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REVIEW

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Selective factors in the evolution of multicellularity in choanoflagellates

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Abstract

Choanoflagellates, unicellular eukaryotes that can form multicellular colonies by cell division and that share a common ancestor with animals, are used as a model system to study functional consequences of being unicellular versus colonial. This review examines performance differences between unicellular and multicellular choanoflagellates in swimming, feeding, and avoiding predation, to provide insights about possible selective advantages of being multicellular for the protozoan ancestors of animals. Each choanoflagellate cell propels water by beating a single flagellum and captures bacterial prey on a collar of microvilli around the flagellum. Formation of multicellular colonies does not improve the swimming performance, but the flux of prey-bearing water to the collars of some of the cells in colonies of certain configurations can be greater than for single cells. Colony geometry appears to affect whether cells in colonies catch more prey per cell per time than do unicellular choanoflagellates. Although multicellular choanoflagellates show chemokinetic behavior in response to oxygen, only the unicellular dispersal stage (fast swimmers without collars) use pH signals to aggregate in locations where bacterial prey might be abundant. Colonies produce larger hydrodynamic signals than do single cells, and raptorial protozoan predators capture colonies while ignoring single cells. In contrast, ciliate predators entrain both single cells and colonies in their feeding currents, but reject larger colonies, whereas passive heliozoan predators show no preference. Thus, the ability of choanoflagellate cells to differentiate into different morphotypes, including multicellular forms, in response to variable aquatic environments might have provided a selective advantage to the ancestors of animals.

KEYWORDS

choanoflagellate, multicellularity, predator avoidance, *Salpingoeca*, suspension feeding, swimming

1 | INTRODUCTION

Over 600 million years ago, the first multicellular animals, the Urmetazoans, evolved from their single-celled ancestors, setting in motion all subsequent animal evolution (Armstrong & Brasier, 2005 Knoll & Lipps, 1993; Schopf & Klein, 1992). The ability to form multicellular colonies is thought to be an important transition state in the lineage leading to animals (e.g., Brunet & King, 2017; King, 2004; Mikhailov et al., 2009; C. Nielsen, 2008). What selective pressures might have favored the evolution of multicellularity in the ancestors of animals? Without a time machine, it is impossible to study directly the function and ecological interactions of extinct organisms. Therefore, the best we can do is to make sensible choices of living organisms that can be useful model systems for helping us to understand how extinct organisms might have worked. If we discover mechanisms responsible for performance differences

that can affect ecological interactions and fitness in living model systems, we can make more informed inferences about possible selective pressures at the time of animal origins.

Choanoflagellates are protozoans that share a common ancestor with animals, as shown by molecular phylogenetic and comparative genomic analyses (Brunet & King, 2017; King et al., 2008; Richter & King, 2013; Ruiz-Trillo, Roger, Burger, Gray, & Lang, 2008; Shalchian-Tabrizi et al., 2008; Torruella et al., 2015; Valentine & Marshall, 2015). A number of species of choanoflagellates have unicellular life stages and can also form multicellular colonies (e.g., King, 2004; Leadbeater, 2015) by cell division (Dayel et al., 2011; Fairclough, Dayel, & King, 2010). Because colony formation is found in diverse choanoflagellate lineages, it is possible that colony formation was present in the last common ancestor of animals and choanoflagellates (e.g., Carr, Leadbeater, Hassan, Nelson, & Baldauf, 2008). Therefore, choanoflagellates are being used as model systems to study the evolution of multicellularity in the ancestors of Urmetazoans (e.g., Brunet & King, 2017; Brunet et al., 2019; Carr et al., 2008; King, 2004; Richter & King, 2013; Steenkamp, Wright, & Baldauf, 2006). By understanding mechanisms responsible for performance differences between single-celled versus colonial choanoflagellates, we can gain insights about possible selective advantages of being multicellular for the protozoan ancestors of animals (e.g., Richter & King, 2013; Roper, Dayel, Pepper, King, & Koehl, 2013; Ruiz-Trillo et al., 2008). Although there may have been a selective advantage to being multicellular when the ancestors of Urmetozoans and choanoflagellates evolved, the existence of both unicellular and colonial choanoflagellates today suggests that there may be different environmental conditions under which unicellular or multicellular forms perform better and that natural selection may have favored the ability to change form in response to environmental cues, rather than simply favoring multicellularity. The purpose of this review is to examine mechanisms affecting the performance of some important functions that can affect fitness (swimming, feeding, and avoiding predation) by unicellular versus multicellular choanoflagellates.

Heterotrophic microbial eukaryotes that eat bacteria are important components of aquatic food webs (e.g., Azam et al., 1983; Ohtsuka, Suzaki, Horiguchi, Suzuki, & Not, 2015). Many microbial eukaryotes are unicellular, while others form multicellular colonies, but the consequences to feeding performance or predator avoidance of being single-celled versus multicellular are not yet well understood. Choanoflagellates that produce both unicellular and multicellular forms permit us to study the effects of colony formation on the performance of these trophic functions within a single species, thereby avoiding unknown sources of variability involved in comparing unicellular species of some protozoans with colonial species of others. Thus, the principles learned from choanoflagellates about the performance of single cells versus multicellular colonies may shed light on mechanisms affecting ecological interactions of aquatic protozoans, as well as on the evolutionary origins of animals.

1.1 | Choanoflagellate morphology and colony formation

Choanoflagellates are eukaryotic flagellates with a distinctive morphology that resembles the structure of the feeding cells (choanocytes) of sponges (e.g., James-Clark, 1868; Fjerdingstad, 1961; Laundon, Larson, McDonald, King, & Burkhardt, 2019; Leys & Degnan, 2002). Each choanoflagellate cell and sponge choanocyte has an ovoid cell body with a single apical flagellum surrounded by a collar of actin-filled microvilli (Figure 1; e.g., Karpov & Leadbeater, 1997; 1998; Leadbeater, 2015). The flagellum produces water currents that carry bacterial prey to the collar, where they are trapped on the microvilli, phagocytosed, and transported to the cell body (Dayel & King, 2014; Lapage, 1925; Pettitt, Orme, Blake, & Leadbeater, 2002). Although there is a debate about whether choanoflagellate cells with collars and sponge choanocytes are homologous (e.g., Mah, Christensen-Dalsgaard, & Leys, 2014; Naumann & Burkhardt, 2019; Pozdnyakov, Sokolova, Ereskovsky, & Karpov, 2017; Sogabe et al., 2019), the conserved structure and function of such "collar cells" in choanoflagellates and sponges suggest that collar cells were present

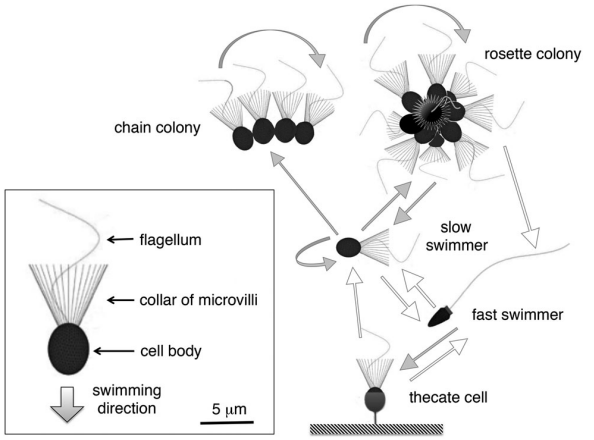

FIGURE 1 Diagrams of the different morphologies of unicellular and multicellular stages of *Salpingoeca rosetta* (drawn based on information in Dayel et al. (2011) and Nguyen et al. (2019)). A choanoflagellate cell has an ovoid body that is pushed through the water by a single flagellum surrounded by a collar of microvilli (insert). Slow swimmers, which can be haploid or diploid, have this morphology, as do cells that form the chain and the rosette colonies. Multicellular colonies are formed by cell division, not by aggregation. Thecate cells also have the morphology shown in the insert but secrete an extracellular cup (theca) that is attached to the substratum by a stalk. Fast swimmers have a bullet-shaped cell body, a very reduced collar, and a long flagellum. Although much remains to be learned about the life cycle of *S. rosetta*, the arrows show transitions between different stages (based on Brunet and King (2017), Dayel et al. (2011), and our observations). Arrows with gray fill show transitions that have been observed, arrows with white fill show inferred transitions, and the production of gametes by haploid slow swimmers and the mating to produce diploid slow swimmers are not shown (Brunet & King, 2017)

in Urmetazoans and in the last common ancestor of choanoflagellates and animals (Brunet & King, 2017; Brusca & Brusca, 2002; Knoll, 2011; Richter & King, 2013). Collar cells have also been documented in many other animal phyla (reviewed by Brunet & King, 2017).

King developed use of the choanoflagellate, *Salpingoeca rosetta*, which can be unicellular and can form multicellular colonies, as a model system to study the evolution of animal multicellularity (Brunet & King, 2017; Fairclough et al., 2013; King, 2004; King et al., 2008; Richter & King, 2013). *S. rosetta* has a complex life cycle and can take on diverse morphotypes (Brunet & King, 2017; Dayel et al., 2011; Figure 1). Slow swimmers, which have the collar-cell morphology, can be haploid or diploid. When nutrients are limiting, haploid cultures of slow swimmers become diploid due to anisogamous mating between small uniflagellate cells with collars: round "male" cells fuse with slightly larger ovoid "female" cells (Brunet & King, 2017; Levin & King, 2013; Woznica et al., 2016; Woznica, Gerdt, Heulett, Clardy, & King, 2017), and chondroitin lyases released by bacteria (*Vibrio fischeri*) induce such mating (Woznica et al., 2016; Woznica et al., 2017). Fast swimmers, which have a bullet-shaped cell body, a very reduced collar, and a long flagellum, are thought to be a dispersal stage (Dayel et al., 2011; Leadbeater, 2015). They aggregate in patches of bacteria and in regions of low pH (an indicator of high bacterial density; Mino, Koehl, King, & Stocker, 2017) and can rapidly attach to surfaces using filopodia (Brunet & King, 2017). These attached cells become thecate cells, which have the same morphology as slow swimmers, but the cell body sits in an extracellular cup (theca) that is attached to a surface by a stalk (Dayel et al., 2011).

Multicellular colonies of *S. rosetta* are produced when slow swimmers undergo serial cell divisions and the sister cells remain attached to each other (Dayel et al., 2011; Fairclough et al., 2010; Laundon et al., 2019), thus these colonies are examples of clonal development, not aggregation (reviewed by Brunet & King, 2017). Since all cells in a clonally-developed colony are genetically the same, competition and cheating between cells is less likely than in colonies formed by aggregation (e.g., Buss, 1987; Grosberg & Strathmann, 2007), and clonal development is used by all the lineages in which obligate multicellularity with differentiated cell types and regulated development evolved (reviewed by Knoll, 2011). The plane of cell division in choanoflagellates is along the apical–basal axis, traversing the apical pole where the collar of microvilli is located (Leadbeater, 2015). *S. rosetta* can form chain colonies or spherical rosette colonies composed of collar cells whose flagella point outwards (Figure 1). Cells in rosette colonies are held together by extracellular matrix (ECM), filopodia, and cytoplasmic bridges (Alegado et al., 2012; Laundon et al., 2019). Three-dimensional (3D) reconstruction of transmission electron micrographs of serial sections of rosette colonies showed that within a colony, there are ranges of cell sizes and of cell–cell contacts via cytoplasmic bridges, and revealed the presence of elongated cells that might represent a differentiated cell type (Laundon et al., 2019). Molecular cues (sulfonolipids) from certain bacteria (*Algoriphagus machipongonensis*) trigger rosette colony formation (Alegado et al., 2012; Woznica et al., 2017), suggesting molecular mechanisms by which bacteria may have contributed to the evolution of multicellularity, and providing a way to reliably produce rosette colonies in the laboratory.

There is great diversity in the morphology of choanoflagellate colonies (reviewed by Leadbeater, 2015). Not only do colonies change in form as they produce more cells, but colonies of *Choanoeca flexa* sp. nov. can rapidly change their structure by active actomyosin-mediated contraction, switching from a cup shape with the flagella pointing into the cup interior when the colonies are in the light, to a cup with the flagella pointing outwards when put in the dark (Brunet et al., 2019). The cells in a *C. flexa* colony are attached to each other via their collars, rather than by the ECM, filopodia, and cytoplasmic bridges that hold the cell bodies together in *S. rosetta* rosette colonies.

Many recent studies of choanoflagellates have used colony formation as a simple model system to study the evolution of the molecular toolkit for cell signaling and adhesion necessary for the development of animal multicellularity, to explore the coupling of sensory input and contractile behavior, or to investigate the mechanisms by which animals and beneficial bacteria interact (reviewed by Brunet & King, 2017; Brunet et al., 2019; Richter & King, 2013). Here, we complement those reviews with a summary of organismal-level research comparing the performance of unicellular with multicellular choanoflagellates to study possible selective factors that might have affected the evolution of multicellularity in the ancestors of animals.

1.2 | Proposed selective advantages of being multicellular

A number of ideas have been proposed about the selective factors that might have favored the evolution of multicellularity in the ancestors of choanoflagellates and animals. One adaptive explanation for the transition to multicellularity is that cooperative feeding by members of a colony improved the ability to capture unicellular prey or nutrients (e.g., Cavalier-Smith, 2017; Koschwanez, Foster, & Murray, 2011; Short et al., 2006; Stanley, 1973). It has also been argued that an important selective factor leading to the evolution of multicellularity was the relative difficulty that protozoan predators may have had in capturing large multicellular colonies compared to smaller unicellular prey (e.g., Boraas, Seale, & Boxhorn, 1998; Fenchel, 2019; Richter & King, 2013; Stanley, 1973). Swimming performance affects both feeding effectiveness and susceptibility to predation in a number of ways. Resources (e.g., bacterial prey or dissolved substances) are patchily distributed on fine scales in natural bodies of water (reviewed by Stocker & Seymour, 2012), and the ability to travel to and remain in resource-rich patches depends on swimming speed and on chemotactic or chemokinetic changes in swimming behavior. However, rapid swimming also increases the likelihood that microorganisms encounter predators (e.g., Crawford, 1992; Fenchel, 1982a; Shimeta & Jumars, 1991), but may also enhance their ability to escape (e.g., Matz & Jürgens, 2005). Of course, there may not have been just one factor that favored the formation of multicellular colonies (Fenchel, 2019), or the initial

evolution of multicellularity in the ancestors of animals might simply have been due to fixation by genetic drift of a neutral character (Brunet & King, 2017).

1.3 | Trophic performance of choanoflagellates depends on hydrodynamics

Swimming, capturing prey, and interacting with predators by choanoflagellates are all hydrodynamic processes. The pattern of fluid flow around an organism and the mechanisms by which it generates the forces to locomote through a fluid or to create currents past itself depend on the magnitude of inertial forces relative to viscous forces. The ratio of inertia to viscosity for a particular flow situation is the Reynolds number ($Re = \rho UL/\mu$, where U is velocity, L is a linear dimension of the organism or structure, and ρ and μ are the density and viscosity, respectively, of the fluid; e.g., Vogel, 1994). A disturbance produced in a fluid will tend to persist if inertial forces predominate, thus for large organisms like humans or whales that operate at high *Re*, the flow of air or water is turbulent. In contrast, for small organisms operating at low *Re*, any disturbance to the fluid tends to be damped out by the viscous resistance of the fluid to undergoing shear deformation. Therefore, the flow around microscopic organisms is laminar (i.e., the fluid moves smoothly around the body in layers between which there is no significant mixing) and is reversible in space and time (e.g., Happel & Brenner, 1965; Koehl, 1981; Lauga & Powers, 2009; Purcell, 1977; Vogel, 1994). Because humans operate at high *Re*, our intuition about how fluids move is not helpful in understanding the hydrodynamics of microscopic organisms such as choanoflagellates, which operate at *Re*'s of order 10^{-4} (Pettitt et al., 2002; Roper et al., 2013).

Microorganisms swimming at low *Re* affect fluid motion at large distances relative to their body size (e.g., Cheer & Koehl, 1987; Koehl, 1981; Vogel, 1994). The fluid in contact with a moving object does not slip relative to the object's surface, hence a velocity gradient develops in the fluid around the structure. The smaller or slower the object (i.e., the lower its Re), the thicker this layer of sheared fluid is relative to the size of the structure. Most of the published observations of water flow near microorganisms are artefacts caused by the proximity of the floor and ceiling of the chamber in which the observations were made (Liron & Blake, 1981; Pepper, Roper, Ryu, Matsuidara, & Stone, 2010), so useful measurements of the hydrodynamics of choanoflagellates must be made for cells or colonies middepth in chambers deep enough to minimize interference of the chamber walls with the flow produced by the organisms.

2 | EFFECTS OF MULTICELLULARITY ON FORAGING AND RESISTANCE TO PREDATION

By comparing the hydrodynamic performance of unicellular choanoflagellates with that of multicellular colonies, we can test various ideas about possible selective advantages of multicellularity at the time of animal origins.

2.1 | Swimming performance

Examples of the swimming trajectories and speeds of *S. rosetta* unicellular slow swimmers, unicellular fast swimmers, and multicellular rosette colonies are shown in Figure 2. Slow swimmers with ovoid cell bodies and large collars swim at speeds of ~5–30 μm/s (Mino et al., 2017; Nguyen, Koehl, Oakes, Bustamante, & Fauci, 2019), whereas fast swimmers, which have very short collars, longer flagella, and bullet-shaped bodies, swim at speeds in the range of ~35–80 μm/s (Kirkegaard, Marron, & Goldstein, 2016; Mino et al., 2017; Nguyen et al., 2019).

Colonies swim slowly (~5–20 μm/s; Kirkegaard et al., 2016) and are more likely to swim around in circles than are single cells (Figure 2). The mechanisms responsible for these differences in swimming performance have been analyzed by high-speed videomicrography of the kinematics of and flow fields produced by choanoflagellates swimming in chambers large enough to minimize wall effects, and by mathematical models of the hydrodynamics of choanoflagellate swimming.

When a free-swimming unicellular choanoflagellate in the water column waves its flagellum, it is pushed through the water with the cell body on the leading end (Figure 1), thus the direction of water flow *relative to* the cell is from the cell body towards the collar (Figure 3, top). Choanoflagellates are eukaryotes, so dynein-driven microtubule sliding produces waves of active bending that move

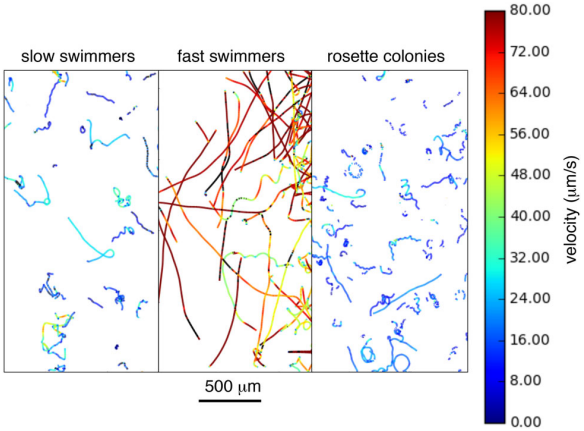

FIGURE 2 Trajectories of *Salpingoeca rosetta* slow swimmers, fast swimmers, and rosette colonies swimming in vessels 3 mm wide by 0.1 mm deep, digitized from videos shot at 30 frames/s through an inverted Nikon T2e compound microscope with a CCD camera (PCO 1600, Cooke) at a magnification of ×4 (videos made by M. Koehl, N. King, and R. Stocker). Custom software using OpenCV version 2.4 was used to track the choanoflagellates, and these data were analyzed with the SciPy software stack. Colors indicate instantaneous velocities (scale on right). CCD, charge-coupled device [Color figure can be viewed at wileyonlinelibrary.com]

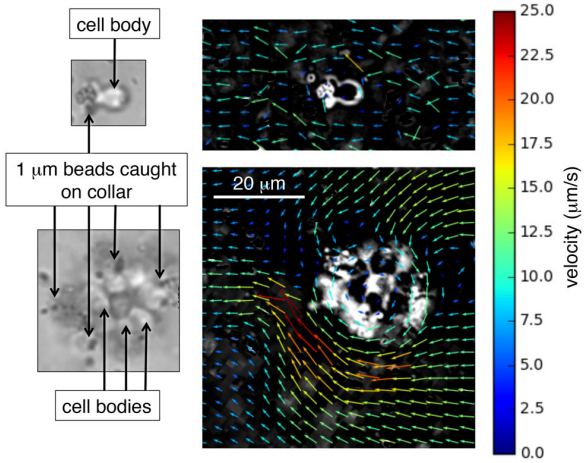

FIGURE 3 Water velocity vectors relative to a slow swimmer (upper right) and a rosette colony (lower right) of *Salpingoeca rosetta*. Videos recorded at 100 frames/s and lasting 16 s were made using a Fastek Hi-Spec1 2G Mono camera system through a Leica DM 2500 compound microscope with a ×100 oil immersion lens (videos made by K. Wong and B. Cummings). The choanoflagellates were in the flat-bottomed well (245 μm depth, 1500 μm diameter) of a depression slide, and the focal plane was 80 μm below the coverslip. Images to the left are from single frames of the videos, where the cell bodies of the choanoflagellates and beads caught on their collars can be seen. Particle-tracking velocimetry of flow markers (neutrally-buoyant polystyrene beads, diameter = 1 μm) was used to measure instantaneous water velocities. Custom software using OpenCV version 2.4 was used to track particles and features of the choanoflagellates, and these data were analyzed with the SciPy software stack. The instantaneous velocity vectors of the particles relative to the laboratory reference frame were rotated to align with the direction of travel of either a single cell or of a colony. Then, the instantaneous velocity of the choanoflagellate cell or colony was subtracted from the particle velocities to determine the velocities of the particles relative to the choanoflagellate. An overlaid grid was then used to bin all the instantaneous vectors and to calculate a mean water velocity relative to the choanoflagellate cells for each gridpoint over the duration they were followed (16 s). The colony shown was rotating counterclockwise, so the flow relative to the cells was clockwise. Colors indicate instantaneous water velocities relative to the choanoflagellate cells (scale on right) [Color figure can be viewed at wileyonlinelibrary.com]

along the flagellum from base to tip. The flagellum of *S. rosetta* beats in one plane (Dayel & King, 2014), and measurements made on high-speed videos showed that slow swimmers use a sinusoidal flagellar beat that has a constant wavelength, but increases in amplitude along the flagellum (Nguyen et al., 2019). In contrast, the long flagellum of a fast swimmer beats at a lower amplitude and with a longer wavelength than used by a slow swimmer (Nguyen et al., 2019).

Mathematical models of the hydrodynamics of choanoflagellate swimming can be used to run "experiments" that cannot be done with living organisms: various morphological or kinematic parameters can be varied independently to quantify the consequences of each to performance. Nguyen et al. (2019) used a 3D computational fluid dynamic (CFD) model that explicitly included the cell body, collar, and flagellar dynamics to analyze the diverse unicellular forms of *S. rosetta*. We found that longer microvilli reduce speed, cell shape only affects speed when the collar of microvilli is very short, and a longer flagellum increases speed. The model also showed that single cells with short flagella locomote more rapidly if they use the high-amplitude small-wavelength flagellar waveform of slow swimmers, whereas cells with long flagella swim faster if they use a low-amplitude long-wavelength waveform of fast swimmers.

When cells are tethered to each other in a chain or rosette colony, their flagella point in different directions, and, thus, the forces they produce can cancel each other and result in slower swimming than by single cells (Roper et al., 2013). There is variation in the angles between neighboring cells in chain colonies (Roper et al., 2013) and in the orientation and spacing between cells in rosette colonies (Laundon et al., 2019) of *S. rosetta*, so when they beat their flagella, the colonies produce uneven flow fields and tend to rotate (Figure 3, bottom). Unlike *Volvox* spp., which are algae that form spherical colonies of cells that beat their flagella in a co-ordinated direction and swim rapidly (~500 μm/s; Short et al., 2006), multicellular *S. rosetta* do not beat their flagella in a coordinated fashion (Kirkegaard et al., 2016; Roper et al., 2013) and swim slowly along noisy helical paths (Figure 2; Kirkegaard et al., 2016). Thus, the formation of multicellular colonies by *S. rosetta* does not improve the swimming performance. However, the hydrodynamics and swimming performance of other colony configurations should be explored. For example, the cup-shaped colonies of *C. flexa* swim more rapidly when they invert into tight cups with the flagella pointing outwards than they do when they relax into the flatter, more open configuration with the flagella pointing inwards (Brunet et al., 2019).

2.2 | Finding patches of resources

To succeed in heterogeneous environments, organisms must move through the habitat and use search behaviors that maximize exposure to limiting resources (Grünbaum, 1998). The ability to locate and exploit patches of resources is especially important in environments where background resource availability is low, such as in aquatic habitats, where planktonic microorganisms regularly experience background concentrations of limiting nutrients or prey that are below the critical limits required for optimum growth (e.g., Leising & Franks, 2000; Mullin & Brooks, 1976). Dissolved nutrients and bacteria are patchily distributed in the water column, with hotspots of rich resources on the scale of hundreds to thousands of microns (reviewed by Stocker & Seymour, 2012) due to exudates from small bodies such as phytoplankton and organic "marine snow" particles (e.g., Kiørboe and Jackson, 2001; Smriga et al., 2016), and to resource-rich filaments swept off substrata by turbulent currents (e.g., Crimaldi & Koseff, 2001; Koehl, Strother, Reidenbach, Koseff, & Hadfield, 2007). Do unicellular and multicellular choanoflagellates differ in their patch-finding performance?

The recent development of microfluidic technology (e.g., Weibel, DiLuzio, & Whitesides, 2007) has enabled studies of

choanoflagellates swimming behavior in response to realistic patches of bacteria or dissolved substances (Kirkegaard et al., 2016; Mino et al., 2017). Fast swimmers of *S. rosetta* aggregate in dense patches of bacteria and in patches of seawater with pH of 6–7, a level of acidification characteristically produced by concentrated patches of bacteria (Mino et al., 2017). In contrast, the slowly-moving multicellular colonies and the unicellular slow swimmers do not respond to patches of bacteria or to low pH. Since fast swimmers have reduced collars and do not feed, their behavior of aggregating in local regions of lowered pH may be a mechanism by which these rapidly-moving unicellular dispersers can find particles or surfaces rich in prey onto which they can attach and become thecate cells (Figure 1; Mino et al., 2017). Although multicellular colonies of *S. rosetta* do not aggregate in patches of prey, they do show "aerotaxis" (i.e., they accumulate in oxygen-rich patches), as do fast swimmers (Kirkegaard, Bouillant, Marron, Leptos, & Goldstein, 2016). Analysis of the trajectories of the colonies suggested that they use "stochastic taxis" (i.e., they turn less when moving up the oxygen concentration gradient, which biases their motion towards regions of higher oxygen). Thus, although multicellular *S. rosetta* do show chemokinetic behavior, only the rapidly-moving unicellular fast swimmers use pH signals to aggregate in locations where bacterial prey might be abundant.

2.3 | Capture of bacteria

Feeding success not only depends on finding patches of prey but also on the rate at which prey can be captured from the water surrounding a choanoflagellate cell. It has been proposed that multicellular choanoflagellate colonies might produce stronger feeding currents that increase the rates of capture of bacterial prey by cells in the colony when compared to unicellular choanoflagellates (e.g., Cavalier-Smith, 2017; Koschwanez et al., 2011; Short et al., 2006; Stanley, 1973). The nutrient uptake rates of cells in *Volvox* spp. colonies is enhanced by the coordinated beating of their flagella (Short et al., 2006).

The feeding rates of various choanoflagellates have been measured, including unicellular freely-swimming (Shimeta, Jumars, & Lessard, 1995) and attached (Fenchel, 1982c) forms, and swimming (Kreft, 2010) and sessile (Fenchel, 2019) colonies. Feeding rates of choanoflagellates and other flagellates depend on the concentration of bacterial prey. At low concentrations, feeding rate is encounter-limited, whereas at high concentrations, feeding rate plateaus, limited by the speed with which a flagellate ingests and processes the bacteria (Fenchel, 1982b; Shimeta & Jumars, 1991; Shimeta et al., 1995). Therefore, studies comparing the feeding rates of cells in choanoflagellate colonies of different sizes with those of single cells of the same species have been run for short-time periods before the cells saturate (Fenchel, 2019; Kreft, 2010). In these studies, the number of fluorescently-labeled particles or bacterial prey captured by each cell have been counted. The choanoflagellate *Codosiga botrytis* forms hemispherical colonies attached to the substratum by a stalk. Colony formation does not increase the bead capture rates of cells of *C. botrytis* (Fenchel, 2019). Cells in cup-shaped colonies of *C. flexa* capture more prey when the flagella point inwards than they do when the flagella point outwards (Brunet et al., 2019). In contrast, cells in freely-swimming rosette colonies of *S. rosetta* form food vacuoles at higher rates than do unicellular slow swimmers, indicating that cells in colonies capture prey at higher rates than do single cells (L'Etoile & King-Smith, 2020). Furthermore, cells in rosette colonies of *S. rosetta* capture more bacteria per cell per time on average than do unicellular slow swimmers or sessile thecate cells, but the variation between capture rates of cells within a colony is high (Kreft, 2010). Water velocities measured relative to the collars of slow swimmers and of cells in rosette colonies show that some cells in colonies that spin as they swim encounter much faster flow than do other cells in the colony or than do unicellular slow swimmers (Figure 3). Thus, the differences in individual feeding rates of cells within a freely-swimming rosette colony may be determined in part by the flux of prey-carrying water past their collars.

A choanoflagellate cell feeds by beating its single flagellum, which draws water to the collar of prey-capturing microvilli that surrounds the base of the flagellum (Figures 1 and 3). The collar was thought to be a sieve that strains bacteria from the water flowing through it (e.g., Fenchel, 1982a; Orme, Otto, & Blake, 2001; Pettitt et al., 2002), but mathematical models of flow at low *Re* near finite arrays of microscopic cylinders (Cheer & Koehl, 1987) and near the collar of microvilli on a choanoflagellate (Nguyen et al., 2019) show that very little water flows between neighboring microvilli in a collar. (Exceptions to this are choanoflagellate cells surrounded by an extracellular lorica that forces all the water propelled by the flagellum to flow through the collar rather than along it; L. Nielsen et al., 2017). Whether water flows along or though the collar, bacteria carried within one prey radius of the collar have the potential of being caught, and the flux of water through this capture zone of a cell's collar is used as a proxy for feeding rate in mathematical models of choanoflagellate hydrodynamics. Early approaches to simplifying the geometry of a unicellular choanoflagellate to model the flow produced by its flagellum (e.g., Orme, Blake, & Otto, 2003; Orme et al., 2001; Pettitt et al., 2002) are reviewed by Nguyen et al. (2019). More recent models comparing water flux to single cells versus to cells in colonies treat the flagellum as a point force (Roper et al., 2013) or a row of point forces (Kirkegaard & Goldstein, 2016) that create the flow. Unfortunately, these models have produced conflicting results. Roper et al. (2013) calculated that anistropically-arranged cells in chain colonies create long-range feeding currents that produce a greater flux of water per cell than is produced by unicellular slow swimmers. In contrast, the model of Kirkegaard and Goldstein (2016) indicates that the flux of water to single cells that can swim more rapidly than multicellular choanoflagellates is greater than to the cells in chain or rosette colonies. Neither of these models included the hydrodynamic effects of the collar. More recent models of the hydrodynamics of unicellular choanoflagellates have used CFD to include details of cell and collar morphology and flagellar kinematics (Nguyen et al., 2019; L. Nielsen et al., 2017). The CFD model of *S. rosetta* showed that the flux of prey-carrying water into the

collar capture zone is greater for slow swimmers than for sessile thecate cells, and that ignoring the hydrodynamics of the collar (as the earlier models have done) overestimates flux of water to the capture zone and exaggerates the benefit to feeding performance of swimming versus being attached (Nguyen et al., 2019). Perhaps using a similar CFD approach to modeling flow produced by cells with collars in colonies could resolve the discrepancies of the earlier models. However, it is still not clear how cells in colonies interact to affect feeding currents and flux of water to their collars, or how the arrangement of those cells affect the feeding performance.

The flux of water to the collar of a choanoflagellate cell is not a perfect measure of feeding performance. Factors such as prey size and motility, size and spacing of microvilli, feeding current velocity, and adhesion of prey to microvilli, all can affect prey capture (Rubenstein & Koehl, 1977; Shimeta & Jumars, 1991), but these factors have not yet been studied for choanoflagellates. Thus, although much remains to be learned about the hydrodynamics and prey capture by cells in choanoflagellate colonies of different configurations, it is already clear that becoming multicellular does not always increase the feeding rates of cells in colonies.

2.4 | Vulnerability to predation by protozoans

Before multicellular animals evolved, the predators on the ancestors of choanoflagellates and animals would have been other protozoans. Fossils, chemical biomarkers, and molecular analyses indicate that diverse heterotrophic protozoans (including ciliates, flagellates, and amoebae) evolved before multicellular animals (e.g., Armstrong & Brasier, 2005; Li et al., 2007; Parfery, Lahr, Knoll, & Katz, 2011; Porter, 2001; Schopf & Klein, 1992). The various mechanisms by which swimming, crawling, and sessile protozoan predators of different morphologies capture prey (reviewed by Arndt et al., 2000; Fenchel, 1986; Sleigh, 1991) have been categorized by Sleigh (2000) into functional types: (a) motile raptors actively capture prey with, for example, pseudopodia or tentacles, (b) suspension feeders create a feeding current that delivers prey to capture surfaces, and (c) passive predators intercept prey that swim or drift into them. Are there differences between unicellular and multicellular choanoflagellates in their susceptibility to being eaten by protozoans using each of these modes of prey capture?

A variety of approaches are used to study predator–prey interactions of protozoans. A common technique is to measure the rates at which prey are removed from a volume of water by a population of predatory protozoans (e.g., Fenchel, 1980; Jakobsen & Hansen, 1997; Jonsson, 1986; Landry, 1994). However, video studies that reveal the organismal-level processes involved in prey capture by individual protozoans are more useful for determining the mechanisms responsible for differences in feeding rates (e.g., Boenigk, Matz, Jürgens, & Arndt, 2001; Stoecker, Gallager, Langdon, & Davis, 1995; Strom & Buskey, 1993; Taniguchi & Takeada, 1988; Wu, Boenigk, & Hahn, 2004; Roberts et al., 2011). Recent studies using videomicrography to measure interactions between unicellular slow swimmers or multicellular rosette colonies of *Salpingoeca helianthica* with protozoan predators are revealing how the feeding mode of the predator determines whether single cells or colonies are more vulnerable to being eaten.

Protozoan prey and predators can sense each other via mechano- and chemoreception (e.g., Buskey & Stoecker, 1989; Echevarria, Wolfe, Strom, & Taylor, 2014; Jakobsen, 2002; Jakobsen, Everett, & Strom, 2006; Karpenko, Railkin, & Servain, 1977; Machemer, 2001). Behavioral studies showed that diverse protozoans respond to hydrodynamic signals (reviewed in Visser, 2001). Particle-tracking velocimetry of flow produced by *S. rosetta* shows that the region of water disturbed by rosette colonies as they swim is much bigger than the region disturbed by unicellular slow swimmers (Figure 4). Therefore, single cells should be more hydrodynamically cryptic than colonies, and, thus, less vulnerable to raptorial predators. The benthic raptorial predator, *Amoeba proteus*, does not react to unicellular *S. helianthica*, that swim nearby (Figure 5a), but responds to approaching rosette colonies by rapidly extending pseudopodia that capture the colonies (Figure 5b,c).

Differences in the susceptibility of unicellular versus multicellular choanoflagellates to predation by some types of protozoans

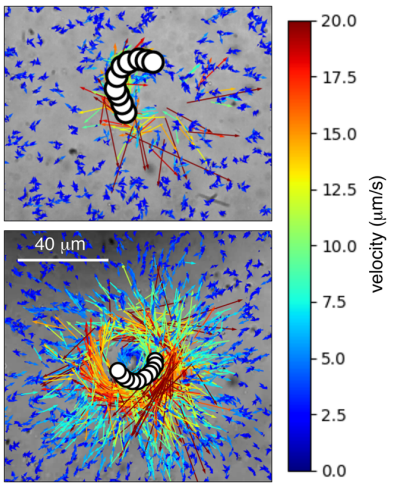

FIGURE 4 Water velocities relative to the laboratory reference frame (i.e., hydrodynamic signals that might be sensed by a predator) produced by a slow swimmer (top) and a rosette colony (bottom) of *Salpingoeca rosetta*. Particle tracking velocimetry of 1 μm beads was used to measure instantaneous water velocities in videos recorded at 100 frames/s by a Fastek Hi-Spec1 2G Mono camera system through a Leica DM 2500 compound microscope with a ×100 oil immersion lens (videos made by K. Wong and B. Cummings). The choanoflagellates were in the flat-bottomed well (245 μm depth, 1500 μm diameter) of a depression slide, and the focal plane was 80 μm below the coverslip. Custom software using OpenCV version 2.4 was used to track particles and choanoflagellates and these data were analyzed with the SciPy software stack. The white circles indicate the positions of the choanoflagellate at 0.3 s intervals, with each successive position shown on top of the previous position (diameter of circles does not reflect cell or colony size). Colors indicate instantaneous water velocities relative to the laboratory (scale on right) [Color figure can be viewed at wileyonlinelibrary.com]

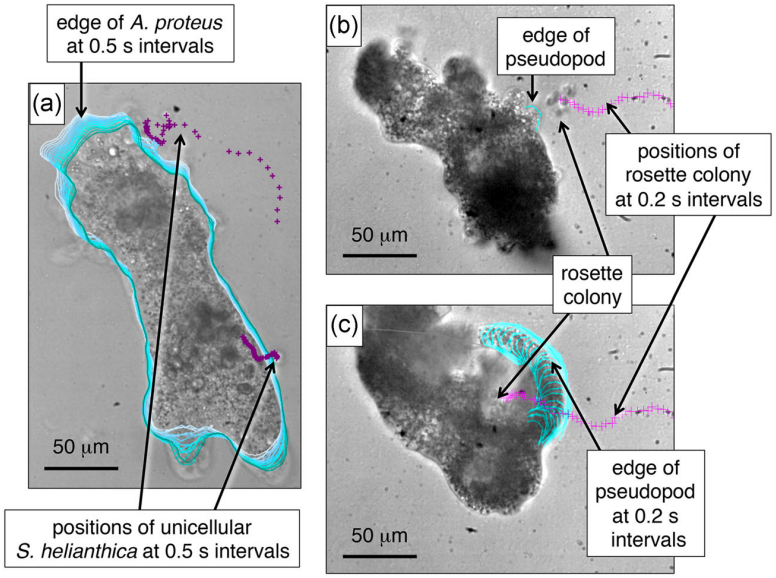

FIGURE 5 Videos of *Amoeba proteus* and *Salpingoeca helianthica* were recorded at 5 frames/s using a Fastec Hi-Spec1 2G Mono camera system through a Leica DMLS compound microscope at a magnification of ×40 (videos made by K. Wong and B. Cummings). The organisms were in the flat-bottomed well (0.7 mm depth, 15 mm diameter) of a depression slide, and the focal plane was 120 μm below the coverslip. Custom software using OpenCV version 2.4 was employed to track the choanoflagellates and the perimeter of the *A. proteus* (a) and the pseudopod (b and c). (a) The tracks of two unicellular *S. helianthica* are shown near an *A. proteus*, which did not respond to the choanoflagellates. (b) Track of a rosette colony of *S. helianthica* approaching an *A. proteus*. The video frame shown is the last one before the pseudopod of the predator responded to the colony. (c) Tracing of the leading edge of a pseudopod of the *A. proteus* shown in (b) as it encircles the rosette colony. This video frame shows the colony after it was captured by the pseudopod [Color figure can be viewed at wileyonlinelibrary.com]

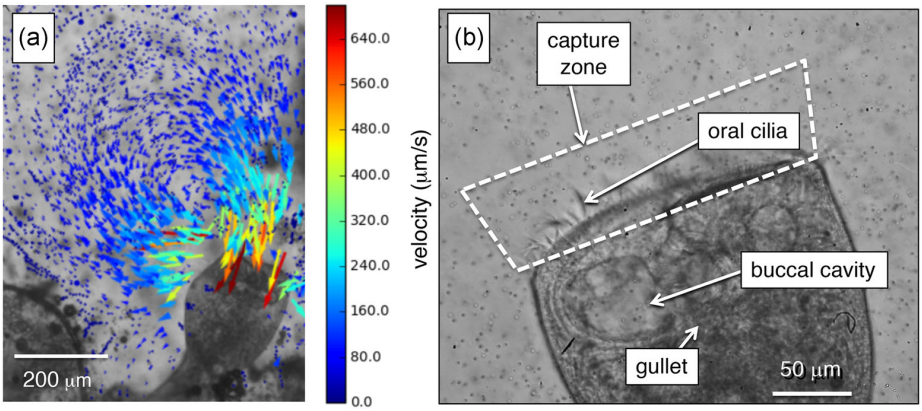

FIGURE 6 Videos of *Stentor coeruleus* were recorded at 50 frames/s using a Fastec camera Hi-Spec1 2G Mono camera system through a Leica DMLS compound microscope at a magnification of ×10 (a) or ×40 (b) (videos made by D. Weiler). The organisms were in the flat-bottomed well (0.7 mm depth, 15 mm diameter) of a depression slide, and the focal plane was 150 μm (a) or 120 μm (b) below the coverslip. (a) Part of the feeding current produced by a stationary *S. coeruleus* attached to a piece of detritus. Custom software using OpenCV version 2.4 was used to track particles (neutrally-buoyant l μm polystyrene beads) and these data were analyzed with the SciPy software stack to calculate water velocities relative to the stationary predator. Colors indicate instantaneous water velocities relative to the *S. coeruleus* (scale on right). (b) Frame of a video of a *S. coeruleus*. Beating oral cilia created a current that carried prey into the capture zone. The height of the capture zone was 1.5 times the length of oral cilia and its width was determined by tracing the paths of the polystyrene beads (black dots) that were carried into the area encircled by the oral cilia. Choanoflagellate prey were transported through the capture zone into the buccal cavity, where unicellular *Salpingoeca helianthica* were moved into the gullet and engulfed in vacuoles more often than larger colonies, which were carried away by the excurrent flow (Weiler, 2015) [Color figure can be viewed at wileyonlinelibrary.com]

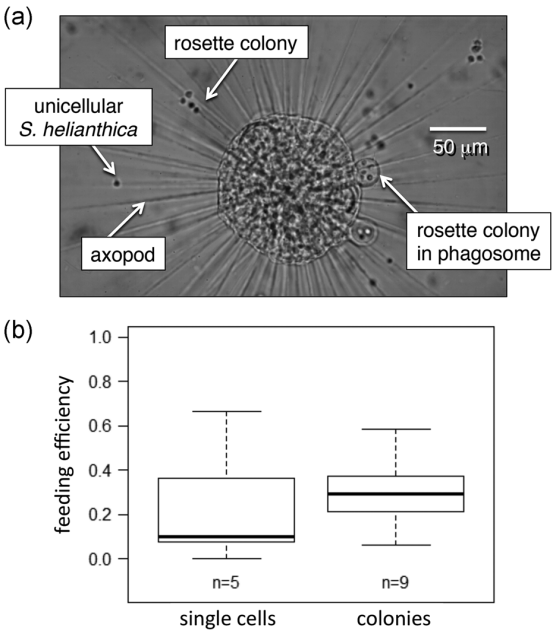

FIGURE 7 (a) Frame of a video of an *Actinosphaerium nucleofilum* feeding on unicellular and colonial *Salpingoeca helianthica* recorded at 5 frames/s using a Fastek Hi-Spec1 2G Mono camera system at a magnification of ×40 through a Leica DMLS compound microscope (video made by W. Kumler). The organisms were in the flat-bottomed well (0.7 mm depth, 15 mm diameter) of a depression slide, and the focal plane was 120 µm below the coverslip. (a) Prey are captured on the long, slender axopodia, transported to the cell body along the axopodia, and engulfed into phagosomes at the cell body. (b) Feeding efficiency of *A. nucleofilum* on unicellular *S. helianthica* and rosette colonies of *S. helianthica* (values in graph calculated from data in, in revision). Box plots show the medians, first quartiles, and ranges of the data (*n* is the number of individual *A. nucleofilum* predators tested). There was no significant difference between the feeding efficiency on single cells and on rosette colonies (Wilcoxon Rank Sum two-tailed test, $p > .05$)

may simply be due to the fact that colonies are larger than single cells. It has been suggested that multicellularity enabled the ancestors of animals to escape in size from protozoan predators (e.g., Boraas et al., 1998; Fenchel, 2019; Richter & King, 2013; Stanley, 1973). Various heterotrophic protozoans are unable to consume large prey (e.g., Fenchel, 1986; Jonsson, 1986; Verity, 1991). Furthermore, the presence of protozoan predators induces certain unicellular algae to form multicellular colonies (Boraas et al., 1998; Jakobsen & Tang, 2002; Kapsetaki & West, 2019), suggesting that increasing size by becoming multicellular is a defense against protozoan predators. Conversely, encounter rates of motile prey with predators increase with prey size (e.g., Crawford, 1992; Fenchel, 1982a, 1984; Rubenstein & Koehl, 1977; Shimeta & Jumars, 1991). Although diverse heterotrophic protozoans show size-selective feeding, some favor large prey and others small prey (e.g., Fenchel, 1980; Jonsson, 1986; Pfandl, Posch, & Boenigk, 2004).

The sessile suspension-feeding ciliate, *Stentor coeruleus*, produces a feeding current (Figure 6a) that is faster than *S. helianthica* can swim and that sweeps both unicellular and colonial prey into the buccal cavity (Figure 6b). These predators engulf most of the small choanoflagellates into the gullet while rejecting most of the large colonies. The feeding efficiency of a predator is the number of prey captured per number encountered; in the case of ciliates, feeding efficiency is the proportion of the prey that entered the capture zone (Figure 6b) that are taken into the gullet. There was a significant negative association between the feeding efficiency of *S. coeruleus* and the number of cells in the choanoflagellate prey they consumed, whether the prey were alive or dead (Kendall's Tau, $p < .05$; Weiler, 2015). Therefore, increasing size by forming multicellular colonies does reduce the vulnerability of choanoflagellates to being eaten by such ciliates.

The passive heliozoan predator, *Actinosphaerium nucleofilum*, traps prey that contact its long axopodia, which transport those prey to the cell body where they are engulfed into phagosomes (Figure 7a). The feeding efficiency of *A. nucleofilum* is the ratio of prey engulfed by phaogosomes to the number that entered into the sphere of water occupied by the axopodia. As expected, a greater proportion of the rosette colonies of *S. heliantica* bumped into axopodia than of unicellular slow swimmers. However, more colonies than single cells were lost during transport to the cell, so there was no difference in the feeding efficiency of this passive predator on unicellular versus multicellular choanoflagellates (Figure 7b; Kumler and Koehl, 2018).

Escape behaviors by protozoans have been documented. For example, experiments with steady siphon flow show ciliates and flagellates execute rapid escape "jumps" if water deformation rate exceeds a threshold (Jakobsen, 2002), and behavioral studies showed that flagellates and ciliates can change direction or accelerate if they bump into something (Jakobsen & Hansen, 1997; Machemer & Deitmer, 1985). In contrast, *S. helianthica* was not observed to use escape maneuvers when interacting with the predators studied thus far.

These studies reveal that colony formation by choanoflagellates can either raise or lower the risk of predation, or can have no effect, depending on which protozoan predators are abundant.

3 | CONCLUSION

Studies comparing the performance of unicellular and multicellular stages of choanoflagellates reveal tradeoffs between swimming versus feeding performance, and between susceptibility to raptorial versus suspension-feeding protozoan predators. This suggests that the ability of cells to differentiate into different morphotypes (including multicellular forms that can change shape by growth or by rapid contraction) in response to spatially heterogenous and temporally varying aquatic environments might have provided a selective advantage to the ancestors of animals. Thus, the groundwork would have been laid not only for the ability to become multicellular, but also for cell differentiation.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

This is a review article and thus research data are not shared.

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