INVESTIGATION OF CONTACT-BASED CUES MEDIATING FOOD UPTAKE IN THE MARINE TINTINNID CILIATE *FAVELLA* SP.

A Thesis

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Sean Cobb

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ABSTRACT

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FOOD UPTAKE IN THE MARINE TINTINNID

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Marine ciliates are important consumers of marine phytoplankton and represent a vital link in the food web. These unicellular grazers exhibit complex feeding behaviors and can be highly selective in their prey choices. Recently, Echevarria et al. (2016) showed that the marine tintinnid ciliate *Favella* sp. preferentially ingested natural dinoflagellate prey *Heterocapsa triquetra* over prey proxies (polystyrene beads), which appeared to be mediated by contact-based cues at the cytostome, the cell's oral apparatus. In the present study, I investigated the nature of this contact-based recognition. I offered a variety of neoglycoprotein-coated 15 μm polystyrene microspheres alongside natural prey to *Favella* sp. and observed ingestion over 10-20 min. Although uncoated beads, or those coated with only the chemical linker or protein, were ingested at very low rates, beads coated with mannose and fructose were ingested as much as or more than natural prey. Beads coated with other sugars (fucose, galactose, methyl-mannopyranoside, lactose, mannitol, glucose, and sucrose) were ingested at intermediate rates.

Furthermore, pre-incubation with 10-50 μM dissolved sugars could inhibit uptake of live prey in a specific manner. I examined the genome of *Tetrahymena*, a model ciliate, for potential sugar-binding cell surface proteins and found a number of potential candidates. These results suggest that ciliates may employ sugar-binding proteins as part of their mechanism for prey recognition, similar to many other organisms. Prey selection likely depends on other surface molecules such as proteins, and my results also suggest an explanation for the paradoxical observation that additions of dissolved small side-chain amino acids can inhibit feeding by *Favella* and other marine grazers.

CHAPTER I

INTRODUCTION

Microzooplankton Regulate Primary Production

Microzooplankton (zooplankton 20-200 μm) are a large and diverse group of eukaryotic protists that are accepted as the main predatory pressure on planktonic primary production, consuming up to 60–70% of the mass (Calbet, 2004; Schmoker et al., 2013). They are important intermediaries in the aquatic food chain, converting nanoplankton (2-20 μm) and picoplankton (0.2-2 μm)─inorganic matter that is too small to be filtered by crustacean zooplankton─into a particle size which is readily available for predators higher up the food chain. This acts to decrease the export of primary production both in terms of sinking and/or consumption by higher trophic levels. The trophic prosperity of microzooplankton is dependent on a diverse range of feeding strategies, such as phagotrophy, osmotrophy, chlorokleptoplasty, or mixotrophy (Echevarria et al., 2014). Although the food grasping behavior of phagotrophic raptorial feeders evolved to ingest preferred prey and reject toxic ones, the exact mechanisms of their associated sensory biology is poorly understood (Buskey and Stoecker, 1988; Taniguchi and Takeda, 1988).

Alveolate microzooplankton (dinoflagellates and ciliates) are prime candidates for representing microzooplankton when constructing plankton models (Sherr, 1988). Ciliates are a member of the superphylum, Alveolata, which is a group of unicellular eukaryotes characterized by a layer of flattened membranous sacs subtending the plasma membrane. They are described

by the presence of cilia, or hair like organelles, which allow for a wide range of feeding and swimming behaviors during times of food scarcity or abundance.

The most commonly studied ciliates are *Paramecium* and *Tetrahymena*. While useful in presenting conserved sensory biology, they are not adequate representatives of their entire group. They are bacteriovores that live in freshwater and are morphologically different than many marine ciliates due to the adaptive differences in food selection. Freshwater alveolate research is far from complete and the marine counterpart still has yet to identify the process behind prey selection in ciliates. The molecular basis of feeding in marine ciliates still remains largely unknown. The ciliate tintinnid, *Favella* sp., has recently been presented as a model of marine microzooplankton grazers due to its distribution, protective lorica, and size (Echevarria et al., 2014). Using *Favella* as a model, the machinery underlying ingestion in marine ciliates can begin to take shape.

Overview of Feeding Process in Tintinnids

Ciliates feed on smaller organisms, such as bacteria and algae, by sweeping particles into the oral groove by modified oral cilia. In *Favella*, a ring of adoral membranelles surrounds the oral cavity which puts them in the subclass of ciliates known as oligotrichs. Within the oral cavity is the cyostomal region (mouth), at the base of which vacuoles form. The adoral membranelles beat, creating a current that brings prey to the cytostome as well as propelling the ciliate forward; thus, locomotion and grazing in tintinnids is linked. Feeding by tintinnids can be divided into distinct steps, which may be governed by chemical, physical or biological cues (fig. 1).

Contact and Capture

Contact is primarily determined by the probability of particle encounter rate and

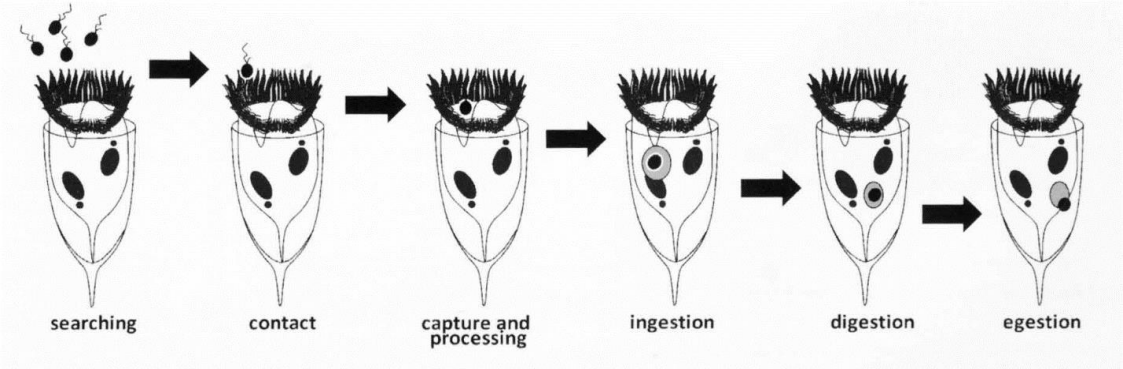

Figure 1. Tintinnid ciliate feeding schematic. When contact occurs at the cilia and cytostome, a decision is made to determine if the particle is sufficient for ingestion. If ingestion occurs, the cytostome helps to pinch off the resulting vacuole, which then travels down the cytopharynx and fuses with lysosomes, where it is digested. Waste is then excreted out of the cytoproct during egestion (Montagnes et al., 2008).

searching. *Favella* will contact ideal prey more frequently than toxic prey of similar size and concentrations, suggesting that contact is in part mediated by dissolved chemical cues (Verity, 1991).

Prey that collide with the oral cilia are often not captured (Blackbourn, 1974). Receptor-ligand interactions may aid in the selection process, as shown in the rejection of toxic *Heterosigma akashiwo* after contact at the cytostome (Montagnes et al., 2008; Taniguchi and Takeda, 1988). In mixtures of two prey species, preferred selection of non-toxic prey is observed, while in single, toxic-prey experiments, ingestion will still occur (Graham, 2008).

The Cytostome

A key feature of the cytostome is the nature of the undulating membrane made of modified cilia, which is found in active ciliate predators (Chambers and Dawson, 1925; Nisbet, 1984). Though the features of these cytostomal movements remain largely unexplored, they are suggestive of employing food-gripping functions, acting as a propulsion system to move prey through the cytostome, as well as joining the microtubule-reinforced walls of the cytopharynx

together to permit fusion of the cytopharyngeal pouch membranes to form a sealed vacuole (Chalmers and Pekkola, 1918; Minchin, 2003; Smith-Somerville and Buhse, 1984; Tucker, 1968). Early ciliate ancestors probably had inherited this from a flagellate ancestor, using the undulating membrane for locomotion, ingestion, and for the formation of a cytopharyngeal tube (Eisler, 1992). Recently, it was found that rhythmic depolarizations regulate cytostomal contractions in *Favella* (Echevarria et al., 2016).

Early ciliate research shows that cytostome architecture is connected with metabolism and functions as part of a neuromotor system (Kofoid, 1920). Rees (1921) determined that the neuromotor apparatus in *Paramecium* connects with the outer membranelles and the cytopharynx, as it was found that upon damaging the neuromotor apparatus, both types of membranelles ceased normal function. In *Tetrahymena*, the cytostome is connected to calcium-filled alveolar sacs and the plasma membrane by a network of microtubules (Sattler and Staehelin, 1979). This suggests that the alveolar sacs, the outer membranelles, the cytopharyngeal membranelles, and the neuromotor apparatus are all connected in ciliates with an undulating membrane.

Ingestion

Phagocytosis is central to the origin of Eukaryotes, allowing for the procurement of bacterial endosymbiotes which gave rise to mitochondria. Ingestion begins at the cytostome and is regulated by several key factors. Chemosensory and mechanosensory mechanisms, as well as metabolic pathways and secondary messenger systems, form the central components of the phagocytic process. It is well documented in macrophages, where it is employed to disable pathogens, but poorly understood in alveolates. Ciliates use vegetative phagocytosis, which is the classical eukaryotic microbial uptake of food particles (Lewis and O'Day, 1996). In this process,

receptor clustering occurs upon particle binding, which generates a phagocytic signal; however, the preceding chemical recognition system has not yet been studied in marine ciliates.

The formation of food vacuoles from existing membrane may be the limiting step in feeding when food is abundant. After the particle enters through the cytostome, a vacuole is formed around either single (Taniguchi and Takeda, 1988) or multiple prey (Stoecker and Govoni, 1984). The vacuole travels through the body towards the cell posterior, where it becomes digested by acids and enzymes after fusing with lysosomes along the way. Later, neutralizing alkaline enzymes further digest the food and finally the waste is excreted out of the cell via the cytoproct during egestion.

Bioelectric Control of the Feeding Process

Particle handling and recognition behaviors define the selective process in *Favella* and is an exhibition of prey preference. The initial processing of prey can last anywhere from 0.2-100 sec, depending on the species. Tintinnids are capable of selecting against prey that have been captured (Buskey and Stoecker, 1988). During rejection, particles may be expelled from the cytostome by a reversal of cilia (Taniguchi and Takeda, 1988). While the shape and size of prey are major determining factors by planktonic ciliates, some species have the ability to select cells over non-living particles and certain species over another, despite similar dimensions (Taniguchi and Takeda, 1988). *Favella* prefer dinoflagellates over other similarly-sized prey, non-toxic species over toxic ones, and beads over heat-killed dinoflagellates (Buskey and Stoecker, 1988). Chemical recognition studies have yet to be explored and the exact mechanism behind the rejection process is unknown, but it occurs after physical contact within the oral apparatus.

Echevarria et al. (2016) explored the bioelectric control of swimming and feeding of *Favella*. They showed that mechanically-stimulated depolarizations occur at the membranelles and cytostome. Before the cilia depolarizes, there is a short lag phase, while a longer more variable lag phase occurs after contact at the peristome (the region surrounding the cytostome). During this longer lag phase, *Favella* rejects microspheres, but ingests *Heterocapsa* due to an unknown process. This suggests that although mechanical cues are used to capture prey particles and move them to the cytostome, contact-based chemoreception at the cytostome is used to detect and selectively ingest live prey (fig. 2).

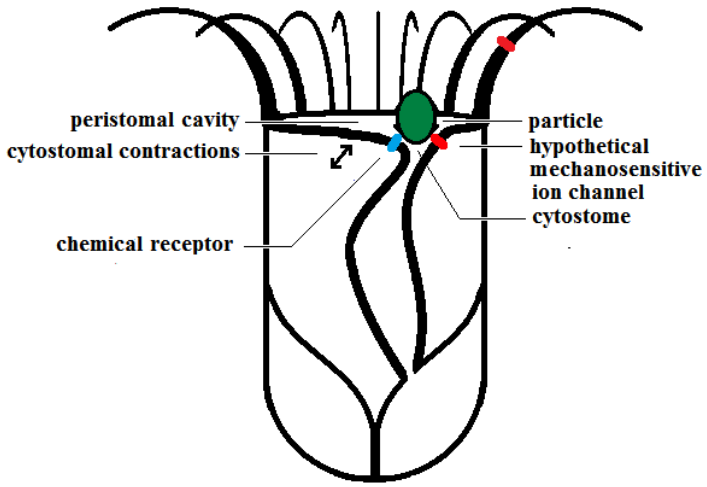

Figure 2. Diagram of contact in the oral area of *Favella*. Particles enter the cytostome and stretch hypothetical mechanically-gated ion channels, resulting in an electric response after a lag phase. During this lag phase, chemical receptor proteins may asses the surface of the particle. Finally, either hyperpolarization (ingestion) or depolarization (rejection) occurs.

Contact-Based Recognition of Prey Sugars Regulates Ingestion

Although the nature of such receptors is not yet known, there is growing evidence that carbohydrate-binding receptors play an important role in the protist feeding process, though

investigations of sensory biology in planktonic ciliates leading to population-level feeding dynamics are sparse (Petri Jr et al., 2002; Sakaguchi et al., 2001; Svobodová et al., 1997; Ucko et al., 1999; Wootton et al., 2007). Surface glycoconjugate moieties help predators to recognize prey and are involved in many other physiological processes including nonself recognition, endocytosis, and cellular differentiation (Ramoino et al., 2001).

Protein ligand-mediated phagocytosis is present in many well-studied organisms. The macrophage mannose receptor, which plays a role in innate immunity, is a calcium-gated lectin which recognizes mannose carbohydrates on the surface of microorganisms and mediates phagocytosis (Cambi et al., 2005). A range of eukaryotic organisms employ mannose-binding lectin (MBL), such as amoeba, which use these lectins as well as galactose/N-acetylgalactosamine (Gal/GalNAc) lectin to uptake prey (Allen and Dawidowicz, 1990; Gao et al., 1997).

Lectins are highly specific binding proteins which can be used for a diverse range of applications, such as conjugation, nuclear division, cellular morphogenesis, trichocyst (thin thread) discharge and membrane transport (Ramoino et al., 2001). Glycoproteins are most abundant on the cell surface, but they are also present on intracellular organelles, including food vacuoles, the Golgi apparatus, and nuclear membranes (Ramoino, 1997). Glycoproteins are commonly studied by using fluorescent derivatives of plant lectins which allows researchers to visualize a change in glycoprotein expression. For example, *Paramecium* glycoproteins vary with different feeding states (Ramoino, 1997). *Euplotes vannus,* a marine hypotrich ciliate (characterized by bundles of cilia on the ventral surface), binds to concanavalin A (ConA), wheat germ agluttinin (WGA), peanut agluttinin (PNA) and other lectins (Roberts et al., 2006).

Wootton et al. (2007) found that the marine dinoflagellate *Oxyrrhis marina* uses a Ca^{2+}-dependant mannose-binding lectin to recognize and phagocytose phytoplankton prey. They used polystyrene microspheres of similar size to naturally preferred prey, but conjugated with various sugars or sugar derivatives, and observed feeding selection of beads coated with mannose-BSA over GalNAc coated beads. With additions of 20 μM dissolved mannose, grazers were unable to discriminate between either, showing that the chemical signal can be blocked.

Study Objectives, Questions, and Hypotheses

The contact-based chemical basis for selective phagocytosis of live prey by *Favella* (Echevarria et al., 2016) stipulates that *Favella* use chemical information on the surface of prey particles to initiate phagocytosis. Such 'eat me' recognition signals likely include specific sugars and amino acids, but have yet to be described in marine ciliates. In the present study, I investigated this in *Favella* by quantifying ingestion of natural and artificial prey. I modified the cell surface of natural prey (the dinoflagellate *Heterocapsa triquetra*) by various treatments (heat, chemicals), and prepared artificial prey (polystyrene microspheres) with different sugar coatings. I addressed five main questions:

1. Does cytostomal contraction rate vary during feeding and in starved vs. sated cells?
2. How do feeding rates on artificial prey compare with natural prey?
3. How do dissolved and contact-based cues affect the uptake of prey?
4. How are these rates affected by modifying natural prey surfaces, or by coating artificial prey surfaces with chemical cues?
5. In the *Favella* transcriptome, what potential receptors and transporters exist that might help regulate metabotropic control of feeding?

To answer these questions, I designed experiments to test 3 hypotheses:

1. Artificial prey coated with 'eat me' cues will be eaten along with natural prey, while those lacking specific cues will be rejected.
2. Artificial prey coated with 'eat me' cues will be ingested at higher rates than those coated with other cues.
3. Adding dissolved solutes similar to those that stimulate feeding when bound to prey will swap out the ‘eat me’ signal and inhibit feeding of natural prey.

CHAPTER II

MATERIALS AND METHODS

Cultures

Favella sp. cultures (originally isolated from Puget Sound, WA) were gifts from Dr. Suzanne Strom, Shannon Point Marine Lab, Western Washington University, and Michael Echevarria, North Carolina State University, Wilmington. Cells were grown in dim light at 16 °C in filtered, autoclaved Fort Bragg seawater for early experiments and San Francisco seawater for bead and dissolved cue experiments. Prey were grown on f/2 media and included *Heterocapsa triquetra* (dinoflagellate; abbreviated as Ht in this thesis), *Mantoniella squamata* (prasinophyte) and *Isochrysis galbana* (haptophyte), and were fed to *Favella* in a 4:1:0.01 cell ratio, respectively. After exhausting prey supply, *Favella* became transparent and were ready for feeding tests.

Feeding

Favella that were semi-starved (less than 24 hrs after feeding) could initially hold many more vacuoles than starved cells. In cells that contained over 14 vacuoles, boundaries became blurred by superimposed chlorophyll and very bright fluorescence. In dense cultures, cells exhibited gravitaxis. These cultures were preferred for experiments. A minimum density of 25 cells/mL, pipetted from the top of the water column, produced optimal specimens with the maximum feeding rate. Lower concentrations of cells resulted in a sub-maximum feeding rate. Density of *Favella* was determined by counting individual cells per mL of culture per well of a 24 well plate.

Feeding experiments involving carboxylic beads followed a modified guideline provided by Wootton et al. (2007) and were conducted as a single-blind study. Unless otherwise stated, experiments were done at 50:1 prey (artificial and/or live) to predator ratio, in starved conditions, at 16 C°. Ingestion rates of microspheres were measured using a light microscope to count vacuoles.

Microscope, Pictures and Videos

To circumvent vacuole ambiguity under brightfield, the fluorescent properties of chlorophyll was used to count vacuoles. Micrographs were taken with sensitivity set at 100 and exposures set at 1/700 sec for bright field (BF) and 1/4.5 sec for fluorescence. For cells with many vacuoles, the exposure was set to 1/6 sec.

Brightfield videos used a light setting of 4.5 and a gain of 6.5 when at 100X. At 10X, light was set to 4 and gain, 2. For phase contrast images, the maximum light setting was used. Before each picture was taken, background color was automatically adjusted.

Quantifying *Favella* Cytostomal Contractions

A cytostomal contraction was marked by pronounced anterior movement of the peristomal ring, adjacent to the cytostome. Vacuoles and other organelles were also seen to move during this type of contraction. Separate, smaller and faster, contractions also occurred in proximity, but were not accompanied by organelle movement. These were not counted. Video recordings were played back for 30 sec and the total number of contractions were counted, then divided by 30 to calculate rates in Hz.

Slides were prepped by creating a square of vaseline/mineral oil mix onto a slide. Using a suction micropipette, *Favella* were captured under the dissecting microscope at a low

light setting and transferred to the slide, then a coverslip was gently placed on top of the square, allowing for just enough compression to immobilize cells for video recording.

Fixing Cells

Glutaraldehyde (0.5% final) was initially used to fix cells, but caused the cell to exocytose recently ingested prey. *Favella* were extremely sensitive to fixing agents. I found 10% ethanol to be a better fixative.

Heterocapsa triquetra Surface Treatments

10 mL of Ht culture was poured into a beaker to prevent contamination of stock, then density was determined with a hemocytometer. Ht was pipetted into 3 separate 1.5 mL tubes, labeled "0.01 % Triton X-100" (900 µL Ht), "0.18 % Glutaraldehyde" (994 µL Ht), and "heat" (1 mL Ht). 100 µL of 0.1% TritonX-100 was added to its respective tube to reach a concentration of 0.01%. The tube was incubated for 30 min. 6 µL of 30% glutaraldehyde was added to its respective tube to reach a final concentration of 0.18%. This tube was incubated for 20 min. The "heat" tube was placed in a beaker filled with simmering DI water atop a hot plate for 20 min.

After incubation periods, the tubes were spun down at 3000 rpm for 5 min. 500 µL of the supernatant was gently pipetted out, and the tubes were refilled with ciliate medium. The tubes were then triturated, a few drops were pipetted onto glass slides for microscopy, while the rest were used for feeding observation.

For Con A binding microscopy, Ht were preincubated with 50 mM Con A for 1 hr, then filtered through a 10 µm mesh with f/2 media. After 30 min, feeding rate was measured. For feeding tests, Ht cells were incubated in 20 µg mL^{-1} Con A for 30 min, then spun down at 1000 rpm for 5 min. 700 µL of supernatant was pipetted off, then 700 µL filtered seawater was added

back in. The tube was vortexed and allowed to incubate at 16 °C for another 30 min before being fed to *Favella.*

Preparation of Neoglycoconjugate Beads

Fluorescent neoglycoconjugate beads were prepared according to Luyai et al. (2009) with modifications (fig. 3). Briefly, 2 mg of free reducing glycan were mixed with 100 µL freshly prepared 4-nitrophenyl anthranilate (NPA; Oakwood Chemicals) solution (0.35 M in 7:3 v/v DMSO:acetic acid) and 100 µL $NaCNBH_3$ (Fisher) solution (1M in 7:3 v/v DMSO:acetic acid) in a 1.5 mL snap-cap microfuge vial. Reductive amination occurred by heating the mixture at 65 °C for 2 hr and was quenched by addition of 1 mL acetonitrile. After cooling at −20 °C for 2 hr, the reactions were centrifuged at 10,000 *g* for 5 min to precipitate glycan-NPA (GNPA). The supernatant was discarded and the pellet desalted with a C18 Sep-pak (Waters Corporation). Unconjugated sugars were eluted with water and GNPA with acetone. After evaporation of solvent, these were conjugated to protein (BSA, Fisher) by adding 100 µL of a 5 mg mL^{-1} solution in PBS (0.1M, pH 8.5 with 1% DMSO), giving a 10:1 GNPA:BSA molar ratio and incubated overnight at 40 °C. Conjugated GNPA-BSA was then purified and concentrated by ultrafiltration (10,000 MWCO), and was finally conjugated to 15 or 20 µm polystyrene microspheres (Polysciences X) that had been coated with carboxymethylcellulose (CMC), using the N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride coupling method (EDAC; #AC17144-0010) and a coupling buffer (50 mM MES, pH 5.2). Total adsorbed protein was calculated by difference from measurements before and after conjugation (NanoDrop, Thermo Fisher). Coated microspheres were stored at 4 °C in storage buffer (10 mM Tris, pH 8.0). Fluorescence of the microspheres was monitored by microscopy (Olympus BX-51) using a DAPI filter.

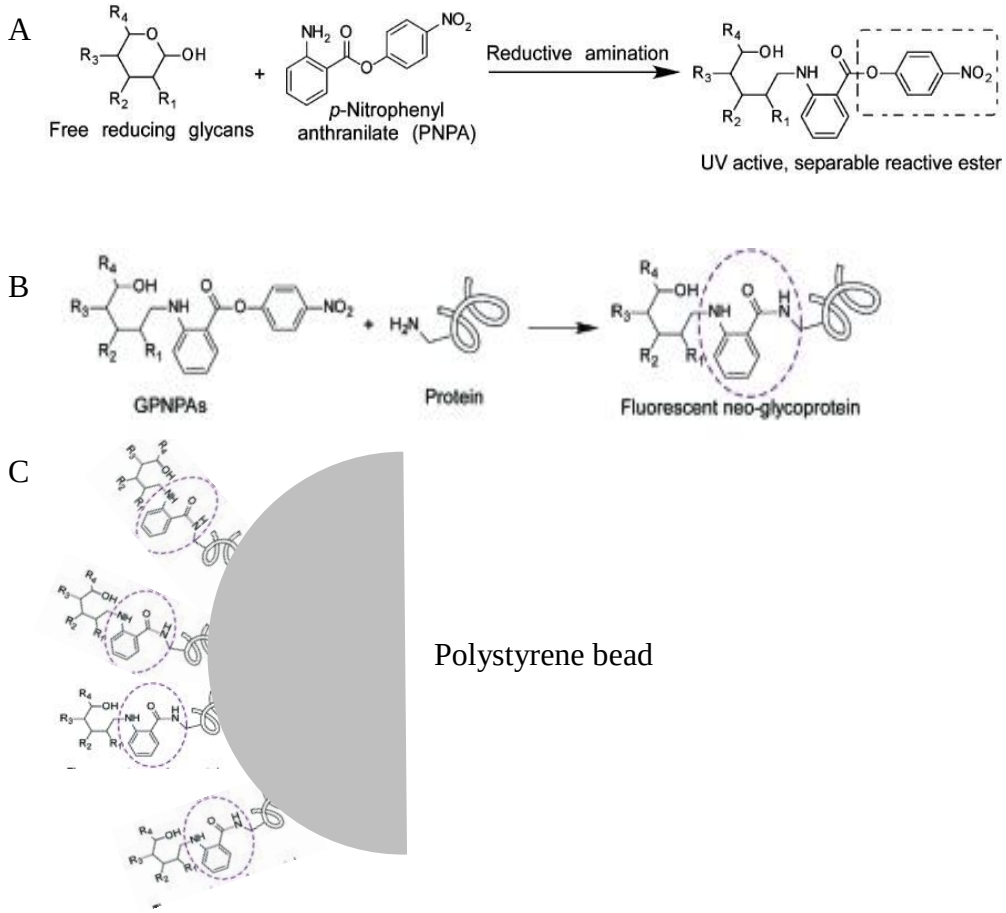

Figure 3. Preparation of neoglycoconjugate beads. (A) Reducing sacharrides were linked via reductive amination to NPA to make a fluorescent conjugate, which was then incubated with protein (BSA) to create the neo-glycoprotein (B). Finally, the glycoprotein was covalently attached to polystyrene microsphere beads (C). Modified from Luyai et al. (2009).

Dissolved Sugar and Amino Acid Feeding Tests

Dissolved sugar and amino acid tests were blind studies. 9 soluble compounds at 3 different concentrations were pipetted into 2 mL of *Favella* culture, which had been incubated in 28 wells of two 24 well plates for 20 min. 10 min feeding tests were simultaneously done at a 50:1 prey to predator ratio. Cells were fixed with a final solution of 10% ethanol and vacuoles were counted.

Bioinformatics Analysis of Recognition Proteins in Ciliates

The Tetrahymena Genome Database (TGD) was searched for lectins, G-protein-coupled receptors (GPCRs), transporters, and other surface proteins that may interact with carbohydrates, glycoproteins, or amino acids. Keyword searches that yielded no headlines or domains were not included in the results. The amino acid sequences of proteins were examined with the protein Basic Local Alignment Search Tool (pBLAST) and checked for homology in other eukaryotes. Inversely, known amino acid and sugar receptors or transporters were pBLASTed with search term set to "ciliates". These proteins consisted of the lectins found in the TGD as well as amino acid-binding GPCRs, periplasmic binding proteins (PBPs), ATP-binding cassette (ABC) transporters, and solute carriers (SLCs). Function and homology were formatted as a table; sequences bearing the lowest E-value had the most significance.

CHAPTER III

RESULTS

Characterizing Feeding Rates on Natural Prey *Heterocapsa triquetra*

I initially characterized *Favella* ingestion rates on natural prey (Ht) by observing the functional response: the number of cells ingested per unit time as a function of prey density. This was done by incubating starved *Favella* with different ratios of prey cells and tracking uptake over time by counting food vacuoles with chlorophyll fluorescence. Starved *Favella* can typically ingest a maximum of ~15 *H. triquetra* cells (fig. 4).

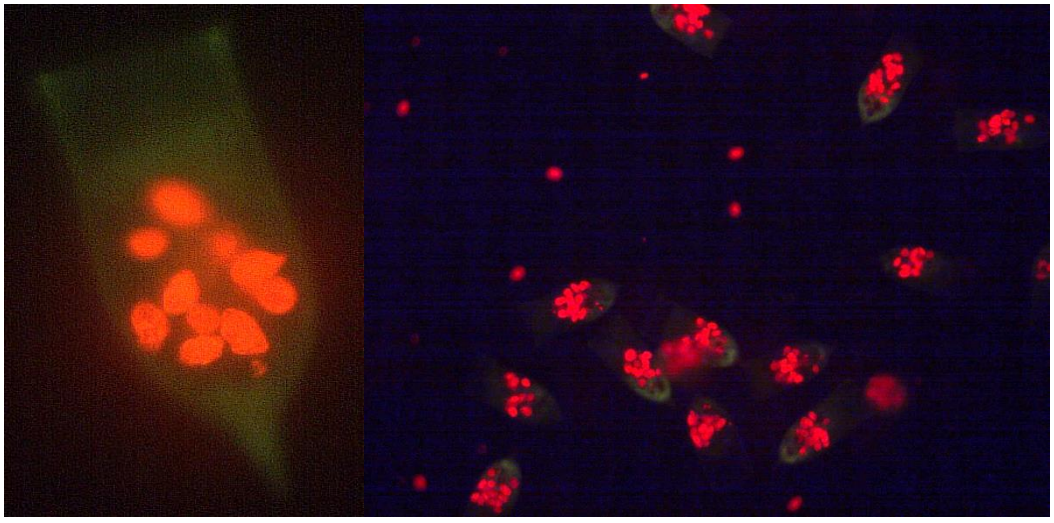

Figure 4. Examples of quantifying ingested prey over time. Epifluorescence micrographs of *Favella* with blue excitation reveal ingested Ht as red chlorophyll autofluorescence. Shown are results at 200:1 prey: predator. Left: after 10 min, 10 Ht cells are visible inside a *Favella* cell (100X). Right: after 70 min, most *Favella* are saturated with ~15 prey (10X). Individual uningested prey are also visible. (Source: 7/15/13)

When prey density was saturating (~200 prey/predator), satiety occurred in ~20-30 min; fig 5A). Below saturation, the time to reach satiety increased as *Favella* spent additional time encountering prey. Graphing the initial rate of prey capture over time gave a functional response (fig. 5B). Based on initial tests, I observed that a ratio above 50:1 prey: predator would give ~linear uptake over 10-20 min, so 50-100:1 ratios were used for subsequent tests of modified prey and artificial prey proxies.

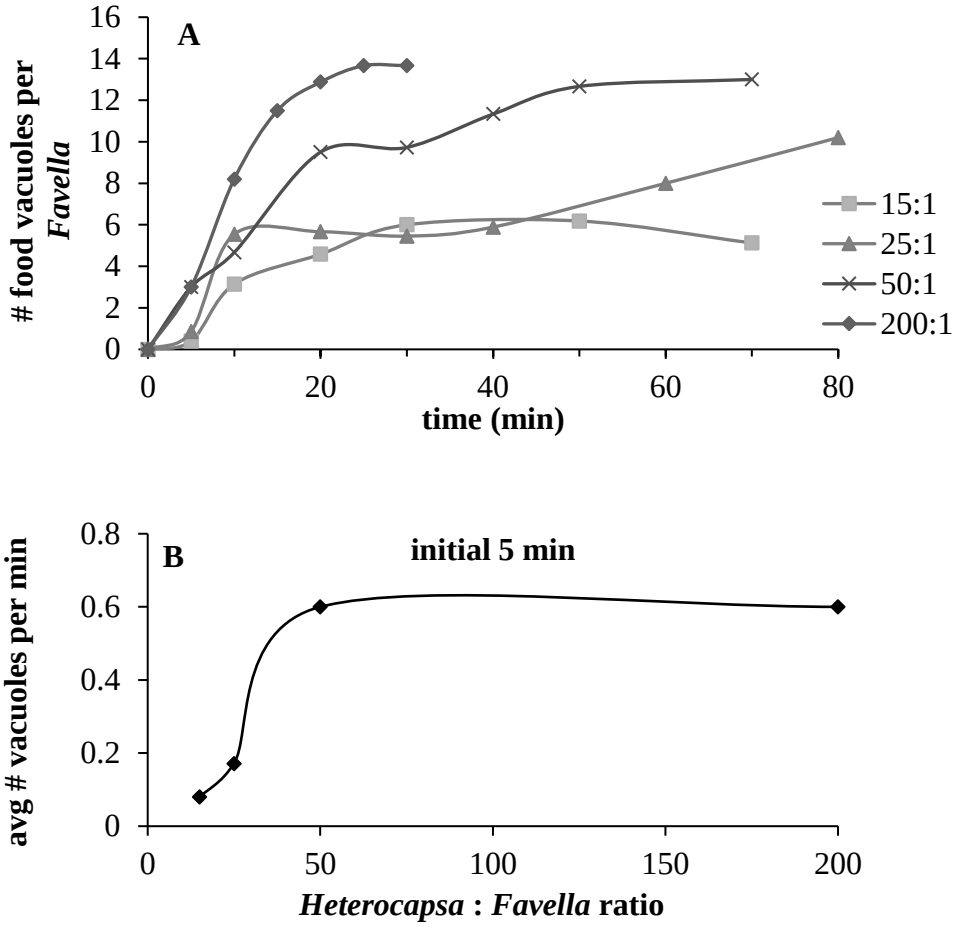

Figure 5. Determination of *Favella* feeding functional response. (A) Examples of ingestion over time. Ratios are # prey per *Favella*. Starved *Favella* ingest 12-14 Ht per cell over 20-30 min at saturation (50-100 Ht per *Favella*). (B) *Favella* become saturated at 50:1 prey to predator ratio for the first 5 min. (Source: feeding rate, 7/15/13)

Changes in Cytostomal Contraction Rates During Feeding on *Heterocapsa*

Starved *Favella* showed cytostomal contraction rates of ~2-4 Hz, possibly higher under intense light (fig. 6). As *Favella* fed, contraction rate slowed gradually as cells became filled with prey, then rose again (fig. 6). However, these changes were not significant.

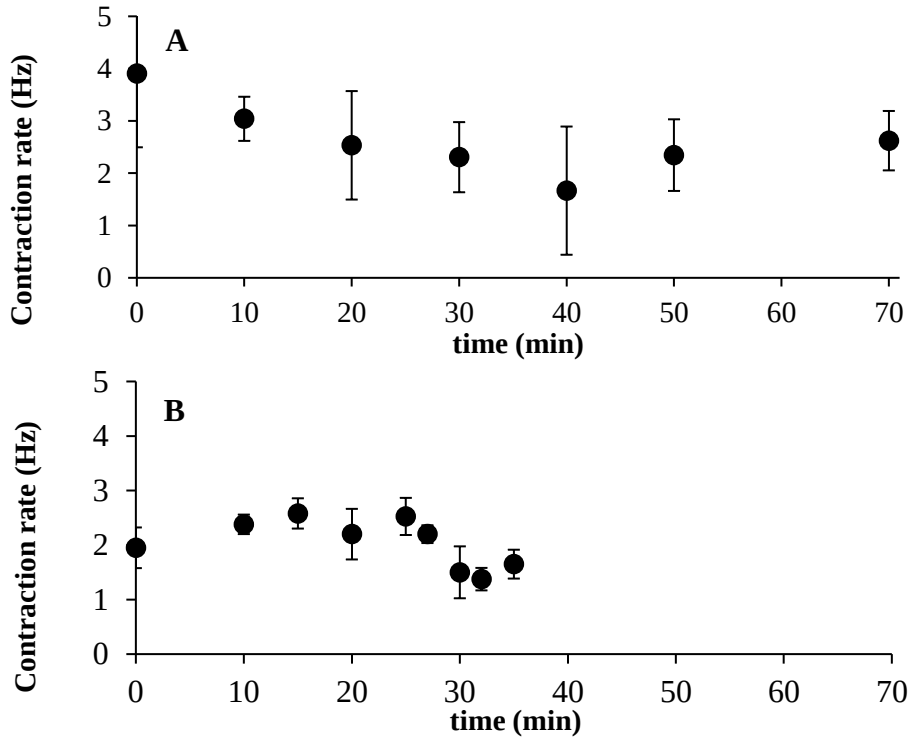

Figure 6. Cytostomal contraction rate decreases temporarily after feeding. Shown are average of cells; error bars show the standard deviation. (A) Experiment 6/13/13; 3 *Favella*/mL, 100:1 prey to predator ratio, high light. (B) Experiment 9/30/14; 13 *Favella*/mL, 100:1 prey to predator ratio, low light. (Source: (A) feeding rate 6/13/13; (B) feeding rate 9/30/14)

Feeding on *Heterocapsa* with Surface Modifications

I ran an initial trial which probed the impact that grossly modified Ht surfaces had on *Favella* feeding rate, using treatments of heat, detergent, or protein cross-linking fixation. Changes were observed in swimming behavior (data not shown) but were largely insignificant in

feeding rates (fig. 7). Ht incubated in 0.01% glutaraldehyde initially caused cell death in *Favella* and had to be washed three times, leading to a comparable feeding rate to the control. 0.01% Triton X-100 caused maneuvers and sinking in *Favella,* which eventually died after 1 hr. This treatment reduced the clear outer coating of Ht and *Favella* swam with increased avoidance maneuvers after feeding more than 10-20 min. Heat-treated cells appeared to be eaten the least, although the difference was still barely significant. Ingestion of heat-treated prey caused longer durations of backward swimming.

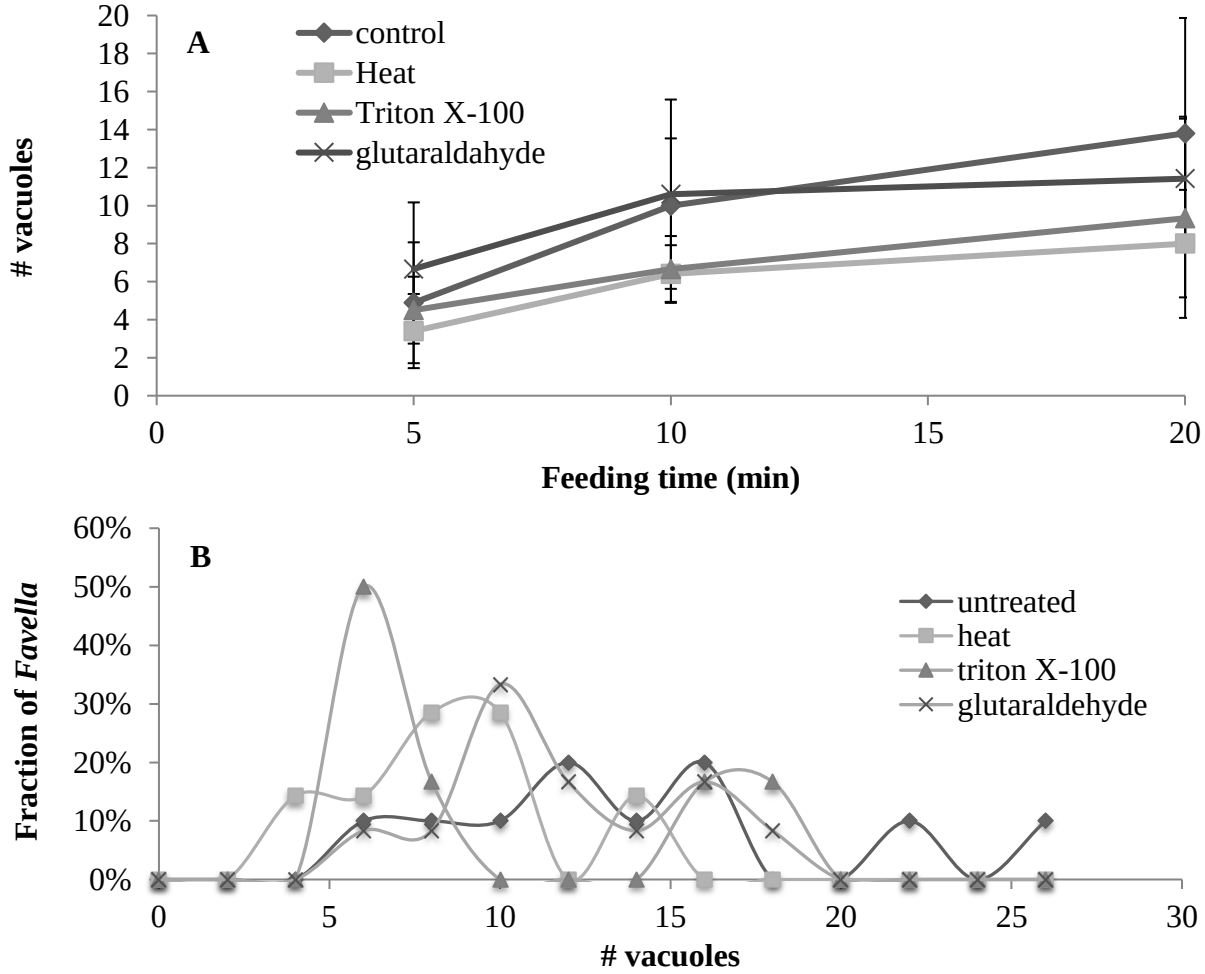

Figure 7. Ht treatment (heat, detergent, or protein cross-linking) effect on ingestion (A) Feeding rate on treated Ht over time, with standard error. (B) Histogram showing fraction of *Favella* in the total sample with number of vacuoles formed after 20 min of feeding. (source: Ht surface treatments on feeding rate, 8/6/15).

Feeding on Artificial Prey: Polystyrene Microspheres

I next offered *Favella* artificial, polystyrene prey (PS microspheres/beads) of similar size (15 μm or 20 μm) to their natural prey (Ht). Microspheres were coated with either methylcellulose (MC) or carboxymethylcellulose (CMC) after Echevarria et al. (2016) and were offered in equal concentrations─either separately or along with live prey. Co-uptake of beads and live prey could be observed by a combination of brightfield and fluorescence microscopy (fig. 8).

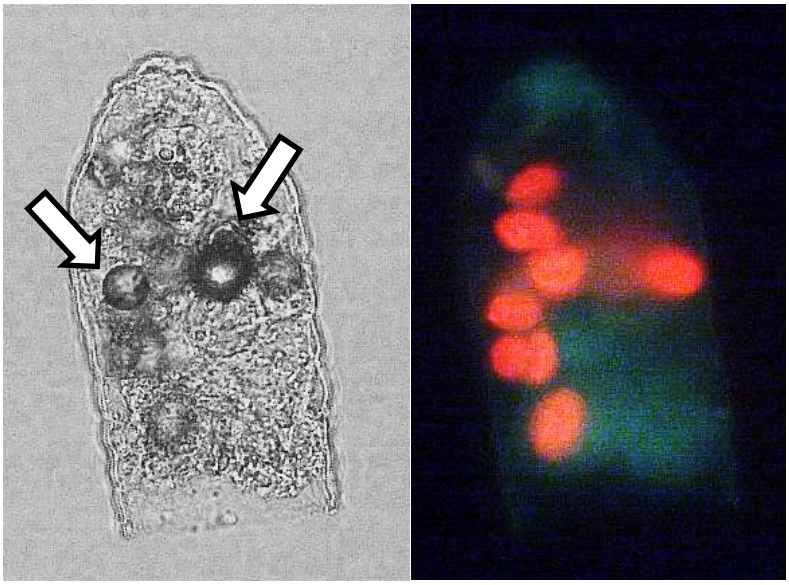

Figure 8. Preferential ingestion of live prey vs. control beads. Left: BF image shows two 15 μm beads ingested after 20 min (arrows). Right: epifluorescence shows 8 Ht cells ingested during the same time (red). (Source: 11/28/15)

In single-prey tests over 10-20 min, all beads were eaten, but at significantly lower rates than live prey (fig. 9A). This was also true in triple prey coincubation tests (fig. 9B). If incubated long enough, *Favella* would eventually ingest beads until they became full, and would subsequently egest them over a period of 8 hrs (data not shown).

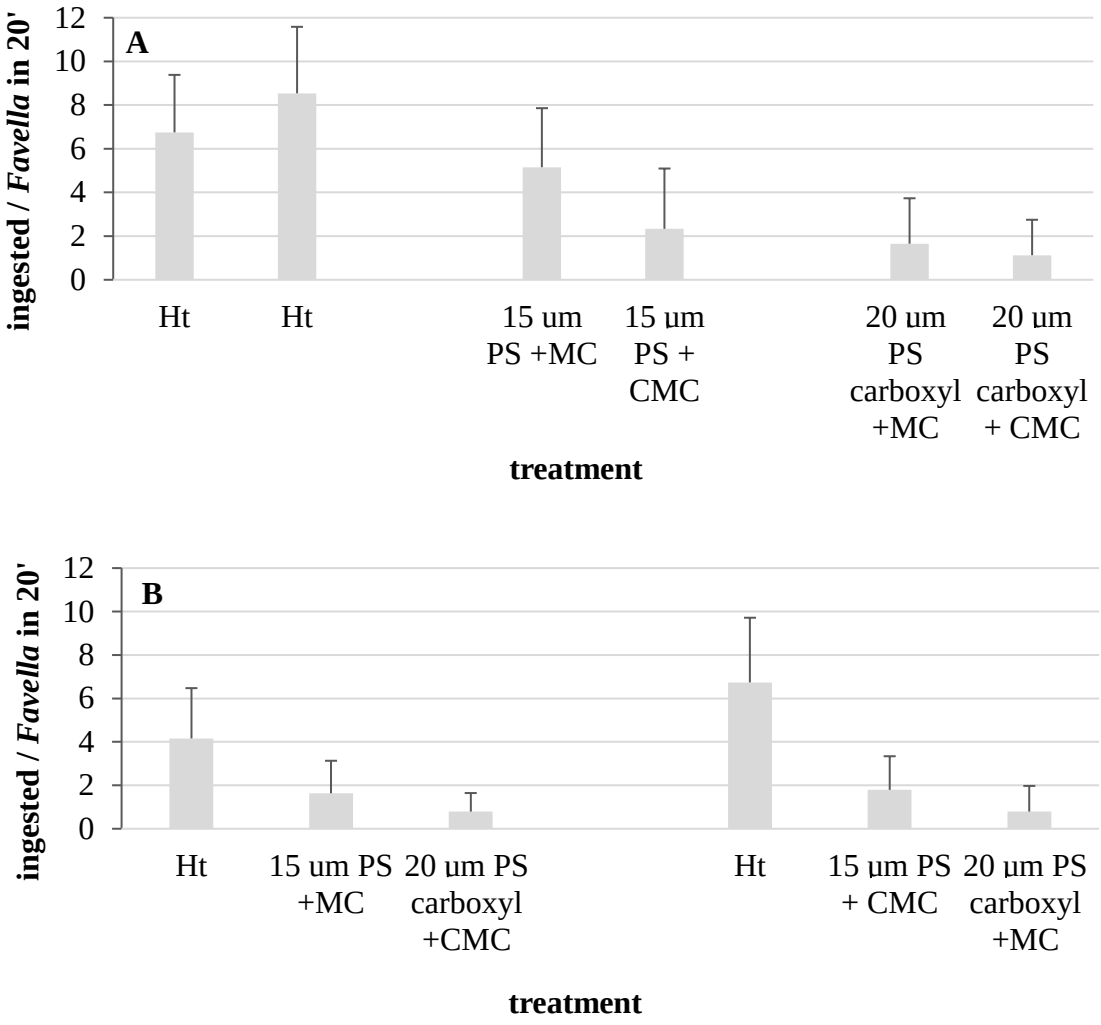

Figure 9. Preferential ingestion of live prey vs. control beads over 20 min. Bars show averages and error bars standard deviations of 12-23 *Favella* for each treatment. (A) Each bar represents a single-prey (*Heterocapsa* or bead) test. (B) Mixed-prey (*Heterocapsa* + both beads) coincubation test. The sum of the three bars equals the number of vacuoles a single *Favella* ingested on average. (Source: 11/28/15).

Microspheres were then modified with specific sugars conjugated to the surface after the method of Luyai et al. (2009) to determine if contact-based cues would affect ingestion rate. Uncoated microspheres, or those coated with BSA alone, exhibited little or no fluorescence under UV excitation (fig. 10A, B). However, beads with the fluorescent NPA linker—either alone (fig. 10C) or conjugated to sugars (fig. 10D)—exhibited the expected fluorescence.

All bead and sugar feeding experiments were blind and conducted in equal concentrations of Ht or microspheres. An initial test in early March, 2016 showed that NPA-coated beads were highly discriminated against. In contrast, mannose-coated beads were ingested half as much as Ht, while fructose-coated beads were eaten as much as Ht (fig. 11). Subsequent tests did not always give such clear-cut results. Increasing the number of observed *Favella* usually resulted in similar patterns, even when treatment comparisons appeared non-significant (fig. 12A). This was usually due to the influence of a few outlier cells. Analyzing results as histograms showed clear discrimination against ingesting uncoated, or control-coated beads (fig. 12B).

Treatment of microspheres with sugars resulted in variable ingestion rates, which were consistent across multiple experiments (figs. 13-15). Two sugars (fructose, mannose) consistently resulted in ingestion rates as high, or higher, than live prey, while several other sugars (galactose, lactose, mannitol, mannopyranoside, glucose) increased ingestion rates above untreated beads. Still other sugars (fucose, sucrose) resulted in no increase. These results were repeatable over time (table 1). Offering mixtures of live and artificial prey resulted in selective ingestion of live prey when artificial prey were not coated with preferred sugars, but no selection when preferred sugars were used (fig. 16). This suggests that specific prey cell-surface sugars act as 'eat me' cues.

Table 1. Summary of glycoconjugate bead feeding experiments.

Date	Treatments	Results
3/4/2016	NPA, mannose, Fructose	NPA, mannose ingested least
3/22/2016	none, BSA, Ht	Ht > BSA > beads preferred
4/3/2016	all	Ht > glucose > fructose > galactose
4/5/2016	all	mannose/fructose ≥ Ht
4/25/2016	NPA, mannose, fructose, Ht	NPA beads are discriminated against

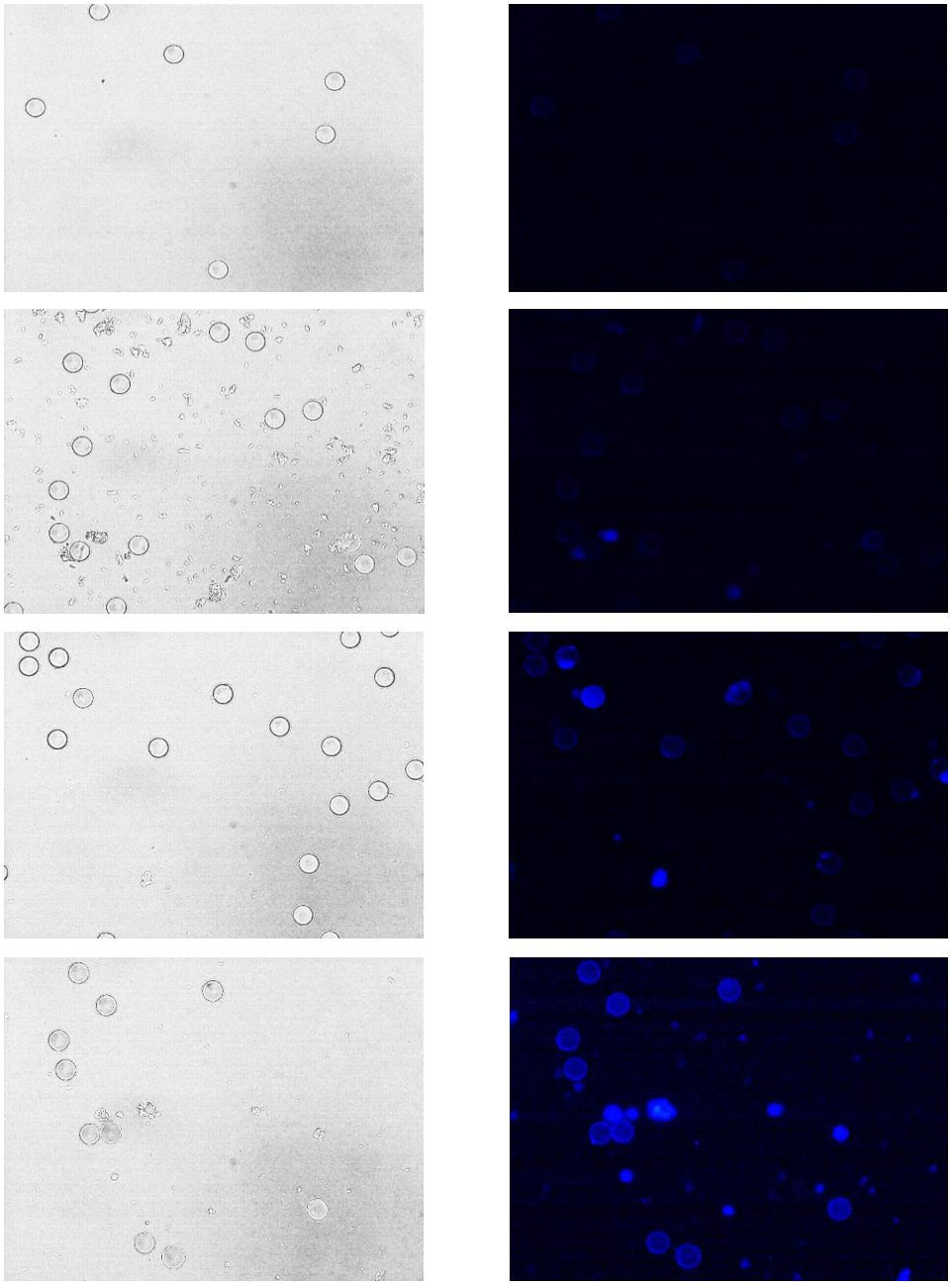

Figure 10. Carboxylate polystyrene microspheres (400X). Left: brightfield; right: fluorescence with DAPI filter set, 1/5s exposure. Treatments: (A) uncoated beads; (B) beads coated with BSA only; (A) beads coated with NPA-BSA, but no sugar; (D) beads coated with (B) glucose-NPA-BSA.

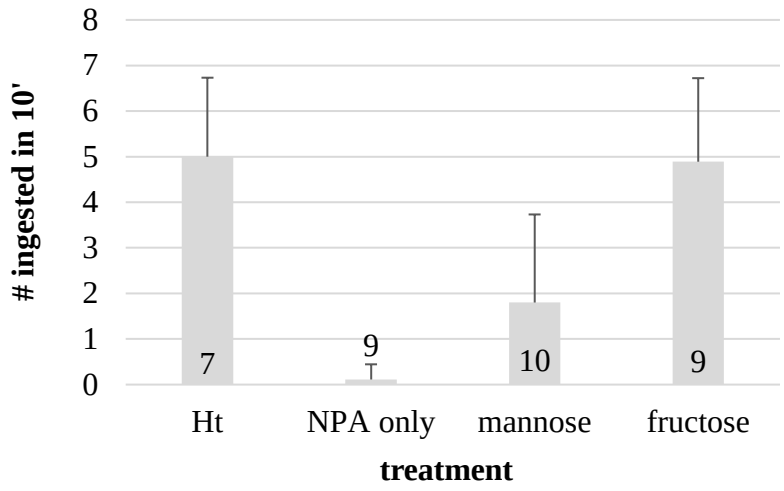

Figure 11. Initial feeding test with neoglycoconjugate or control beads. Numbers in bars show the number of *Favella* observed to calculate means, with error bars denoting ± 1 standard deviation (source: bead feeding, 3/4/16).

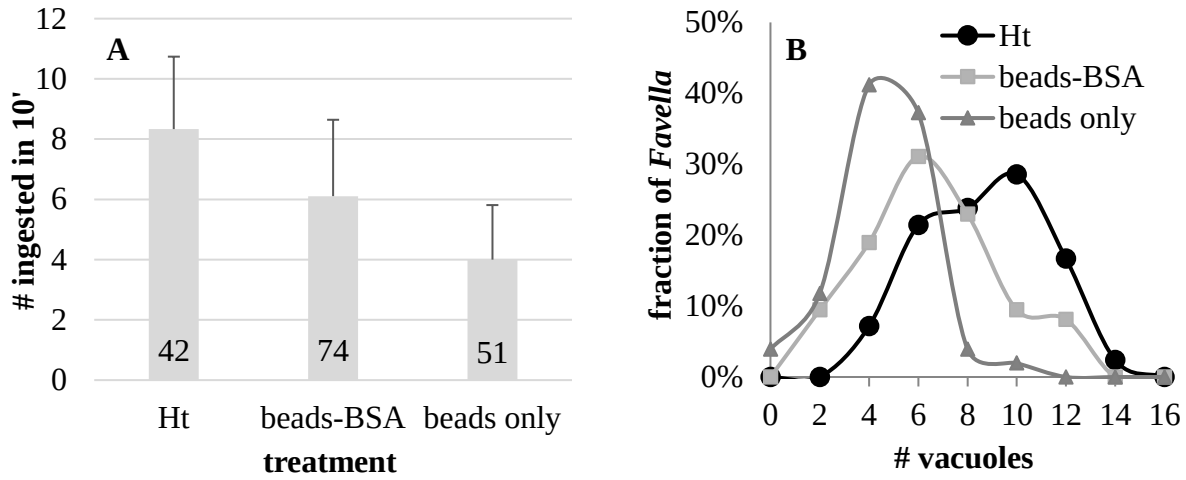

Figure 12. Feeding experiment on live prey (Ht) vs. control beads over 10 min. (A) Individual prey tests of Ht, protein-coated beads, and naked beads. Numbers in bars show the numbers of *Favella* observed to calculate means, with error bars denoting ± 1 standard deviation. (B) Histogram plot of the fraction of *Favella* that ingested a given number of prey. (source: bead feeding, 3/22/16).

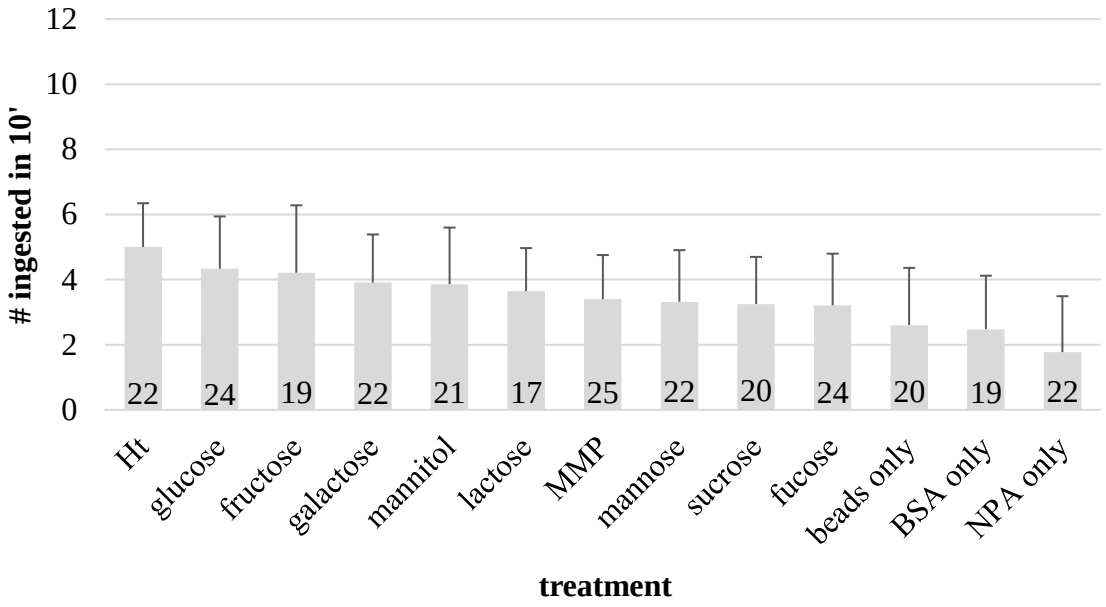

Figure 13. Feeding on 12 treated beads, arranged by feeding preference. Numbers in bars show the numbers of *Favella* observed to calculate means, with error bars denoting $\pm$ 1 standard deviation. MMP = methyl mannopyranoside. (source: bead feeding, 4/3/16)

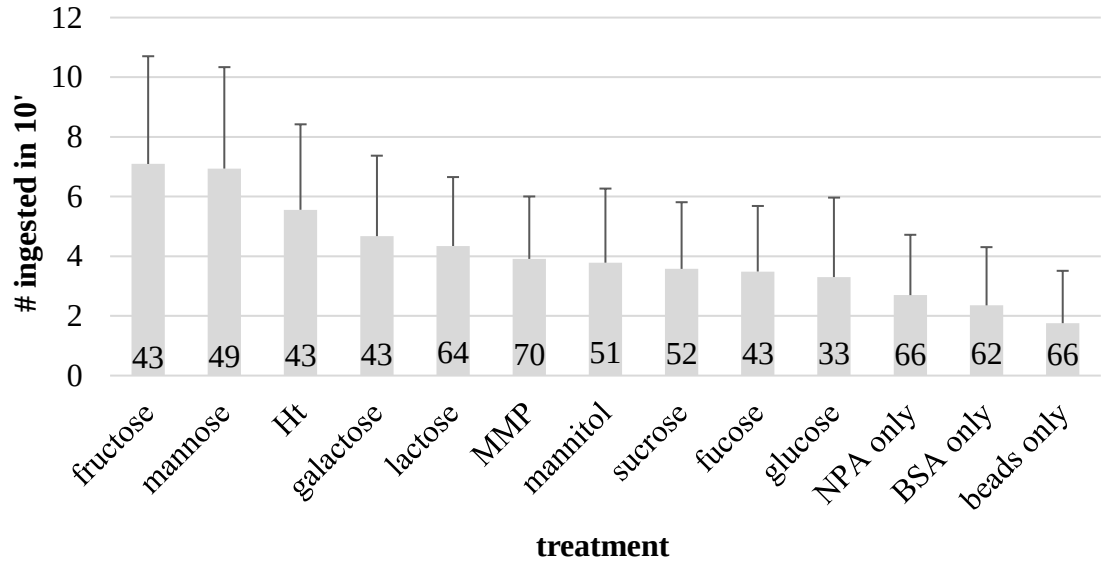

Figure 14. Feeding on 12 treated beads, arranged by feeding preference. Numbers in bars show the numbers of *Favella* observed to calculate means, with error bars denoting $\pm$ 1 standard deviation. MMP = methyl mannopyranoside. (source: bead feeding, 4/5/16)

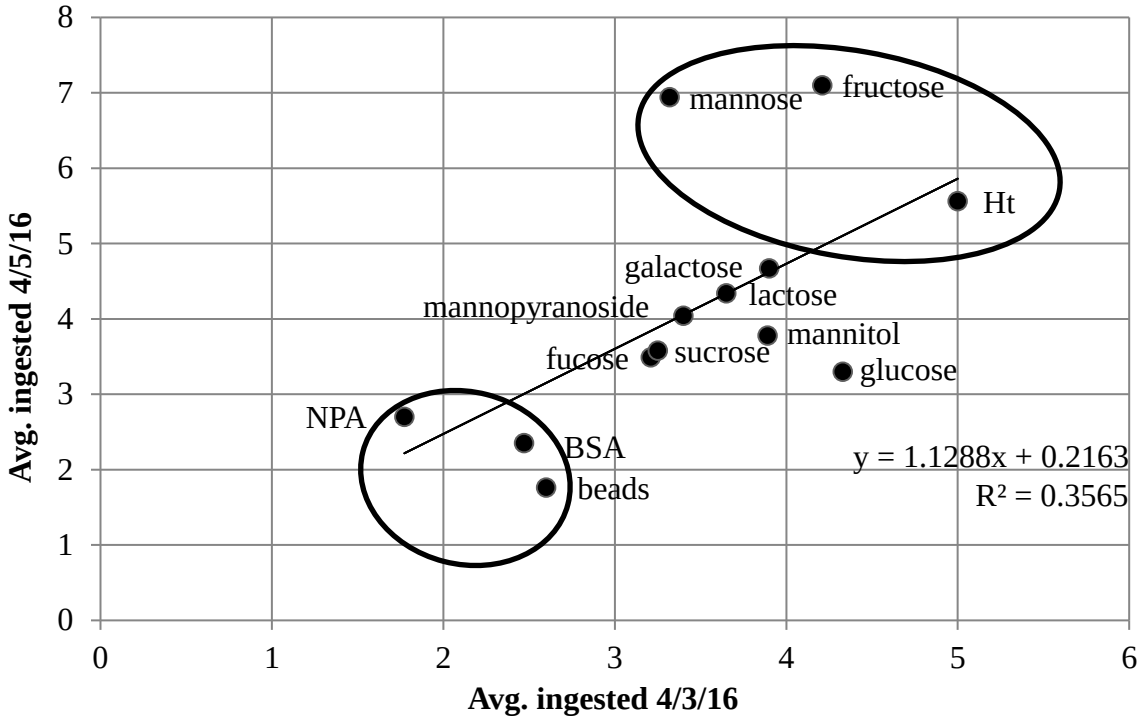

Figure 15. Experiments 4/3/16 and 4/5/16 compared. Sugar-coated beads are ingested more frequently than NPA or BSA-coated beads, and naked beads. Mannose and fructose-covered beads are eaten as much or more than Ht.

Dissolved Sugar and Amino Acid Feeding Inhibition on *Heterocapsa*

Amino acids are known chemical cues which are present on the surface of live cells, and they have been shown to inhibit feeding in *Favella* in their dissolved form (Strom et al., 2007). Although amino acids and sugars are markedly different, the chemical cues they provide may result in the same phagocytic mechanism. Adding dissolved amino acids to the sugar tests provided an additional control, and showed an inhibition in feeding rate in a size-dependent manner consistent with previous research. A preliminary experiment on May 19, 2016 with 10-1000 μM fructose showed detectable inhibition at 10 μM, and profound inhibition at >100 μM (fig. 17).

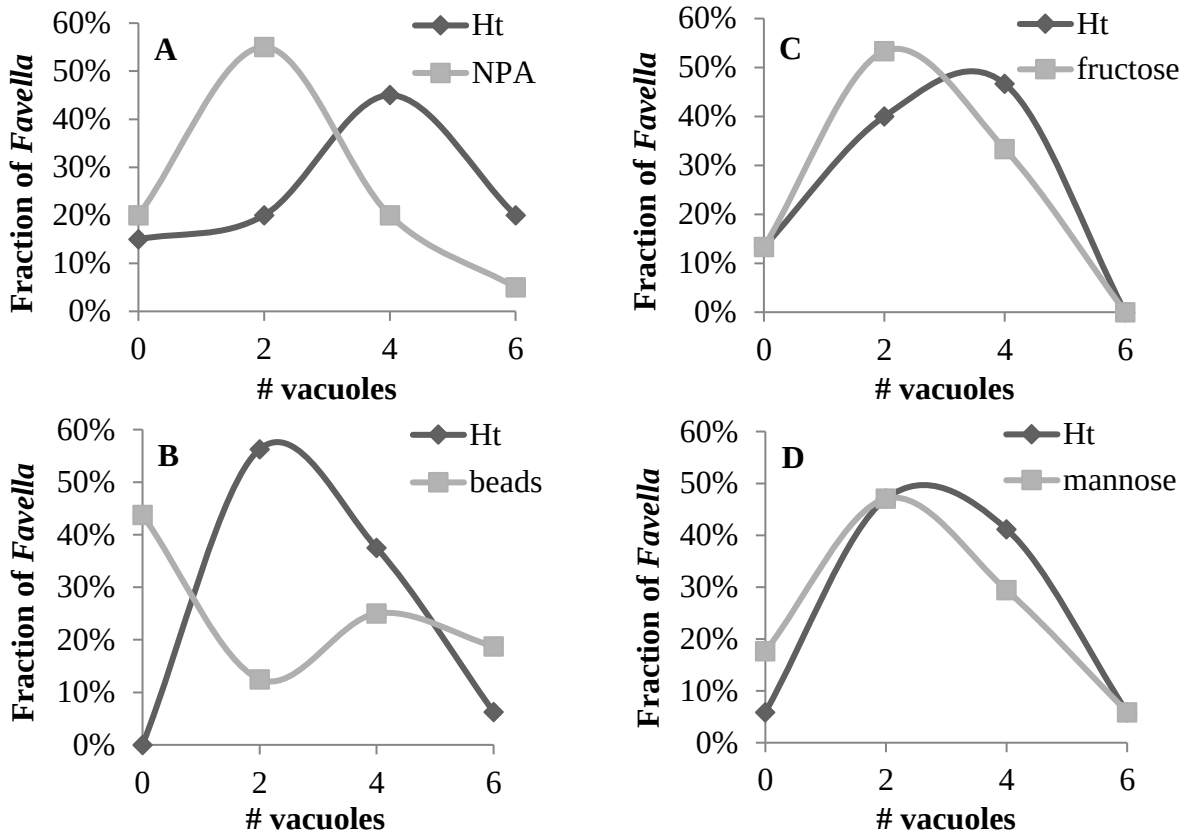

Figure 16. Paired coincubation studies of Ht and treated beads. Ht, in combination with NPA beads (A) or untreated beads (B), were offered to *Favella* at 25:1 prey to predator ratios simultaneously (50:1 total). More vacuoles contained Ht over naked beads and NPA beads. Fructose and mannose-covered beads (C, D), as well as Ht, were ingested in equal amounts. (Source: bead feeding, 4/25/16)

A second test on May 29 (table 2), using other sugars and proline, showed variable inhibition by sugars and strong inhibition by proline. Although sucrose showed feeding inhibition in some experiments, this was erratic and not consistent in other feeding experiments. Mannose did consistently inhibit feeding, though not as much as fructose.

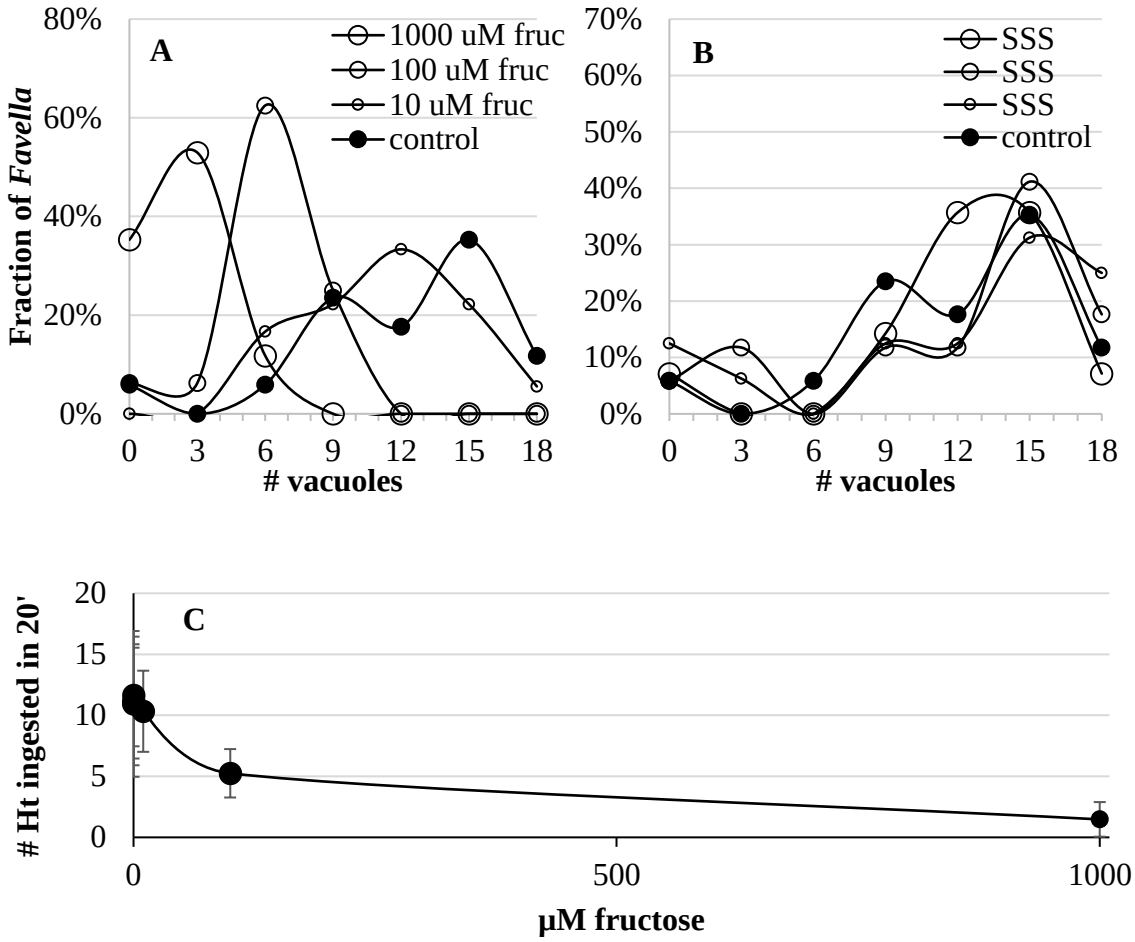

Figure 17. Inhibition of feeding on *Heterocapsa* by dissolved fructose over 20 min (A) Histogramof ingestion incubated with micromolar concentrations of dissolved fructose. (B) Dilutionsof Sigma Sea Salts (SSS) had no effect on feeding. Fructose inhibited feeding in a dose-response (C). Error bars show standard deviation of means. (source: sugar feeding 5/19/16).

Table 2. Summary of feeding inhibition on *Heterocapsa triquetra* by dissolved cues.

Date	Treatments	Results
5/19/2016	fructose (10-1000 µM)	fructose inhibits
5/26/2016	mannose, fucose, sucrose, proline (10-1000 µM)	sugar inhibition intermediate proline inhibits feeding
6/20/2016	proline, fructose, serine, mannose, arginine, fucose, tryptophan, sucrose (2-50 µM)	serine, proline, fructose inhibit feeding strongly; mannose intermediate

A final test with lower concentrations (2-50 µM) of sugars or amino acids showed several distinct responses. The amino acids proline and serine were strongly inhibitory at 50 µM (fig. 18A), as was fructose (fig. 18B). All showed profound inhibition at 20 µM and partial inhibition at 10 µM (not shown). Mannose was slightly inhibitory at 50 µM (fig. 18B), while arginine, tryptophan, fucose and sucrose showed little if any effect (fig. 18C).

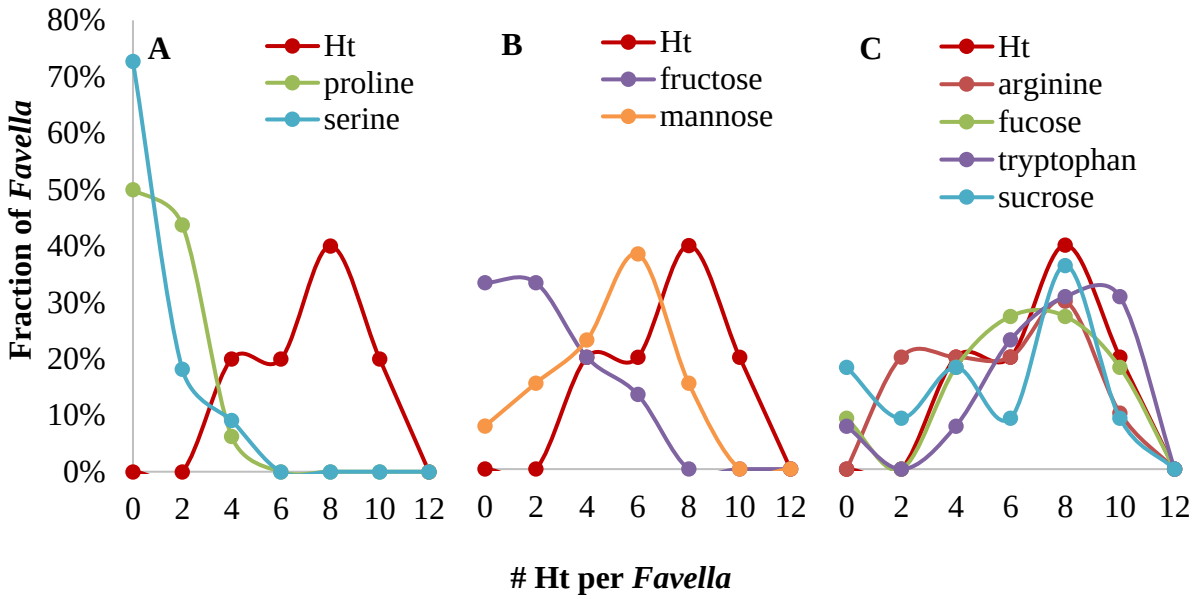

Figure 18. Feeding on *Heterocapsa* in 50 µM dissolved substances over 10 min. Histogram of ingestion frequencies show small amino acids strongly inhibited ingestion (A), while fructose and mannose moderately inhibited feeding (B). Arginine, fucose and sucrose did not affect feeding; however, tryptophan increased feeding (C). (Source: sugar test, 6/20/16).

Bioinformatics Exploration of *Favella* Receptors and Transporters

The *Tetrahymena* genome showed many lectin domains, many of which belonged to MBLs, though most were uncharacterized (table 2). PBLAST results of *Tetrahymena* lectin genes showed conserved sequences involved in signal transduction and adhesion (table 3).

Table 3. Chemical binding domains found in TGD.

Domain ID	Domain description	Gene name
Carbohydrate surface receptors		
PF01419	Jacalin-like lectin domain	TTHERM_00646960 (+9)
PF03954	Hepatic lectin, N-terminal domain	TTHERM_00127060 (+1)
PF00652	ricin-type beta-trefoil lectin domain	TTHERM_00492400 (+1)
PF00059	Lectin C-type domain	TTHERM_00237320
PF01453	D-mannose binding lectin	TTHERM_00191140 (+2)
PF02140	Galactose binding lectin domain	TTHERM_00019610
PF07554	Uncharacterized Sugar-binding	TTHERM_00666580 (+63)
pfam08016	PKD2, possible sour taste, C-type lectin	TTHERM_00348280 (+1)
PF08037	attractin, weight control, C-type lectin	TTHERM_00688300
PF02010	REJ, sperm binding, C-type lectin	TTHERM_00633070 (+51)
pfam13385	Con A-like lectin	49 homologues
PF00219	Insulin growth factor binding protein	TTHERM_00224470
IPR019019	H-type lectin domain	TTHERM_000223430 (+11)
PF02018	Carbohydrate binding domain (rhoptry)	TTHERM_00678140
PF03427	Carbohydrate binding domain (family 19)	TTHERM_01144980 (+1)
PF05296	Mammalian taste receptor protein (TAS2R)	TTHERM_00641100 (+5)
PF09064	Thrombomodulin-like fifth domain, EGF-like	TTHERM_00550950 (+30)
Other signaling surface receptors		
PF03392	Insect pheromone binding family	TTHERM_00979790
PF04549	CD47 integrin associated protein	TTHERM_00490660 (+6)
PF00002	7 transmembrane receptor (secretin family)	TTHERM_00633170 (+5)
PF00001	7 transmembrane receptor (rhodopsin family)	TTHERM_00633170 (+27)
PF09067	Erythropoietin receptor	TTHERM_00716160 (+1)
PF08395	7tm Chemosensory receptor (gustatory, odorant)	TTHERM_01444900 (+3)
PF06011	Transient receptor potential (TRP) ion	TTHERM_00105210 (+34)
PF03549	Translocated intimin receptor (Tir) intimin-binding	TTHERM_00486330

Table 4. PBLAST of lectins found in *Tetrahymena* genome.

Tetrahymena gene	Search	Protein	ID	Organism	AAs	Score	E-value
TTHERM_00492400	*Mammalia*	rab GTPase	XP_005363997.1	*Microtus ochrogaster*	1023	106	1.00E-22
	Non-alevolate	rab6 GTPase	ESL06902.1	*Trypanosoma rangeli*	381	110	1.00E-23
TTHERM_00191140	*Mammalia*	mannose lectin	CEP00913.1	*Plasmodiophora brassicae*	236	135	4.00E-33
	Non-alevolate	mannose lectin	WP_056043352.1	*Rathayibacter*	659	131	3.00E-29
TTHERM_000223430	*Mammalia*	FRAS1	XP_016068520.1	*Miniopterus natalensis*	3979	92	2.00E-16
	Non-alevolate	FRAS1	XP_009991776.1	*Chaetura pelagica*	3876	95.1	3.00E-16
TTHERM_000057199	*Mammalia*	PCSK5	XP_004713893.2	*Echinops telfairi*	1830	152	9.00E-35
	Non-alevolate	PCSK5	XP_004713893.3	*Aquila chrysaetos canadensis*	1796	154	2.00E-34
TTHERM_000492408	*Mammalia*	none					
	Non-alevolate	Ricin B lectin	WP_021341844.1	*Saccharopolyspora erythraea*	951	72.8	2E-10
TTHERM_000417959	*Mammalia*	PCSK5		*Camelus ferus*	1859	329	2.00E-88
	Non-alevolate	S/T protein kinase		*Fonticula alba*	2745	710	0
TTHERM_000415818	*Mammalia*	FRAS1	EPQ02198.1	*Myotis brandtii*	4236	139	4.00E-33
	Non-alevolate	FRAS1	EPQ02198.2	*Cyprinodon variegatus*	3955	118	5.00E-23
TTHERM_000415817	*Mammalia*	PCSK5	XP_014418358.1	*Camelus ferus*	1859	104	2.00E-20
	Non-alevolate	furin protease 2	XP_014418358.2	*Stegodyphus mimosarum*	973	114	2.00E-22
TTHERM_000415815	*Mammalia*	none					
	Non-alevolate	none					
TTHERM_000415816	*Mammalia*	none					
	Non-alevolate	none					
TTHERM_000191139	*Mammalia*	PCLO X5	XP_011910160.1	*Cercocebus atys*	4964	104	4.00E-06
	Non-alevolate	actin binding	XP_011910160.2	*Polysphondylium pallidum*	266	114	1.00E-25
TTHERM_000527078	*Mammalia*	S/T protein kinase	XP_009498144.1	*Fonticula alba*	2471	162	4.00E-36
	Non-alevolate	FRAS1	XP_009498144.2	*Fukomys damarensis*	4004	140	8.00E-31
TTHERM_000527079	*Mammalia*	FRAS1	XP_004653532.1	*Jaculus jaculus*	3978	143	1.00E-31
	Non-alevolate	S/T protein kinase	XP_004653532.2	*Fonticula alba*	2471	163	1.00E-36
TTHERM_001035764	*Mammalia*	PCSK6	XP_016010328.1	*Rousettus aegyptiacus*	932	45.8	0.009
	Non-alevolate	PCSK6	XP_016010328.2	*Rousettus aegyptiacus*	932	45.8	0.15
TTHERM_000270380	*Mammalia*	PCSK5	XP_014936616.1	*Acinonyx jubatus*	1845	77.8	3.00E-12
	Non-alevolate	PCSK5	XP_014936616.2	*Astyanax mexicanus*	1879	80.5	9.00E-12

CHAPTER IV

DISCUSSION

The results of this study provide further evidence for contact-based biochemical recognition of prey by planktonic alveolates. Moreover, such contact-based recognition also suggests a mechanism to explain the surprising observation of inhibition of feeding in the presence of micromolar range dissolved amino acids and other organics by Strom et al. (2007).

Experimental Difficulties that Might Bias Observations

Behavioral observations of *Favella* capturing and processing prey, including the cytostomal contraction experiments, were likely affected by my primitive observation conditions. Although I tried to minimize light and heating during observations of cells in microwell containment under brightfield or phase contrast, both happened to a variable degree, and likely impacted cytostomal contraction rates and possibly prey handling results. In bright light, avoidance reactions are common and feeding rate is low (Song et al., 1980). In contrast, Echevarria et al. (2016) used more sophisticated observational techniques, such as tethering individual cells with suction pipettes and perfusing with temperature-controlled solutions.

My bulk feeding tests were conducted in small (mL) but not micro-volumes, and under more controlled light and temperature conditions, so ingestion rates of live prey and beads are probably accurate. However, it is also possible that experimental artifacts affected my results. For example, I observed some uptake of control microspheres coated only with methyl

cellulose or carboxymethyl cellulose (fig. 9), in contrast to Echevarria et al. (2016), who reported complete rejection of beads following contact at the cytostome.

Echevarria et al. (2016) observed tethered cells perfused with seawater and offered beads for short (min) time periods, whereas I used bulk additions. My observation of increased ingestion of beads over long time periods suggests that beads may adsorb solutes from solutions containing positive feeding cues. Given enough time, *Favella* will ingest all treated beads until they are full, but will expel them after 8 hrs with 30-70% mortality. It is likely that other differences in experimental setup exist, but despite these differences, I largely confirmed prior results, as well as the effect of micromolar dissolved amino acids on feeding reported by Strom et al. (2007).

Contact-Based Feeding Observations

Cytostomal contraction rates decrease slightly during feeding. During forward swimming, the pulsations of the cytostome, driven by rhythmic depolarization (Echevarria et al., 2016), were 2-4 Hz in starved cells (fig. 6). Cytostomal contractions are thought to be involved in processing prey during phagocytosis. Following introduction of prey and commencement of feeding, contraction rates decreased, but only after 10-20 min, allowing *Favella* to completely fill with prey at saturating prey densities (fig. 5). This suggests that ingestion may be partly controlled by negative feedback from digestion, although contraction rates only decreased modestly and were likely affected by experimental artifacts such as light and heat, as behaviors were consistent within a particular culture, but varied among experiments. It is interesting to note that during retraction of *Favella* into the lorica, governed by mechanostimulated action potentials (Echevarria et al., 2016), cytostomal contractions ceased for the duration of the retraction (1-3

sec), only resuming after the cell began to re-emerge from the lorica and recommence forward swimming. As contractions became more frequent, the cell emerged more quickly. During this time, obviously, feeding could not occur.

In some cells, swimming stopped after prey capture, while in others, swimming continued. In some cases of the latter, the prey was seen to be rotated around several axis in the cytostome, sometimes for up to 30 sec, allowing the entirety of the prey surface area to be sampled. Thus, cytostomal contractions may serve to both grip and gather information of the prey surface prior to phagocytosis, while becoming less useful at satiety. Since both contraction rate and swimming speed are linked by electric regulation, changes in contraction rate may simply be a byproduct of decreasing swimming speed, which allows *Favella* to stay within a food patch in response to rapid increases in food concentration (Buskey and Stoecker, 1988).

Ingestion of Microspheres can be Induced by Specific Sugar Coatings

Feeding Favella artificial prey (polystyrene microspheres the same size as Ht), or microspheres treated with control chemicals used for preparing neoglycoconjugates (NPA, BSA), did not result in significant ingestion. Thus, I confirmed the observation by (Echevarria et al., 2016) that artificial prey are not ingested, although this work was not as complete. In contrast, Favella ingested polystyrene beads that were coated with specific sugars (fructose and mannose) as much, or even more, than live prey. Beads coated with other sugars, including mono- and disaccharides, or polyols, were ingested less than live prey, but more than control beads, and glucose-treated beads showed variable ingestion. These observations were generally repeatable over time (fig. 15) and occurred in both single-prey and mixed-prey experiments (fig. 16). These results support those by Wootton et al. (2007) on the dinoflagellate Oxyrrhis marina,

suggesting that prey cell-surface sugars or glycoproteins act as chemical cues to facilitate phagocytosis by lectin-like proteins at the cytostomal area in marine alveolates.

Many species of marine microalgae bear glycans on their surfaces. Many, if not most, dinoflagellates have mannose residues, as detected by Concanavalin A (ConA) binding (Aguilera and Gonzalez-Gil, 2001; Cho, 2003; Costas et al., 1993; Costas and Rodas, 1994; Logan et al., 2010). Although I know of no reports testing lectin binding to *Heterocapsa*, in preliminary observations ConA appeared to bind and agglutinate *Heterocapsa* (not shown). However, the lectin stocks employed were very old, and glutaraldehyde fixation autofluorescence impaired detection, so this needs to be repeated. Detection of other sugar groups on dinoflagellate surfaces appears more variable, and may change according to nutrient conditions, especially for amino sugars (Kremp and Anderson, 2004). Such sugars may be used for specific interactions; for example, the coral endosymbiont *Symbiodinium* contains N-acetyl galactsosamine residues on its surface (Logan et al., 2010), which are used in establishing endosymbiosis by lectin-binding proteins in the host (Jimbo et al., 2013; Kuniya et al., 2015). Unfortunately, no commercial source of fructose-binding lectin is available. Although lectins demonstrate the ability to bind to both mannose and fructose (Van Wauwe et al., 1975), such proteins have only recently been reported in literature (Cheung et al., 2009; Rafiq et al., 2014), so little is known about the occurrence of this sugar, which was the most stimulatory tested.

Several reports of filter-feeding bivalves suggest these animals use lectin-like mechanisms to contact-detect prey. Espinosa et al. (2009) were able to agglutinate microalgal prey with mucus from *Crassostrea virginica*, the Eastern oyster, and showed that they ingested glucosamide-BSA-coated plastic microspheres, but not BSA-coated microspheres. Espinosa et

al. (2010) further showed that the blue mussel, *Mytilus edulis*, preferentially ingested glucosamide-BSA- and mannopyranosylphenyl-BSA-coated plastic microspheres, and detected a novel C-type lectin expressed in the mucocytes of the gills, labial palps and mantle.

My attempts to modify *Heterocapsa* surface proteins with treatments such as heating, cross-linking by glutaraldehyde, or detergents (Triton X-100) generally reduced feeding compared to control prey, but did not show significant changes (**fig. 7**). It is difficult to assess exactly what changes took place in these treatments, which also likely created artifacts from incomplete washing of treatment solutions. However, it is notable that heat treatment alone caused the most reduction in feeding, which is not surprising since many phagotrophic protists discriminate against heat-treated prey (Caron, 2001)

Contact-Based Feeding Cues can Explain Amino Acid Inhibition

The observation that ingestion of Ht could be prevented by incubating with the same dissolved sugars (10 – 50 μM, table 2, fig. 18) that stimulate feeding on artificial prey suggests that an excess of 'eat me' cues in dissolved form blocks the ability of *Favella* to use the cue on a prey cell. This may explain prior observations that micromolar dissolved amino acids can profoundly inhibit feeding in *Favella*, but only those with small side chains (Strom et al., 2007). Such observations were surprising, since amino acids, as well as their derivatives (DMSP) have often been shown to be feeding attractants for a variety of marine protists (Dacey and Wakeham, 1986; Martel, 2006; Seymour et al., 2010).

An experiment by Huber (2008) described amino acid uptake into *Favella* after a brief period of binding to the cell surface. She showed that inhibitory amino acids do not selectively bind to the cytostomal area, but to the entire somatic membrane. This suggests

additional roles for amino acids, which can act as nutrients, signals, and osmolytes in many other organisms. This prompted me to leave various dissolved cue treatments unfed over a period of 72 hrs. Untreated *Favella* died within 24 hrs; however, *Favella* incubated in fructose, mannose, arginine, fucose and tryptophan died within 48 hrs. Only cells incubated in proline survived after 48 hrs, but later died before the third day. This is especially interesting, considering that proline was the most inhibitory compound in feeding tests. Although the binding and transport of dissolved amino acids and sugars inhibit phagocytosis, they may be utilized as an alternate energy source.

Glycan Receptors

The *Tetrahymena* Genome Database (TGD) revealed a variety of carbohydrate-binding surface receptors (Table 3). The largest sum of search results belonged to several domains. The thrombomodulin-like domain, which helps in adhesion and blood coagulation in mammals, was present in 31 different genes. There were 52 genes encoding the receptor for egg jelly (REJ) domain. This domain has a largely unknown function, but contains two C-type (calcium-gated) lectin carbohydrate-recognition modules. Although C-type lectins are diverse and abundant in ciliates, they were absent in the genes containing the uncharacterized sugar binding domain, which, according to the summary description, has no enzymatic properties and most likely binds to N-acetyl glucosamine. However, the genes which this domain belongs to have a wide array of functions, ranging from osmoprotective transporters to DNA enzymes. The non-calcium gated jacalin-like lectin domain is known to bind to galactose or mannose/glucose, but has hypervariability in the binding site loops in plants, allowing for great diversity in receptor binding (Raval et al., 2004). The calcium-gated mannose binding lectins (MBLs), which

Wootton et al. (2007) showed was required for phagocytosis in *Oxyrrhis marina*, had several different domains in *Tetrahymena*, and was the only sugar-specific binding lectin other than galactose, though it is likely that the sour-tasting receptors bind to glucoside.

MBLs are an integral component of the innate immune system found in plants, animals and primitive multicellular organisms, helping them to identify and phagocytose foreign microbial invaders through the lectin pathway of the complement system. For an in depth review of MBLs, see Eddie Ip et al. (2009). MBLs can bind to phospholipids, nucleic acids and non-glycosylated proteins, enabling the host to bind to a wide range of microbial targets. They are capable of intracellular signaling through activation of proteases by conformational changes, and can cooperate with other membrane receptors such as CD93. BLASTing the CD93 protein with search term "ciliates" reveals some homology (E-value: 6e-06) with an insulin-like binding receptor, like that found in TTHERM_00224470. BLASTing TTHERM_00224470, while excluding ciliates, results in a large number of proprotein convertase hits, showing that these proteins have both enzymatic and carbohydrate-binding functions (data not shown).

BLAST results of *Tetrahymena* lectins showed large proteins with multiple conformational subunits, having homology with various enzymatic proteins involved in intracellular signaling and protein modification (Table 4). In ciliates, C-type lectins containing the REJ domain may aid in mating and self-recognition, while the other C-type lectins, specifically those that bind to mannose, may mediate phagocytosis by playing a role similar to the lectin pathway in the complement system.

Bioinformatics analysis shows that MBLs are ancient receptors and facilitators of phagocytosis that evolved early in Eukaryotes. Furthermore, the presence of a possible CD93

homologue in ciliates strongly suggests that many variants of MBLs and other carbohydrate-recognizing surface receptors coordinate together to bind to a wide range of prey surface cues, allowing a complete profile of the particle surface at the cytostome to be ascertained. In conjunction with ionic mechanisms, phagocytosis of desirable food particles can be precisely controlled by *Favella.*

Amino Acid Receptors

My initial research focused on possible mechanisms for the inhibition of feeding by small amino acids. I hypothesized that *Favella* may have a protein similar to the mammalian T1R1/T1R3 umami receptor, which binds to small amino acids (Nelson et al., 2002), but did not find any homologues. Metabotropic glutamate receptors also had no significant orthologs in ciliates, either at NCBI or in model genomes (*Tetrahymena*), and several class A rhodopsin-like GPCRs that bind amino acids (olfactory, pheromone, and calcium sensor receptors) were also absent. Lampert et al. (2011) describes the first GPCR found in ciliates, which is involved in chemoattraction to bacterial cues, but metabotropic amino acid signaling in metazoa clearly evolved after separation of opisthokonts from alveolates.

In contrast, I found there appears to be a number of ionotropic glutamate/glycine receptors in ciliates (tables 5-6), in which amino acid binding opens the channel and changes the membrane potential in the cell. In addition, I found orthologs of GABA A-type ionotropic receptors (table 7), which Bucci et al. (2005) have shown modulate swimming behavior in *Paramecium*. This is not surprising, considering ciliate genomes have revealed extensive diversification of ion channel protein families (Martinac et al., 2008).

Table 5. Ion channel pBLAST: Ionotropic glutamate receptors in ciliates.

Protein	Organism	AAs	Score	E-value	Result ID
NMDA1 4a	*Rattus norvegicus*	885	39.3	3.00E-04	EJY82491.1
NMDA receptor 1	*Drosophila*	997	39.7	3.00E-04	EJY82491.1
AMPA receptor GLUR1	*Columba livia*	902	51.2	1.00E-05	EAR90882.2
AMPA receptorGLUR2	*Macaca fascicularis*	883	50.4	2.00E-05	EAR95125.2
AMPA receptor GluR4	*Homo sapien*	902	47.4	2.00E-04	EAR90882.2
Kainate KA1	*Macaca fascicularis*	861	44.3	8.00E-06	EAR95125.2
Kainate KA2	*Rattus norvegicus*	979	38.9	0.084	XP_001015370.1

Table 6. Ion channel pBLAST: Ionotropic glycine receptors in ciliates.

ID	Organism	AAs	Score	E-value	Result ID
AAF49298.2	*Drosophila*	686	49.3	3.00E-05	EJY69607.1
EFA02903.1	*Tribolium castaneum*	505	72.4	1.00E-12	EJY74701.1
ETN66465.1	*Anopheles darlingi*	328	52.4	1.00E-06	EJY74701.1
NP_000162.2	*Homo sapiens*	449	94	1.00E-19	EJY69607.1
NP_000815.1	*Homo sapiens*	497	81.3	3.00E-15	EJY82222.1
NP_036700.1	*Rattus norvigicus*	452	99.8	2.00E-21	EJY80027.1

Table 7. GABA A-subtype ion channel-like proteins found in ciliates.

Protein	Organism	AAs	Score	E-value	Result ID
(GABA)A α 6	*Mus musculus*	453	70.5	4.00E-12	EJY80027.1
(GABA)A β 2	*Xenopus tropicalis*	474	89.4	5.00E-18	EJY80027.1
(GABA)A β 3	*Xenopus tropicalis*	475	88.2	1.00E-17	EJY80027.1
(GABA)A γ 2	*Homo sapien*	467	75.1	2.00E-13	EJY74701.1
(GABA)A δ	*Mus musculus*	449	79.3	6.00E-15	EJY69607.1
(GABA)A δ	*Homo sapien*	452	78.2	2.00E-14	EJY80027.1
(GABA)A ρ	*Rattus norvigicus*	474	88.6	7.00E-18	EJY85082.1
(GABA)A ρ 2a	*Danio rerrio*	475	90.9	1.00E-18	EJY85082.1
(GABA)A ρ 1	*Bos taurus*	421	89.4	3.00E-18	EJY85082.1

Summary

1. I confirmed prior observations that *Favella* discriminates strongly against artificial prey vs. live prey, although I observed some ingestion of untreated plastic microspheres.

2. Treatment of microspheres with control chemicals used for preparing neoglycoconjugates (NPA, BSA) did not increase ingestion. However, treatment of microspheres with sugars resulted in dramatic changes in ingestion rates. Several sugars (fructose, mannose) resulted in ingestion rates as high, or higher, than live prey. Several other sugars (galactose, lactose, mannitol, glucose) increased ingestion rates above background. Still other sugars (mannopyranoside, sucrose, fucose) resulted in no increase. Offering mixtures of live and artificial prey resulted in selective ingestion of live prey when artificial prey were not coated with preferred sugars, but no selection when preferred sugars were used.

3. Ingestion of live prey could be prevented by incubating with excess dissolved sugars (10 – 100 μM) in a specific manner.

4. The inhibition of feeding by small side-chain AAs observed by Strom et al. (2007) was confirmed. I suggest that analogous to inhibition of feeding by dissolved sugars, this likely is due to swamping out an ‘eat me’ signal from prey-surface proteins.

5. Ciliates have lectin-like proteins, and many amino acid-gated ion channels, but no mammalian-like amino acid GPCRs. The machinery to sense and respond to contact cues appears to be similar to phagocytosis in the innate immune system of higher eukaryotes.

Recommendations for Future Studies

Several avenues of research should be taken to further the science of microzooplankton feeding. The next steps in protist chemical recognition studies are to first determine if the higher eukaryote and prokaryote phagocytic processes arose from a common

ancestor. Then, the exact ionic mechanisms behind lectin binding and phagocytosis need to be evaluated in order to confirm the conceptual working model of depolarization and ion flux (Echevarria et al., 2014). This can then be combined with signal transduction studies, which would inevitably lead to studies involving calcium release by the alveolar sacs. Finally, the nature of cytostomal contractions need to be examined to determine if they are employed as a food-gripping structure, or if they are used for non-mechanical processes such as breathing or osmotic regulation. The merging of these components will then provide a clearer idea of how raptorial protist cell physiology determines feeding behavior, thus affecting the marine carbon cycle.

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APPENDIX

Table 8. List of experiments.

#	Date	Title	Treatment	Results
1	1/13/2013	Shpakov, 2011	alt. data visualization	no relationship between a.a. case study and cyclases
2	2/26/2013	cytostomal contractions	fed	feeding decreases frequency of contractions.
3	3/4/2013	cytostomal contractions	fed	feeding decreases frequency of contractions.
4	3/21/2013	toxins	EtoH, Lugol's, Glut, K Cy.	glut worked best
5	3/22/2013	feeding rate	50:1, 200:1	feeding rate abnormally slow
6	3/26/2013	feeding rate	50:1, 200:2	feeding rate abnormally slow, functional response made
7	6/13/2013	cytostomal contractions	contractions fed,starved	contractions decrease as fed
8	6/16/2013	cytostomal contractions	fed	feeding decreases frequency of contractions.
9	6/27/2013	Wilks and Sleigh, 2004 review	alt. data visualization	binding goes from outside to inside
10	7/9/2013	lectin binding micrographs	Con A, hyperosmosis	Con A bound to membranelles, cells bloated
11	7/10/2013	Feeding rate at different ratios	15, 25, 50, 200 :1 ratios	Fed normally
12	7/15/2013	Feeding rate	15, 25, 50, 200 :1 ratios	fed normally, functional curve (low *Favella* density however)
13	7/18/2013	Feeding rate	15, 30, 60 :1 ratios	30 cells/mL above avg feeding rate
14	8/15/2013	cytostomal contractions	temp, luminosity	light has affect on contraction rate
15	8/15/2013	consolidated contractions	fed vs starved	starved show slightly higher contraction rate
16	1/20/2014	Shpakov, 2011	review	aa on cyclases
17	2/2/2014	a.a. feeding vs T1R3 activity	review	correlation
18	4/25/2014	bioinformatics	BLAST, TGD	no amino acid taste receptors
19	8/4/2014	stinson beach sampling	fav pop growth	dark + fed grew then died, light + unfed grew
20	9/11/2014	feeding rate	many ratios	concentration of *Favella* low, normal results
21	9/18/2014	contractions	fed	marked decrease at 30 min
22	9/30/2014	contractions	alt. data visualization	only correlation is with feeding time
23	11/5/2014	fav division rate	dark/light/fed/unfed	dark and fed grew, others did not
24	5/24/2014	SF samples	diff locations	fav found on coast more than in bay
25	11/5/2014	feeding rate	many diff ratios	Fav still slightly low concentration, normal results
26	11/15/2014	*Favella* gravitaxis	inverted culture or not	fav exhibit gravitaxis, more concentrated unmixed, in light
27	11/16/2014	*Favella* agreggation	fed/unfed	fav exhibit agreggation
28	2/24/2015	lag and log phase feeding	low/high [Ht]	fav feed much faster in log phase
29	7/9/2015	con A binding feeding	feeding with con A	Con A on *Favella* inhibited completely
30	7/17/2015	con A binding and feeding	feeding with con A	Con A prevents feeding when bound to *Favella*
31	7/22/2015	con A binding and feeding	feeding with con A	con A on fav inhibited
32	7/22/2015	con A fav/ht micrographs		dropbox
33	7/30/2015	Mannopyranoside feeding rate		no effect
34	8/6/2015	Ht surface treatments on feed	heat, glut, triton,	no effect
35	8/10/2015	dissolved sugars feeding	various sugars	no effect
36	8/10/2015	population growth	sugars, con A	mannopyranoside showed increased division
37	10/28/2015	videos of feeding	beads/Ht	beads had more ping ponging

38	11/24/2015	bead feeding	A-F	A,C,D inhibition of feeding
39	12/11/2015	bead feeding	A-F	all beads were lower, no clear trend, control ate too fast
40	3/4/2016	bead feeding	1,5,6	1 inhibited most, 5 some
41	3/8/2016	bead feeding	tubes 1-12	no clear trend
42	3/12/2016	bead feeding	tubes 1,3,9	no clear trend
43	3/17/2016	bead feeding	tubes 7 and 10	seemed to eat 7 more than Ht, 10 the same
44	3/22/2016	bead feeding	beads 11 and 12	12 least, 11 some, ht most
45	1/13/2016	bead feeding	tubes 2-5	no clear trend, diff sized beads
46	4/3/2016	bead feeding	tubes 1-12	2,6 preferred. 3,4,5,7,8,9,10 medium. 1,11,12 not preferred
47	4/5/2016	bead feeding	tubes 1-12	averages, looks like bell curve when ordered 1-12 (except ht)
48	4/25/2016	bead feeding	1,5,6,12	histogram shows 1 and 12 selected against
49	5/2/2016	bead feeding	tubes A, H, I, K	A, H, I inhibited
50	5/19/2016	sugar test	tubes 1 and 9, (A-C)	1 A and B not preferred
51	5/26/2016	sugar test	tubes 2-5	5 showed inhibition
52	5/30/2016	sugar test	tubes 1-9	1,3,9 showed inhibition
53	6/20/2016	sugar test	tubes 1-9	1,2,3,4, showed inhibition (1 should not)