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Rapid firing rates from mechanosensory neurons in copepod antennules

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Abstract Small detection distances coupled with rapid movements require copepods to respond to stimuli with behavioral latencies on the order of milliseconds. Receiving adequate sensory information in such a short time necessitates extremely rapid firing rates of the efferent receptors. Here we show that copepod mechanoreceptors can fire at frequencies up to 5 kHz in response to fluid mechanical stimuli. Neural activity at these frequencies enables these animals to code for a range of fluid velocities thus providing important information regarding the nature of different fluid disturbances.

Introduction

Whether escaping predators, pursuing mates or attacking potential prey, rapid behavioral responses often are critical to the survival of individual animals. As natural selection hones an animal's ability towards shorter behavioral latencies, there must be a concomitant increase in either the transmission speed for sensory data or a decrease in the amount of sensory information necessary on which to base these behavioral responses. The biological and physical environment of pelagic copepods requires highly discriminate and yet rapid behavioral responses. Living at low Reynolds numbers, chemical stimuli are transported to animal sensors largely through the slow process of laminar fluid displacement and Fickian diffusion (Moore et al. 1999). Similarly, mechanical stimuli are attenuated quickly by viscous dampening causing fluid velocity to decrease with distance cubed (Fields and Yen 2002). As such, copepods often do not detect other individuals until they are within a few body lengths of each other and yet these animals still elicit rapid directed behavioral responses. For example, *Euchaeta rimana*, a predatory copepod, detects and identifies the fluid motion (Fields and Yen 2002) created by prey and initiates an attack within 5 ms. Similarly, the tandem hops during the "mating dance" of some copepod species occur within 1 ms of each other (M. Doall, personal communication) and escape responses from other fluid mechanical stimuli are initiated within 1.5 ms (Lenz and Hartline 1999). In addition to the need for rapid reactions, each of these scenarios requires a unique behavioral response that necessitates accurate discrimination of the stimuli.

Responding in an ecologically appropriate manner with a 1- to 5-ms behavioral latency presents a unique challenge. Rapid and accurate behavioral responses of copepods require that three fundamental conditions be met. First, the neural impulses must have time to travel from the receptor site to the target motor region. Second, the neural impulses must contain adequate information to differentiate stimuli from each other and, third, they must provide information to determine the 3-D location of the stimulus source. How does the neural system in copepods generate enough information in such a short time interval? Calanoid copepods possess numerous antennal sensors (Strickler and Bal 1973; Friedman and Strickler 1975; Yen et al. 1992; Fields et al. 2002). Depolarization of the nerve cell results from the deflection of the associated mechanosensory hair. Disturbance size is encoded by the number of cells simultaneously stimulated, whereas fluid velocity, at the level of the individual sensor, is encoded by the firing rate (Adrian 1928). The rate and angle of deflection are likely to provide the animal with information regarding the velocity and potentially the acceleration of the fluid signal. Compared to other crustaceans (Wiese 1976), copepod mechanoreceptors are at least an order of magnitude more sensitive, which allows them to detect fluid velocities as small as 20 $\mu m\ s^{-1}$ with a hair tip displacement of only 10 nm at 1 kHz (Yen et al. 1992)

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and a displacement velocity of 0.5 mm s^{-1} at frequencies of 100 Hz (Fields et al. 2002). This high sensitivity provides these animals with early detection of a potential predator allowing maximum time to collect information and initiate a behavioral response.

To achieve both stimulus differentiation and behavioral latencies of 1–5 ms, individual neurons must fire at a minimum frequency of 250–1,000 Hz to generate the requisite 2-spike minimum to differentiate a signal from spontaneous activity. Graded responses to fluid velocity, required for finer discrimination of signal strength, require higher frequencies. For example, coding a second or third magnitude of fluid velocity within the same 1.0-ms constraint would demand minimum frequencies of 2,000 and 3,000 Hz, respectively. Given these frequencies are much higher than those known for other animals, we investigated the firing rates of copepod mechanosensory neurons.

Methods

Animals

Gaussia princeps were collected from the research vessel Pt. Sur operated by the Monterey Bay Aquarium Research Institute (MBARI). Midwater trawls were taken at a depth of 600–800 m. Animals were transported by air in ice-filled containers to our laboratory at Georgia Institute of Technology (GIT). At GIT, animals were kept in the dark at 4°C and maintained in concentrations of five animals a liter on a diet of freshly hatched *Artemia* nauplii for up to 5 weeks.

Experimental setup

The experimental apparatus consisted of (1) an optical pathway to record setal motion (filmed using standard video), (2) a stimulus (water jet—filmed using high speed video 2,000 Hz), and (3) a system to extracellularly record nerve firing rates. The timing of the stimulus and the collection of physiological data were coupled with an accuracy of 10 μs through an externally generated transistor–transistor logic (TTL; + 5-V electronic signal) pulse.

Recording setal motion Setal deflection was filmed digitally at 2,000 Hz and an equivalent shutter speed of 1/40,000 s (Motion Scope PCI 2000s; Redlake Imaging). Illumination was achieved using two fiber-optic lights with one positioned parallel, and the other perpendicular, to the camera.

Water jet A small water jet was used to stimulate individual setae. The water jet was created using a 1-mm outer diameter (OD) capillary tube pulled to an OD of 150 μm and an inner diameter (ID) of 75 μm at the tip. The pipette was filled with ambient fluid, which was then expelled forcibly by compressed air at a pressure ranging from 14 kPa (2 psi) to 344 kPa (50 psi) using a microinjection system (Picospritzer II; General Valve division of Parker Hannifin). The duration that the solenoid remained open was maintained at 13 ms, and the velocity of the water jet was modulated by varying the pressure behind the solenoid valve. The size and precision of the jet allowed stimulation of individual setae, and these protocols produced a monotonic increase in fluid velocity at the locations of the setae that ranged from 1 to 500 mm s^{-1}. Previous work has shown that fluid disturbances created in this manner mimic the natural fluid disturbance created by swimming and escaping copepods (Yen 2000).

Neurophysiology In preliminary experiments, we found that amputated antennules gave similar recordings as whole animal preparations. The amputated antennule was preferred since it allowed us to individually test each antennule, and because the antennule could be positioned much more easily than the entire animal. The antennule was ablated between the second and fifth segments, and neural activity was recorded following the technique described by Gassie et al. (1993). Briefly, the base of the antennule was grasped (clamped) with stainless steel forceps and lowered into the experimental vessel through a 13×20 mm (depth×diameter) Plexiglas tube. The Plexiglas tube, which was suspended just above the water surface, was filled with mineral oil (4 ml) until all the water was displaced from the tube. The forceps and the first eight to ten segments of the antennule were then drawn out of the seawater into the electrically insulated oil layer. The electric potential was recorded at a sampling rate of 48 kHz between the forceps and a silver reference wire (placed in the seawater) using standard extracellular recording techniques (Fields et al. 2002). This method allows the detection of all the neural traffic that passes the oil-water interface traveling between the distal and proximal regions of the antennule. We used an additional technique in a number of cases to verify the accuracy of results obtained by the forceps clamping method. These recordings were accomplished by inserting a tungsten electrode (2 MΩ; FHC, Bowdoinham, Me., USA) and a silver reference wire directly into the proximal end of the antennule. The antennule was lowered into the seawater without the oil layer, until the cut basal end was just above the water surface. Neural responses received using both techniques were sorted based on their waveform characteristics (i.e., amplitude, rise time, offset slope) using commercially available software (Datawave; Longmont, Colo., USA) and matched to the waveform library created by the tactile stimulation (see description below). Individual setae were stimulated in two ways. First each seta received tactile stimulation to survey nerves that signaled above background noise and to generate an experiment-specific library of the waveform of the individual neurons associated with each seta. Once the library had been created, the mouth of the pipette was aimed normal to the seta and discharged in an incremental fashion. The

pipette tip was positioned at a distance ranging from 150 to 800 μm from the seta. Neural data were collected for a total of 100 ms prior to stimulation to determine the rate of spontaneous activity, and 1.4 s post stimulation. Only cells whose action potential characteristics could be matched to our templates were used for frequency analysis.

Results

Within a single preparation, action potentials varied in amplitude from ~350 to ~800 μV with spike widths ranging from 0.20 to 0.28 ms. Spontaneous activity within the cells was consistently less than 0.25 action potentials/min. Our sampling rate of 48 kHz worked well to capture the shape of the action potential by providing a minimum of ten points per action potential. The data clearly shows that the different neurons can be discriminated based on their signatures (Fig. 1), with amplitude and slope providing the most discriminatory power. All the different setae we tested fired at exceptionally high frequencies. Single neurons fired at a maximum frequency of 5,000 Hz (Fig. 2a) and sustained frequencies of 3,000–4,000 Hz for up to 4 ms (Fig. 2b). These rapid rates were recorded using both an external clamp method and an internal tungsten probe (Fig. 2c) demonstrating that they are independent of the recording method.

The application of 10^{-8} M tetrodotoxin (TTX, Sigma) solution directly to the excised region of the antennule completely and reversibly blocked the propagation of action potentials generated during the mechanical stimulation of individual setae (Fig. 3). Since TTX is known to block gated Na^+ channels, these results confirm that the recorded action potentials are biologically derived and propagated along the axon by highly selective Na^+ channels. Spike propagation ceased within 30 s of the toxin application and complete washout of the toxin took approximately 36 min, at which point the activity of the neuron was fully restored to pre-toxin levels.

In the absence of the toxin, increasing the pressure behind the solenoid increased both the instantaneous spike frequency (Fig. 4a) and the duration of the high frequency portion of the spike train (Fig. 4b). Whereas the instantaneous frequency rapidly increased to a plateau of 5 kHz and then remained constant, the duration of the high frequency response continued to increase over the pressure range we used to drive the water jet. The properties of our water jet mimic a potential prey item such as a copepod (Yen 2000) that rapidly increases its velocity during an escape (Lenz and Hartline 1999). The more rapid the jump, the greater the force applied

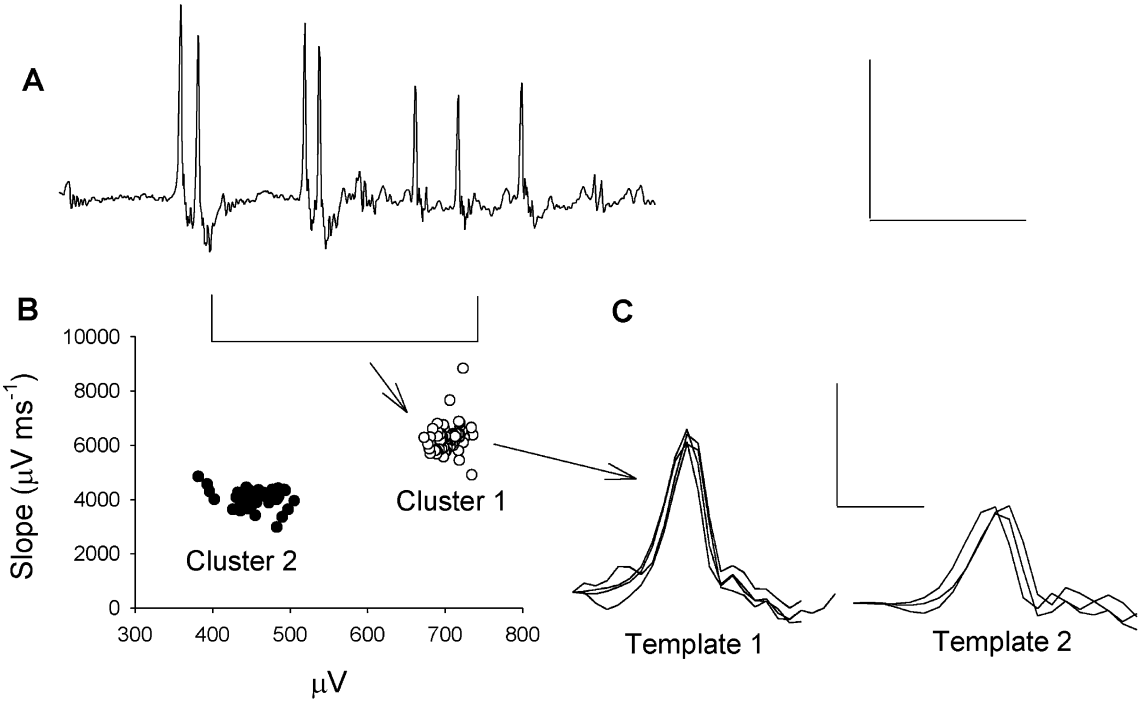

Fig. 1a–c Waveform characteristics of action potentials recorded from two different seta along the antennule of *G. princeps* in response to controlled tactile stimulation. **a** A 25-ms recording showing impulses with different characteristic shapes. Scale bar represents 500 μV on the ordinate and 5 ms on the abscissa. **b** Action potentials from the entire experiment are sorted into clusters based on their waveform characteristics. Individual impulses are sorted into distinct clusters based on peak amplitude and slope. The large amplitude action potentials correspond to cluster #1 while the small amplitude ones refer to cluster #2. **c** Action potentials clusters were averaged into template waveforms to create an experiment specific library of setal responses. The template is used to identify the response of individual nerve cells to a more general stimulus such as the water jet. Scale bar represents 500 μV on the ordinate and 0.2 ms on the abscissa

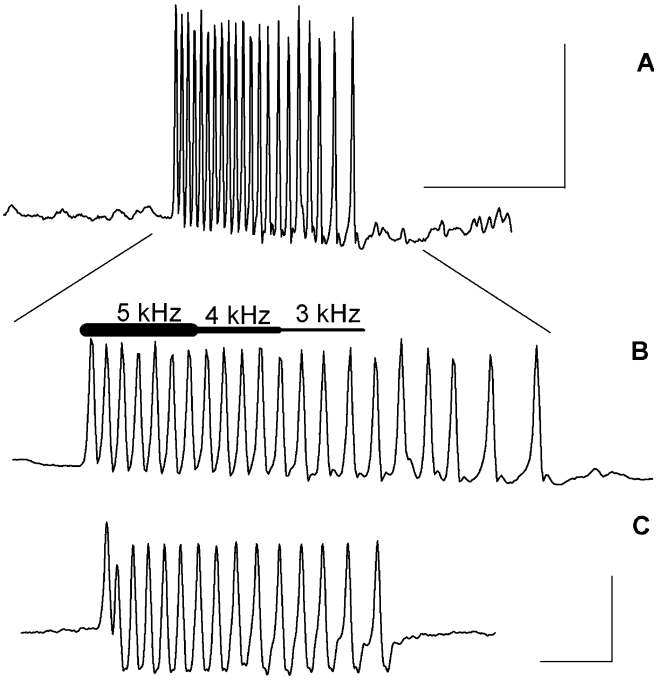

Fig. 2 Recording from seta #8 of *G. princeps* using an external recording techniques (**a**) and an internally positioned tungsten probe (**c**). **a** Time series of the action potentials recorded from a large distal seta in response to a 500 mm s^{-1} water jet (50 psi with the solenoid open for 13 ms). The stimulus was oriented such that the seta was stimulated to move distally. Scale bar represents 500 μV on the ordinate and 5 ms on the abscissa. **b** Expanded view of the impulse train. Note the high frequency response (3–5 kHz) was sustained for over 3 ms. **c** Recording from the same seta using a tungsten electrode (2 MΩ: FHC) and a silver differential electrode inserted in the proximal region of the antennule near the head. Scale bar represents 500 μV on the ordinate and 1 ms on the abscissa

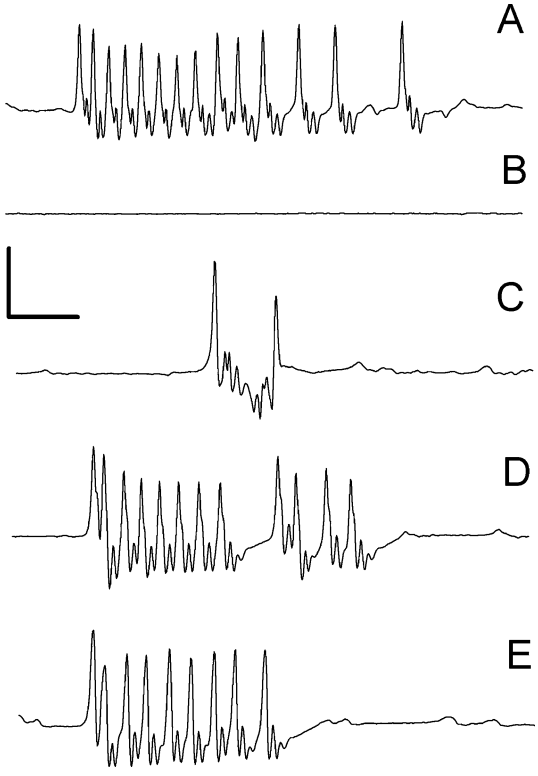

Fig. 3a–e A time series of the neural response of an individual seta of *G. princeps* to direct mechanical stimulation when challenged with TTX. Recordings were made every 2–4 min with the antennule lowered into the water bath for wash-out between stimulations. Scale bar represents 500 μV on the ordinate and 2 ms on the abscissa. **a** The response of the seta #7 to direct tactile stimulation. **b** The response of seta #7 after applying tetrototoxin (TTX: 10^{-8} M; Sigma) directly to the amputated side of the antennule. No response was recorded for the first 18 min of washout. **c** Neural response after 20 min of continuous washout where activity returned at a significantly lower frequency than during the pre-TTX recordings. **d** and **e** At 30 and 36 min the instantaneous firing frequency resumed pre-TTX levels. These recordings were taken using a tungsten electrode inserted into the antennule as previously described

to the surrounding fluid and the larger its velocity. The stimulus-response function depicted in Fig. 4 indicates that *Gaussia* mechanosensory neurons are able to distinguish different naturally occurring fluid disturbances.

Discussion

Detecting prey or avoiding predators is fundamental to the survival of organisms, and many animals derive a selective advantage by increasing their ability to rapidly evade predators or attack quickly moving prey. The pressure for rapid responses has often led to special mechanical and neurophysiological designs (Alexander 1995) to increase behavioral speed and diminish response time, which have evolved independently across numerous taxa (Gronenberg 1996). For example, large-diameter nerve fibers in numerous invertebrate species, such as the giant squid (Young 1939), crayfish (Wiersma et al. 1953) and cockroaches (Palka et al. 1977), provide decreased behavioral response times through increased conduction rates. Similarly, many vertebrates, and some evolutionary lines of invertebrates such as copepods (Davis et al. 1999), have independently evolved myelin or myelin-like structures surrounding nerve cells to rapidly conduct sensory or motor information.

In copepods, predatory, escape and mating behaviors all require extremely short latencies in conjunction with rapid behavioral acts. Errors, particularly in regard to predator avoidance, clearly suffer strong selection pressure. Premature or unnecessary escape reactions are metabolically expensive (Alcaraz and Strickler 1988) and attract the attention of both visual (Brewer and Coughlin 1996) and mechanoreceptive predators. Copepods also must be able to process sensory information rapidly and quickly determine whether to attack the source of the stimulus, escape from it, or attempt to mate with it.

Differences in sensor morphology along the antennules have been shown to be fundamental to the ability of copepods to detect complex flow structures with spa-

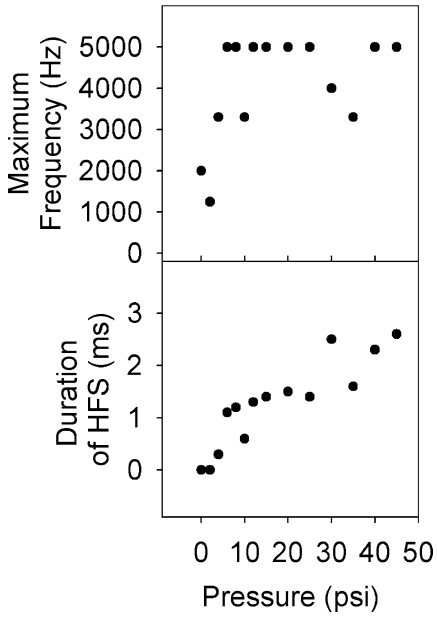

Fig. 4 Characteristics of the action potential train in response to increasing signal strength. Varying pressure behind the solenoid valve (2–50 psi), while maintaining the solenoid remained open for 13 ms, controlled the velocity of the fluid signal. Fluid velocity at the setae ranged from approximately 1 mm s^{-1} up to 500 mm s^{-1}. A stimulus-response function to fluid velocity was apparent in both the maximum instantaneous frequency (*upper panel*) and in the duration of the HFR (> 2,500 Hz) (*lower panel*)

tially variable velocity components. The antennules of *G. princeps* consist of 23–24 free segments with segments X and XI partly fused. With the exception of the distal tip, each segment is adorned with one to three sensory structures each singly or dually innervated (Weatherby and Lenz 2000; Fields et al. 2002). Fields et al. (2002) showed that different sensors are preferentially tuned to detect particular ranges of fluid velocities. Copepods encode fluid velocity and the spatial structure of the velocity field by the activities of their sensory neurons (Fields and Yen 2002) along their cephalic appendages. Variations in the magnitude of the stimulus velocity are coded for in the firing rate of the individual sensory neuron (Fields et al. 2002, this work), whereas its spatial properties including direction are likely to be derived from temporal changes in the firing rate and the relative timing of discharge of adjacent sensors (Erulkar 1972; Fields et al. 2002). Furthermore, by comparing the relative timing of discharge of adjacent sensors, a single action potential from multiple neurons can provide information concerning the spatial pattern of a signal if there is a temporal difference in their onset time. However, without an independent measure of size, a relatively large but slow moving fluid disturbance that excites adjacent setae with a given lag time would be indistinguishable from a small rapidly moving disturbance that also stimulates adjacent setae with a similar delay. As a result of this ambiguity, a single sensory neuron must be able to code for differences in fluid velocity or some other aspect of the intensity of the fluid motion, whereas a simultaneous measurement from different mechanoreceptors provides information concerning the spatial properties of the flow.

In copepods, the ability to discriminate different fluid velocities is limited by the interplay between the maximum firing rate, neuronal transmission speed and the minimum behavioral latency period that the animal can have and still remain ecologically viable (i.e., capture prey and escape predators). With the recent discovery of myelin-like structures within the antennules of some copepods (Davis et al. 1999) and the short distances over which the nerve signals must travel, the calculated transmission time is small compared to the observed latency periods of most behavioral responses (Weatherby et al. 2000). This suggests that the minimum reaction time is governed by the firing rates in individual cells. Thus, neurons with relatively slow firing rates can support only behaviors requiring little information concerning stimulus parameters or those behavior patterns with relatively long latency periods. Rapidly firing neurons are required in order to encode accurate intensity measurements within a relatively short period. This is an important point given the short (1–5 ms) behavioral latency periods reported for copepods.

The data presented here provides the underlying mechanism for how copepods are able to distinguish among stimuli and still exhibit such short behavioral latencies. A firing frequency of 5,000 Hz enables a single sensor to potentially encode a great deal of information about the fluid motion even within 1–2 ms. The ability to discriminate different fluid disturbances increases if the behavior has a longer latency or if animals use more complex algorithms (e.g., those based on instantaneous nerve impulse frequencies that vary within the nerve impulse train).

Gaussia mechanosensory neurons show a graded response to stimulus variation (pressure) that mimics natural variation in biologically relevant fluid disturbances (Yen 2000). The duration of the high frequency response, and to a lesser extent, instantaneous firing rate, both vary with the force driving the water motion (Fig. 4b). The observed graded response along with the rapid firing rates support the hypothesis that, in *Gaussia* mechanosensory neurons, sufficient information is relayed rapidly enough to code for stimulus intensity within the short behavioral latency period. Once the firing rate peaks, information pertaining to the stimulus strength is still provided by the duration of the high-frequency response (HFR; Fig. 4b). Since increasing the force (pressure) driving the water jet will simultaneously change several aspects of the resulting disturbance (velocity, acceleration, duration), further study on the linkage between fluid motion and setal motion is required to determine how differences in fluid motion are encoded by the physiological response.

Firing rates of 5,000 Hz exceed previously reported values for copepods by an order of magnitude (Yen et al. 1992). However a re-analysis of data from Yen et al. (1992: Fig. 4) and Lenz et al. (2000), suggest that me-

chanosensors in *Acartia fossae* and *Pleuromamma xiphias* may also discharge at instantaneous spike frequencies exceeding 1,000 Hz, and that mechanosensors in *Labidocera madurae* and *Undinula vulgaris* are capable of generating the very-high-frequency responses reported in this study. Although rapid firing rates have been reported in other animals, primarily vertebrates (Feng 1991; Brumberg et al. 2000; Raman and Bean 2001), our values are roughly two to seven times higher.

The rapid firing rates observed here are likely the result of the ecological pressure to perform quick behavioral responses, and partially resolve how these plankters receive adequate information on which to base behavioral decisions. Similar to other arthropod mechanosensors (e.g., spider slit sensilla) and most mechanoreception systems, the events recorded in this study most likely are initiated through mechanically gated ion channels (Hudspeth 1989; Weatherby and Lenz 2000) and propagated along the axon by highly selective Na^+ channels (Höger et al. 1997). The underlying physiological or anatomical solutions that permit such rapid spike frequencies, while seemingly based on general processes found throughout other organisms, require further studies.

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