Feast or flee: bioelectrical regulation of feeding and predator evasion behaviors in the planktonic alveolate Favella sp. (Spirotrichia)

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ABSTRACT

Alveolate (ciliates and dinoflagellates) grazers are integral components of the marine food web and must therefore be able to sense a range of mechanical and chemical signals produced by prey and predators, integrating them via signal transduction mechanisms to respond with effective prey capture and predator evasion behaviors. However, the sensory biology of alveolate grazers is poorly understood. Using novel techniques that combine electrophysiological measurements and high-speed videomicroscopy we investigated the sensory biology of Favella sp., a model alveolate grazer, in the context of its trophic ecology. Favella sp. produced frequent rhythmic depolarizations (~500 ms long) that caused backward swimming and are responsible for endogenous swimming patterns relevant to foraging. Contact of both prey cells and non-prey polystyrene microspheres at the cilia produced immediate mechano-stimulated depolarizations (~500 ms long) that caused backward swimming, and likely underlie aggregative swimming patterns of Favella sp. in response to patches of prey. Contact of particles at the peristomal cavity that were not suitable for ingestion resulted in MSDs after a lag of ~600 ms, allowing time for particles to be processed before rejection. Ingestion of preferred prey particles was accompanied by transient hyperpolarizations (~1 s) that likely regulate this step of the feeding process. Predation attempts by the copepod Acartia tonsa elicited fast (~20 ms) animal-like action potentials accompanied by rapid contraction of the cell to avoid predation. We have shown that the sensory mechanisms of Favella sp. are finely tuned to the type, location, and intensity of stimuli from prey and predators.
INTRODUCTION

Alveolate grazers (ciliates and dinoflagellates) are key members of marine plankton communities and as members of the microzooplankton consume ~60% of global marine primary production in the world’s oceans (Schmoker et al., 2013). They therefore mediate regeneration of nutrients in the surface oceans (Dolan, 1997; L’Helguen et al., 2005) and act as a trophic link between phytoplankton and larger metazoan consumers in marine food webs. The ecological success of alveolate grazers is underpinned by complex behaviors they have evolved to capture preferred prey efficiently, reject toxic and unpalatable prey, while avoiding predators (Taniguchi and Takeda, 1988; Buskey and Stoecker, 1989; Broglio et al., 2001). To effectively capture prey and avoid predation, alveolate grazers must be able to sense a range of hydrodynamic, mechanical, chemical, and electrostatic signals produced by prey and predators, and integrate these signals via signal transduction mechanisms to respond with effective prey capture or predator evasion behaviors. Predators and prey of alveolate grazers can generate similar mechanical stimuli so the sensory mechanisms of alveolate grazers must also be sophisticated enough to discern between the two. Despite their importance, the sensory mechanisms of alveolate grazers are poorly understood.

The marine ciliate *Favella* sp.¹ (Fig. 1A) is a cosmopolitan member of the plankton community that has well described trophic behaviors (Stoecker and Sanders; 1985, Buskey and Stoecker, 1988; Taniguchi and Takeda, 1988; Buskey and Stoecker, 1989; Stoecker et al., 1995; Strom et al., 2007; Stoecker, 2013) and has morphological and behavioral characteristics that are representative of marine planktonic ciliates; they are therefore an excellent model for studying the sensory mechanisms underlying the trophic ecology of marine alveolate grazers (Montagnes, 2013, Echevarria et al., 2014). *Favella* sp. exhibit aggregative swimming patterns in the presence of chemical and mechanical stimuli from preferred prey that are believed to allow them to increase residence time in prey patches that typically occur in the environment (Buskey and Stoecker, 1989). Increased frequency of ciliary reversals that cause transient backward

¹The genus *Favella* has been recently redescribed and some former *Favella* spp. placed in the new genus *Schmidingeralla* Agatha S & Strüder-Kypke MC (2012) Reconciling cladistic and genetic analysis in choreotrichid ciliates (Ciliphora, Spirotricha, Oligotrichia). *J Eukaryotic Microbiol* 59: 325-350. For consistency with the ecological literature, and due to uncertainties in species-level identification in previous studies, we retain the name *Favella* throughout this review.
swimming and turning events in response to mechanical stimuli appear to underlie these aggregative swimming patterns (Buskey and Stoecker, 1988; Stoecker et al., 1995). However, the sensory mechanisms that underlie these ciliary reversals are unknown. Once food particles have been successfully contacted they are processed at the peristomal cavity where they are either rejected or consumed depending on the characteristics of the prey surface (Tanguchi and Takeda, 1988; Stoecker et al., 1995; Montagnes et al., 2008). *Favella* sp. are also capable of performing predator avoidance behaviors. In response to predation attempts by a predatory dinoflagellate, *Favella azorca* contract into the lorica, although dinoflagellates are still able to extract them (Uchida et al., 1997). The lorica may be effective as protection from copepods; in feeding experiments with a copepod, crushed loricas containing intact cells were observed at the end of the experiment (Stoecker and Sanders, 1985).

Although *Favella* sp. exhibit complex behavioral responses to predators and prey the cellular mechanisms that underlie these responses are unknown. In contrast to marine ciliates, the sensory mechanisms of model freshwater ciliates such as *Paramecium* and *Tetrahymena* have been well studied. Although *Favella* sp. may be expected to utilize similar conserved sensory mechanisms, it is likely that they implement them in different ways due to their different morphological and ecological characteristics. Nevertheless, it is known that ionotropic mechanisms—those governed by voltage-, chemo-, or mechano-sensitive ion channels—are important in regulating the behaviors of ciliates (Preston and Van Houten, 1987, Shiono and Naitoh, 1997, Grønlien et al., 2011). For example ionotropic mechanisms are known to be important in sensory processes that relate to prey capture in ciliates. Contact of the freshwater ciliate *Tetrahymena vorax* with prey cells elicits ciliary reversal and backward swimming that are regulated by transient depolarizations (Grønlien et al., 2011). Ionotropic mechanisms may also be important in regulating predator evasion behaviors. Contractile behavior of the freshwater ciliate *Vorticella* sp. upon mechanostimulation is regulated by rapid all-or-nothing action potentials (Shiono and Naitoh, 1997). Many ciliates contain extrusomes, membrane bound organelles that can be ejected from the cell to deter predators and immobilize prey, whose release is coordinated by the bioelectrical activity of the cell (Iwadate et al., 1997, Harumoto et al., 1998, Rosati and Modeo, 2003). Similar ionotropic mechanisms may function in regulating predator evasion and prey capture behaviors in *Favella* sp.
To investigate the sensory capabilities of *Favella* sp., electrophysiological measurements and high-speed video microscopy were performed in the presence of artificial and live prey, and predatory copepods to investigate the bioelectrical regulation of prey capture and predator evasion behaviors. Using this powerful combination of techniques we investigated signal transduction from environmental stimuli to behavioral response in an ecologically relevant context, providing a deeper understanding of the sensory mechanisms possessed by marine alveolate grazers.

**MATERIALS AND METHODS**

**Maintenance of *Favella* sp., *A. tonsa*, and prey cultures**

*Favella* sp. and the phytoplankton cultures *Heterocapsa triquetra*, *Mantoniella squamata*, and *Isochrysis galbana* were obtained from the Shannon Point Marine Center, Anacortes, WA. The phytoplankton *Tetraselmis* sp. was obtained from Michael Finiguerra at the University of Connecticut, Groton, CT. *Favella* sp. cultures were maintained in 200 ml batches at 14–16 °C in filtered seawater (30 ppt) supplemented with a dilute trace metal solution (Strom, et al., 2007). *Favella* cultures were fed with prey and sub-cultured every 3–4 days. Phytoplankton were maintained in 40 – 1000 ml batches in filtered 36 ppt seawater supplemented with f/2 nutrients and Guillard’s vitamins (Guillard, 1975) at 14–16 °C and 50–80 μmol photons m⁻² s⁻¹ on a 12:12 light: dark cycle. Phytoplankton were sub-cultured every 1–2 weeks. The copepod predator of *Favella* sp., *A. tonsa*, was obtained from the aquaculture supplier, AlgaGen (VeroBeach, FL). *A. tonsa* were maintained in 2 L batches at 14–16 °C in filtered 33 ppt seawater at 14–16 °C and 50–80 μmol photons m⁻² s⁻¹ on a 12:12 light: dark cycle. They were fed *Tetraselmis* sp. and *I. galbana* every 3–4 days. For physiological experiments examining interactions of *Favella* sp. and *H. triquetra*, *H. triquetra* were prepared immediately prior to use by gentle filtration through 3 μm pore size Whatman polycarbonate filters (General Electric, Pittsburgh, USA) followed by two rinses in artificial seawater (ASW; 450 mM NaCl, 30 mM MgCl₂, 16 mM MgSO₄, 8 mM KCl, 10 mM CaCl₂, 2 mM NaHCO₃, 10 mM HEPES, pH adjusted to 8.0 with NaOH), to remove any dissolved chemical cues present in the supernatant.
**Electrophysiology**

Electrophysiological measurements of the bioelectrical activity *Favella* sp. were obtained using sharp electrode current clamp and voltage clamp recordings of whole cell membrane potential ($V_{\text{mem}}$) and currents, respectively (Fig. 1A). *Favella* sp. were prepared for electrophysiology by rinsing cultures 3 times in ASW by reverse filtration through a through 40 μm nylon mesh cell strainer (Corning Life Sciences, Maine, USA) before being placed in a recording chamber equipped with gravity fed perfusion that was secured to the stage of an Olympus IX 71 microscope. A Peltier cooled stage (fabricated in-house) maintained the recording chamber at 14–16 °C for experiments where behaviors and electrical activity of *Favella* sp. were recorded simultaneously. All other experiments were performed at room temperature (~20°C). Cells were tethered by the base of the lorica (Fig. 1A) using a micro suction pipette fabricated from non-filamented borosilicate glass capillaries (1.5 mm outer diameter. x 0.86 mm inner diameter, Sutter Instruments, Novato, California) using a microelectrode puller (Narishge, Tokyo, Japan) and bent into a position that allowed optimal imaging and access to cells with sharp electrodes. Sharp recording electrodes (10–20 MΩ) were fabricated from filamented borosilicate glass capillaries (1.5 mm outer diameter x 0.86 mm inner diameter, Sutter Instruments, Novato, California), coated in beeswax to minimize stray capacitance, filled with 1 M KCl and inserted into an electrode holder (Harvard Apparatus, Holliston, MA) that was mounted onto the headstage of an Axon 900A voltage clamp amplifier (Molecular Devices, Sunnyvale, CA). The electrode was positioned using a Sutter MP-285 motorized micromanipulator (Sutter MP, Novato, CA) until contact was made with the lateral surface of the cell, just posterior to the ring of membranelles. Electrical access to the cell was accomplished using a brief (< 30 ms) capacitance overcompensation.

Single electrode current and voltage clamp experiments were recorded using Clampex software (Molecular Devices, Novato, CA). Single electrode current clamp switching frequency was generally between 5–15 kHz and voltage clamp gain was generally between 0.3–3. To determine the voltage response of *Favella* sp. to negative current injection and the passive membrane properties of *Favella* sp. hyperpolarizing step protocols were used that injected 1 nA pulses of negative current for 120 ms. The passive membrane properties of *Favella* sp. were calculated by calculating resistance and time constant ($\tau$) from the voltage response to injection of negative current. Action potentials (AP) and rhythmic depolarizations (RD) (2 stereotypical
patterns of bioelectrical activity in *Favella* sp.) were elicited by injecting 5–10 nA current for 10–20 ms. Cells were perfused with 0.8, 4, 8, and 40 mM KCl ASW while measuring free running \(V_{\text{mem}}\) to determine the role of \(K^+\) in regulating resting potential and on spontaneous electrical activity. Experiments that examined the relationship between electrical activity and behaviors were performed without perfusion to avoid mechanostimulation by the flow of media around the cell.

Data were analyzed off-line in Clampfit 11.0 (Molecular Devices, Novato, CA). The characteristics of spontaneous electrical activity and electrical activity in the presence of prey were determined using threshold analysis tools of the software. The characteristics of APs evoked by positive current injection were measured using manual cursors.

**Behavioral observations and analysis**

Simultaneous electrophysiological and video acquisition allowed us to determine how the bioelectrical phenomena described above co-ordinate behavior in *Favella* sp. High-speed (up to 250 fps) video recordings were accomplished using a Fastec Inline 250M camera (Fastec Imaging, San Diego, CA) camera with 320 x 228 pixel resolution. Camera acquisition was triggered with a TTL pulse from the electrophysiology software for simultaneous electrophysiology and behavioral recordings. In order to quantify spontaneous and evoked behavioral characteristics of *Favella* sp., videos were analyzed using MiDAS Player (Ver. 2.2.1.1., Xcitex Incorporated, Cambridge, MA) and Metamorph Basic (Ver. 7.7.4.0., Molecular Devices, Inc., Sunnyvale, CA) software to define regions of interest (ROI). ROI’s were drawn around areas occupied by structures of interest e.g. cilium, cell body, peristomal cavity or stalk. Threshold analysis tools in Clampfit 11.0 (Molecular Devices, Novato, CA) were used to measure changes in average pixel intensity value as the cell structure moved into and out of the ROI. Pixel intensity values were normalized to a scale of 100 and inverted for the purposes of data presentation.

**Responses to natural and artificial prey**

In order to determine how the bioelectrical activity of *Favella* sp. co-ordinates feeding behaviors, electrophysiological and high-speed video recordings were obtained in the presence of natural and artificial prey (polystyrene microspheres). *Favella* sp. were exposed to either the preferred prey dinoflagellate *H. triquetra* or 15 µm polystyrene microspheres (Polysciences, Inc., Warrington, PA) at concentration of 15,000–30,000 mL\(^{-1}\) while simultaneously recording...
membrane potential and behavior. Microspheres were coated with methylcellulose to neutralize surface charge (Stoecker et al., 1995) and were the same equivalent spherical diameter as *H. triquetra* and therefore simulated mechanical stimulus from prey while controlling for chemical prey cues. Interactions of *Favella* sp. with microspheres and *H. triquetra* were manually scored via frame-by-frame observations to determine the sequence of events from time and location of particle contact, bioelectrical response, to behavioral event.

**Interactions of *Favella* sp. with copepod predators**

Behavioral interactions of free swimming *Favella* sp. with the copepod predator *A. tonsa* were investigated using high-speed (125 FPS) video microscopy. Prior to experiments *A. tonsa* cultures were rinsed twice with ASW by reverse filtration through 40 μm pore size nylon cell strainers and starved for 15–20 hours. Immediately prior to experiments ciliates and copepods were rinsed twice with ASW by reverse filtration through 40 μm pore size nylon cell strainers (Corning Life Sciences, Maine, USA). 30 individuals of *Favella* sp. and 6 late stage copepodite to adult female *A. tonsa* were combined in a 3.5 mL spectrophotometry cuvette and the volume was adjusted to 1 mL with ASW. Cuvettes were imaged through the side using an Olympus SZX12 dissecting microscope at 12.5x magnification. High-speed (125 fps) video recordings were accomplished using a Fastec Inline 250M camera (Fastec Imaging, San Diego, CA) with 640 x 478 pixel resolution. Cuvettes were observed for 0.5 h and all events where ciliates were captured by copepods were recorded. Behavioral interactions between copepods and ciliates were manually scored as “captures and ingests *Favella* sp.” or “captures and releases *Favella* sp.”. The amount of time it took *Favella* sp. to emerge from the lorica post-release was also measured.

**Statistical tests**

All differences in means were tested using a 1 way ANOVA followed by multiple comparisons with Tukey’s HSD test. To determine the relationship between the length of rhythmic depolarizations and behavioral events linear regressions were performed. All statistical analyses were performed with Sigmaplot Software version 11 (Systat Software, Inc.). All quantitative measures are given as mean ± standard deviation.
RESULTS

Bioelectrical characteristics of *Favella* sp.

The passive membrane properties of *Favella* sp. were similar to previously investigated ciliates (Table S1). All *Favella* sp. cells produced spontaneous RDs (n = 34 cells) (Figs. 1B, C), and occasionally rapid ‘all or nothing’ APs were observed (Fig. 1B, C) (n = 4, events analyzed = 4). RDs had a frequency of 0.153 ± 0.1 Hz, peak amplitude of 27.4 ± 2.2 mV and an average length of 618 ± 183 ms (n = 5, events analyzed = 58). When cells were voltage clamped at resting $V_{\text{mem}}$ (-62 mV), spontaneous inward currents were recorded that underlie the RDs observed in current clamp (Fig. 1D).

A strong dependence of $V_{\text{mem}}$ upon external [K$^+$] was observed, with a Nernstian depolarization (50 mV change in $V_{\text{mem}}$ per decadal change in external [K$^+$]) of $V_{\text{mem}}$, over the concentration range between 4–40 mM KCl ASW (Fig. 2A, B). $V_{\text{mem}}$ deviated from the Nernstian relationship in <4 mM KCl ASW, with cells exhibiting only a slight hyperpolarization when perfused with 0.8 mM KCl ASW (Fig. 2A, B). Interestingly, changing external [K$^+$] changed the characteristics of RDs. The duration and peak depolarization of RDs in all [K$^+$]$_{\text{ext}}$ concentrations was similar. However, decreased external [K$^+$] led to decreased amplitude of the small hyperpolarizations that occurred at the end of RDs (p<0.05, ANOVA, Tukey’s HSD) (Fig. 2C, Table S1). In contrast, high external [K$^+$] (40 mM KCl ASW) caused APs to be generated at the rising phase of the RDs (Fig. 2D). These AP-RDs were much shorter than RDs and do not exhibit hyperpolarizations following the depolarized phase of the event (n = 4, events analyzed = 51) (Fig. 2D, Table S1). They almost always preceded bursts of APs induced by the depolarized state (up to 3 Hz) (Fig. S1) that were accompanied by whole-cell contraction into the lorica (not shown).

Under normal ionic conditions, positive current injection pulses elicited depolarization and either RDs or APs (Fig. 3A, B) that were identical to spontaneous RDs and APs. Over the range of positive current injection protocols tested there did not appear to be a correlation between length (10–20 ms) or amplitude (5–10 nA) of injection and type of response elicited (AP or RD). In contrast, negative current injection elicited a typical RC voltage response (Fig. 3C). APs were elicited in 7 cells where 5–10 nA current was injected for 10–20 ms (Fig. 3B). APs did not occur until $V_{\text{mem}}$ reached an average depolarization threshold of -3.7 ± 17.10 mV (n = 3,
events analyzed = 3) and $V_{mem}$ at peak after-hyperpolarization was $-76.50 \pm 6.75$ mV ($n = 3$, events analyzed = 3). The time to peak depolarization from start of the evoked AP was very rapid at $6.92 \pm 2.40$ ms ($n = 7$, events analyzed = 7).

**Regulation of swimming behavior by rhythmic depolarizations**

Spontaneous RDs always resulted in ciliary reversals, peristomal cavity contractions, and stalk bending events, (Figs. 4, 5, 6A), which all began within 70–110 ms of the start of RDs (Fig 6A). RDs lasted approximately the same length of time as the events they regulated (between 600-750 ms, Figs. 6A) ($n = 4$, events analyzed = 58): there was a strong 1:1 correlation ($r^2=0.89$, $p<0.001$) between the length of RDs and the period of time cilia were in reverse beating. There were weaker correlations between length of RDs and duration of peristomal cavity contractions ($r^2=0.23$, $p<0.001$) and stalk bending ($r^2=0.17$, $p=0.007$) (Figs. 5). During forward beating the instantaneous ciliary beat frequency (CBF) was $27.7 \pm 1.5$ Hz ($n = 5$, events analyzed > 10,000) and during reversed beating it was slightly higher at $33.3 \pm 7.5$ Hz ($n = 5$, events analyzed > 500) (Fig. S2). Forward beating transitioned to reverse beating very rapidly (within approximately 45 ms) ($n = 5$, events analyzed = 48).

**Behavioral interactions of Favella sp. with prey are differently regulated by contact with cilia and peristome**

Contact of *H. triquetra* with the cilia of *Favella* sp. invariably resulted in depolarizations and behaviors (ciliary reversals, peristomal cavity contractions, and stalk bending) ($n = 4$, events analyzed = 13) that were very similar to RDs (Figs. 6B, 7A, Movie S1). Identical responses were observed when *H. triquetra*-sized polystyrene microspheres (15 μm) that were used as artificial prey to control for the effect of chemical cues present on the surface of natural prey particles (Figs. 6C, 7B), and these events are thus termed ‘mechano-stimulated depolarizations’ (MSDs). There was a very short lag of $29.8 \pm 17.9$ ms ($n = 4$, events analyzed = 13) between contact of *H. triquetra* with cilia and MSDs, with a nearly identical lag observed with beads (Figs. 6B–C, 7A–B, Movie S1).

Polystyrene microspheres were never observed to be ingested on contact with the peristomal cavity ($n = 5$ events analyzed = 6) (although one cell contained a microsphere that was ingested before video recording started [Fig. 6B, D]). Instead, a significant lag occurred before a depolarization was elicited that resulted in ciliary reversal and ejection of microspheres from the peristomal cavity (Figs. 6E, 7D). In some cases when *H. triquetra* contacted the
peristomal cavity, they also elicited this response (Fig. 6C). Although the lag between particle contact at the peristomal cavity and subsequent MSD was longer for *H. triquetra* (750 ± 552 ms) than for microspheres (596 ± 777 ms) lag times were highly variable and therefore not statistically significant. However, in other instances, contact of *H. triquetra* with the peristomal cavity resulted in ingestion after a processing period of 1.01 ± 0.84 s (n = 7, events analyzed = 7) that invariably resulted in hyperpolarization that began 212.1 ± 384.7 ms (n = 3, events analyzed = 3) after ingestions started, lasted 1700 ± 800 ms (n = 8, events analyzed = 8), and had peak amplitudes of 3.5 ± 0.8 mV (n = 8, events analyzed = 8) (Figs. 6F, 7E, Movie S2). No change in CBF was observed during hyperpolarizations associated with prey ingestion (n = 4, data not shown) suggesting swimming is unaffected. The striking discrimination against ingesting microspheres suggests that ingestion and associated hyperpolarization are mediated by chemical cues present on the cell surface, but not on the microspheres.

**Action-potential regulated contraction serves as predator evasion behavior in *Favella* sp.**

Capture of *Favella* sp. by *A. tonsa* resulted in contraction of *Favella* sp. into the lorica (Fig. 8). *Favella* sp. were released 73 ± 25 % of the time and ingested the rest of the time (6 experiments, n = 16 events). Released ciliates were invariably contracted into the lorica, but re-emerged and resumed swimming after 10.6 ± 3.8 s (6 experiments, n= 5 events (Fig. 8).

The bioelectrical basis of this predator evasion behavior was investigated using sharp electrode current clamp recordings and simultaneous high-speed video microscopy. Spontaneous and evoked APs caused cessation of cilia beating and rapid contraction into the lorica (Figs. 6F, 9, Movie S3). The coupling between electrical event and behavioral response was extremely rapid with contraction starting 12.2 ± 5.9 ms after the initiation of AP (n = 7, events analyzed = 7) with cells reaching 95% of their full contraction at 121 ± 77 ms (n = 5, events analyzed = 5). Cells gradually recovered to pre-AP state after 8.8 ± 2.0 s (n = 5, events analyzed = 5) (Figs. 6F, 9, Movie S3).
DISCUSSION

Passive membrane properties and resting membrane potential of *Favella* sp.

*Favella* sp. exhibited a resting membrane potential that was more negative than previously studied marine and freshwater ciliates reported in the literature (Table S2). This indicates their resting permeability and the electromotive force across the plasma membrane are determined by somewhat different conductances. The passive membrane properties of *Favella* sp. show a high input capacitance in comparison with other ciliates (Table S2) that likely reflects their large cell size and large membrane surface area. Additionally, their input resistance was rather low compared to other ciliates (Table S2) suggesting a high resting ionic permeability. Similar to other eukaryotes, resting permeability to K\(^+\) was important in determining resting \(V_{\text{mem}}\) in *Favella* sp. The near Nernstian relationship of \(V_{\text{mem}}\) and \([K^+]_{\text{ext}}\) at concentrations above 8 mM KCl indicated \(V_{\text{mem}}\) was primarily determined by permeability to K\(^+\) ions. However, below 8 mM KCl the non-linear curve suggests permeability to ions other than K\(^+\) contributes to resting \(V_{\text{mem}}\).

Bioelectrical regulation of swimming behavior

The bioelectrical activity of *Favella* sp. is critical in regulating endogenous swimming patterns (Fig. 10). *Favella* sp. exhibited frequent spontaneous RDs that regulated ciliary reversals, stalk bending, and peristomal cavity contractions. Ciliary reversals result in periods of backward swimming in free-swimming cells, and stalk bending changes the orientation of the cell and would therefore affect swimming angle. Peristomal cavity contractions may function in clearing the surface of the peristomal cavity of particles during ciliary reversals. The strong correlation between length of RDs and length of ciliary reversal indicates that *Favella* sp. can modify the length of RDs and ciliary reversals based on environmental and endogenous signals.

RDs have been observed in several ciliates (Machemer, 1970, Lueken et al., 1996) including the marine benthic spirotrich *Euplotes vannus* in which RDs mediate alternating periods of slow and fast walking and backward walking behavior (Machemer, 1970, Lueken et al., 1996). Increases in inward conductance of Ca\(^{2+}\) and decreases in outward conductance of K\(^+\) appear to be important in mediating RDs in *E. Vannus* (Lueken et al., 1996). The specific ionic conductances responsible for RDs in *Favella* sp. are unknown but the importance of voltage-gated channels in mediating RDs in *Favella* sp. was suggested by the fact that transient
depolarizations and ciliary reversals were elicited by the injection of positive current (Fig. 3A). We observed increased after-hyperpolarization with decreases in $[K^+]_{\text{ext}}$ indicating that $K^+$ channels were important in returning the cell to resting $V_{\text{mem}}$ following RDs (decreased $[K^+]_{\text{ext}}$ resulted in an increased electromotive force of $K^+$ from *Favella* sp.). The cellular mechanisms that regulate the rhythmicity of RDs in *Favella* sp. and other ciliates have not been identified. The high frequency of RDs and backward swimming events in *Favella* sp. and presence of these mechanisms in multiple taxa indicates they most likely play an important role in structuring ciliate search patterns for food particles and in determining encounter rates with prey and predators.

The rate of ciliary beating in *Favella* sp. was also regulated by bioelectrical activity of the cell. The increased reverse CBF of *Favella* sp. during RDs will correspond to increases in swimming speed in free-swimming cells. Intracellular Ca$^{2+}$ dynamics are most likely involved in regulating ciliary beating in *Favella* sp. In *Paramecium tetraulia* Ca$^{2+}$ dynamics determine the frequency and direction of ciliary beating with Ca$^{2+}$ concentrations greater than 1 μM causing a reversal of ciliary beating in *Paramecium* sp. (Nakaoka et al., 1984, Plattner et al., 2006). Powerful Ca$^{2+}$ buffering systems must also be present in the cilia that return cilia to resting Ca$^{2+}$ levels following depolarization induced ciliary reversals (Husser et al., 2004). The cyclic nucleotides cAMP and cGMP are also important in regulating CBF and direction and have interactive effects with Ca$^{2+}$ (Bonini and Nelson, 1988; Noguchi et al., 2004). Similar mechanisms may regulate ciliary beating in *Favella* sp. Higher CBF will lead to increased swimming velocities and increased encounter rates with prey but will also increase the susceptibility of *Favella* sp. to detection by predators.

**Bioelectrical activity regulates behavioral responses of *Favella* sp. to prey cues**

Although earlier studies linked RDs and ciliary movements, a novel observation in the present study is the coordination RDs with other behaviors such as peristomal contractions (Fig. 5B) and stalk bending. Moreover the differences in bioelectrical signaling at the cilia and peristome during prey handling indicate that *Favella* sp. are able to discriminate the type and location of stimuli from prey. Contact of *H. triquetra* and 15 μm microspheres at the cilia elicited almost instantaneous MSD- mediated ciliary reversals whereas contact of *H. triquetra* with the peristomial cavity elicited variable responses, sometimes resulting in hyperpolarization-mediated ingestions and sometimes resulting in MSD-mediated ciliary reversals preceded by a lag period.
In contrast, contact of 15 μm microspheres at the peristomal cavity invariably elicited ciliary reversals preceded by a lag period (Fig. 10B–C). We hypothesize that spatially distinct classes of ion channels and receptors, allowing for sophisticated prey handling behaviors, regulate interactions of planktonic alveolates, such as *Favella* sp., with prey.

MSD-mediated ciliary reversals upon particle contact at the cilia may allow the cell to briefly re-orientate to a particle on the periphery of the cell that could not otherwise be directly ingested. MSD-mediated reversals could also result in increased encounter rates of *Favella* sp. with prey due to the patchy small-scale distributional patterns of phytoplankton in nature (Mitchell et al., 2008). We propose that the ciliary reversals produced by MSDs increase residence time of *Favella* sp. within food patches by decreasing their diffusivity and thus may contribute to mechano-stimulus induced aggregative swimming behavior previously reported for *Favella* sp. (Buskey and Stoecker, 1988; Buskey and Stoecker, 1989) (Fig. 10B). Therefore this is the first demonstration of the cellular basis of population level responses in *Favella* sp. Mechanostimulated aggregative swimming behaviors may be an important planktonic alveolate foraging strategy.

The production of MSDs is presumably due to the activation of mechanosensitive ion channels (Fig. 10B). In *E. vannus* the bioelectrical regulation of depolarizations and backward movement events induced by mechanical stimulus has been well described (albeit in a non-ecological context). In these cells mechanical stimulus at the anterior results in an influx of Ca$^{2+}$ and Mg$^{2+}$ through stretch activated ion channels and Na$^+$ through Ca$^{2+}$ dependent Na$^+$ channels resulting in a reversal of the cirri (compound cilia responsible movement) beating direction that results in backward movement (Krüppel and Lueken, 1990, Krüppel et al., 1995). The $V_{\text{mem}}$ of *E. vannus* is returned to resting level by the efflux of K$^+$ from slower voltage gated K$^+$ channels (Krüppel et al., 1995). As with RDs the involvement of voltage-gated channels in mediating MSDs in *Favella* sp. is suggested by the fact that transient depolarizations and reversals may be elicited by the injection of positive current (Echevarria et al., 2014).

Ciliary reversals are rarely elicited upon contact of *Favella* sp. with smaller non-preferred prey; therefore smaller particles (<4 μm) do not elicit aggregative changes in swimming in swimming behavior (Buskey and Stoecker, 1988, Stoecker et al., 1995). This suggests smaller particles do not produce enough force on contact with membranelles to activate mechanosensitive ion channels. The mechanosensitivity of *Favella* sp. and other alveolates may
therefore be tuned to sense optimally sized prey items, supporting the role of MSD-mediated ciliary reversals in capturing and encountering prey.

In contrast with ciliary contact by particles, direct peristomal contact resulted in a lag period before initiation of MSD (Fig. 10B). This may allow particles in the peristomal cavity to undergo a processing period where they are sampled for their surface properties and are subsequently ingested, or rejected via ciliary reversal and backward swimming. The longer response time to particles that contact the peristomal cavity indicates that *Favella* sp. possess different mechanisms that regulate mechanosensitivity at the cilia and peristomal cavity: for example *Favella* sp. may have more sensitive or higher densities of mechanosensors located on cilia than the peristomal cavity. The spatial distribution of ion channels is known to be important to regulating complex behaviors in *Euplotes* sp. and *Paramecium* (Machemer and Ogura, 1979, Krüppel et al., 1993). Particle processing at the peristome most likely includes crosstalk between mechanosensory and chemosensory signaling pathways. The lag between particle contact and depolarization mediated ciliary reversals may give ciliates sufficient time to assess particles with chemosensors on the peristomal cavity and determine whether they are suitable for ingestion. Further work is now required to understand the spatial distribution of sensory mechanisms and how this allows alveolate grazers to perceive mechanical and chemical prey cues during processing and ingestion.

Unlike cilia, detection of particles at the peristomal cavity likely involves chemical information, since 15 μm microspheres were rejected, unlike similarly sized *H. triquetra* (Fig. 10B). Chemical recognition of prey particles is known to be important in allowing *Favella* sp. to identify and ingest favorable prey. For example *Favella* sp. are able to recognize and reject toxic *Heterosigma akashiwo* upon contact, indicating that unfavorable surface compounds may be present on *H. akashiwo* (Taniguchi and Takeda, 1988), while ingesting similarly sized *H. triquetra*. The mechanism by which chemical recognition occurs in *Favella* sp. is unknown, but is likely similar to those known for other ciliates. Sugar-lectin interactions at the interface of prey and predator cell surfaces seem to be a common and important method of prey particle identification by ciliates and other planktonic alveolates (Scott and Hufnagel, 1983, Esteve, 1984, Casci and Hufnagel, 1988, Sakaguchi et al., 2001, Wilks and Sleigh, 2004, Roberts et al., 2006, Wootton et al., 2007). Surface proteins that are attached to the extracellular surface by
glycosylphosphatidylinositol (GPI) are also important in predator-prey recognition processes in freshwater ciliates (Simon and Kusch, 2013).

Recognition of favorable prey particles most likely involves cross-talk between metabotropic (enzyme-linked or second messenger-linked) signaling and ionotropic signaling pathways. G-protein coupled receptor (GPCR) pathways may be important in linking these signal transduction mechanisms (Echevarria et al., 2014). GPCR signaling pathways are critical for chemosensation of prey in ciliates as demonstrated in experiments with the marine ciliate *Uronema* sp. where chemosensory responses to prey were decreased by application of pharmacological compounds that inhibited the GPCR signal transduction pathway (Hartz et al., 2008). These GPCR signaling pathways provide a feedback link to modulate activity of ion channels through the activity of cyclic AMP, and thus the behaviors described above that these channels regulate. For example, genes for adenylyl cyclase were cloned from *P. tetraurelia* and found to localize to cilia, where they potentially regulate K⁺ channels that regulate ciliary movements (Weber et al., 2004). One potential consequence of such a pathway may be the ingestion-mediated hyperpolarization observed in *Favella* sp., which to our knowledge has not been described in the literature. Hyperpolarization may make the cell less excitable, decreasing the likelihood that either RSDs or MSDs will be activated that would cause particle ejection from the peristomal cavity during ingestion as observed by Taniguchi and Takeda where toxic *H. akashiwo* cells were rejected by *Favella* sp. (1988) (Fig. 10C). Thus, membrane hyperpolarization during ingestion may be a general mechanism for allowing planktonic alveolates to processes food particles.

**Bioelectrical activity regulates behavioral responses to predator cues**

Our research represents the first demonstration of behavioral interactions of *Favella* sp. with copepod predators. Previous research suggests that copepods may extract *Favella* sp. from the lorica during predation (Stoecker and Sanders, 1985) indicating that the lorica is an awkward size or shape for ingestion or non-nutritive (the lorica is highly refractory in nature and does not degrade in the presence of strong acids and bases [Dolan et al., 2013]). We showed that upon capture *Favella* sp. contracted into the lorica and were often released after being captured. Therefore contraction into the lorica may prevent copepods from detecting the nutritive cell inside and impede extraction. Upon release by copepods *Favella* sp. remained contracted for several seconds, not beating their cilia, instead sinking through the water column. This might
decrease their hydrodynamic signature and decrease the encounter rate with predators that still occupy the area. These results suggest mechanostimulated contraction into the lorica is a predator evasion behavior that could be common in the plankton given the prevalence of loricated organisms.

Contraction into the lorica was directly regulated by APs (Fig. 10D). Evoked AP-mediated contractions in tethered cells and contractions caused by predation attempts in free swimming cells were approximately the same length of time indicating that similar molecular mechanisms mediate both processes. The ionic mechanisms underlying changes in $V_{\text{mem}}$ during AP production are unknown although voltage activated channels are most likely involved because current injection resulted in the rapid (ms) excitation-contraction coupling. In addition, spontaneous depolarizations in low $[K^+]_{\text{ext}}$ changed into AP-RDs in higher $[K^+]_{\text{ext}}$; a change that is likely due to a shift in $V_{\text{mem}}$ sufficient to reach the threshold for voltage gated cation channels responsible for APs. How predation attempts trigger the escape response is unclear although similar classes of mechanosensitive ion channels that are involved in production of MSDs during interactions with prey are most likely activated on contact of *Favella* sp. with predators. However, stronger mechanostimulus by predators may activate more ion channels, or a different class of mechanoreceptors resulting in a greater depolarization that reaches the threshold necessary for all-or-nothing APs (Fig. 10D). Such threshold dependent sensory mechanisms likely underpin distinct prey capture and predator evasion behaviors exhibited by alveolate grazers.

Although there is a clear relationship between fast APs and whole cell contraction in *Favella* sp., the mechanism is yet to be determined. A similar excitation-contraction mechanism has been well studied in the benthic ciliate *Vorticella* sp. albeit in a non-ecological context. Stimulation of mechanoreceptors in the cell body results in an all or nothing rise in $Ca^{2+}$, immediately followed by an all-or-nothing rises in $V_{\text{mem}}$ and contraction of *Vorticella* sp. along its stalk (Katoh and Kikuyama 1997; Shiono and Naitoh, 1997). This rapid rise in $Ca^{2+}$ is due to an influx of $Ca^{2+}$ from membranous tubules that surround the stalk via a calcium induced calcium release mechanism (CICR). Calcium interacts with contractile proteins in the stalk (spasmins), resulting in contraction (Routledge, 1978; Katoh and Naitoh, 1994; France, 2007). Whether a similar mechanism functions in *Favella* sp. remains to be tested (Echevarria et al., 2014). Efflux of $K^+$ ions may be important in returning cells to resting $V_{\text{mem}}$ following action
potentials. This is indicated by the abolishment of after-hyperpolarizations in AP-RDs observed in 40 mM KCl ASW that would result from a decrease in the driving force of $K^+$ due to an increase in $[K]_{ext}$.

**Summary**

*Favella* sp. exhibited complex and diverse bioelectrical and behavioral properties with responses to stimuli dependent on their type and location. Additionally, bioelectrical responses and resulting behaviors were intensity-dependent: from single particle triggered MSD and ciliary reversal, to high frequency APs that resulted in extended contraction into the lorica. This may allow fine-tuned behavioral responses based on the strength of stimuli. Overall these findings show that *Favella* sp. have sophisticated cellular mechanisms that enable them to perceive predators and prey, discriminate between them and respond with appropriate behaviors (Echevarria et al., 2014).

With the techniques developed in this study, it is now possible to examine the behavioral and bioelectrical responses to a range of chemical, mechanical (contact), and hydromechanical (non-contact) cues from both predators and prey, and ultimately the ionic mechanisms and downstream signaling processes such as GPCR signal transduction pathways and CICR mechanisms that link bioelectrical responses to behavior. These mechanisms underpin the integration of multiple environmental cues to maximize fitness in these single-celled organisms that are critical consumers of phytoplankton and play important roles in the marine microbial web.
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AUTHOR CONTRIBUTIONS
M.L.E. participated in development, design and execution of experiments, data analysis, interpretation of results, and in manuscript preparation. G.W. participated in initial experimental design and in writing the manuscript. A.R.T. participated in development and design of experiments, supervision of data collection and analysis, interpretation of results and writing the manuscript.

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Fig. 1. Spontaneous electrical activity of *Favella* sp. **A.** Brightfield micrograph showing *Favella* sp. cell tethered by the loria with a glass suction micropipette for electrophysiological recording (scale bar = 50 μm) **B.** Typical current clamp trace of free running membrane potential (\(V_{\text{mem}}\)) showing two main types of spontaneous electrical activity, rhythmic depolarizations (RDs) and all-or-nothing action potentials (indicated by red boxes). **C.** A detailed example of an RD and AP from the \(V_{\text{mem}}\) trace in **B.** **D.** Voltage clamp recording showing free running membrane current (\(I_{\text{mem}}\)) of a cell voltage clamped at -62 mV. Spontaneous inward currents of up to 10 nA and similar in duration to RDs are observed.
Fig. 2. Effect of [K$^+$] on $V_{\text{mem}}$ and bioelectrical activity of *Favella* sp. A. Effect [K$^+$]$_{\text{ext}}$ on $V_{\text{mem}}$ of *Favella* sp. Perfusion with 0.8 mM KCl ASW causes a slight hyperpolarization in $V_{\text{mem}}$, from 61 mV in 8 mM KCL ASW (dashed line), whereas perfusion with 40 mM KCl ASW causes a dramatic ~30 mV depolarization in $V_{\text{mem}}$. B. Relationship between [K$^+$]$_{\text{ext}}$ and $V_{\text{mem}}$. A roughly linear and Nernstian slope (of 1.2 mV mM K$^+$$^{-1}$) is observed for concentrations above 4 mM K$^+$ while $V_{\text{mem}}$ is relatively insensitive to concentration changes below 4 mM. C. Examples of spontaneous bioelectrical activity in *Favella* sp. under various K$^+$ treatments. From top to bottom panels show examples of RDs in 0.8, 4, and 8 mM KCl ASW, respectively. Their shape is similar in all solutions but after-hyperpolarization (arrows) amplitudes increase with decreasing [K$^+$]$_{\text{ext}}$. D. An action potential – rhythmic depolarization (AP-RD) associated with
the rising phase of the RDs that frequently occur in 40 mM KCl ASW. Unlike in lower $[K^+]_{\text{ext}}$ ASW, AP-RDs in 40 mM KCl ASW do not exhibit hyperpolarizations. The change in spontaneous electrical activity from RDs in low $[K^+]_{\text{ext}}$ to AP-RDs in higher $[K^+]_{\text{ext}}$ is likely due to $V_{\text{mem}}$ depolarization closer to the AP threshold.
Fig. 3. Evoked bioelectrical properties of *Favella* sp. A. RD (top) elicited by positive current injection (bottom) B. AP (top) elicited by positive current injection (bottom) C. RC voltage response (bottom) to injection of negative current during hyperpolarizing step protocol (top).
Fig. 4. Spontaneous bioelectrical activity and associated synchronous behaviors of *Favella* sp.  

A. Micrographs of *Favella* sp. cell immediately prior to a RD depolarization (left) and during a depolarization (right). Regions of interest (ROIs) are drawn around areas covered by an area passed through by a single cilium during normal forward beating (red), cytostomal cavity (blue), a portion of the stalk (green) at resting $V_{\text{mem}}$ B. Free running $V_{\text{mem}}$ of cell showing RDs.  

C-E. Normalized average grey level pixel intensity values that are color-coded to correspond with ROIs in panel A. Pixel intensity values from each greyscale image were inverted and normalized such that values of 100 represent maximal coverage of ROI by cell feature in ROI and 0 represents minimal coverage of ROI by cell feature.  

C. Normalized pixel intensity values derived from ROI passed through by a single cilium during normal forward swimming. Troughs in graph represent periods of ciliary reversals.  

D. Pixel intensity values derived from the ROI over the cytostomal cavity of the cell. Troughs in graph represent periods where contractions of the peristomal cavity occur.  

F. Pixel intensity values derived from an ROI over a portion of the stalk. Troughs in graph represent periods of stalk bending.
Fig. 5. Correlations between length of RDs and length of associated behaviors of *Favella* sp. 58 RD events from 4 cells were analyzed with each color representing values derived from a single cell. Linear regressions were performed to determine the degree of correlation between RD length and length of behaviors, and were performed on event lengths for all cells pooled together. There was a strong 1:1 correlation between length of the RD and the length of time spent in ciliary reversal (top). Correlations between length of RD and length of peristomal contraction (middle) and stalk bending (bottom) events were weaker.
Fig. 6. Temporal relationships between bioelectrical events and behaviors they regulate in *Favella* sp. A-G. Grey lines represent start and end time of respective events. Error bars at beginning and end start times represent standard deviation (S. D.) of start times of events relative to 0 s, and S. D. of length of events, respectively. B, D. * There was high variability in the lag
between *H. triquetra* (S. D. = ± 0.552 s) and 15 µm polystyrene microsphere (S. D. = ± 0.777) contact with the peristomal cavity and time to the start of depolarization, therefore error bars are omitted for clarity of presentation. **F.** Red diamond represents average time of 95% contraction into the lorica.
Fig. 7. Bioelectrical regulation of prey processing and ingestion in *Favella* sp. Top panels. A-E. Micrographs showing interactions between *Favella* sp. and particles (scale bar = 50 μm). **Bottom panels.** $V_{\text{mem}}$ traces showing bioelectrical activity that regulates behavioral interactions shown in top panels. Red arrows on $V_{\text{mem}}$ traces indicate times where corresponding images were taken. Blue arrows indicate direction particle is moving in A-D. Contact of *H. triquetra* (A) and 15 μm polystyrene microspheres (B) with cilia results in immediate MSDs and ciliary reversals. **C-D.** Contact of *H. triquetra* (C) and 15 μm polystyrene microspheres (D) with the peristomal cavity results in MSDs and ciliary reversals after a lag. **D.** Ingestion of *H. triquetra* results in hyperpolarization. Red circle indicates position of *H. triquetra* cell prior to (left micrograph) and after (right micrograph) ingestion.
Fig. 8. *Favella* sp. responses to predation attempt by *A. tonsa*. Scale bar = 500 μm. A. Immediately prior to attack jump by copepod. B. Immediately after attack jump. Ciliate has been captured and is being processed. C. Immediately after release of ciliate by copepod. Ciliate is contracted into lorica. D. Immediately after ciliate has reemerged from lorica and has started swimming again. Red arrows show location of ciliate. Time values indicate time elapsed relative to panel A (0 s).
Fig. 9. Evoked action potentials cause prolonged whole cell contraction and cessation of ciliary beating. A. Micrographs of Favella sp. cell immediately prior to an action potential (left) after an action-potential contraction (middle) and after recovery from an action potential-mediated contraction (right). Regions of interest (ROIs) are drawn around area covered by the uncontracted cell body (red), a fully contracted cell body (blue), and by an area passed through by a single cilium (green). B. $V_{\text{mem}}$ before (1) immediately after (2) and at recovery (3) from AP-mediated contraction. * indicates injection of 10 nA current for 20 ms to elicit AP. C. Expanded section of $V_{\text{mem}}$ trace from between cursors 1 and 2. D-F. Pixel intensity values from each ROI in the greyscale image were inverted and normalized such that values of 100 represent maximal coverage of ROI by cell feature in ROI and 0 represents minimal coverage of ROI by cell feature. Normalized average grey level pixel intensity values are color-coded to correspond with ROIs in panel D. ROI for Uncontracted cell. The beginning of contraction is measured as the point when threshold values decrease sharply to minimal values from the maximal normalized value of 100. Recovery from contraction is measured as the point at which threshold values return to maximal normalized values. E. ROI for contracted cell. The time of full contraction is
measured as the point at which these values reach their maximum amplitude. F. ROI for cilium. Time that cilia cease beating is measured as the time where high frequency oscillations in threshold intensity due to ciliary beating cease. Time that cilia resume beating is measured as the time at which these oscillations resume.
**Fig. 10.** Relationships between behaviors and ecological processes (left column) and the bioelectrical events that regulate them (right column) A. Backward swimming events during endogenous swimming (left) are regulated by RDs B. Backward swimming occurs upon contact of prey particles with cilia and non-preferred prey particles (red particle) with the peristomal cavity (left). These behaviors may regulate aggregative swimming behaviors in patches of
preferred prey and rejection of non-preferred prey. MSDs (middle) regulate these backward swimming behaviors. C. Ingestion occurs upon contact of preferred prey particles at the peristomal cavity (left). Hyperpolarizations (middle) underlie ingestion events. D. Contraction into the lorica occurs upon capture of Favella sp. by predators (left). Contractile behavior is mediated by action potentials (middle).