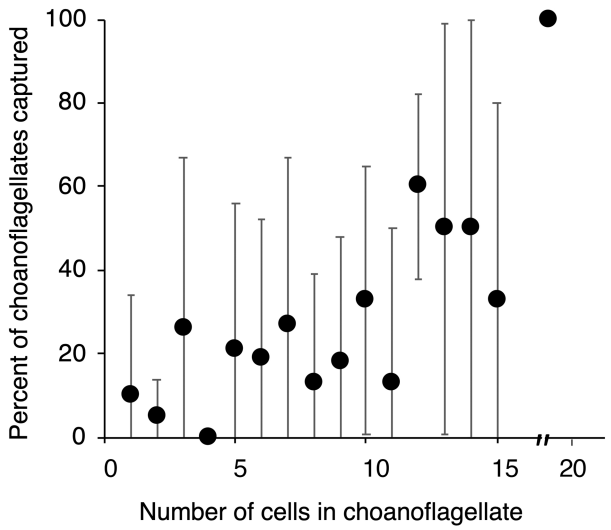

FIGURE 2 Percent of choanoflagellates within 100 µm of the surface of an *Ameoba proteus* that were captured by the *A. proteus* (means ± SD). There was a significant positive correlation between the percent of choanoflagellates captured by *A. proteus* and the number of cells in a choanoflagellate (one-sided Kendall's tau rank correlation test, $p = 0.017$, $\tau = 0.130$, data for 22 *A. proteus* and 171 *Salpingoeca helianthica*).

An example of the kinematics of the pseudopodia of *A. proteus* during the capture of *S. helianthica* prey is shown in Figure 3. Pseudopodia formed in response to choanoflagellates before the prey touched the surface of the predators. However, not all of the prey in the CZ stimulated the formation of pseudopodia by the predator. Furthermore, an *A. proteus* pursuing one prey sometimes responded to another prey in the CZ. When an *A. proteus* did respond to a choanoflagellate, a second pseudopodium often formed after the first pseudopodium was initiated, and sometimes a third pseudopodium developed after that. The speed of the tip of an extending pseudopodium was not steady. Rather, a pseudopodium showed pulses of extension every few seconds. The pseudopodia extended toward the prey, and eventually encircled the prey (Figure 3), forming a food vacuole containing the prey when the tips of the pseudopodia fused (Figure 1C). Therefore, we examined aspects of predator behavior that might have contributed to their size-selective feeding: the distance of prey when pseudopodium formation was initiated, the percent of prey ignored by predators already in pursuit of other prey, the speed of pseudopodium extension, the number of pseudopodia formed and the time intervals between their initiation, and the loss of prey that were being pursued.

Examples of the trajectories of swimming *S. helianthica* and the velocity vectors of the water they disturbed as they swam are shown in Figure 4. There was no significant difference between the swimming speeds of unicellular (mean = 9.8 µm/s, SD = 4.9, $n = 31$ single cells) versus colonial *S. helianthica* (mean = 12.9 µm/s, SD = 5.4, $n = 16$ colonies; One-way ANOVA with post-hoc Tukey HSD test, $p > 0.05$, $F = 10.2_{3,56}$). However, when multicellular colonies of *S. helianthica* swam, they produced faster flow in the water around them than did unicellular *S. helianthica*, and the disturbances in the water covered greater distances (Figure 4). This observation suggests that *A. proteus* might be more likely to perceive and respond to large choanoflagellate colonies than to single cells or small colonies. We also examined whether *S. helianthica* executed escape maneuvers in response to being pursued by pseudopodia, either by changing their swimming speed or the straightness of their trajectories.

Responses by *A. proteus* predators to *S. helianthica* prey

Distance of prey when predator responded

Large multicellular colonies of *S. helianthica* were farther away from the surfaces of *A. proteus* when they stimulated the formation of pseudopodia by the predators than were small colonies or unicellular choanoflagellates (Figure 5).

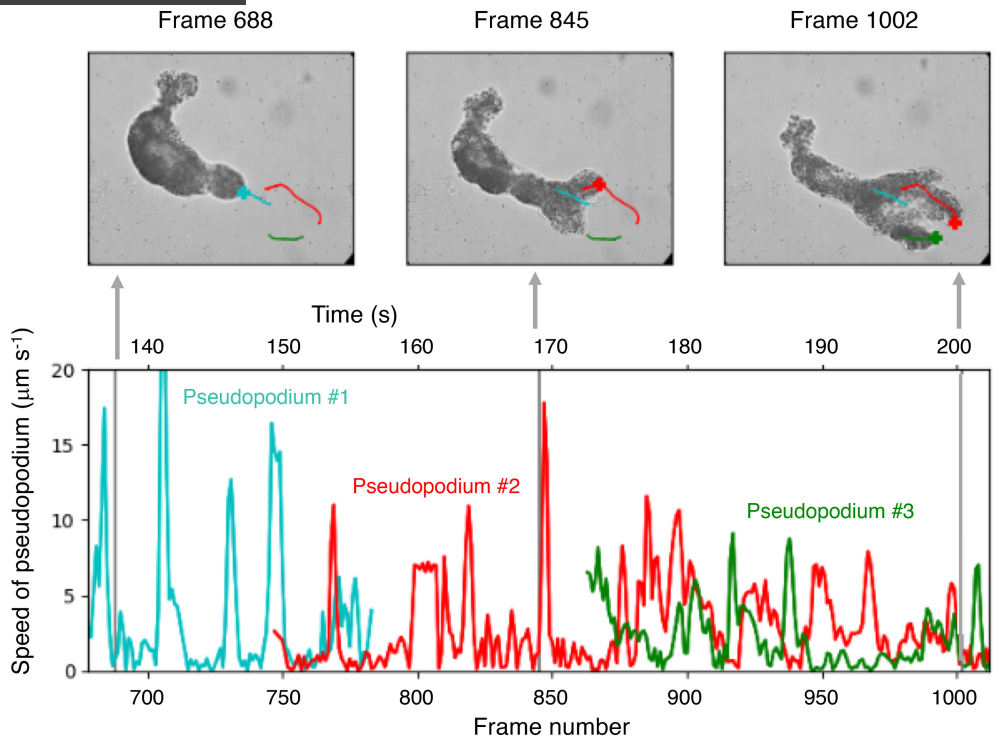

FIGURE 3 Example of the kinematics of pseudopodia of *Amoeba proteus*. The speeds of three pseudopodia are plotted as a function of time (frame number, and seconds from the start of the video). Frames of the video at different times during the process are shown above the graph, and gray vertical lines on the graph indicate when those frames were taken. The colored trajectories on the video frames indicate the paths of the tips of each pseudopodium (aqua shows pseudopodium #1, red shows pseudopodium #2, and green shows pseudopodium #3). The "+" on a trajectory shows the position along the trajectory of the tip of the pseudopodium in that frame of the video. Each pseudopodium undergoes pulses of rapid extension at roughly 10–30 s intervals.

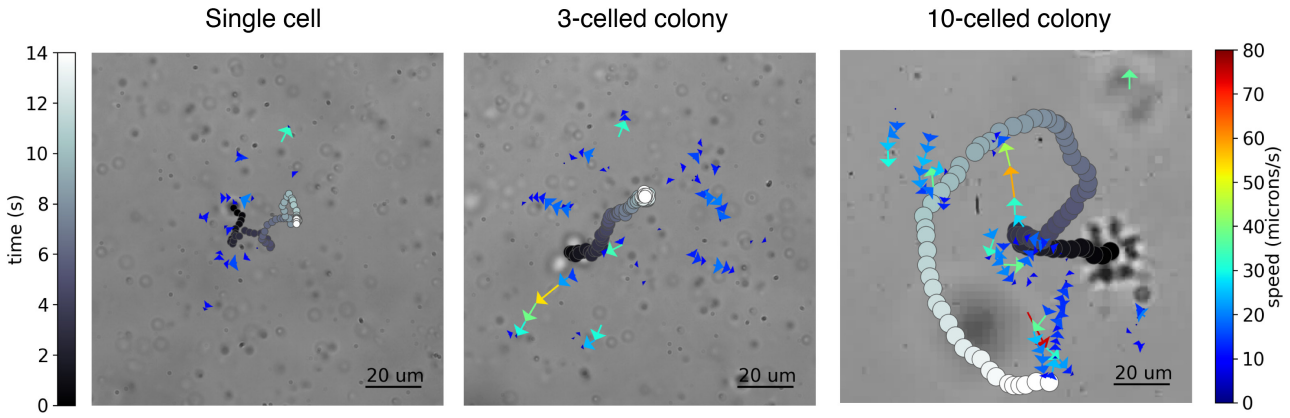

FIGURE 4 Examples of trajectories of water disturbances produced by swimming *Salpingoeca helianthica* of different sizes (single cell, small colony of three cells, and large colony of 10 cells). The position of the *S. helianthica* at 0.2 s intervals is shown by circles (shade of gray indicates time since the start of the tracking; scale shown on left). The frame of the video shown is the last frame of the 14 s trajectory. Velocity vectors of water motion produced by the choanoflagellates as they swam are shown in the water (color scale shown on right). To indicate the distances at which the choanoflagellates disturbed the water, only velocities ≥10 μm/s are shown.

Prey ignored by predators while capturing other prey

We tallied whether or not pseudopodia formed in response to choanoflagellates within 100 μm of *A. proteus*. Very few prey ("first prey") that entered the CZ of *A. proteus* that were not already pursuing other prey were ignored (mean % ignored = 12%, SD = 25, n = 64 *A. proteus*). However, if *A. proteus* were already pursuing prey, then subsequent *S. helianthica* entering the CZ ("second prey") were more likely to be ignored. The percent of second prey ignored was significantly higher than the percent of first prey ignored for unicellular *S. helianthica* (one-sided Mann–Whitney U test, p = 0.002, W = 48, n = 15 *A. proteus* encountering first prey and 16 *A. proteus* encountering second prey), for small colonies

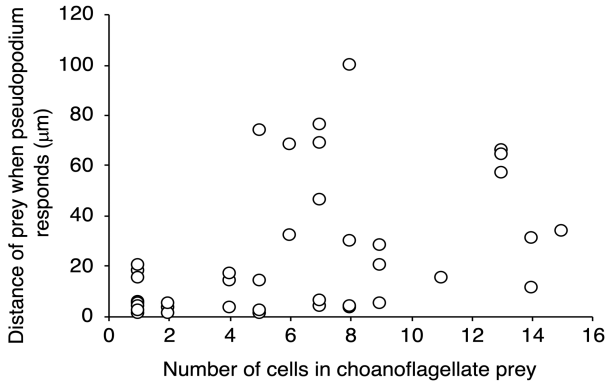

FIGURE 5 Distance of *Salpingoeca helianthica* prey from the surface of an *Amoeba proteus* when the first pseudopodium of the *A. proteus* started to form. There was a significant positive correlation between the distance of a choanoflagellate from an *A. proteus* when its first pseudopodium responded and the number of cells in the choanoflagellate (one-sided Kendall's tau rank correlation test, $p = 0.017297$, $\tau = 0.246595$, $n = 40$ choanoflagellates).

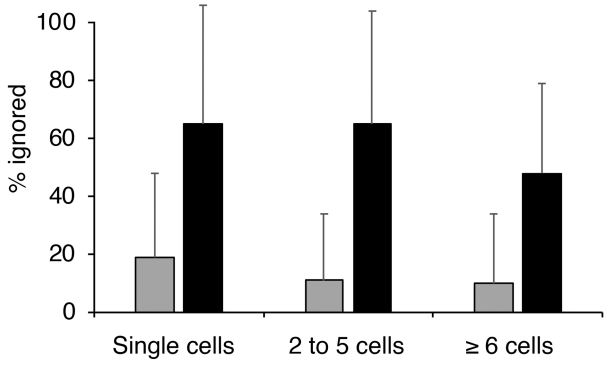

FIGURE 6 Percent of *Salpingoeca helianthica* within 100 µm of the surface of an *Amoeba proteus* that are ignored by the *A. proteus* (i.e. that do not induce the formation of a pseudopodium) when the *A. proteus* was not responding to other prey (gray bars) and when the *A. proteus* was already responding to another *S. helianthica* (black bars). Prey were pooled into three size categories (single cells, small colonies of 2–5 cells, and large colonies of ≥6 cells) so that each size category had a large number of events. If the *A. proteus* were already pursuing other prey, there was a significant negative correlation between the percent of choanoflagellates ignored and the size of the choanoflagellates (one-sided Kendall's tau rank correlation test, $p = 0.017$, $\tau = -0.226$, $n = 63$ *A. proteus*).

($p = 0.00004$, $W = 62$, $n = 21$ *A. proteus* encountering first prey and 19 *A. proteus* encountering second prey), and for large colonies ($p = 0.000007$, $W = 145$, $n = 28$ *A. proteus* encountering first prey and 28 *A. proteus* encountering second prey). The larger the choanoflagellate, the less likely it was to be ignored by *A. proteus* that already were extending pseudopodia in response to other prey (Figure 6).

Extension speed of pseudopodia

There was no correlation between the size of the *S. helianthica* prey and the mean extension speed (Figure 7A)

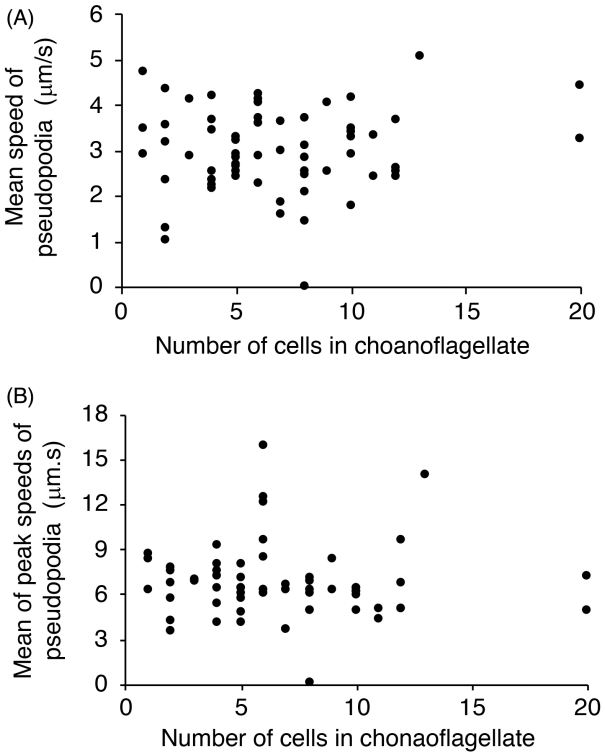

FIGURE 7 Extension speeds of pseudopodia of *Amoeba proteus* in response to *Salpingoeca helianthica* of different sizes (cell number). (A) Mean pseudopodium extension speed plotted as a function of choanoflagellate size (number of cells). There was no positive correlation between mean pseudopodium extension speed and the number of cells in a choanoflagellate (one-sided Kendall's tau rank correlation test, $p = 0.3623$, $\tau = 0.032$, $n = 61$ pseudopodia). (B) Mean of the peak speeds of all the pulses of extension of a pseudopodium plotted as a function of choanoflagellate size (number of cells). There was no positive correlation between peak pseudopodium extension speed and the number of cells in a choanoflagellate (one-sided Kendall's tau rank correlation test, $p = 0.812$, $\tau = -0.081$, $n = 61$ pseudopodia).

or the peak extension speed (Figure 7B) of the pseudopodia of *A. proteus* responding to the choanoflagellates.

Number and timing of pseudopodia

There was no correlation between the size of the *S. helianthica* and the number of pseudopodia of *A. proteus* that formed in response to the choanoflagellates (Figure 8). There also was no correlation between the size of the *S. helianthica* and the time between the initiation of the first and second pseudopodia of *A. proteus* (Figure 9A), or between the initiation of the second and third pseudopodia (Figure 9B).

Loss of prey being pursued

There was no correlation between the size of the *S. helianthica* being pursued by pseudopodia and the percent

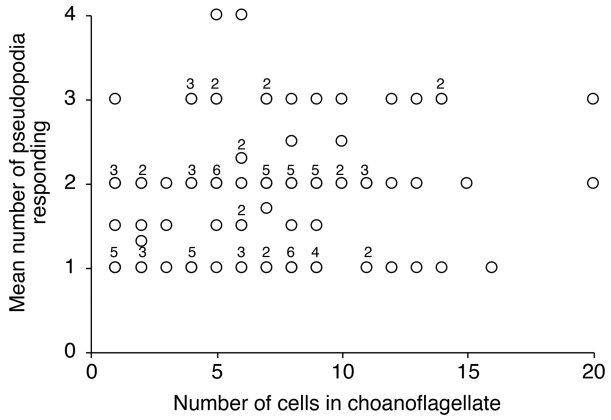

FIGURE 8 Number of pseudopodia of *Amoeba proteus* that are formed in response to *Salpingoeca helianthica* of different sizes (number of cells). Each point represents the mean for an individual *A. proteus* of the number of pseudopodia responding to choanoflagellates of a specific size. Numbers above the circles indicate the number of data points superimposed at that value. There was no positive correlation between the number of pseudopodia responding to a choanoflagellate and the number of cells in the choanoflagellate (one-sided Kendall's tau rank correlation test, $p = 0.134$, $\tau = 0.112$, $n = 108$).

of those prey lost by the predator (two-sided Kendall's tau rank correlation test, $p = 0.628$, $\tau = -0.039$, $n = 102$ choanoflagellates).

Responses of *S. helianthica* to *A. proteus*

Some videos showed *S. helianthica* colonies swimming both before the predator reacted to them, and then after the first pseudopodium began to extend toward them before the colonies were encircled by pseudopodia. In those cases, we could determine whether or not *S. helianthica* changed their swimming in response to extending pseudopodia that were not yet restricting the choanoflagellate motion. The swimming speeds of *S. helianthica* colonies before *A. proteus* responded to them were not significantly different from the swimming speeds of those colonies when pseudopodia were extending toward them but had not yet started to encircle them (one-way ANOVA, $p = 0.448$, $F = 0.622_{1,10}$). Similarly, the straightness indices of the trajectories of the colonies did not change when pseudopodia began to extend toward them (two-sided Mann–Whitney *U* test, $p = 0.11$, $W = 23$, $n = 10$ colonies). Thus, it appears that *S. helianthicus* colonies do not have an escape response to approaching pseudopodia. Unfortunately, because unicellular *S. helianthica* were closer to the predator than colonies when pseudopodia began to extend, there were no trajectories during pseudopodium extension toward single cells that were long enough to determine their swimming speeds or straightness indices before they were encircled.

When swimming freely, unicellular and multicellular *S. helianthica* swam at similar speeds, but the trajectories

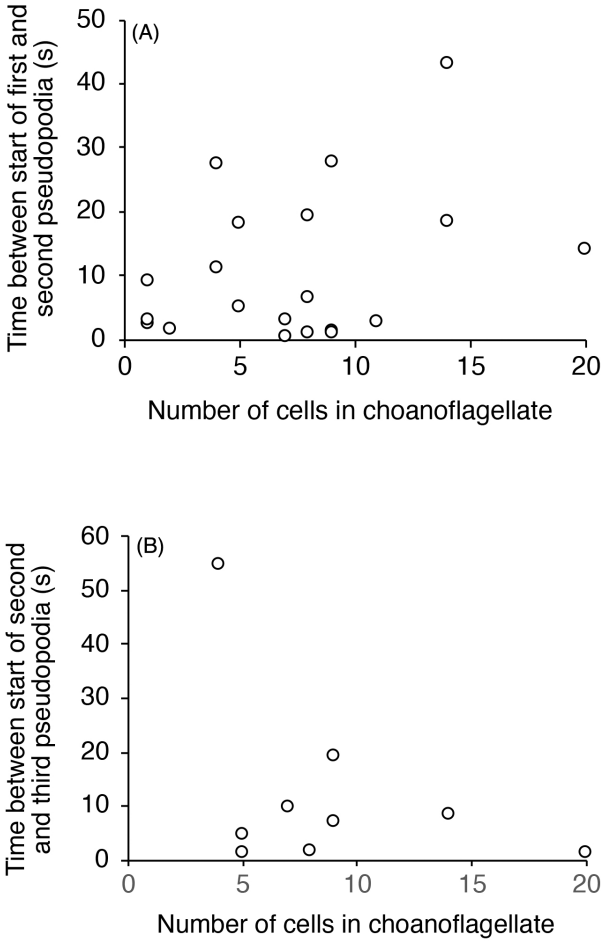

FIGURE 9 Time between initiation of the first pseudopodium and the second pseudopodium (A), and between initiation of the second pseudopodium and the third pseudopodium (B) of *Amoeba proteus* in response to *Salpingoeca helianthica* prey of different sizes (cell numbers). There was no positive correlation of the time between the initiation of the first and second pseudopodia with the number of cells in the choanoflagellate (two-sided Kendall's tau rank correlation test, $p = 0.359$, $\tau = 0.157$, $n = 20$ *A. proteus*), or of the time between the initiation of the second and third pseudopodia with the number of cells in the choanoflagellate (two-sided Kendall's tau rank correlation test, $p = 0.458$, $\tau = 0.203$, $n = 10$ *A. proteus*).

of single cells were straighter than those of colonies (Figure 10). However, when their motion was constrained after they were encircled by pseudopodia, both single cells and colonies swam more slowly and their paths became less straight (Figure 10).

DISCUSSION

Multicellularity increased susceptibility to predation by a raptorial ameboid predator

We found that multicellular choanoflagellates, *S. helianthica*, were more susceptible than unicellular choanoflagellates to capture by the raptorial predator, *A. proteus*. This result runs counter to the idea that forming

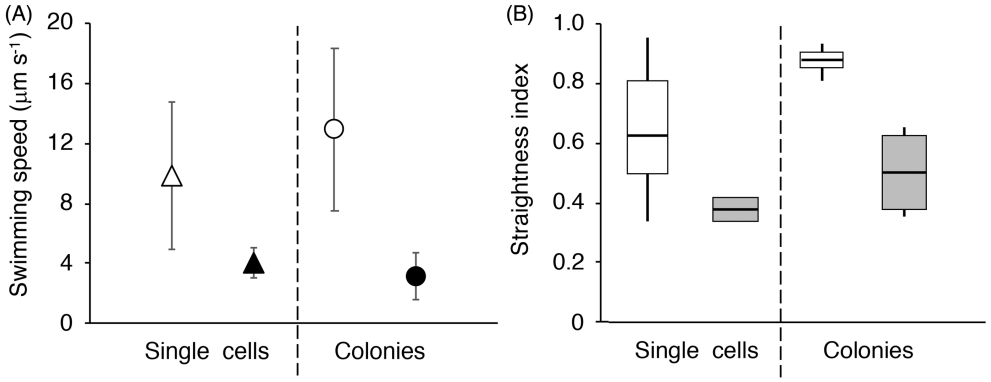

FIGURE 10 Swimming of *Salpingoeca helianthica*. (A) Swimming speed of single cells (triangles) and colonies (circles) when not encircled by pseudopodia of *Amoeba proteus* (open symbols) and when being encircled by pseudopodia of *A. proteus* (black symbols). Unicellular choanoflagellates swam significantly more slowly when being encircled by pseudopodia than when not being encircled by pseudopodia ($p = 0.036$), as did colonies ($p = 0.001$), but the swimming speeds of single cells and colonies were not significantly different from each other, both when swimming freely ($p = 0.141$) and when being encircled by pseudopodia ($p = 0.900$; one-way ANOVA with posthoc Tukey HSD test, $F = 10.23_{3,56}$). (B) Straightness index of the trajectories of *S. helianthica* when not encircled by pseudopodia of *A. proteus* (white bars) and when being encircled by pseudopodia of *A. proteus* (gray bars). The straightness indices of unicellular choanoflagellates were significantly higher when they swam freely than when they were being encircled by pseudopodia ($p = 0.006$), as were the straightness indices colonies ($p = 0.003$), but the straightness indices of single cells were significantly higher than for colonies, both when swimming freely ($p = 0.01018$) and when being encircled by pseudopodia ($p = 0.00002$; Kruskal–Wallis test with posthoc Dunn's test, $H = 20.41_{3,41}$)

multicellular colonies enabled the ancestors of choanoflagellates and animals to escape in size from predation by microbial predators (e.g. Boraas et al., 1998; Fenchel, 2019; Richter & King, 2013; Stanley, 1973). The observation that the passive heliozoan predator, *Actinosphaerium nucleofilum*, consumed unicellular and multicellular *S. helianthica* at the same rate (Koehl, 2020; Kumler et al., 2020) also is inconsistent with the hypothesis that the selective advantage of multicellularity in the ancestors of animals was reduced susceptibility to predation. In contrast, large colonies of *S. helianthica* were rejected by the suspension-feeding ciliate, *Stentor coeruleus*, while small colonies and unicellular choanoflagellates were readily engulfed (Koehl, 2020; Weiler, 2015). Therefore, there appears to be a tradeoff between avoidance of capture by raptorial predators (single cells are less susceptible) versus by suspension-feeding predators (colonies are less susceptible).

Possible selective advantages of forming multicellular colonies

There may have been multiple factors that favored the formation of multicellular colonies in the ancestors of choanoflagellates and animals (Brunet & King, 2017; Fenchel, 2019; Koehl, 2020). Although it has long been thought that predation was an important selective pressure leading to the evolution of multicellularity in animal ancestors, it has also been suggested that colonial ancestors were able to produce stronger feeding currents and capture more particulate food per time than could single cells (e.g. Cavalier-Smith, 2017; Koehl, 2020; Koschwanez et al., 2011; Short et al., 2006; Stanley, 1973). Fenchel (1986) showed that protozoan suspension feeding is more effective if the organisms do not translate through the water, either because they are attached to solid surfaces or because they form suspended colonies that swim very slowly through the water as they draw in water from different directions. However, mathematical hydrodynamic models of the feeding currents produced by unicellular versus colonial choanoflagellates yielded conflicting results. Roper et al. (2013) found that the flux of prey-carrying water into the CZs of choanoflagellate cells in chain colonies of certain configurations was greater than for single cells. In contrast, Kirkegaard and Goldstein (2016) found no enhancement of flux for cells in chains or in rosette colonies (balls of cells with their flagella pointing outward) compared with unicellular choanoflagellates. Experiments showed no effect of multicellularity on feeding rates for choanoflagellates that form hemispherical colonies attached to the substratum by a stalk (Fenchel, 2019), whereas other studies revealed that freely swimming rosette colonies captured more bacteria per cell per time than did unicellular swimmers or unicellular thecate choanoflagellates attached to surfaces (Kreft, 2010; L'Etoile & King-Smith, 2020). Measurements of choanoflagellate swimming showed that rosette colonies traveled more slowly than did unicellular choanoflagellates (Kirkegaard et al., 2016; Koehl, 2020), but water velocities measured relative to the collars of unicellular swimmers and of cells in rosette colonies showed that some of the cells in colonies encountered much greater water flux than did the single cells (Koehl, 2020). These studies suggest that there may be a trade-off between swimming versus feeding performance, and they show that the geometry of colonies determines whether or not cells in colonies

are more effective suspension feeders than unicellular choanoflagellates.

The distribution of bacteria and microbial eukaryotes in aquatic environments is spatially patchy and varies with time (e.g. Raina et al., 2022; Stocker, 2012), so we suggest that animal ancestors that had the ability to switch between being unicellular or multicellular in response to the abundance in the water around them of bacterial prey, and of raptorial or suspension-feeding predators, might have had a selective advantage over purely unicellular forms. The choanoflagellate *S. rosetta* is induced to form colonies in response to certain chemical cues from bacteria (Alegado et al., 2012; Ireland et al., 2020), but whether choanoflagellates are induced to form colonies by abundant suspension-feeding predators or to be unicellular by abundant raptorial predators is not yet known.

Aspects of predator behavior affected by prey size

The distance at which *S. helianthica* stimulated pseudopodium formation by *A. proteus* correlated with the size (number of cells) of the choanoflagellates (Figure 5). Furthermore, large colonies of *S. helianthica* were more likely than small colonies or single cells to induce a feeding response by *A. proteus* that were already pursuing other prey (Figure 6). The flow fields produced by large swimming colonies of *S. helianthica* were bigger than those produced by smaller choanoflagellates (Figure 3). The bigger the flow field produced by a swimming organism, the greater the distance its hydrodynamic signal can be detected and the farther its odors are carried. *A. proteus* sense both hydrodynamic and chemical signals produced by prey (e.g. Kepner & Taliaferro, 1913; Prusch & Britton, 1987; Schaeffer, 1917; Weisman & Korn, 1967). Thus, our data suggest that a mechanism responsible for the preferential feeding on large choanoflagellates by *A. proteus* is the greater likelihood that the predators can sense the flow and/or odor fields produced by large multicellular colonies than by single cells and small colonies.

Other aspects of the behavior of pseudopodia did not vary with prey size. For example, the number of pseudopodia that formed (Figure 8) and the timing between their formation (Figure 9) were not affected by the number of cells in *S. helianthica*. The percent of the prey that were lost by the predator during the capture process also did not vary with prey size. Furthermore, the number of cells in *S. helianthica* prey did not affect the kinematics of the extension of pseudopodia. We found that pseudopodium extension by *A. proteus* was pulsatile (Figure 3), with peak speeds during pulses of extension ranging from ~3 to 17 mm s^{-1} (Figure 7B), and with time-averaged extension speeds of ~1–5 mm s^{-1} (Figure 7A). These mean speeds are in the same range as published crawling speeds of *A. proteus* (Cameron et al., 2007; Folger, 1925; Mast & Prosser, 1932; Mast & Stahler, 1937; Miyoshi et al., 2003), but are faster than lamellipod extension by *Dictyostelium* amoebae (Schindl et al., 1995). We found that neither the peak nor mean speeds of pseudopodia varied with prey size (Figure 7).

Behavior of unicellular and multicellular prey

There was no difference between the swimming speeds of unicellular versus colonial *S. helainthica*, (Figure 10A). Multicellular choanoflagellates, *S. rosetta*, do not beat their flagella in a coordinated fashion (Kirkegaard et al., 2016; Roper et al., 2013), and thus do not show the rapid swimming achieved by spherical colonies of *Volvox* spp. that are composed of cells that beat their flagella in a coordinated direction (reviewed in Koehl, 2020). The swimming speeds and straightness indices we measured for unicellular and colonial *S. helianthica* were similar to those reported by Kumler et al. (2020). Furthermore, single cells and rosette colonies of *S. helianthica* swam at the same range of velocities, respectively, as did collared single cells (Mino et al., 2017; Nguyen et al., 2019) and rosette colonies of *S. rosetta* (Kirkegaard et al., 2016; Koehl, 2020). Rosette colonies of *S. rosetta* swam along noisy helical trajectories (Kirkegaard et al., 2016) that were not as straight as the paths of single cells (Koehl, 2020), and colonies of *S. helianthica* also had less straight trajectories than did single cells (Figure 10B).

When encircled by pseudopodia of *A. proteus*, *S. helianthica* swam more slowly than when swimming freely (Figure 10). The viscous resistance of water to being sheared determines the hydrodynamic forces experienced by microscopic organisms, the layer of sheared water around a microscopic swimmer is large relative to the size of the organism, and stationary surfaces can slow down the motion of microswimmers that are many body lengths away (e.g. Vogel, 1994). Thus, the viscous resistance of the water to being sheared between swimming choanoflagellates and nearby surfaces of pseudopodia is most likely the mechanism responsible for the reduction in the swimming speed of encircled prey. A similar reduction in speed has also been measured for *S. helianthica* swimming between the axopodia of heliozoans (Kumler et al., 2020).

S. helianthica did not perform escape maneuvers or change their swimming speed in response to being pursued by pseudopodia, so the size-selective feeding by *A. proteus* was due to predator behavior rather than to the escape performance of prey. *S. helianthica* also did not execute escape responses to passive heliozoan predators (Kumler et al., 2020) or to suspension-feeding ciliates (Koehl, 2020; Weiler, 2015).

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