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MECHANORECEPTION IN ZOOPLANKTON FIRST ANTENNAE: ELECTROPHYSIOLOGICAL TECHNIQUES

Donald V. Gassie, Petra H. Lenz, Jeannette Yen and Daniel K. Hartline

ABSTRACT

We describe methods for delivering calibrated mechanical displacements to antennal mechanoreceptors in zooplankton while simultaneously recording related neural traffic. Mechanosensory neural responses to small water displacements (0.01–1 μm) were studied over a wide frequency range (30 to >3000 Hz) Receptors could be localized and properties (thresholds, phase locking, habituation) examined. These methods have been tested on calanoid copepod first antennae (antennules), but may be suitable for other preparations. Extracellular recordings are made by holding the animal in stainless steel forceps and raising it into a layer of mineral oil, leaving one of the first antennae projecting into the underlying sea water. Nerve impulse traffic is recorded between the forceps and a chlorided silver wire in the seawater. Antennae are stimulated by water displacements produced by a vibrating sphere attached to either an electromagnetic or piezoelectric transducer. A fiberoptic sensor continuously monitors displacement. A computer-controlled waveform generator and amplifier drive the transducer with various frequencies, amplitudes and waveforms. The amplified sensor output and neural activity are digitized for later analysis.

Behavioral and morphological studies have indicated that mechanoreception may be critical to pelagic copepods for prey detection, predator avoidance and mate recognition. Sensory capabilities of copepods and other zooplankton have been inferred from behavior (Giguère and Dill, 1979; Haury et al., 1980; Buskey, 1984; Kirk, 1985; Strickler, 1985; Gill, 1985) because their small size has limited study of the underlying neural mechanisms. The electrophysiology of sensory mechanisms in larger, benthic crustacea (mostly decapods such as lobster and crayfish) has been successfully studied (Breithaupt and Tautz, 1990; Bush and Laverack, 1982 (review)). Structural evidence (Strickler and Bal, 1973; Friedman, 1980; Yen et al., 1992; Lenz and Yen, 1993) suggests similar mechanisms exist in copepods, but it is not yet known whether they operate in a like manner.

Here we present a method for recording extracellular nerve impulses in calanoid copepod first antennae. Mechanoreceptor neural activity was elicited by water movements caused by a vertically oscillating, transducer-driven sphere (computer-controlled). A displacement sensor continually monitored actual transducer motion. Both neural activity and sensor output were digitized and stored on a computer system for display and later analysis. Although this particular methodology has been used exclusively with copepods, some of the techniques described may have more general application, e.g., other small aquatic arthropods, other appendages, or other functions (chemoreception, motor systems).

This paper is organized into three major topics: the computer, stimulating, and recording systems. For each section there is a discussion of general requirements, and the specific implementation that we have chosen.

COMPUTER SYSTEM. Used to coordinate the stimulation and recording systems, and for post-experiment data analysis.

STIMULATING SYSTEM. Used to create a stimulus waveform and output it to a stimulating device which provides calibrated water displacements for mechanical stimulation.

RECORDING SYSTEM. Used for positioning the copepod antenna and for acquiring,

amplifying, and digitizing nerve impulses for display and analysis by the computer system.

COMPUTER SYSTEM

General Considerations.—Our initial studies (Yen et al., 1992) were done with only a function generator, digital oscilloscope and flat-bed plotter. However, it became apparent that the large volume of data obtained would require a digital computer for efficient analysis. Given the computer, it was then logical for it to control stimulus-delivery and data acquisition hardware, allowing direct measurement of signal parameters. We chose to do this in real time (rather than offline from tape-recorded data) to save steps and have the data readily available for consideration as the experiment progressed. Many standard microcomputer systems (e.g., IBM or MacIntosh) would be suitable for the general requirements of this methodology. Perhaps more critical is the software available for producing stimuli, running the experiment and analyzing data.

Implementation.—We chose an available IBM PC-AT clone system (which limited other choices to compatible hardware and software). The main experiment-control software was CSCOPE, written by Dr. Bradley R. Jones, and already in use for neurophysiology research in our laboratory. The CSCOPE menu was used to control the attached Scientific Solutions TL-125 data acquisition hardware. It could be used for initiating experimental protocols and storing the data for later display and analysis. It provided the synchronizing pulses for sending a waveform to the transducer or starting data acquisition by the A/D converter. A second software package written by Dr. Jones, ANAPLT, converted data files into ASCII for use by our own analysis programs (written in BASIC).

STIMULATING SYSTEM

Stimulation requires the generation of suitable electrical waveforms to drive a transducer that accurately moves a sphere or other stimulus (Fig. 1). Actual displacements can be monitored in real time or inferred from pre- and post-experiment calibration of the stimulating system.

Waveform Generation.—GENERAL CONSIDERATIONS. A variety of stimulating waveforms are necessary for studying the full range of copepod receptor properties. A system for providing this can be implemented in one module or in several, inside the computer or separately. For example, software might be used to design all waveforms, store them in the computer, and selectively output them via a D/A converter (the only external hardware needed). At the other extreme, all waveforms might be created and stored within an external, special-purpose function generator which would need only a manual or computer-generated start pulse to output a selected waveform. The latter reduces potential conflicts among subsystems controlled by the computer, but may also be less flexible.

COPEPOD MECHANORECEPTION CONSIDERATIONS. Evidence indicates that copepod antennal mechanoreceptors are sensitive to vibration frequencies in the kilohertz range (Yen et al., 1992). A suitable stimulator needed to produce mechanical displacements of substantial amplitude (microns) at frequencies up to 5 kHz. It was also necessary to stimulate at less than 50 Hz, to simulate slower mechanical signals, e.g., those associated with appendage beat rates, or flow-field changes. Thus, waveform generation required an accurately timed sequence of voltages, changing rapidly enough to represent sine waves of several kilohertz

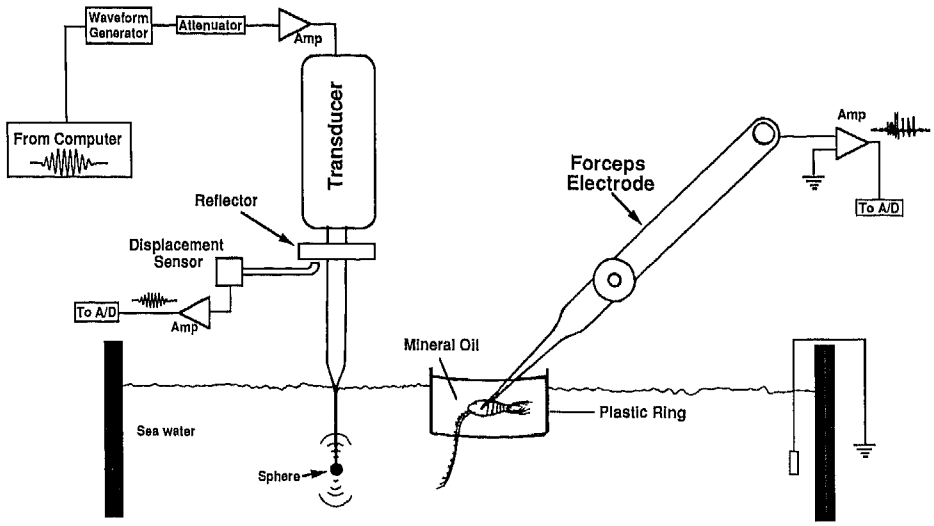

Figure 1. Schematic diagram of the major components of the experimental set-up. Fluid displacements are generated by a vertically oscillating sphere (attached to transducer). Waveforms generated by the computer are downloaded into a waveform generator, then scaled and amplified to drive the transducer. Transducer movement is monitored at the reflector by a fiberoptic displacement sensor. The amplified sensor output goes to an A/D converter, and the resulting digitized signal is then stored in the computer. The copepod is held in a stainless steel forceps electrode, with only the antenna projecting into the seawater. The neural activity from the forceps electrode is amplified and then digitized for computer storage.

(20–40 points per cycle was desirable, although with suitable filtering, fewer points would suffice).

IMPLEMENTATION. We used a mixture of computer-dependent and stand-alone hardware, reflecting availability of equipment, costs and time constraints. The central piece of hardware was an R4000 arbitrary waveform generator (Rapid Systems, Inc., Seattle, WA), which accepted a downloaded waveform from the computer and then output it in response to a gate pulse from the data-acquisition program.

DESIGNING A WAVEFORM. Waveform characteristics were specified using PIPER a program written in QuickBASIC® by K. Raquel Sanborn. This information was downloaded into the R4000, which can store 16,000 data points.

WAVEFORM OUTPUT. In response to a gating pulse from the CSCOPE software, the R4000 clocks out a stored waveform on its D/A converter at a rate set by the PIPER software. Because a stored waveform has a fixed number of points, output rate depends on waveform duration. For example, a 100-ms (duration) tone pip of 5 kHz, required 160 points per cycle, which was ample for accurate waveform representation.

Waveform amplitude is frequently varied during an experiment. Although the R4000 output amplitude can be changed at the computer keyboard, we found it more convenient to scale a fixed output with a precision manual attenuator. The R4000 output could then be set to its highest level (10V) in order to maximize signal size relative to noise level (around 1mV).

TYPICAL WAVEFORMS. Figure 2 shows examples of waveforms used to study neural phenomena such as threshold, adaptation, and phase-locking (Lenz and Yen, 1993). Figure 2A shows a tone pip (a sine wave which is amplitude-mod-

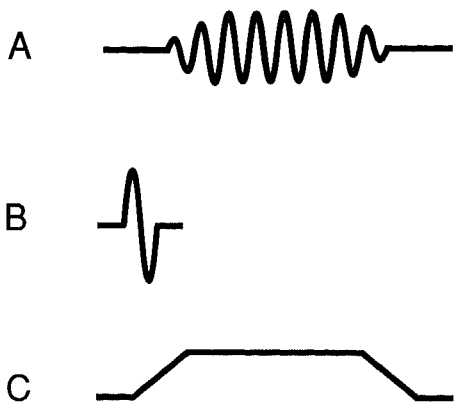

Figure 2. Examples of waveforms used for stimulation. A. Ramp changes in tone pip amplitude minimize high frequency overtones which might interfere with threshold determination. B. The single cycle is used to determine response latencies. C. The trapezoidal shape provides constant velocity during ramp periods (for the piezoelectric transducer).

ulated by a trapezoidal function), with its gradual increase and decrease in amplitude on either side of a period of constant amplitude. This waveform minimizes higher frequency components associated with abrupt changes (e.g., Wiese and Marschall, 1990). Frequency, duration and envelope shape can be specified.

A single sine wave cycle (Fig. 2B) can be used for latency measurement. A third useful waveform consists of a trapezoidal function (Fig. 2C), which gives constant-velocity movement during the "on" and "off" ramps (with piezoelectric transducers). In addition to the downloaded waveforms from PIPER, the R4000 also has built-in waveforms (e.g., continuous sine waves) which can be accessed from the PIPER menu and used to test and calibrate the system.

Stimulator

General Considerations.—The stimulator system must accurately transduce the electrical waveform into mechanical motion, to generate displacements that can be sensed by mechanoreceptors. The stimulator must contain at least three components: a transducer, a matching amplifier and a means (such as a sphere) for delivering the displacements to the receptor (Fig. 1). Additionally, if distance between stimulator and preparation is to be varied, a positioner is required.

Two types of transducers are readily available and suited to moving a sphere under computer control: moving-coil electromechanical devices (e.g., Coombs and Janssen, 1990) and various types of piezoelectric devices (e.g., Corey and Hudspeth, 1980). The moving-coil type can usually give larger displacements. The associated power amplifier must be able to handle inductive loads. Piezoelectric transducers usually have a higher frequency response. They require a high-voltage amplifier that can handle capacitive loads. The manufacturer of the transducer often sells an amplifier matched to the device.

A sphere, attached to the transducer by a narrow-diameter, rigid shaft, was used to create a dipole pattern of water displacement for stimulating mechanoreceptors (e.g., Coombs and Janssen, 1990 in fish lateral line, or for theoretical considerations, Kalmijn, 1988). When the sphere is displaced vertically, it produces water movement (near-field displacement) which falls off as the cube of distance (Bergeijk, 1967):

$$d_r = Da^3(\cos\theta)/r^3 \quad (1)$$

$$d_\theta = Da^3(\sin\theta)/2r^3 \quad (2)$$

where d_r is the radial component and d_θ is the tangential component of displacement and θ is the vertical angle. D, a and r are the sphere displacement, radius and distance, respectively. Some alternatives to a sphere include a human hair (Gill, 1985) or wire in contact with individual hairs (Yen et al., 1992), a water jet (Saunders and Szymko, 1989), and movement of the antenna itself relative to a stationary environment (Yen et al., 1992).

Implementation.—We have used both moving-coil (Ling Model 203) and piezoelectric (Burleigh PZL-15) transducers. Use of both types gives a capability for working around resonance frequencies specific to one type and allows for stimuli of sufficient amplitude in the range of 30 Hz to >4 kHz. The moving-coil transducer was driven by a low-noise high fidelity audio power amplifier (Proton AA-1150). The piezoelectric transducer was driven by a Burleigh PZ-150 amplifier, designed for a capacitive load and also capable of providing a voltage bias (e.g., +50V) needed by the transducer.

The stimulator hardware was mounted on a vertical positioner, which was itself mounted on a horizontal positioner. This arrangement allowed for adjustment in x, y, and z axes. We have used several types of stimulator end-pieces to generate displacements. In Lenz and Yen (1993) a 3-mm diameter plastic sphere mounted on a narrow shaft (Fig. 1) was used to produce dipole water displacements at variable, calibrated distances from an antenna. Distance between the sphere and the antenna was measured by using an eyepiece reticle in the microscope (r in equations 1 and 2). In Yen et al., (1992) antennae were moved directly by attaching the forceps to the electromagnetic transducer to provide receptors with motion relative to the stationary water movement. In some cases a stiff wire was used to directly stimulate an antenna or its setae.

Displacement

Displacement Sensor.—There are two general ways to determine the accuracy of transducer movement: 1) pre- and post-experiment calibration of the stimulator assembly and 2) direct monitoring of the stimulator during experimental runs. Ideally one would want to monitor the nanometer displacements of setae and/or water directly, but this is not always practical. Nanometer displacement gauges are usually based on capacitance, laser interferometry (Tautz et al., 1981), hot-film anemometry (Coombs et al., 1989) or light reflectance. In Yen et al., (1992), a capacitance-based device was used to calibrate the set-up prior to experimentation.

In Lenz and Yen (1993), the displacement was continuously monitored during experimental runs by a fiberoptic sensor (88N, Philtec, Inc., Arnold, MD) that measured light reflected from a polished target mounted on the shaft connecting the sphere to the transducer (Fig. 1). This instrument provided a linear change in voltage for displacements up to 33 μm, with a sensitivity of 21.2 nm/mV. The calibration chart supplied by the company was checked against a micromanipulator drive calibrated in microns, and found to be in good agreement (within 5%). The output of the monitor was amplified, A/D converted, and stored to provide a continuous, calibrated verification of vertical displacement for the entire record of neural activity.

Resonances.—Resonances in the stimulating system must be identified, then either

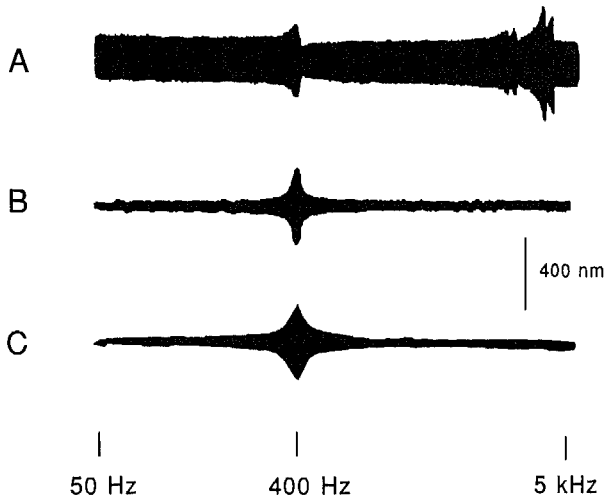

Figure 3. Example of resonance in a piezoelectrically-driven stimulator (50 Hz to 5 kHz, logarithmic sweep). A. Vertical motion (z-axis), fairly flat response except at 400 Hz and near 5 kHz. B. and C. Lateral movement (from same stimulus) measured at two points 90 degrees apart. Response at 400 Hz (same as vertical) indicates point of resonance, to be avoided during an actual experiment.

eliminated or avoided. The fiberoptic gauge used for monitoring vertical displacements during an experiment (Fig. 1) was also used offline to determine the extent of lateral excursions of the tip of the shaft during resonances. A cube-shaped target reflector (only slightly larger than the sphere) was attached to the end of the shaft. Lateral motion was measured in two perpendicular directions to allow computation of the lateral movement vector. Up/down movement was also measured for correlation with lateral movement at resonant points.

Figure 3 shows some typical resonance characteristics for our piezoelectric stimulator. A particularly large vertical resonance near 400 Hz was also associated with substantial lateral movement (Fig. 3). Stimulation frequencies near the resonances were avoided. Resonant points can be moved by altering a physical characteristic such as shaft length or sphere density.

Recording System

Preparation Arrangement.—Copepod Considerations. Copepods have very sensitive mechanoreceptors, so extraneous vibration must be minimized. A loosely mounted stimulator can generate resonances that propagate throughout the set-up. Footsteps, air movement from air conditioning ducts, outside traffic, and even loud talking can be above mechanoreceptor thresholds. Alternative routes for transmission of mechanical stimuli to the receptors from the stimulator must also be eliminated.

Implementation. The copepod was securely held in stainless steel forceps (Fig. 1), mounted on (but electrically isolated from) a micromanipulator to allow positioning in three planes. The antenna shaft was positioned parallel to the sphere motion to minimize the contribution of any lateral sphere movement and to eliminate the radial component of vertical sphere movement (see dipole equations 1 and 2, where equation 2 becomes: $d = 1.69D/r^3$, where r is measured in mm). We used an air-damped isolation table (Technical Manufacturing Corporation,

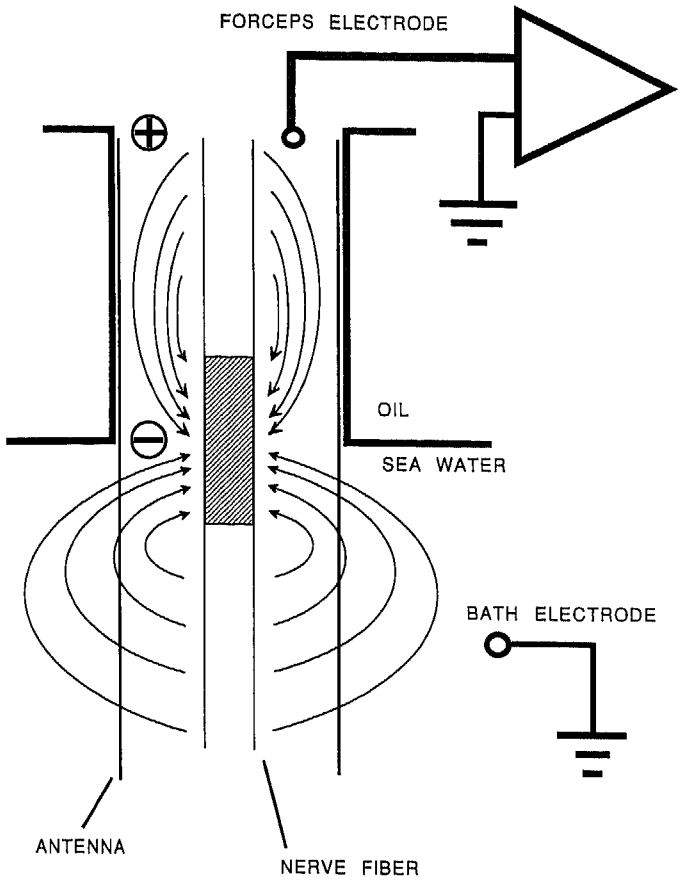

Figure 4. Schematic representation of current flow from an impulse in the antenna. The oil restricts current flow, reducing shunting and causing a voltage drop across the resistive extracellular medium. This leads to a positive swing of the forceps electrode with respect to the bath. The diagram is simplified, and does not incorporate the resistivity of the cuticle.

Peabody, MA; TMC Model 63-531-06) and sound dampening acoustic foam on surrounding walls to lessen environmental vibration and reduce spurious responses. The manipulator holding the forceps electrode and the seawater dish (Fig. 1) were attached to a steel base-plate, which was placed on shock mounts on the table. The stimulator was mounted on a separate shock-mounted plate. Control runs with the stimulating sphere in the air were made to ensure that only direct routes of transmission occurred.

Recording Neural Activity.—General Considerations. Two electrodes and a system for amplifying the signal and converting it to digital form (for use by the computer) are the basic requirements for extracellular neural recording. The electronic components necessary for this could be separate or combined in various ways, and they could be inside or outside of a computer. Shielding for electrical noise may be needed. Tape recorders, hard disks, chart records or other storage means are useful for post-experiment data analysis.

Implementation. Neural activity (Fig. 5) in copepod antennae was recorded

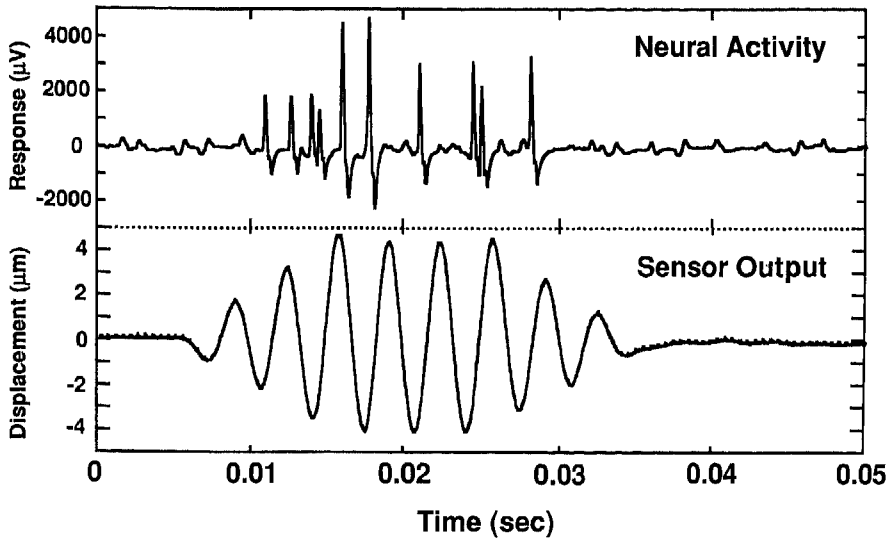

Figure 5. Example of antennal neural response in *Pleuromamma xiphias* to a 300 Hz pip stimulus from a moving coil transducer (recorded from displacement sensor). Maximum peak-to-peak fluid displacement at the antenna was ca. 70 nm.

by using the stainless steel forceps holding the copepod as one electrode and a chlorided silver wire bath ground as the other (Figs. 1, 4). A manipulator-mounted plastic ring was used to maintain a layer of mineral oil in the center of a sea-water bath. The copepod was attached to the forceps electrode, and then drawn up into the mineral oil. One antenna was allowed to protrude into the water below. Recording took place near the oil/water interface.

Currents entering the active site of an impulse generate voltage drops across the resistive extracellular medium, especially that restricted by the oil. This leads to a positive swing of the forceps electrode with respect to the bath as the impulse passes the oil-water interface, followed by a return to baseline and a slight undershoot or biphasic shape as impulse currents become completely confined to the antenna in oil (Fig. 4).

The recording site was varied by moving the antenna up or down through the oil/water interface. The wide range of spike amplitudes (<50 mV to 5 mV) was handled by an amplifier with switchable gains of 1,000 and 10,000. The amplifier output went to an A/D converter (Scientific Solutions T1-125; Axon Instruments, Foster City, CA), which was triggered by the CSCOPE program to start converting either slightly before (for pre-trigger events), or simultaneously with, the stimulus output.

Converted data were monitored on a triggered storage oscilloscope and temporarily stored on a hard disk, where they were available for display on the computer screen by CSCOPE. Later, data were archived on a WORM-type optical disk.

REPRESENTATIVE DATA. Figure 5 shows records from the extracellular amplifier, along with simultaneously recorded output from the displacement gauge. The stimulus is a trapezoid-modulated 300 Hz sine wave causing a 9 mm (70 nm at the antenna) peak-to-peak displacement of the stimulating sphere. Background activity, consisting of small (<400 μV) spikes, is present both before and after

the stimulus. Four msec after the onset of sphere movement and 0.5 msec after the third peak (which may have triggered it), a 2 mV spike is fired. Thereafter, additional spikes are fired, often time- locked to successive peaks in the stimulus pattern. The varying amplitudes of spikes represent different sensory units and/or the superposition of simultaneously-fired spikes with smaller amplitudes. Data analysis programs are used to determine the firing time and amplitude of each spike as well as the timing and amplitudes of sphere displacement (Lenz and Yen, 1993).

Discussion

Because of their small size, copepods provide some challenges for electrophysiological research. The forceps electrode recording technique, adapted from other neurophysiological work (Welsh, et al., 1968) is a simple and easy method for recording nerve impulses from the antennae of these small crustaceans. We have recorded successfully from animals as small as *Acartia fossae* (1–1.5 mm prosome length; Yen et al., 1992), and recordings can be made from either detached antennae or the whole animal.

The dipole stimulus, adapted from lateral line work (Harris and van Bergeijk, 1962), has the advantage that it can be used to study the behavioral responses of free-swimming or tethered animals, as well as the sensory properties of mechanoreceptors. In addition to lateral line, the technique has been applied to studies of mechanoreception in benthic decapods (Taylor, 1968) and krill (Wiese and Marschall, 1990), but not to the smaller planktonic copepods.

In our application of the electrophysiological technique, we have been investigating the basic properties of the sensory hairs, e.g., threshold sensitivities, latencies, phase locking, habituation. Some of these applications are described elsewhere in this volume (Lenz and Yen, 1993). With little modification it should be feasible to: 1) correlate individual units with individual hairs; 2) record responses to chemical stimuli; 3) investigate possible interactions between mechanical and chemical stimuli; and 4) examine responses to "natural" stimuli such as other free-swimming copepods.

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ADDRESSES: (D.V.G.) (P.H.L.) (D.K.H.) *Békésy Laboratory of Neurobiology, Pacific Biomedical Research Center, University of Hawaii, Honolulu, Hawaii 96822;* (J.Y.) *Marine Sciences Research Center, State University of New York, Stony Brook, New York 11794-5000.*