Georgia Journal of Science

2019

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FINDINGS CONSISTENT WITH NONSELECTIVE FEEDING IN
Tetrahymena pyriformis

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ABSTRACT

In amoeboid cells, food particles are engulfed only after receptors on the phagocytic cell's membrane bind to ligands on a particle's surface. Ciliates also feed via phagocytosis but, instead of enveloping particles, some ciliates take them up through a complex, permanent, funnel-shaped, feeding apparatus. It is unclear whether receptor-ligand interactions are needed to trigger the process. If ciliates were shown to feed selectively on certain particles over others, based on the particles' surface properties, then receptor-ligand interactions would likely play a role in phagocytosis. The literature includes few reports of such selectivity. To further investigate this issue, we chose to study feeding preference in the ciliate *Tetrahymena pyriformis*. We fed *Tetrahymena* mixtures of orange and green, fluorescent, 3 µm, polystyrene beads at two concentrations. One of the two types of beads was coated with bovine serum albumin. Authors were blinded to experimental conditions. We found no evidence of a preference for coated or uncoated beads at either concentration. We also found no trend toward the development of selective feeding as cells acquired more beads over time. Although we cannot rule out the possibility that *Tetrahymena* feeds selectively, we did not find convincing evidence of such selectivity when *T. pyriformis* is given a choice between uncoated beads and those coated with albumin. Our results failed to demonstrate a role for molecular recognition when *Tetrahymena* engages in phagocytosis.

Keywords: Alveolata, alveolate, Ciliophora, filter-feeding

INTRODUCTION

Phagocytosis is best understood as it occurs in amoeboid cells that serve as model organisms (Cardelli 2001; Alberts et al. 2002, p. 747). The process is triggered by molecular recognition; receptors on the phagocytic cell's membrane bind to ligands on the surface of the particle to be ingested. The cell then envelopes the particle. Phagocytosis in some ciliates is likely to differ mechanistically as they consume solid material through a permanent, funnel-shaped, oral apparatus (Verni and Gualtieri 1997). It is unclear if molecular recognition is necessary to trigger phagocytosis in these organisms. In well-studied amoeboid cells, receptor-ligand interactions allow for selective consumption of preferred material (Aderem and Underhill 1999; Cardelli 2001). If ciliates were shown to prefer certain particles over others, based on the particles' surface properties, then receptor-ligand interactions would likely play a role in phagocytosis.

Feeding selectivity has been studied in ciliates by comparing feeding rates when cells were provided with single types of particles, and by giving cells multiple types of

particles simultaneously and looking for a preference. Authors have examined selectivity between different types of natural prey (Taniguchi and Takeda 1988; Pfister et al. 1998; Hamels et al. 2004; Thurman et al. 2010; Dopheide et al. 2011), between nutritive and nonnutritive particles (Mueller et al. 1965; Elliot and Clemmons 1966; Pfister et al. 1998; Grønlien et al. 2002; Boenigk and Novarino 2004), and between nonnutritive particles that differed in their surface properties or composition (Sanders et al. 1998; Dürichen et al. 2016). Selective feeding appears to be established in some cases but not in others. Dürichen et al. (2016), in particular, stated that *Tetrahymena pyriformis* feeds more rapidly on beads coated with bovine serum albumin (BSA) than on uncoated beads. To further investigate preferential feeding, we chose to determine whether the ciliate *T. pyriformis* feeds preferentially when given a choice between uncoated polystyrene beads and those that are coated with BSA.

MATERIALS & METHODS

Three micrometer diameter, hydrophobic, fluorescent, orange and green polystyrene beads were obtained from Phosphorex, Inc (products 2218 and 2105B; Hopkinton, Massachusetts, USA). Some were left uncoated while others were coated with BSA. Because covalent linkage caused clumping, beads were coated via passive adsorption as follows. Beads were washed in phosphate-buffered saline (PBS) to remove surfactant and exposed to 0.8 mg BSA/ml of PBS for 2 h with agitation. Beads were then rinsed several times and the washings were retained. The concentration of BSA in the washings was determined by measuring absorbance at 280 nm and comparing it to that of standards. By knowing the initial quantity of BSA, and the volume and concentration of BSA in the washings, we were able to determine how much BSA remained adsorbed onto the beads and the walls of the microcentrifuge tubes. We performed the same procedure without beads to calculate how much BSA remained attached to the tubes alone. Subtraction yielded estimates of the quantity of BSA adsorbed onto the beads, 8−13 μg protein/mg of beads.

We used Thermo Scientific's bicinchoninic acid protein assay kit to ensure that the BSA remained adsorbed onto the beads (product 23227, Rockford, Illinois, USA). The assay relies on the ability of proteins to reduce Cu^{2+} to Cu^+. The latter absorbs light at 562 nm and creates a purple solution. Because fluorophores can reduce copper and create false positives, beads were first treated with the kit's assay solution for a week to bleach the fluorophores. Beads were then washed thoroughly, treated with BSA as described above or exposed to PBS instead, and washed again. The solutions were frozen and thawed, because the same was done with experimental aliquots. The beads were washed again and then exposed to the assay solution for 1 day. A microcentrifuge tube that contained no beads was also subjected to the protein coating procedure so that we could account for the BSA that became adsorbed onto the inside of the microcentrifuge tubes.

Tetrahymena pyriformis (referred to as *Tetrahymena* below) was obtained from Carolina Biological Supply Company (Burlington, North Carolina, USA). Cells were maintained in Carolina's *Tetrahymena* medium. One to three days prior to testing, cells were washed and moved to commercially-available spring water to ensure a high initial rate of uptake (Ricketts 1971) and to eliminate any surfactant present in the culture medium. Surfactant could cause the BSA to separate from the beads.

Tetrahymena was exposed to beads at a total concentration of either 9×10^6 beads/ml or 45×10^6 beads/ml for 45 min at the low concentration and 5 min for the high one. Either the orange or green beads were coated with protein. Investigators did not know which, and it varied among the trials. After the incubation period, cells were killed through the addition of enough Lugol's solution (product C90034, Carolina) to reach a final concentration of 10%. In each experimental trial, 10 *Tetrahymena* cells that contained at least five beads were located randomly and the number of orange and green beads was determined (Figure 1). To ensure random sampling, investigators started in
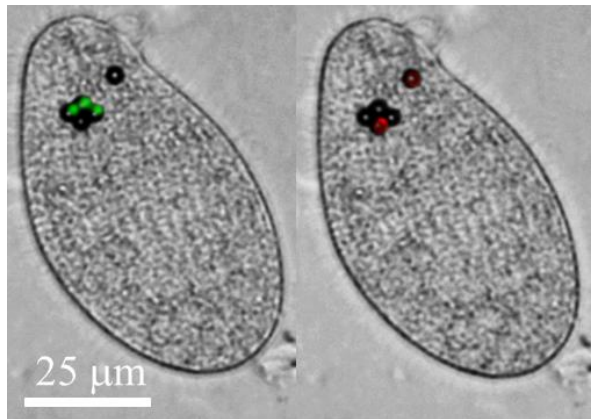

Figure 1. Overlay of phase contrast and fluorescence images of the same *Tetrahymena*. Of the five beads present, three are green and two appear red—the false color assigned to the orange beads.

the upper left corner of the coverslip, scanned over to the right side, then down one field of view, scanned to the left, and so on. Forage ratios (see below) from those 10 cells were averaged with that average being a single datum or replicate in the analysis. Since there were two concentrations, and two types of coated beads, there were four groups. There were 12–16 replicates in each.

It proved impossible to dilute the beads so that the two types of beads were present at equal concentrations. Thus, we obtained concentrations that were as equal as possible, and corrected for the remaining difference with Ivlev's forage ratio, *FR* (Lechowicz 1982). Restated in terms of beads, forage ratio is as follows

$$FR = \frac{B_i/B_T \text{ (cell)}}{B_i/B_T \text{ (solution)}}$$

wherein B_i is the number of beads of interest in a cell (numerator) or the solution (denominator) and B_T is the total number of beads in those two locations. If $FR = 1$ there is no preference. For most of the analysis, the beads of interest were the green ones. We analyzed whether the protein coating influenced the preference for green. If we had considered the coated beads to be the beads of interest, then we would have had to compare the results of the four groups to the hypothetical outcome of 1 with four single sample *t* tests. By analyzing how BSA coating influences the selectivity for green beads, we were able to compare the results of each group to the others', with analysis of variance. This strategy minimized the cumulative type I error rate.

RESULTS

Tetrahymena appears to prefer green beads to orange ones. The mean forage ratio for green was greater than a value of 1 at both bead concentrations, regardless of which type of bead was coated with BSA (Figure 2). This seeming preference for green beads

could have resulted from the fact that they are easier to count than the orange ones when tightly packed into vacuoles. Since sometimes the green beads were coated with BSA, while other times it was the orange ones, our results are unbiased, in spite of the slight difference in brightness between the two types of beads.

We found no evidence that the presence or absence of BSA influenced the apparent preference for green at either concentration (Figure 2A, 2-way ANOVA: main effect of coated vs. not coated, $F_{1,51} = 2.354$, $P = 0.131$; interaction of concentration and coated vs. not coated, $F_{1,51} = 0.294$, $P = 0.294$). Selection for BSA-coated beads would have resulted in higher forage ratios for green beads when they were coated than when they were not (Figure 2B), which did not happen. Selection for uncoated beads would have led to the opposite outcome.

There was no indication that *Tetrahymena* demonstrates a preference for coated or uncoated beads at any level of bead consumption (Figure 3). As cells acquired larger numbers of beads, they approached equal numbers of both kinds.

Results of the bicinchoninic acid protein assay show that BSA remains adsorbed to the beads in spite of them being frozen, thawed, and washed many times (Figure 4). A small amount of protein may have been lost with prolonged freezing.
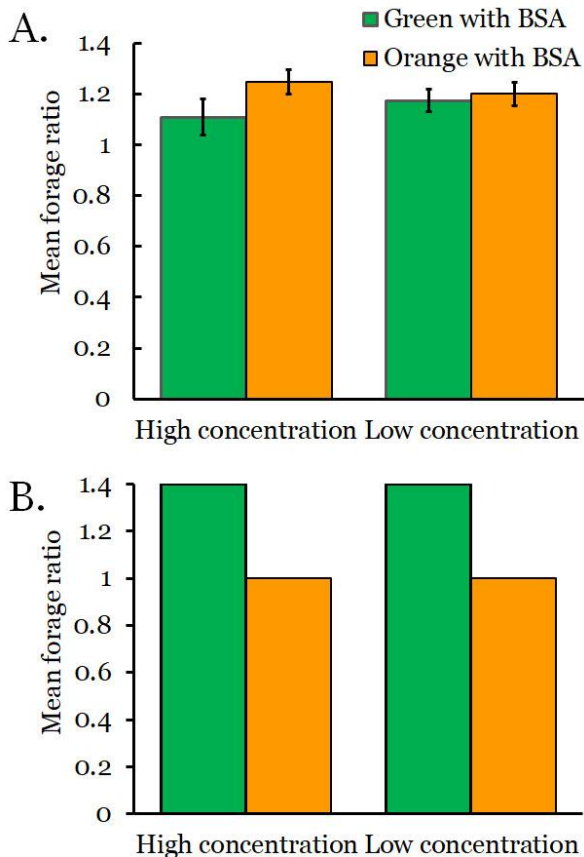

Figure 2. A – Coating beads with BSA has no clear effect on *Tetrahymena*'s seeming preference for green beads, i.e., the average forage ratios do not differ significantly from each other. Bars show standard error of the mean. B – Hypothetical results in which *Tetrahymena*
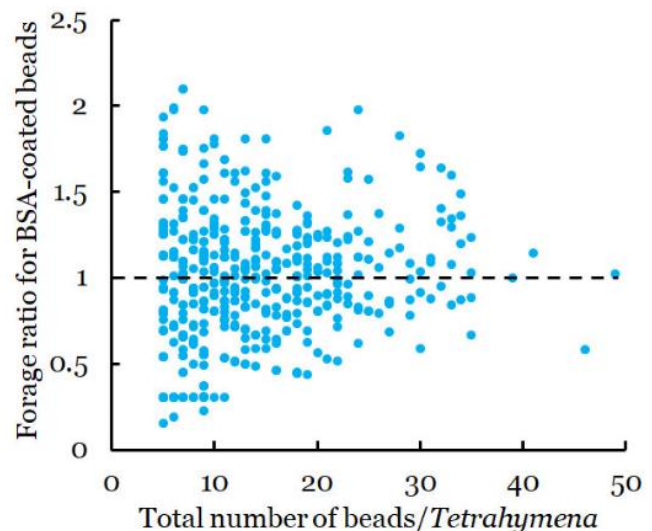

Figure 3. Preference for BSA-coated beads and the total number of beads consumed for every *Tetrahymena* cell examined. If *Tetrahymena* cells acquired selectivity as they filled up with beads, forage ratio would trend upward or downward as more beads were engulfed. Instead, there is no convincing trend.
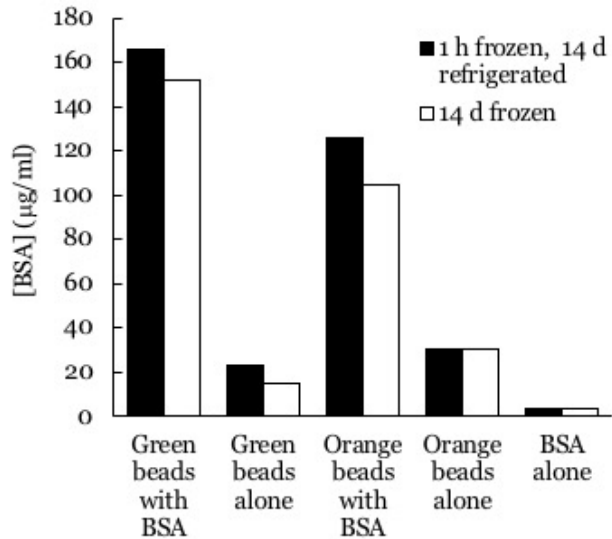
Figure 4. Protein coated beads retain their coat after freezing and washing. To obtain protein quantities, we determined absorbance at 562 nm and compared the results to those obtained from protein standards.

DISCUSSION

Selective feeding has been widely investigated in protists (Montagnes et al. 2008). In many cases, food preference is brought about by constraints placed upon the phagocytic organism. Particles may be too large or small to be trapped or engulfed (Fenchel 1980; Jonsson 1986). Likelihood of capture and consumption can also be influenced by prey behavior. For example, *Stylonychia* can prey most effectively on mobile organisms, perhaps because they are most likely to be trapped through filter feeding (Pfister and Arndt 1998). A prey's physical properties may also influence the likelihood of it being preyed upon. *Euplotes* may be largely resistant to consumption by *Stylonychia* because of *Euplotes*'s rigid cortex (Pfister and Arndt 1998).

In other cases, phagocytic cells actively bring about their own selective feeding. Ciliates can use chemotaxis to locate patches of preferred prey and chemokinesis to remain within them (Paffenhöfer et al. 2007; Montagnes et al. 2008). *Strombidium* can distinguish between different diatom species at close proximity, but without contact, and selectively attack preferred prey. The selected species are likely identified through chemoreception (Hamels et al. 2004).

Few studies have focused on contact recognition in filter-feeding ciliates. In well-studied amoeboid cells, phagocytosis is triggered by the binding of receptors on the cell's membrane to ligands on the surface of the particle to be engulfed, a kind of molecular recognition (Cardelli 2001; Alberts et al. 2002, p. 747). The observation that some ciliates appear to consume particles indiscriminately (Boenigk and Novarino 2004; Perez et al. 2016) allows that phagocytosis in these organisms could be triggered by the mechanical stimulus of particle capture alone (Muller et al. 1965). Some, however, have obtained evidence of selective ingestion based on a particle's surface properties. Sanders (1988) found that *Cyclidium* fed more rapidly on latex beads that were coated with protein than when the beads were carboxylated. Dürichen et al. (2016) determined that particle composition affects the rate of ingestion in *Tetrahymena pyriformis*. Thurman et al. (2010) showed that when *T. pyriformis* is given a choice between two species of equally sized bacteria it preferentially selects one of them. Grönlien et al. 2002 found that *Tetrahymena vorax* feeds selectively in its macrostome form; it captures both latex beads

and its prey *T. thermophila*, but it only engulfs its prey. Our findings are more similar to what Grönlien et al. (2002) observed in *T. vorax*'s microstome stage, seeming nonselectivity. Others have also described *T. pyriformis*'s feeding as nonselective (Muller et al. 1965; Boenigk and Novarino 2004; Perez et al. 2016).

We found no evidence that particle concentration influences whether or not *T. pyriformis* feeds selectively. In contrast, some nanoflagellates feed selectively at high concentrations of prey and inert particles, consuming only the prey, but feed nonselectively when concentrations are lower (Boenigk et al. 2002). A caveat regarding our work is that it is difficult to decide which concentrations to test. So, we chose not only to look at different concentrations, but also the degree to which cells had fed. Would cells packed with large numbers of beads show an indication of preferential feeding? We found no evidence to that effect. There appears to be a trend towards preference for BSA-coated beads in cells with 20 or more beads, but the trend is weak (Figure 3); the mean forage ratio for cells with 20 or more beads was 1.07.

There are a number of explanations as to why some authors have found selective filter-feeding in ciliates while others failed to do the same. *T. vorax*'s macrostome stage clearly feeds preferentially, but the mechanism of phagocytosis likely differs from that found in the microstome form and in other ciliates, for which many authors have reported consumption of nonnutritive beads. Also, it is possible that we would have obtained different results had we coated beads with other substances or tested nutritive as well as nonnutritive particles. In addition, there may be methodological reasons why authors differ in their findings. In our experience, feeding rate is difficult to gauge in *T. pyriformis* because it varies widely among individual cells. Dopheide et al. (2011) made similar observations. To compare feeding rates, they must be quantified. Another issue is that not all authors indicate that they have avoided pseudoreplication (see Hurlbert 1984). When organisms share an environment, like a microcentrifuge tube, the data obtained from those organisms are not independent of each another. Those data cannot be used as individual values when testing a null hypothesis. So, each of our tubes provided just one number for statistical analysis, an average forage ratio.

Many have been fascinated by ciliates since they were first described by Antoni van Leeuwenhoek in 1676. Ehrenberg was so impressed by their complexity that he described them as having organs and organ systems (Hausmann et al. 2003, p. 8). Since then, *Paramecium* has become a mainstay in teaching settings and *T. pyriformis* has acquired the status of a model organism (Ruehle et al. 2016). Although many ciliates have been studied extensively, little is known regarding the mechanics of phagocytosis—a fundamental biological process. Our findings, and those of some others, allow that phagocytosis may be triggered entirely by a mechanical stimulus.

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