DEVELOPMENT OF MECHANOSENSORY RESPONSES IN MYELINATED COPEPODS

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ABSTRACT

This thesis will examine the mechanosensory capabilities of juvenile copepods from an essential prey species in the North Atlantic food web, *Calanus finmarchicus*. An important underlying component of these interactions is myelination of axons associated with decreased response latencies, yet myelination has not been observed in electron micrographs of copepod nauplii. Electrophysiological recordings from juvenile copepods are presented and their analysis found that myelin is consistently present in CII and later copepodite stages (CI copepodids could not be obtained for recording). Two uni-directional mechanosensory units were distinguished in all developmental stages studied using a controlled hydrodynamic stimulus, while a third slower non-mechanosensory unit was observed. Adults were found to be the most sensitive to mechanosensory stimuli, with thresholds of water displacement (not velocity, as reported in other copepods) of less than a nanometer. While copepodids were less sensitive, much longer antenna:prosome ratios were observed in CII copepodids.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

μV = Microvolt

A1 = 1st Antenna

A/D = Analog to Digital converter (DAQPad, Texas Instruments)

A. hudsonica = *Acartia hudsonica*

A. tonsa = *Acartia tonsa*

Amp. = Amplifier

ANOVA = Analysis of Variance

Atten. = Attenuator

CI = CI Copepodid

CII = CII Copepodid

CIII = CIII Copepodid

CIV = CIV Copepodid

CV = CV Copepodid

C. finmarchicus = *Calanus finmarchicus*

C. pacificus = *Calanus pacificus*

dB = decibel

e. g. = exempli gratia

et al. = et alii

Expt. = Experiment date (month/day/year)

G. princeps = *Gaussia princeps*

Hz = Hertz

ℓ = liter

mm = millimeter

ms or msec = millisecond

nm = nanometer

P. xiphias = *Pleuromamma xiphias*

Spp. = Unclassified species within a genus

TTP = Time-to-Peak

U. vulgaris = *Undinula vulgaris*

PREFACE

This thesis begins with background information on how the life cycle of copepods relates to predator-prey interactions and physiological capabilities. Chapter 2 provides a thorough description of the methods utilized for electrophysiologically studying the mechanosensory capabilities of these exceptionally sensitive myelinated copepods. The purpose of Chapter 3 is to summarize the study in the form of a publishable research paper, while also briefly re-stating certain points. The data are presented and discussed in the final sections of Chapter 3.

CHAPTER 1. INTRODUCTION

Copepod Life Cycle

In the open ocean, calanoid copepods are the zooplanktonic crustaceans that link primary production of phytoplankton to the nutritional needs of planktivorous fishes. In particular, larval cod and herring have been shown to directly depend on *Calanus finmarchicus* (reviewed by Sundby, 2000), thus it is essential to thoroughly understand the underlying physiology behind these important predator-prey relationships. Predation pressure is a factor for all stages in the copepod life cycle. In particular, the mortality of eggs and naupliar stages is very high in broadcast spawners like *C. finmarchicus* (reviewed by Mauchline, 1998). Exposed to a diverse array of predatory threats, the success of juvenile copepods (copepodids) must also be considered as a critical component in understanding copepod population dynamics (Paffenhöfer, 1998; Eiane *et al.*, 2002). After hatching, *C. finmarchicus* first passes through six naupliar stages, during which structural changes take place in the musculature and nervous system, that lead to the 1st copepodid (CI) stage (reviewed by Marshall and Orr, 1955; Mauchline, 1998). During the copepodite development (CI to CV, then adult), the number of pereiopods (swimming legs) is increased, and external changes in the segmentation of the prosome, urosome, and antennae (Mauchline, 1998; Boxshall and Huys, 1998) have been observed, while developmental changes in the internal anatomy during development have not been thoroughly described (Marshall and Orr, 1955).

Ecological Interactions

Due to high predation pressures, copepods must specialize their escape responses in order to successfully escape from the primary types of predators preying upon them. At the same time, predators can selectively feed upon copepods of specific sizes, thus placing different developmental stages at risk of being preyed upon by different types of predators (reviewed by Paffenhöfer, 1998). As the characteristics of each developmental stage must be considered, one ontogenetic difference is that earlier copepodids have a more centralized mass within their shorter prosomes. This allows them to be more easily entrained in a predatory flow field with less bending of the mechanosensory antennae than a larger copepod; thus placing earlier copepodids at a mechanosensory disadvantage (Kiørboe and Visser, 1999).

Later developmental stages may instead be placed at risk due to their larger sizes. One case of size-specific predation pressure involves interactions with filter-feeding scyphomedusan jellyfish, in which jellyfish predation results in greater mortality for larger copepod species and later developmental stages (Suchman and Sullivan, 2000; Sullivan, 2000). However, Graham and Kroutil (2001) have shown that jellyfish with larger medusae have a greater capture efficiency for smaller copepods, thus copepodids are still at risk to this type of predation threat. Suggesting the possible importance of mechanosensory cues for the detection of jellyfish, Suchman (2000) demonstrated that copepodids were significantly more likely to actively escape from jellyfish that create more hydrodynamic disturbances. Suchman (2000) has also shown that *A. hudsonica* adults tend to actively escape from jellyfish, but younger copepodids are less likely to respond.

Despite the fact that adult copepods are capable of fast swimming movements and escape responses (e.g., Strickler, 1975), Waggett and Costello (1999) have shown that these movements can be exploited by lobate ctenophores. Adult *Acartia tonsa* were more likely to propel themselves into the oral lobes of the ctenophore by their own normal swimming movements, while the ctenophore also uses flow field entrainment to capture nauplii with the auricular feeding current. This demonstrates how a predator may exploit the weaknesses of different copepod developmental stages.

Much smaller and faster than jellyfish, mechanosensitive invertebrate predators such as predatory copepods and chaetognaths often selectively feed upon smaller copepods and nauplii (e.g., Yen, 1983). Furthermore, omnivorous copepods have been shown to prey extensively upon *C. finmarchicus* eggs and young nauplii (Sell *et al.*, 2001). For example, Caparroy *et al.* (2000) hypothesize that the perception distance of a cruising rheotactic predatory copepod (e.g., *Euchaeta elongata*) increases with prey size, yet the capture rate of copepod prey of later developmental stages is less than expected. This may be due to increased prey reaction distances and escape velocities accomplished by later copepodid developmental stages compared to younger stages. Capporoy *et al.* (2000) also predict that the feeding current of suspension-feeding, hovering predatory copepods is more likely to rotate a smaller copepod into a direction that enables a higher capture rate by the predator; thus placing earlier copepodids at even higher predation risk.

In contrast to the case for mechanosensitive predators, it is the later developmental stages that are placed at a higher risk for visual predators, since the greater size of the later stages enhances their visibility (reviewed by Giske *et al.*, 1994). In response to threats from visual predators, Capporoy *et al.* (2000) predict that the mechanosensory detection of fish larvae by copepods depends on the rate of approach of fish, thus placing great importance on mechanosensitivity in adults. Turbulence has been correlated with higher capture rates of *A. tonsa* by fish, suggesting that copepods may depend on still waters for successful escape responses (Mackenzie and Kiørboe, 1995; Clarke *et al.*, 2005). An exceptional mechanosensory processing ability could be beneficial in escaping such a predator, but previous research has not determined how copepods may determine the position of a threat. Furthermore, different predators may require different methods of processing this information. Mackenzie and Kiørboe (1995) have shown that the two commercially important predators of *C. finmarchicus,* cod and herring larvae (reviewed by Sundby, 2004), use two different feeding behaviors. Cod were observed pausing before attacking *A. tonsa*, while herring cruised continuously during foraging.

In addition to being responsive to mechanosensory cues, predatory threats can be reduced in several other ways. Chemosensory cues are utilized by some zooplankton; a phenomenon that is well-studied in *Daphnia* (reviewed by Lass and Spaak, 2003). Cieri and Stearns (1999) have shown that the copepods *A. tonsa* and *A. hudsonica* reduce feeding behaviors when exposed to exudates of a visual predator. In addition, photic cues may be utilized to avoid predators, as in photophobic responses to shadow stimuli (Buskey *et al.*, 1986; Buskey and Hartline, 2003).

In particular, a widespread tactic that is associated with reduced visual predation risk is diel vertical migration (DVM, reviewed by Hays, 2003). By spending the daylight hours in deep waters, zooplankton can avoid the light necessary for detection by visual predators. The amplitude (vertical distance traveled per day) of DVM in *Calanus* *pacificus* has been shown to depend on food density (Huntley and Brooks, 1982), showing that the threat of food depletion may outweigh the threat of visual predation in surface waters. Correlating with greater visual predation risks for adults, the DVM amplitude of *Calanus* increases during copepodid ontogeny (*C. pacificus*, Huntley and Brooks, 1982; *C. finmarchicus*, Eiane and Ohman, 2004). Supporting these explanations, Titelman and Fiksen (2004) used the need to compensate for an increased visibility as an explanation for the observed tendency of the larger stages of many copepod species to occur at greater depth, where they are less visible. An additional adaptation copepods use for decreasing visual predation is evolving a transparent body (reviewed by Giske *et al.*, 1994).

In terms of behavior, earlier stages may benefit more by avoiding the detection of mechanosensitive predators. These threats may be reduced by tactics such as minimizing movements of feeding appendages and pereiopods, as hypothesized by Jiang and Paffenhöfer (2004). In particular, attacks from predatory copepods have been shown to be triggered by quick 'hops' of the prey copepod (Yen, 1982; Yen, 1983). Demonstrating how this behavior may change during ontogeny of *Centropages velificatus* , Paffenhöfer (1998) observed that early copepodid stages swim continuously without using the quick hops that can be detected by predatory copepods. However, a rapid behavioral change takes place during the ontogeny of *C. velificatus*; resulting in an intermittent rapid swimming behavior in adults. Paffenhöfer (1998) suggests that the risks created by the quick hops of adults could be offset by the benefits of quiescent sinking, in which mechanoreceptive capabilities are not affected by noise created with continuous swimming.

Sensory Transduction and Escape Behavior

A rapidly attacking predator necessitates a quick and efficient escape. Accordingly, detection of near-field hydrodynamic disturbances is mediated by a distinct array of stiffened setae on the first antennae (A1) (Singarajah, 1969; Strickler, 1975; Gill, 1985; Yen *et al.*, 1992; Lenz and Yen, 1993), leading to escape jumps of up to 800 mm s^{-1} (unidentified cyclopoid, Strickler, 1975), with response latencies as short as 1.5 ms (*Undinula vulgaris*, Lenz *et al.*, 2000).

The morphology of the antennae relates to the sensitivity of the copepod, and this research focuses on examining the mechnosensitivity of several copepodid stages. One morphological factor is the size of the A1 in relation to the prosome. A longer A1 may provide a greater distance over which water deformations are transformed into relative movements with respect to the prosome, hence providing greater opportunity for detection (Haury *et al.*, 1980; Yen *et al.*, 1992). Along the length of the A1, mechanosensory setae are used for detecting hydrodynamic disturbances (Yen *et al.*, 1992). Previous threshold analyses (Yen *et al.*, 1992; Lenz and Hartline, 1999; Kiørboe *et al.*, 1999) have shown that the property of water movement most closely linked to triggering neuronal or behavioral responses in *Labidocera madurae*, *Acartia fossae, A. tonsa,* and *Undinula vulgaris* (closely related to *Calanus*) is water velocity along the A1. This differs from the sensitivity to displacement typically seen in receptors for hydrodynamic signals found in other crustaceans (e.g., crayfish, reviewed by Wiese, 1988). Also contrary to the uni-directional mechanosensory setae observed in crayfish, prior electrophysiological measurement has shown that the re-identifiable mechanosensory units within the copepod A1 are bi-directionally sensitive to stimuli (Yen *et al.*, 1992; Hartline *et al.*, 1996). Also differing from crayfish, morphological analysis has shown that mechanosensory setae of *P. xiphias* are dually innervated and attached on a single side (Weatherby and Lenz, 2000), rather than on opposing sides (reviewed by Wiese, 1988). Because of these unique characteristics, it has not yet been shown how a copepod can determine the location of a threat.

While mechanosensory thresholds for water velocity have been shown in other copepod species, previous behavioral analysis by Haury *et al.* (1980) was unable to determine whether *C. finmarchicus* was sensitive primarily to thresholds of water velocity or displacement, because the CIV and CV copepodids studied demonstrated mixed results. In this research, the sensitivity and directionality observed in mechanosensory units within the A1 of adult *C. finmarchicus* are described. However, the mechanosensory system may not be fully developed in early copepodid stages (e.g., Suchman, 2000). Accordingly, mechanosensitivity of copepodids was measured in order to determine whether changes independent of body size take place during ontogeny.

Neurophysiology

Within the calanoid copepods, electrophysiological evidence and microscopy has shown that a monophyletic group of superfamilies possesses a system of extensive myelination around the axons of the nervous system (Davis *et al.*, 1999; Lenz *et al.*, 2000; Weatherby *et al.*, 2000). These densely packed lipid membranes can increase conduction velocity by reducing the capacitive surface area of the axolemma, thus reducing the time necessary for charging the capacitive membrane and triggering the opening of voltage-gated channels at the nodes. The insulative myelin sheath also prevents the transverse loss of current during action potentials (Roots, 1984), thus increasing the axonal length constant. This reduction of ionic transfer between the axoplasm and extracellular fluid also reduces axonal metabolic costs up to 10,000-fold by decreasing the amount of pumping needed to maintain ionic balance within the axoplasm (Bullock and Horridge, 1965; Weatherby *et al.*, 2000).

The increase in conduction velocity due to myelination can be limited by the loss of current at certain points. The nodes of Ranvier are necessary for ensuring the propagation of the action potential without degradation, but allow the loss of ions in exposed regions, and also increase the capacitive surface area. Thus, minimizing the size of the node can increase conduction velocity. Rather than the large circumferential nodes of vertebrates, copepods possess focal nodes that limit the exposed region to only a portion on the side of an axon (Weatherby *et al.*, 2000). While ionic loss at focal nodes appears to be prevented by desmosome-like structures in earthworms and prawns (Heuser and Doggenweiler, 1966; Roots, 1984), copepods do not appear to possess these structures, and instead may restrict leakage by intimate association between the myelin membrane and axolemma (Weatherby *et al.*, 2000). Due to the way myelin is spirally wrapped within the internodal regions of vertebrate axons, longitudinal seam-lines may be another possible leakage point. However, these seam-lines have not been observed in the concentric lamellae of copepods (Weatherby *et al.*, 2000).

Reducing current loss contributes to rapid conduction velocity, but the minimal extracellular currents reduce the signal available for electrophysiological recording in myelinated copepods. As shown by a 10-fold smaller amplitude of nerve spikes observed in myelinated copepods (Lenz *et al.*, 2000), this differential amplitude has enabled the current research to infer the presence of myelin in early copepodids using electrophysiological techniques.

Ecological Benefits of Myelin

With the increased conduction velocity due to myelination, shorter behavioral response latencies are observed in myelinated copepods (Lenz *et al.*, 2000). While this decrease is only estimated at 2.4 milliseconds, Lenz *et al.* (2000) suggest that myelin may be a key advantage allowing myelinated copepods to successfully inhabit the bright surface waters of the open-ocean where the risk for visual predation is high. Large and energetically costly DVM cycles may also be minimized in myelinated copepods.

While extensive myelination has been found throughout the nervous system of adult copepods of the myelinated superfamilies (Weatherby *et al.*, 2000), electron micrographs have revealed little or no myelination in copepod nauplii (Davis and Weatherby, unpublished data). Thus ontogenetic differences in myelination may need to be considered in determining how neurophysiological capabilities can affect predator evasion tactics of early developmental stages, as studied electrophysiologically in the current research on copepodids and adults.

CHAPTER 2. DETAILED METHODS

Extracellular Recording

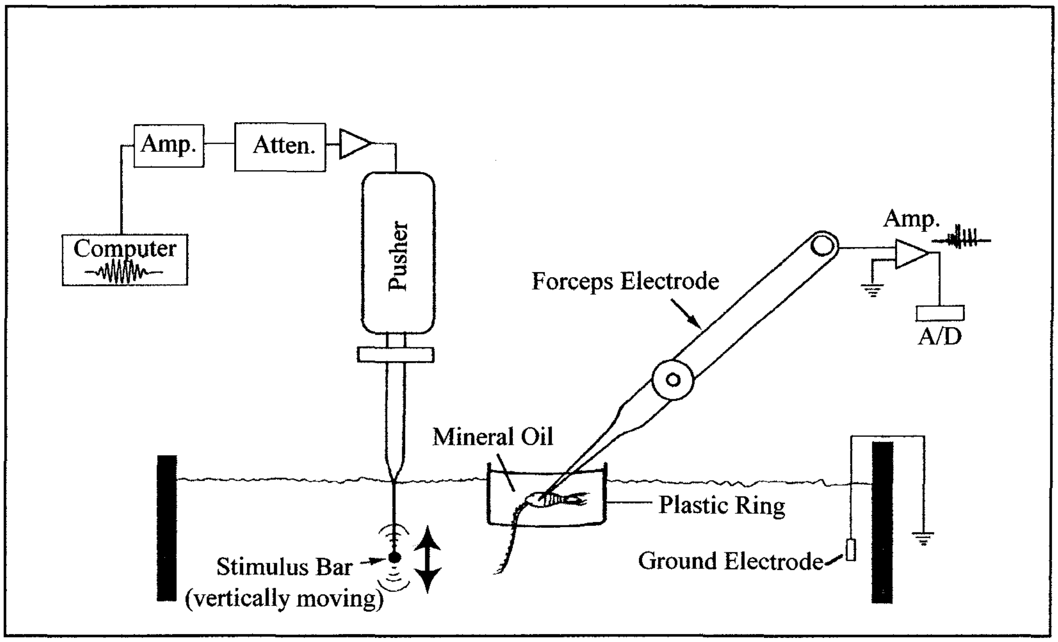

Figure 1. Diagram of Extracellular Recording (adapted from Gassie et al., 1993).

Extracellular recordings were taken from mechanosensory axons in the first antenna (A1), as described by Gassie *et al.* (1993), and shown in Figure 1. The animal is suspended in a layer of mineral oil and held by a forceps electrode, while the A1 protrudes through the oil-water interface into seawater below. The non-conductive mineral oil acts to restrict the extracellular electrical current from the axons to the conductive space inside the A1, effectively creating a large resistor and a measurable voltage drop between the forceps electrode (in electrical contact with the animal's body), and a silver-chloride reference electrode in the seawater bath. Any nerve impulses traveling through the oil-water interface will be recorded as a voltage reflecting current flow across that interface. An amplifier then compares the recorded signal to the electrical signal (noise) at the reference electrode and eliminates most noise from the extracellular recording through "common-mode rejection." Signals were digitized at a sampling rate of 80 KHz in order to discriminate minute differences in the rapid spike forms observed in copepods.

While myelinated copepods are very interesting due to their fast conducting axons and quick responses, electrophysiological study has been limited because the densely packed lipid membranes and minimized axolemma exposure make it difficult to detect extracellular electrical activity; thus spikes of 10-fold smaller amplitude are observed in myelinated copepods (Lenz *et al.*, 2000). Previous copepod neurophysiological research has thoroughly studied non-myelinated copepods due to the relative ease of obtaining a strong signal. By increasing amplification to 10,000-fold and thoroughly shielding the preparation from electrical interference, this research has been able to distinguish multiple units within the mechanosensory discharges of myelinated copepods. Electrical interference was minimized with the use of a Faraday cage constructed from aluminum screens, and by thoroughly grounding and shielding all conductive parts of the experimental apparatus that were not sufficiently shielded by the Faraday cage. This was sufficient for accurate recordings from all animals, except for the CII copepodid. In this case the animal was much smaller and less electrical current could be recorded; thus it was subjected to greater electrical interference than in other preparations. A single source of electrical interference with a regular pattern was then characterized and subtracted mathematically in the presented data from the CII copepodid.

Vibration Isolation

When dealing with the high sensitivity of *C. finmarchicus* to water displacements of less than a nanometer, vibrations of the preparation from numerous sources can introduce error by shaking the surrounding fluid and the forceps that hold the copepod in place. Extensive measures were taken to isolate the animal from vibration following a scheme of vibration dampening structures separated by objects of large mass. This enabled the vibrations to be dissipated within the flexible dampening structures, while the weakened oscillations cannot be easily transferred to the more massive objects above them. This technique was used at multiple points within the structure supporting the preparation. Beginning from ground level, coils of rubber heater hose separated three levels of concrete blocks. Rubber is compressible and allows dampening of vibrations, while the circular nature also allows compression to be absorbed as deformation of the tube. Above the concrete blocks, a Newport (Irvine, CA) benchtop compressed air table (resting on rubber stoppers and a solid sheet of rock) also isolates the preparation from residual vibrations by utilizing the much more compressible nature of gases. Atop the air table, precise rubber vibration isolators called Vibrapods (The Vibrapod Company, St. Louis) were utilized. Each Vibrapod provides a semi-circular base with a rubber thickness specified by the manufacturer to effectively isolate vibrations for supported objects within the weight range of the preparation. Four Vibrapods (Grade #4) supported a concrete block baseplate, upon which 4 Vibrapods (Grade #3) specialized for lighter weights supported the steel baseplate and preparation. While this series of extensive vibration isolation limited most sources of disturbance, mechanosensory responses could still be triggered by large disturbances (such as the rolling of a desk chair, or stomping on the floor). For this reason, activity within the lab was kept at a minimum, and recordings were not taken within 10 seconds of a known response of the copepod to unintended stimuli.

Hydrodynamic Stimulus

In order to quantify mechanosensitivity, a well-controlled and reproducible hydrodynamic stimulus was provided by using a Burleigh (Victor, NY) PZL-015 piezoelectric pusher to control a horizontal stimulus bar. The precise vertical movement of this stimulus bar (a cylinder) placed directly in front of the antenna provided an identical flow field in all planes perpendicular to the transverse axis of the cylinder.

Since the presumed mechanosensory setae on the distal tip of the A1 were positioned on a line through the center of the bar and perpendicular to the vertical axis of bar movement, the near-field water displacement at the tip could be calculated from a simplified dipole equation (Buskey *et al.*, 2000):

$$d_{\theta} = -a^2 D / r^2 \qquad (1)$$

where d is the water movement at a distance r from the center of a cylinder of radius a displaced vertically by a distance D (the negative sign indicates that the water moves in the direction opposite to that of the cylinder). A similar equation holds true for the magnitude of the hydrodynamic disturbance in terms of water velocity and acceleration, enabling tuning curves to be calculated for each component.

CHAPTER 3. SUMMARIZED RESEARCH PAPER

Introduction

The behavior and anti-predation tactics of copepod nauplii and copepodids can play a crucial role in the structure of oceanic food webs (Eiane and Ohman, 2004; Jiang and Paffenhöfer, 2004), yet ontogenetic changes in the mechanosensitivity and neurophysiology that underlie observed differences in the behavior of copepodids (e.g., Paffenhöfer, 1998; Suchman, 2000) have not been thoroughly examined. It has also not yet been determined how copepods can process directional information about a stimulus within the observed response times as low as 1.5 ms (e.g., *Undinula vulgaris*, Lenz *et al.*, 2000); however Fields *et al.* (2002) have suggested that differences in mechanical resistance to bending of setae can lead to directional sensitivity in the non-myelinated copepod *Gaussia princeps*.

Of particular interest is the composition of neural discharges in fast reacting myelinated copepods, which have not been previously studied thoroughly with electrophysiological methods (Lenz *et al.*, 2000). This paper will focus on describing the encoding of directional mechanosensory information in *Calanus finmarchicus*. This copepod species constitutes the major biomass component of mesozooplankton within the North Atlantic Subpolar Gyre, and is a key prey species for many commercially important fishes such as the Atlantic cod and herring (reviewed by Sundby, 2000). It has been thoroughly studied in many aspects (e.g., Marshall and Orr, 1955), with myelination of its nervous system having been first reported by Lowe (1935) and later confirmed with electron microscopic evidence (Barrientos, 1980; Lenz et al., 2000).

As shown by Eiane *et al.* (2002), variations in the types of predator assemblages can result in differential survival rates of *Calanus* spp. developmental stages. Larger copepods, including later stages of *A. hudsonica*, are more susceptible to filter-feeding scyphomedusan jellyfish (Sullivan and Suchman, 2000; Suchman, 2000), yet the behavioral tactics of adults can limit this mortality (Suchman, 2000). Adults are also at a higher risk from visual predators because of their size (reviewed by Giske *et al.*, 1994), while younger stages are at a greater risk from mechanoreceptive invertebrate predators (Yen, 1983; Eiane *et al.*, 2002). Jiang and Paffenhöfer (2004) have described trade-offs involved in species and stage-specific anti-predation behaviors, yet underlying physiological and anatomical changes during copepodid ontogeny have not been thoroughly described. It is necessary to determine how the mechanosensory capabilities may change during ontogeny, thus leading to results such as those observed by Suchman (2000) in which *A. hudsonica* copepodids were less likely than adults to actively escape from a scyphomedusan jellyfish. In the current research paper, the sensitivity of several *C. finmarchicus* copepodid stages was determined with a controlled hydrodynamic stimulus in order to examine how the thresholds of sensitivity change during ontogeny. These results will be correlated with morphological analysis, suggesting how copepodid morphology may be adapted to maximize sensitivity to natural stimuli.

Underlying behavioral and morphological adaptations, the neurophysiological capabilities of mechanosensory axons must be considered. Non-myelinated copepod mechanosensory axons are capable of exceptional firing rates of up to 5 KHz in response to extremely strong stimuli; thus potentially contributing to the rapid processing of sensory information (Yen *et al.*, 1992; Fields and Weissburg, 2004). Demonstrating further neurophysiological adaptation, extensive myelination has been observed throughout the nervous system in a monophyletic group of calanoid copepod superfamilies (Davis *et al*., 1999; Lenz *et al*. 2000; Weatherby *et al*., 2000). Myelination is correlated with shorter response latencies than found in non-myelinated taxa (Lenz *et al*. 2000), therefore predicting an advantage in predator evasion. However, the limited current loss provided my myelination has limited electrophysiological study of myelinated copepods, as shown by the 10-fold smaller amplitude nerve spikes observed in these species (Lenz *et al*., 2000).

While myelin can enable substantially lower metabolic costs of maintaining ionic balance within the axoplasm of the adult copepod (up to 10,000-fold, Bullock and Horridge, 1965; Weatherby *et al*. 2000), the developing copepod must invest energy in developing the multiple layers of densely compacted myelin. Increased conduction velocity may not be as beneficial at an early developmental stage if the risk of visual predation pressure is lower due to small body sizes (see Giske *et al*., 1994; Eiane *et al*., 2002). Furthermore, transmission electron micrographs have shown that the naupliar stages possess little or no myelination (Davis and Weatherby, unpublished data), but extensive myelination is present throughout the nervous system of adults (Weatherby *et al*., 2000). This paper will present evidence of early myelination of mechanosensory axons in the copepodid stages of *C. finmarchicus* through the use of electrophysiology.

Materials and Methods

Collection and Culture

Specimens of *Calanus finmarchicus* were collected with oblique net tows in off-shore waters of the Gulf of Maine (near Mount Desert Rock; 43° 58' N, 68° 8' W) on June 10th, 2004 with a 1.0 m diameter, 500μm mesh plankton net between 100m and the surface. Animals obtained were of CIII copepodid stage or larger. In the lab, *Calanus* spp. were sorted into jars filled with fresh seawater, kept in constant dark, and refrigerated at 4 to 8 °C. Animals were gently transferred with ladles and never poured. Culture jars were kept at an approximate density of 5 animals per liter in 1.9 ℓ, 3.8 ℓ, and 9.5 ℓ containers. Cultures received 50% water changes weekly, and were provided with a concentrated solution of frozen *Tetraselmis* spp. (Aquatic Ecosystems). Responsive individuals with intact antennae were utilized for physiological experiments. In addition, eggs and nauplii were removed from the bottom of culture jars and placed in 3.8 ℓ jars of fresh seawater at 11 to 13 °C with natural sunlight. After approximately 2 weeks, CII copepodids were present. A single responsive CII copepodid was thoroughly examined both electrophysiologically and morphologically, while a second CII copepodidid was only utilized for measurements of A1 and prosome length.

Extracellular Recording

As shown by Figure 1, the glass experimental aquarium (10 x 10 x 5 cm) contained glass microfibre filtered seawater and was cooled to 7-9 °C (11 to 13 °C for CII copepodids) by cold water passing through an external cooling jacket. Due to the high sensitivity of copepods to mechanical disturbance, the experimental apparatus was isolated from vibration by using a compressed air vibration isolation table (Newport, New York) and vibration isolation pods (The Vibrapod Company, St. Louis). Extracellular recordings were made from the A1 of the copepod following the methods described by Gassie *et al.* (1993). Briefly, the prosome was held in place by a forceps electrode, and then drawn upward into a layer of mineral oil with a single antenna protruding downward into the seawater bath. A silver-chloride electrode in the seawater bath provided a ground reference, and the neural activity was recorded from the oil-water interface. This potential difference was amplified by a factor of 10,000, sampled at 80 KHz by a DAQPad 6070E interface (National Instruments), and stored on an IBM-PC compatible computer running LabVIEW 5.1 (National Instruments). Electrical interference was minimized with the use of a Faraday cage constructed from aluminum screens.

Hydrodynamic Stimulus

As described by Buskey *et al.* (2002), a hydrodynamic stimulus was provided by the brief movement of a cylindrical plastic bar of 3 mm diameter positioned 7 to 15 mm in front of the distal tip of the antenna. The stimulus bar was moved in the vertical direction by a Burleigh (Victor, NY) PZL-015 piezoelectric pusher specified by the manufacturer to follow impressed voltages at frequencies up to 3.5 KHz, powered by a Burleigh PZ-150 Amplifier under computer control with LabView software (National Instruments, Austin, TX, with PAcquire-manual program written by Dan Hartline). Vertical displacement calibration was verified using a Philtec (Annapolis, MD) Model D63-A260 fiber-optic displacement sensor.

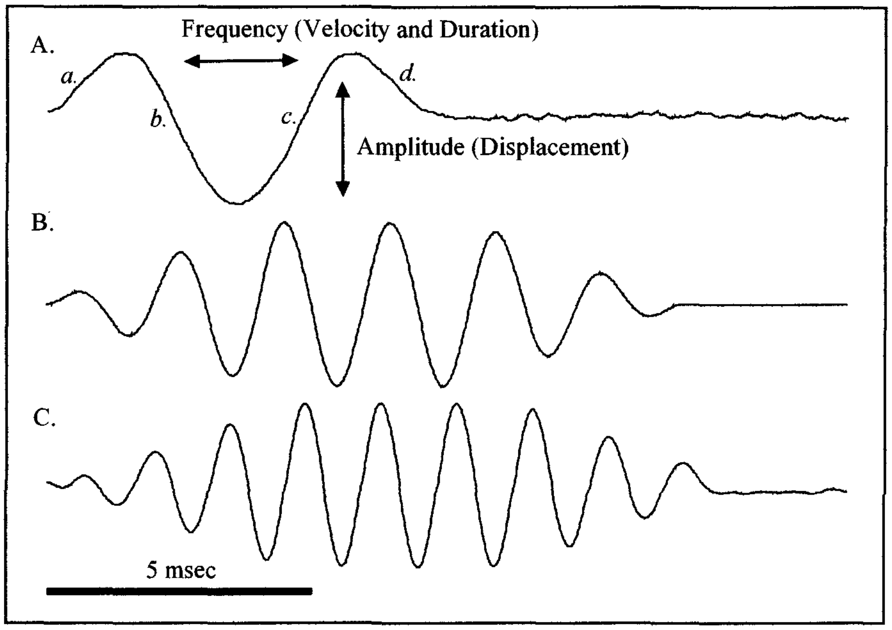

Figure 2. Trapezoidally modulated waveforms indicating the vertical position of the stimulus bar. A.) 1.5 cycle 200 Hz stimulus used for threshold analysis. Arrows indicate the characteristics adjusted to determine mechanosensory thresholds. *a.* corresponds to an initial minor displacement in the distal direction along the A1, *b.* shows the major displacement in the proximal direction, *c.* shows the major displacement in the distal direction, and *d.* shows the minor displacement in the proximal direction. Owing to trapezoidal modulation, peak-to-peak amplitudes for 1.5 cycle waveforms are only 80% of full sine values. B.) 6 cycle 500 Hz stimulus used to study CII copepodid directionality, and C.) 9 cycle, 700 Hz stimulus used to study directionality in other specimens.

The typical stimulus used in threshold experiments was a trapezoidally modulated 1.5 cycle sine wave (See Figure 2), in which the modulating waveform rose linearly for 0.4 cycle, remained constant for 0.7 cycle, and decreased linearly to zero for the remaining 0.4 cycle. The stimulus amplitude was adjusted with a logarithmic attenuator (Hewlett-Packard 350D Attenuator Set, Palo Alto, CA) in 1 dB steps referenced to a peak-to-peak sinusoidal amplitude of 10 µm. The stimulus was adjusted in terms of duration and water velocity by using frequencies ranging from 200 Hz to 2.5 KHz. For determination of directionality, a 9 cycle trapezoidally modulated sine wave with 3 cycle rising and falling phases was provided at a frequency of 700 Hz with at least 10 repetitions, and a 6 cycle 500 Hz stimulus was used for a single CII copepodid with 78 repetitions.

Near-field water displacements surrounding a cylinder of radius *a* moved a distance D in the $\theta = 0$ direction perpendicular to its longitudinal axis, for all planes perpendicular to that axis, can be computed from the dipole equations given by Buskey *et al.* (2000):

$$d_r = a^2 D \cos(\theta) / r^2 \quad (1)$$

$$d_\theta = -a^2 D \sin(\theta) / r^2 \quad (2)$$

where d_r and d_θ are the radial and tangential components of water movement. For all experiments, the stimulus bar was placed directly in front of the mechanosensory setae at the distal tip of the A1 presumed to trigger the largest spikes as well as behavioral escape reactions (Gill and Crisp, 1985; Yen *et al.*, 1992; Lenz and Yen, 1993). At this location, $\theta = 90°$; $\sin \theta = 1$; $\cos \theta = 0$, so from Eqs. (1) and (2), water movements were tangential only, given by:

$$d = -a^2 D / r^2 \quad (3)$$

which was used for calculations. Similar equations hold true for the magnitude of the hydrodynamic disturbance in terms of water velocity and acceleration, enabling tuning curves to be calculated for each component. Pressure waves were ignored since previous evidence has shown that copepods are insensitive to propagated sound (Hawkins and Myrberg, 1983; Lenz and Yen, 1993). The sensitivity thresholds were determined in terms of water displacement, velocity, and acceleration at the distal tip of the A1. In order to verify that the response was not due to the substrate vibrations caused by the stimulus-producing apparatus, the stimulus bar was elevated above the water and the stimulus was repeated to confirm that the mechanosensory response disappeared.

Experimental Protocol

Animals were removed from culture jars with a ladle, captured by gently squeezing their urosome with forceps, and then transferred to the forceps electrode in the experimental aquarium (without lifting the animal through the air-water interference). Animals were secured in the forceps electrode by clamping their prosome dorso-ventrally, and the A1 extended vertically downward. In order to record from only a single antenna, the upward projecting A1 was removed with iridectomy scissors. The animal was then drawn upward into a layer of mineral oil until reaching a position just past the initial curvature of the A1 (See Figure 11), at which point the animal could not easily pull its A1 completely into the mineral oil, nor could the oil-water interface of the seawater easily contact the prosome of the animal. The stimulus bar was then lowered into the seawater bath directly in front of the distal tip of the A1. The distance between the antenna tip and the stimulus bar was measured with an ocular micrometer. After the animal was secured and neural activity was sufficiently stabilized, a series of 700 Hz 1.5 cycle stimuli at various amplitudes were delivered at 10 second intervals in order to determine the lowest amplitude at which an observable response (large mechanosensory unit) could be recorded from the antenna. Threshold was defined as the peak-to-peak amplitude at which 40% to 60% of 10 stimulus presentations were followed by the recording of a large unit. Trials were not counted if it could not be accurately determined whether it was a true response to the stimulus.

Thresholds were first recorded for stimuli delivered at frequencies of 200 Hz and 700 Hz, then a series of 10 or more multi-cycle stimuli were presented to determine the directionality of mechanosensory units. Thresholds were then measured at 500 Hz, 900 Hz, 1200 Hz, 1500 Hz, and 2500 Hz if possible. At least three sample traces were recorded in response to stimuli at the threshold for each frequency. Then a minimum of three recordings were made at an amplitude 3x threshold, and also at 10x threshold if it was possible within the range of the piezoelectric pusher. After the completion of all recordings, the thresholds at 200 Hz and 700 Hz were again measured. If the animal was observed to have a 2 db or higher threshold than previously, then the animal was considered unhealthy, and thresholds for that particular animal were not included in sensitivity analysis.

After the data were recorded, the animal was gently lowered into the seawater, and measurements of prosome length and A1 length were made with an ocular micrometer. The angles of the animal in the horizontal plane and vertical plane were estimated visually. The animal was then released from the forceps and observed to verify health. In two experiments, the animal was instead left in the mineral oil overnight until it could be assumed that it was dead, at which point the baseline electrical noise could be measured. All animals were preserved in a formalin (<5%) solution. Preserved copepods were identified to developmental stage following Mauchline (1998) and Boxshall and Huys (1998). Prosome lengths were compared to limits set by Kwasniewski (2003), ensuring that all animals analyzed were *Calanus finmarchicus*.

Unit Analysis

Action potentials were sorted with template matching and cluster analysis of waveform characteristics to distinguish neural units (reviewed by Lewicki, 1998). In this study, all records from a particular animal were compiled and analyzed off-line with commercially available software (Spike2 v. 5.06, Cambridge Electronic Design). Units were automatically template matched (Wheeler and Heetderks, 1982) by the software that sampled spikes that reached a threshold above the noise level, and created templates (0.15 to 0.25 ms duration) for similar spikes repeated a minimum of 4 times (60 to 100% matching points), and templates were continuously averaged as identified spikes were sorted throughout the dataset for the animal. Principal Component Analysis (PCA) was performed on the template matched clusters, followed by K-Means clustering. Since directionality appeared to be the distinguishing characteristic for each of the large units, these parameters (width, percentage of matching points, number of clusters), were adjusted for all units responding to multi-cycle stimuli until period plots of multi-cycle stimuli revealed the strongest discrimination of directionality for an animal with minimal overlap and spherical distributions. Such automated sorting gave no independent control over the number of templates needed to account for all of the spike shapes detected, and hence resulted in the creation of intermediate templates, for which the directionality could not be distinguished. Spike-forms identified by these templates were merged with those visually resembling other units that responded to stimuli in the same direction, or excluded from analyses if directionality could not be determined. In earlier experiments in which directionality was not tested, mechanosensory units were identified by template matching in order to compare the waveform characteristics of mechanosensory and non-mechanosensory units, but not distinguished by directionality. The slower, spontaneously firing non-mechanosensory units could be easily sorted by less strict methods (e.g., visually, or template matching) due to their frequency of occurrence, but could not be easily observed in multi-cycle stimuli when larger mechanosensory spike-forms were prominent. Using templates of longer duration (0.3 to 0.5 ms) to accommodate their long waveform, spike-forms of these longer units were then compiled from the entire data set (primarily from spontaneous occurrences). Measurements of amplitude, half-height duration (half-width), and rising slope were then taken for all identified spike-forms.

Spike Latency

The latency between the beginning of stimulus presentation and the recording of large spike-forms was measured for all 1.5 cycle stimuli in selected animals. Due to frequent non-responses of units, spontaneous firing, and repeated firings, a latency measurement was only considered for analyses if the unit was distinctly separate from bursts of other units, and within the range typically observed for that frequency. Single responses observed before the stimulus had reached the expected threshold (based on preliminary observations) for the unit or several milliseconds afterward were considered spontaneous, and not included in analyses. In a single CV copepodid in which each directional unit could be readily identified visually in most recordings, total delays from the beginning of stimulus presentation were measured for both the initial and repeated firing of each directional unit.

Results

Sensitivity

In order to understand the relevant hydrodynamic signals for the behavioral reactions of *C. finmarchicus* and its copepodid stages, it is important to quantify how these thresholds may change during ontogeny. Figure 3 shows that all mechanosensory

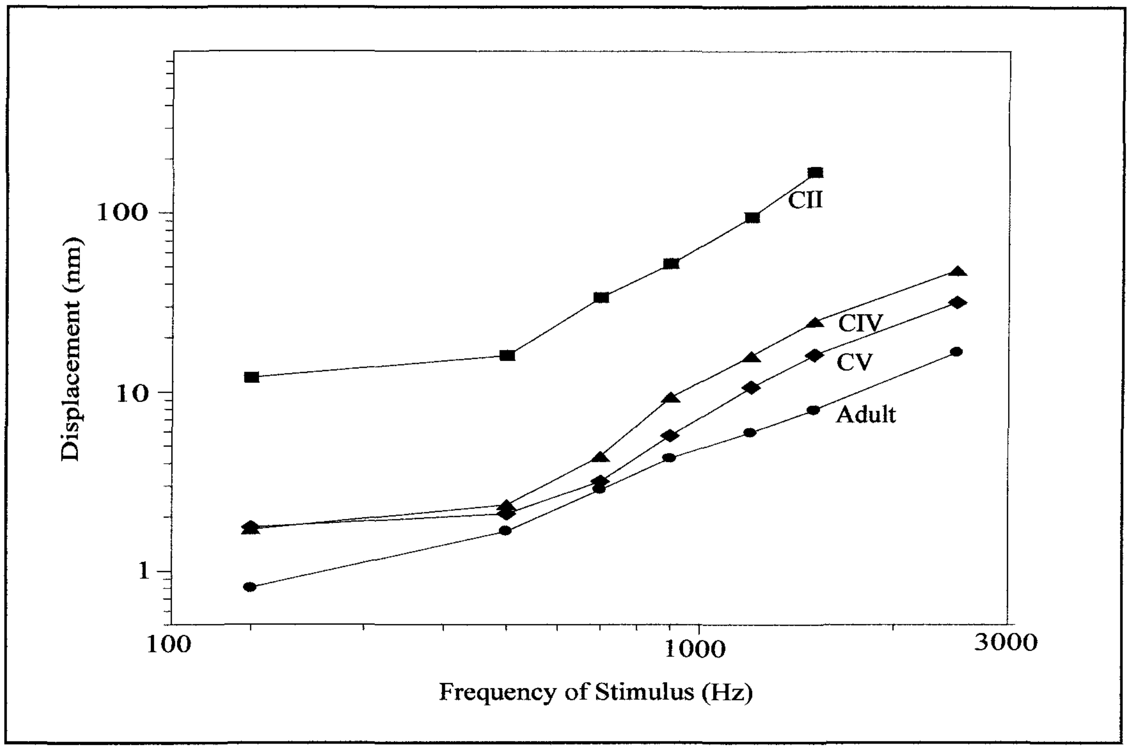

Figure 3. Mean measured mechanosensory thresholds of developmental stages in terms of water displacement at the antenna tip using 1.5 cycle stimuli at selected frequencies. Sample size for CII is 1, CIV is 5, CV is 2, and Adult is 3.

units were most sensitive to 1.5 cycle sine stimuli at frequencies of 200 to 500 Hz. With frequencies above 500 Hz, thresholds for all animals progressively increased with a log-log slope of approximately 2. A fairly constant threshold is seen from 200 to 500 Hz, indicating sensitivity primarily to water displacement. When thresholds were plotted against water velocity or acceleration at the antenna tip, no regions of constancy were observed (not shown).

At their most sensitive frequency of 200 Hz, adults are more sensitive than CIV (Students' two-tailed t-test, $P = 0.01$) and CV copepodids ($P = 0.05$). Adult copepods were also significantly more sensitive than CIV copepodids at 2500Hz ($P = 0.05$). The single CII tested was 10-fold less sensitive than the adults at all frequencies, but the reproducibility of this result has not been demonstrated and could be due to the lack of distal setae (discussed in *Morphology*). However, a behavioral response of A1 flexion-reflex could be triggered in the CII copepodid with stimuli at less than 10x threshold, which was not observed in later copepodid stages, possibly because such large water displacements were not necessary for obtaining 10x threshold recordings from the more sensitive later developmental stages.

Morphology

The relative rate of growth of the antennular sensory organ with respect to the rest of the body impacts the relative sensitivity of the developmental stage to hydrodynamic disturbances. A longer A1 length allows a greater distance from the center of mass over which a given water deformation rate is translated into the bending of mechanosensory setae at the antenna tips, thus leading to a greater sensitivity to perturbation. Measurements of A1 and prosome length showed an allometric growth pattern in which the young copepodid initially had antennae over twice the length of the prosome. As shown in Figure 4, A1:prosome ratios for CII copepodids were significantly greater than those of CIV copepodids (Students' two-tailed t-test, $P = 0.01$), as well as later stages.

No significant differences were found between A1:prosome ratios of later stages. These results appear consistent with the photographs of Marshall and Orr (1955).

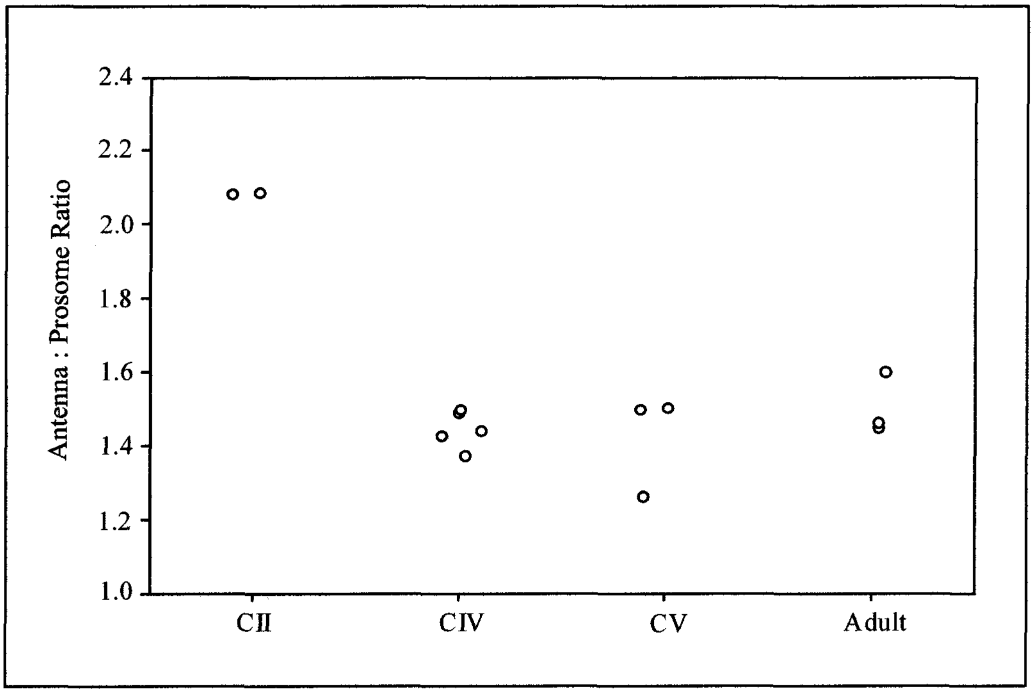

Figure 4. Comparison of A1: Prosome Ratios of developmental stages, with data points showing individual specimens measured.

The distal array of mechanosensory setae has been shown to be responsible for the triggering of giant mechanosensory responses in non-myelinated copepods (Lenz and Yen, 1993); therefore the distal array was examined to determine if all mechanosensory setae were intact. The full distal array observed in adults was also present in CIV and CV copepodids utilized for electrophysiology; however, bent distal setae were observed in 4 of the 5 preserved CIV copepodid specimens studied. Even if bent distal setae in preserved specimens were due to damage in handling, it may demonstrate that the setae of CIV copepodids were less rigid than those of adults, since adults were preserved in the same manner. The single CIV copepodid with a well-preserved distal array was also observed to have the greatest sensitivity for a CIV copepodid (1.3 nm displacements), yet this threshold is still higher than that of all adults measured (0.56, 0.86, and 1.0 nm). In a single CV copepodid with significantly higher thresholds (7.7 nm), the 2nd distal seta appeared broken near the base in the preserved specimen (this animal was omitted from sensitivity analysis for this reason). For the single CII copepodid closely examined both electrophysiologically and morphologically, only two setae extended distally from the tip, not the expected arrangement of 4 setae shown by Huys and Boxshall (1991). It is unclear as to whether the observed variations in setae may have been due to damage in handling.

Unit Composition

Copepod mechanosensory units can encode quantitative information about the hydrodynamic disturbance (Hartline *et al.*, 1996; Fields *et al.*, 2002; Fields and Weissburg, 2004), but directional sensitivity has not been thoroughly established. In the present study, responses often consisted of numerous superimposed units, with at least 3 re-identifiable units or classes of units observed in all animals. The consistent small amplitude (see Table 1) of spikes of all developmental stages studied does not resemble the relatively large 1.5 to 2.4 mV spikes of non-myelinated copepods (Lenz *et al.*, 2000).

Two relatively large, fast mechanosensory units were observed, as well as a clearly distinct smaller and slower non-mechanosensitive unit in all specimens. Categorized as 'Unit U,' the smaller unit could be observed firing frequently in some animals, while rarely in others. Due to the low amplitudes and superpositioning of mechanosensory spikes, the firing rate of Unit U could not be accurately measured.

Table 1. Waveform characteristics (± standard deviation) for all animals studied electrophysiologically.

Developmental Stage	Experiment Date (Month/Day/Year)	Mechanosensory Spikes (Units D &P Combined)				Non-Mechnosensory Spikes (Unit U)			
		N (Spikes)	Amplitude (µV)	Slope (µV/ms)	Half-width (ms)	N (Spikes)	Amplitude (µV)	Slope (µV/ms)	Half-width (ms)
CII	07/25/04	176	37 ± 10	653 ± 335	0.12 ± 0.04	7056	10 ± 4.0	303 ± 228	0.12 ± 0.04
CIV	06/11/04	108	107 ± 14	1990 ± 423	0.09 ± 0.01	448	45 ± 8.6	994 ± 609	0.18 ± 0.03
	06/17/04	135	183 ± 29	2430 ± 415	0.10 ± 0.01	689	89 ± 19	1180 ± 482	0.16 ± 0.03
	06/22/04	138	103 ± 19	1520 ± 268	0.11 ± 0.01	733	44 ± 11	610 ± 246	0.19 ± 0.04
	07/02/04	237	73 ± 13	1220 ± 287	0.11 ± 0.02	301	27 ± 4.5	474 ± 238	0.16 ± 0.03
	07/09/04	147	79 ± 22.5	1280 ± 308	0.09 ± 0.02	311	29 ± 5.9	387 ± 149	0.22 ± 0.04
	07/15/04	43	34 ± 10	570 ± 523	0.09 ± 0.02	188	12 ± 2.4	234 ± 175	0.23 ± 0.07
CIV Mean			97 ± 50	1500 ± 648	0.10±0.01		41 ± 26	647 ± 367	0.19 ± 0.03
CV	06/15/04	207	148 ± 24	2000 ± 459	0.11 ± 0.02	1754	61 ± 17	795 ± 280	0.20 ± 0.04
	06/30/04	37	96 ± 8	1420 ± 201	0.11 ± 0.01	142	38 ± 6.7	484 ± 112	0.16 ± 0.03
	07/16/04	68	50 ± 15	861 ± 238	0.11 ± 0.02	759	14 ± 5.1	335 ± 216	0.17 ± 0.05
CV Mean			98 ± 49	1430 ± 570	0.11		38 ± 24	538 ± 235	0.18 ± 0.02
Adult	07/07/04	268	160 ± 32	1800 ± 1150	0.14 ± 0.04	387	106 ± 17	892 ± 695	0.28 ± 0.09
	07/08/04	111	110 ± 16	1870 ± 357	0.11 ± 0.01	203	45 ± 8.0	581 ± 324	0.21 ± 0.04
	07/24/04	22	51 ± 7	599 ± 127	0.10 ± 0.01	312	25 ± 6.1	303 ± 71	0.12 ± 0.05
Adult Mean			107 ± 55	1420 ± 714	0.12 ± 0.02		59 ± 42	592 ± 295	0.20 ± 0.08

In order to verify that Unit U spike-forms were not noise, it was shown that this unit, as well as all others, was absent from recordings from dead animals. The inconsistent firing and superposition of Unit U likely contributed to the difficulty in sorting larger spikes. As shown in Table 1, for each animal Unit U has significantly smaller amplitudes (42 μV mean for all animals), and longer half-height durations (0.2 ms mean) than the larger mechanosensory spike-forms (90 μV mean, 0.10ms mean, Student's two-tailed t-test, P=0.01), except for the CII copepodid. This inconsistency could be explained by the relatively weak signal strength obtained from the small CII copepodid (0.80 mm prosome length), which made it difficult to distinguish these low amplitude spike-forms from background noise. While the spike-form parameters appear consistent within individuals, the variations in large mechanosensory unit parameters (not directionally distinguished) between developmental stages were not significantly greater than variations among individuals within developmental stages (Table 1, One-way ANOVA, $P > 0.5$).

Analyses of single individuals from the CII, CIV, CV, and adult stages suggested that the large mechanically sensitive spikes were also directionally selective. Analyses of single individuals from the CIV and CV stage suggested that larger spikes were primarily sensitive to water moving past the A1 in the proximal direction (designated "Unit P"), while in the single CII and adult specimens studied directionally, the largest unit was sensitive to stimuli in the distal direction (designated "Unit D"). Template matching (Wheeler and Heetderks, 1982) provided rough evidence of this directionality, yet stricter sorting revealed a smaller unit within recordings from each individual that primarily responded to water movement in the opposite direction, as shown in Figure 5.

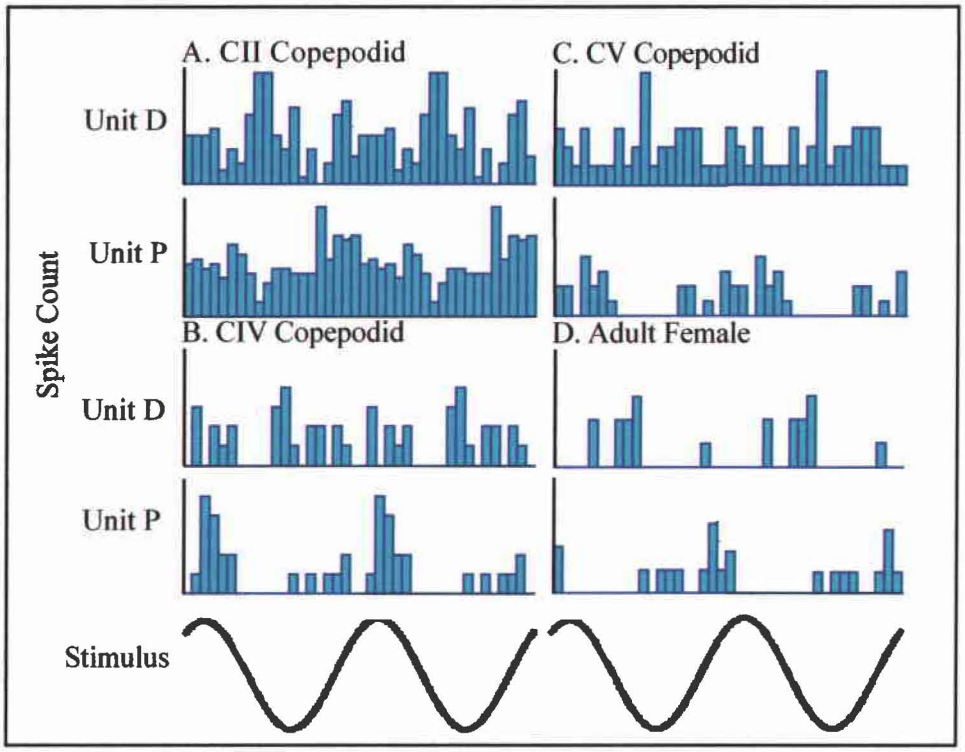

Figure 5. Period plots of at least 10 multi-cycle stimuli used to identify directional mechanosensory units within an animal of each developmental stage studied. The stimulus trace shown represents vertical water movement at the antenna tip, and is time-shifted approximately 1 cycle to account for latency discussed in the 'Spike Latency' section. A.) CII copepodid, Expt. 07/25/04 in response to 12x–threshold 500Hz stimuli. B.) CIV copepodid, Expt. 07/15/04 in response to 3x-threshold 700Hz stimuli C.) CV copepodid, Expt. 07/16/04 in response to 3x-threshold stimuli. D.) Adult female, Expt. 07/24/04 in response to 3x-threshold stimuli.

Clustering of spike-forms that was most effective in distinguishing between Units P and D was based primarily on the curvature of the peak as the source of the most variation (displayed in Figure 8, but not easily discriminated visually), while the fast 80 KHz sampling rate was essential for discriminating these differences. Spikes were then sorted into the nearest means of principle components by Euclidean distance (see Lewicki, 1998) using K-means clustering, while also refining the means to form new templates. Refined templates were then used to identify spike-forms from the raw data, and these strictly sorted units were used for the identifications shown in Figures 5-8. A numerical estimation of spike misidentification (D as P and vice versa) could not be provided with K-means clustering, but frequent overlap of clusters indicates that spike misidentification may be frequent. Using a normal mixtures algorithm to account for distributions (Hartigan, 1975) appeared to result in poorer discrimination of directional sensitivity, thus K-means clustering was used since it was assumed that directionality is the key characteristic of each unit. Despite frequent misidentifications, the mean of identified spike-forms could still be considered accurate. Thus the largest peaks in Figure 5 can be considered to consist primarily of correctly distinguished spike-forms, and these were correlated with the phase of the stimulus that triggers the response of each unit. This enabled the identification of units based on the key characteristic of directionality, while relative amplitudes of mechanosensory units (as used by Hartline *et al.*, 1996 in non-myelinated copepods) varied greatly among individuals. Visual identification was sometimes possible by obvious differences in amplitude or slope, but this was not reliable for all specimens. In forming templates for the CII copepodid, a higher amplitude 12x-threshold 6 cycle stimulus at 500 Hz was used (Figure 2B), with more repetitions than for other animals (78 instead of 10-15) in an effort to distinguish all units despite background noise and the low sensitivity of this individual (11 nm thresholds). With these tactics, it was possible to form better templates for identifying directional units that are partially obscured by superposition events (as seen in Figure 7).

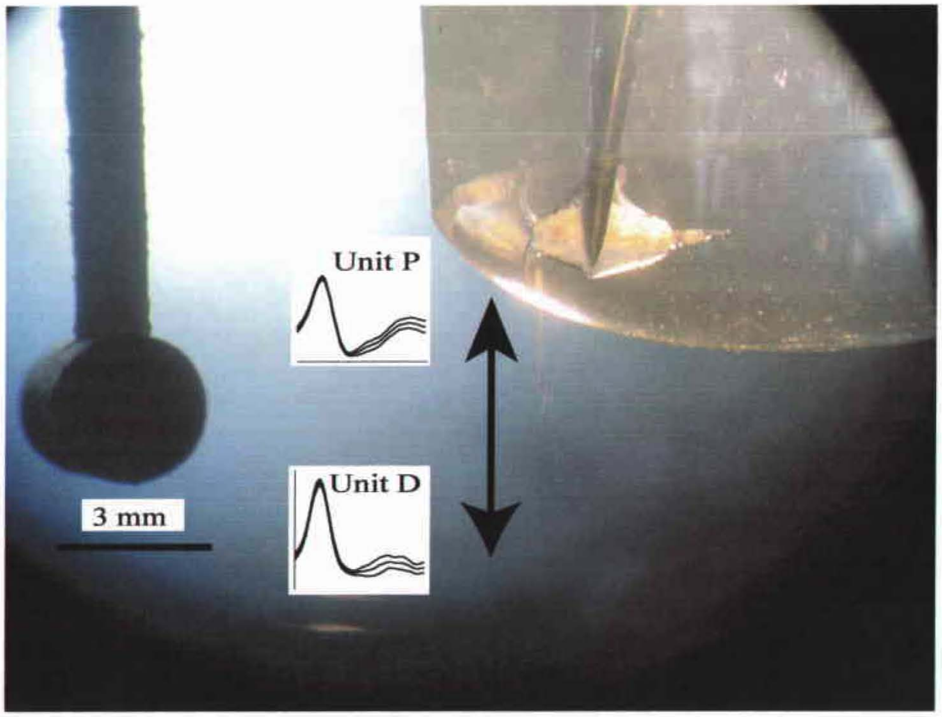

Figure 6. Photograph of preparation of adult female (Expt. 07/24/04) with insets showing spike-forms of directionally identified units (also shown in Figure 8). The arrow indicates the direction of water displacement at the antenna tip likely to trigger each spike-form. Mechanosensory setae cannot be seen in this photograph due to their small size.

An approximate delay of 0.8 ms (further described in next section) was subtracted from multi-cycle recordings in order to determine the true phase of the stimulus that triggers the response. Spike-forms were then identified as Unit D if corresponding to water displacements in the distal direction along the A1, or Unit P if corresponding to displacements in the proximal direction (see Figure 6). It is possible that each directional unit may consist of responses from 2 or more axons, but the measurements did not allow any further categorization. Spontaneous firing due to the high sensitivity of these mechanoreceptors (0.6 - 11 nm displacements, Figure 3) could explain high variability in discharges (see distributions in Figure 5, and variability in firing times in Figure 7).

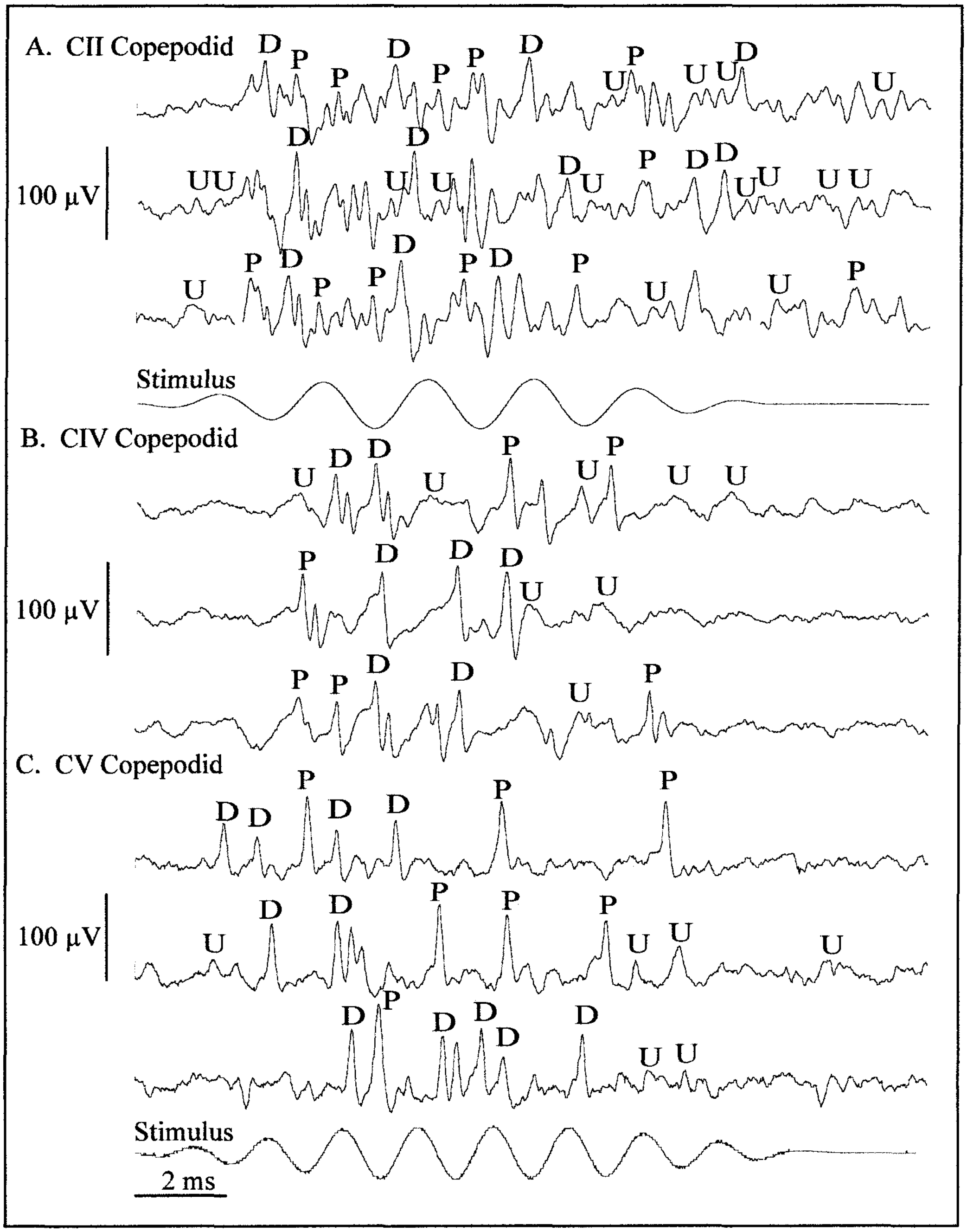

Figure 7. Example recordings from individuals of each copepodid stage studied in response to 3x threshold multi-cycle stimuli, with computer identified units labeled. Stimulus trace indicates vertical movement of water at the antenna tip, however the response is delayed by 1.0 to 1.5 ms (described in 'Spike Latency'). A.) CII copepodid, Expt. 07/25/04 in response to 12x–threshold 500Hz stimuli. B.) CIV copepodid, Expt. 07/15/04 in response to 3x-threshold 700Hz stimuli C.) CV copepodid, Expt. 07/16/04 in response to 3x-threshold stimuli.

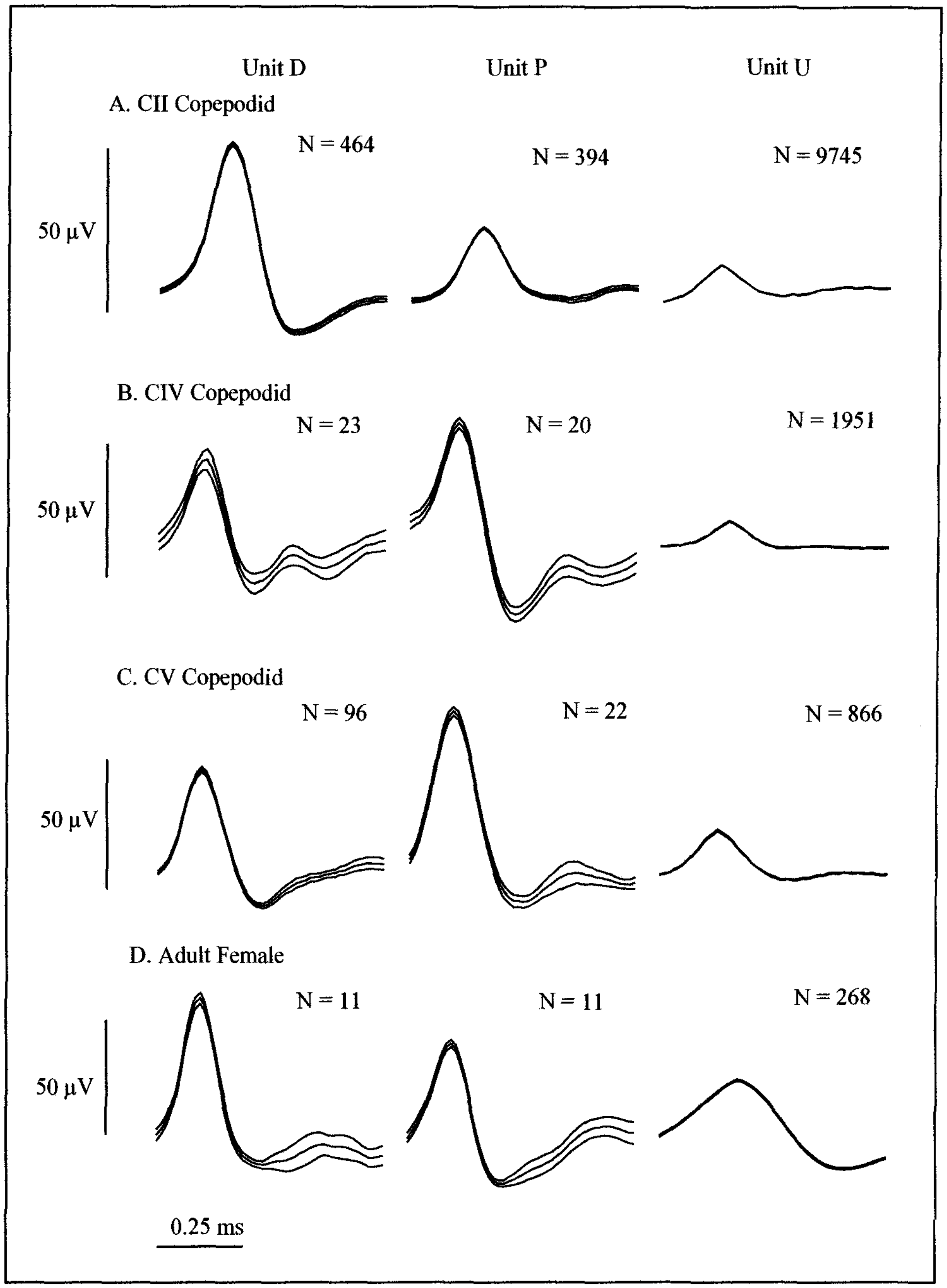

Figure 8. Waveform averages of identified units within single individuals of each developmental stage studied. N indicates the number of spike-forms averaged, and upper and lower lines indicate standard error of the mean (when visible). A.) CII copepodid, Expt. 07/25/04 in response to 12x–threshold 500Hz stimuli. B.) CIV copepodid, Expt. 07/15/04 in response to 3x-threshold 700Hz stimuli. C.) CV copepodid, Expt. 07/16/04 in response to 3x-threshold stimuli. D.) Adult female, Expt. 07/24/04 in response to 3x-threshold stimuli.

Nevertheless, evidence for directionality was observed in a single individual from each of the CII, CIV, CV, and Adult stages. Figure 7 shows several sweeps of responses to long sinusoids, in which sample identifications of all 3 units by Spike 2 software were performed as just described. Note the weak phase-locking.

The resultant waveform averages of all directionally identified units shown in Figure 8 demonstrate the consistent shape of spike-forms, despite variable relative amplitudes between individuals. The waveform averages appear wider in CII copepodids than later developmental stages. Although possibly a developmental difference, this may be explained by the larger stimuli delivered to the CII copepodid. A greater number of overlapping spike-forms (shown in Figure 7) skewed the averages to appear wider.

Spike Latency

Latency of sensory discharge is expected to decrease with both increasing stimulus strength and frequency, which was confirmed in all responses to 1.5 cycle stimuli. However, a particularly long delay of several milliseconds between stimulus on-set and mechanosensory discharges was observed for low frequency (200 Hz) stimuli (see Figure 9). Such a delay could prolong the latency for behavioral responses to sensory signals. The delay could not be easily measured because of the interactions between the effects of amplitude and frequency, combined with the inability to confidently identify each directional unit in all specimens.

In a single CV copepodid, each directional unit could be readily distinguished visually and fit an expected pattern as predicted from earlier trials. For the 1.5 cycle stimulus, both Units P and D usually responded to their corresponding directions of

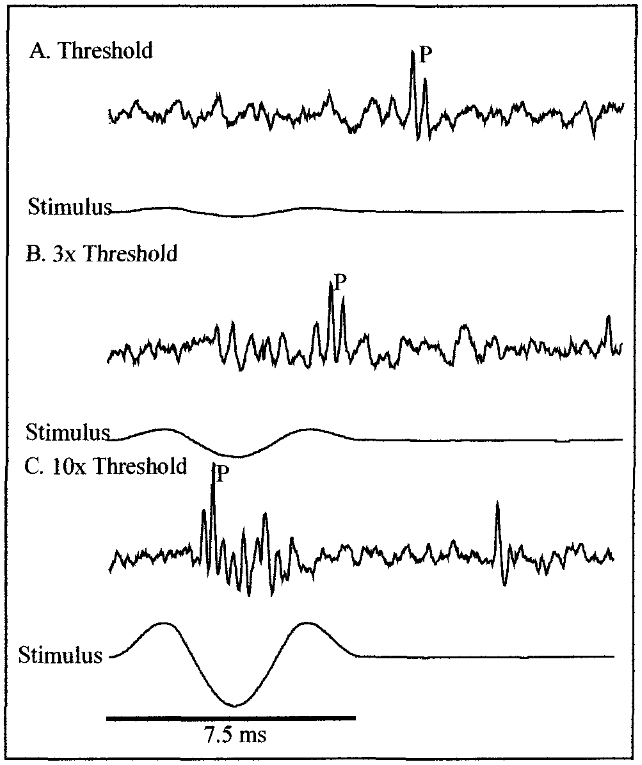

Figure 9. Sample recordings of observed latency in a visually identified Unit P of a CV copepodid (Expt. 07/16/04), demonstrating change in response latency. All firings of Unit P are presumed to be triggered by the major proximal displacement (shown here as the large downward movement of the stimulus bar), but the exact triggering point cannot be determined.

sensitivity. Threshold stimuli appeared to be equally likely to cause a spike-form from Unit P, D or both, as triggered by either of the corresponding major displacements of the stimulus (Figure 2A). For supra-threshold stimuli, response latencies of each unit were decreased, and the likelihood of also triggering a mechanosensory unit due to the minor displacement of the rising and falling portions of the 1.5 cycle stimulus (see Figure 2A) were increased. With high frequencies and 10x threshold stimuli, response latencies of large units were more consistent, and superposition events were more common. The responses of other animals could also be qualitatively described to fit this pattern, but not confidently measured.

In order to quantify latency properties of a spike accurately, it is important to determine the corresponding triggering point on the stimulus waveform. Unfortunately, the observed variable response latency makes it difficult to determine the triggering point of each stimulus frequency and amplitude. If the triggering point was a fixed absolute threshold magnitude for water displacement, then for example, a change in amplitude of the 1.5 cycle 200 Hz stimulus from threshold to 10x threshold could only shift that point on the major proximal displacement by 1.8 ms earlier than at threshold. Instead, Figures 9 and 10 show that the response latency of Unit P is decreased by approximately 6 ms at 10x threshold. This change in delay due to the amplitude of the stimulus varies depending on frequency, as shown as groupings in Figure 9. Figure 9 also shows that the latency of each directional unit is affected by stimulus amplitude in the same way.

In the case of higher frequency stimuli, the peak displacement occurs at an earlier time; resulting in a shorter total latency of as low as 0.33 ms for Unit D in response to 2.5 KHz stimuli at 3x threshold. Figure 11A shows that the total latency of Unit P varies as a linear function of the time-to-peak (TTP) proximal displacement of the stimulus. In response to the larger magnitude displacements of 10x threshold 1.5 cycle stimuli, Figure 11B also shows latency as a linear function of TTP at TTP values below 1.5 ms (500Hz), but at 3.8 ms (200Hz) the response latency is shorter than that expected with a linear function. This shorter delay corresponds to the greater affect of amplitude on latency at 200 Hz, as shown in Figure 9.

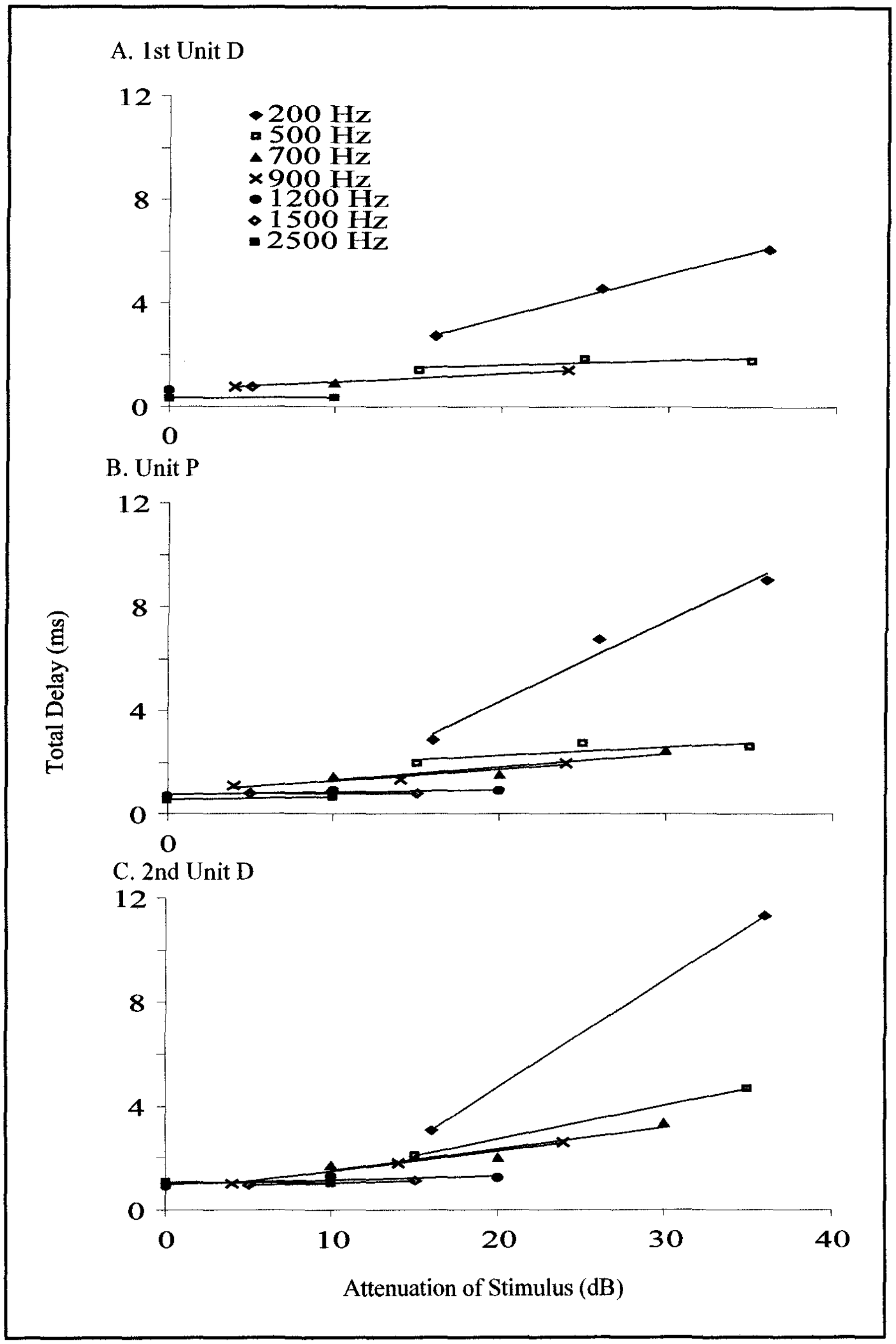

Figure 10. Total response latency of each firing of a visually identified mechanosensory unit in a single CV copepodid, (Expt. 07/16/04) plotted against attenuation of the 1.5 cycle 200 Hz stimulus. A.) Unit D triggered by the initial minor displacement of the stimulus in the distal direction. B.) Unit P triggered by the major displacement of the stimulus in the proximal direction. C.) The 2nd firing of Unit D due to the major displacement of the stimulus in the distal direction.

Thus in response to slower stimuli (frequencies below 500Hz), the latency can be best characterized as decreasing primarily as a linear function of increasing amplitude of displacement (Figure 10). This effect is also seen at higher frequencies, but to a lesser extent. As frequency is increased, the TTP displacement of the stimulus becomes a better predictor of the mechanosensory response latency (Figure 11).

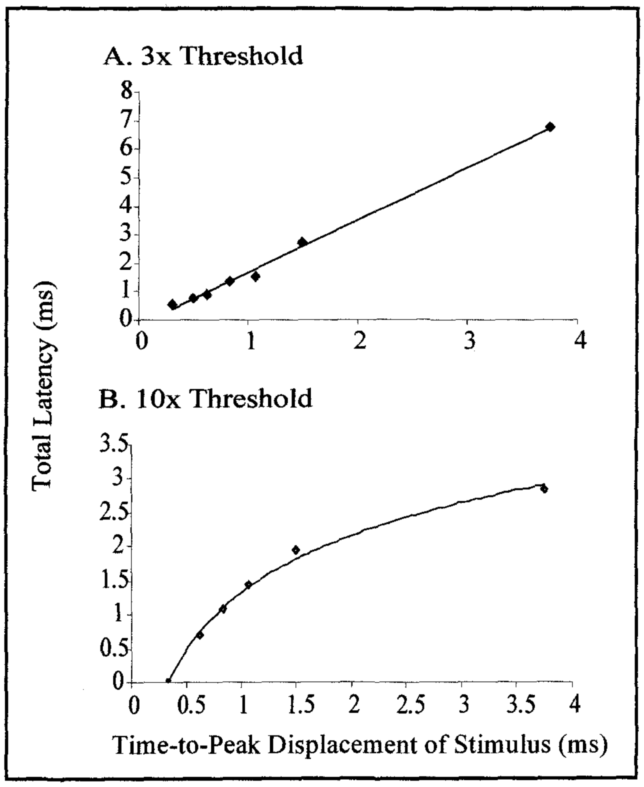

Figure 11. Change in response latency of Unit P associated with a change in time-to-peak (TTP) proximal displacement of each frequency 1.5 cycle stimulus (CV copepodid, Expt. 07/16/04). A.) 3x Threshold, 1.5 cycle stimuli. B.) 10x threshold, 1.5 cycle stimuli.

Discussion

Neurophysiology

This study has examined the mechanosensory neurophysiology of the myelinated copepod *C. finmarchicus*, and ontogenetic changes in morphology and sensitivity. As seen in electrophysiological recordings, the neurophysiological characteristics appear relatively unchanged throughout the copepodid stages. The neural discharges observed in healthy and behaviorally responsive copepodids as early as the CII stage have 50 μV spikes that do not resemble the 10-fold larger spikes previously reported in non-myelinated copepods (Lenz *et al.*, 2000). Spikes are of similar duration in adults and copepodids studied (less than 0.02 ms difference in half-width), suggesting that high conduction velocity and myelination of axons within the A1 have been established as early as the CII copepodid stage.

We have shown that extracellular recording from myelinated copepods can be analyzed to reveal several types of neural units within the A1. Within our limits of resolving each particular unit, it appears that the full complement of unit classes observed in adults is also present in the CII copepodid. The accurate discrimination of these units is made very difficult by the weak and variable signal obtained from a myelinated copepod, combined with their spontaneous activity, high sensitivity, and weak phase-locking capabilities in response to the stimuli utilized in these experiments. Nonetheless, directionality could be observed in all developmental stages tested. Large differences in spike amplitudes among the major mechanosensory units do not consistently occur between developmental stages, thus demonstrating that unit classification by amplitude (e.g., Hartline *et al.*, 1996) is not reliable for these myelinated copepods. It should also be noted that it is possible that directionally sensitive responses may consist of multiple units that are similar in spike-form, but could not be easily distinguished. Histological analysis by Lenz *et al.* (2000) has shown 5 large myelinated axons that could correspond to mechanosensory units within the A1 of the closely related *Undinula vulgaris*, while numerous smaller myelinated axons could correspond to Unit U.

Despite recording difficulties, this study has been the first to examine how *C. finmarchicus* may process directional information, as shown in Figure 11. Similar to displacement sensors seen in other crustaceans (e.g. crayfish, Wiese, 1988), neural discharges correspond to stimuli in either of the two directions that were tested. While crayfish have been reported to accomplish this task by having sensory dendrites attached on opposing sides of the mechanoreceptor, Weatherby and Lenz (2000) have shown that mechanosensory setae of a non-myelinated copepod are dually innervated on a single side. Fields *et al.* (2002) have suggested that mechanical differences in attachment points of mechanosensory setae could enable the encoding of directional information. However, these analyses have not yet been performed on *C. finmarchicus*, thus a different method of encoding directional stimuli may be present.

The response latency of mechanosensory spike-forms observed following the low frequency stimuli not only makes it difficult to determine the exact phase of the stimulus that triggers the mechanoreceptor, but also shows a much longer spike response latency than the short behavioral reaction times of less than 2 ms observed by Lenz *et al.* (2004) in *C. finmarchicus* in response to a large amplitude 1.5 cycle 700 Hz stimuli. Thus *C. finmarchicus* is capable of actively responding to stimuli within 2 ms, but a several millisecond longer delay in mechanosensory discharges must be considered when slower and weaker stimuli are presented. In addition, the effect of the stimulus characteristics on the response latency must also be taken into account. In comparison, Yen *et al.* (1992) reported that spike response latencies of non-myelinated *Pontellopsis* spp. increased by only 1 ms per ms time-to-peak of the stimulus, while observed *C. finmarchicus* response latencies increased by 1.4 ms per ms time-to-peak (in response to higher frequency stimuli in which a linear relationship was shown). While spike latencies of less than 0.33 ms were observed in response to large amplitude and high frequency stimuli, the relatively long latencies in response to slower and weaker stimuli conflict with the prediction that myelination enables short behavioral response times of less than 2 ms, and thus better predator evasion (Davis *et al.*, 1999; Lenz *et al.*, 2000).

However, further implications of this delay that must also be considered. As shown in Figure 9, the latencies of both directional units are similarly affected by the stimulus amplitude. Thus the variable latency of the two units may provide sensory information for determining the relative strengths of stimuli from either direction, yet further experimentation is necessary to examine the integration of directional stimuli. While a longer delay may be acceptable in avoiding a slower predator such as a jellyfish, a faster moving threat such as a rapidly striking larval cod would necessitate a faster response time such as that accomplished by the 0.33 ms delay of Unit D observed at high frequencies.

In addition to directional units, the sensory physiology of Unit U has not yet been studied thoroughly. Presumably, it may correspond to the numerous chemoreceptive aesthetascs on the A1 antennae (e.g., Boxshall and Huys, 1998), but this was not tested. The longer spike duration of Unit U suggests that these axons have different physiological characteristics due to the slower observed spike-forms, thus providing evidence that enhancement of conduction velocity may be preferentially given to mechanosensory axons, rather than other types of sensory axons.

Morphology

While neurophysiological characteristics appear to be conserved through ontogeny, changing morphological characteristics may affect the sensitivity of the mechanosensory system. In a free-swimming copepod, an animal with a longer A1 would have the advantage of increased sensitivity to a given deformation rate at the antenna tip. Smaller copepodids placed at a disadvantage due to their smaller body size may increase their relative sensitivity by possessing longer antennae in relation to their prosome length (2.1:1 in CII copepodids as opposed to 1.4:1 in adults). Such large antennae may hinder a small copepodid in other aspects such as drag during escapes, but the benefits in predator detection may outweigh the costs. As growth proceeds and body size increases, smaller antennae in relation to the prosome may be sufficient for mechanosensory perception in older stages without the added costs of long antennae.

Sensitivity

C. finmarchicus mechanosensory thresholds primarily depend on water displacement at low frequencies, rather than water velocity as previously reported in other copepods (Yen *et al.*, 1992; Hartline *et al.*, 1996). In addition to displacement thresholds, the variable spike latency suggests that sensitivity is affected by stimulus amplitude with low frequency stimuli, while the time of peak displacement is a better predictor of response latency at higher frequencies. These findings are particularly important because the extensive neurophysiological research on non-myelinated velocity-sensitive copepods may not be accurately applied to *C. finmarchicus*. However, Lenz and Hartline (1999) have shown that the closely related *U. vulgaris* is behaviorally responsive to thresholds of water velocity with displacements of 10 to 1000 nm, which is greater than most displacements utilized in this study of the thresholds of only neural discharges from the mechanosensory axons (the first study of its kind on myelinated copepods). This suggests that a great deal of temporal and spatial summation may be necessary before triggering a behavioral response in *C. finmarchicus*.

Absolute sensitivity was shown to increase somewhat during the ontogeny of *C. finmarchicus*, since animals were fixed in the forceps electrode and were not permitted to move with bulk water flow as in a free-swimming situation. Higher mechanosensory thresholds in copepodids may partially explain the observed passive behaviors of *A. hudsonica* copepodids in the presence of jellyfish (Suchman, 2000), while active escape responses may be unnecessary because the two jellyfish studied fed selectively upon larger copepods (Suchman and Sullivan, 2000). Few conclusions about ontogeny can be drawn from the measured sensitivity of the single CII copepodid, but the measured threshold of hydrodynamic disturbance (11 nm) is similar to the most sensitive adult copepods reported in Yen *et al.* (1992), showing that relatively high sensitivity is imparted on a *C. finmarchicus* CII copepodid.

Mechanosensory setae (described in adult *P. xiphias* by Weatherby and Lenz, 2000) added along the medial portion of the A1 during ontogeny (Boxshall and Huys, 1998), as well as more stiffened distal setae in later developmental stages observed by the lack of bending in preserved specimens, could explain the higher sensitivity of adults. One would also expect a larger number of mechanosensory units to be observed in later developmental stages due to increased setation, but there was little evidence for this from the recordings in this study.

Conclusions

This research has been the first to examine the mechanosensory capabilities in relation to the unit composition of mechanosensory discharges during the ontogeny of an ecologically and commercially important myelinated copepod, in which longer antennae may impart greater sensitivity for early copepodids, and myelination of mechanosensory axons is developed as early as the CII copepodid stage. The groundwork has been established for determining how this ecologically essential copepod can process directional stimuli to determine the appropriate escape response. In addition to myelination, the growth of the large and cumbersome mechanosensory antennae may present a challenge for early copepodids. Such investment in mechanosensory capabilities further demonstrates the importance of predator avoidance behaviors throughout the life cycle of copepods. Early copepodids spend much of their time at shallow depths (Eiane and Ohman, 2004), but this may not be due to a relative lack of pressure from visual predators alone. Our results show that the fast mechanosensory capabilities are established as early as the CII copepodid stage, despite the metabolic cost of myelination and antennule growth. However, the threat of invertebrate predation (Eiane *et al*., 2002) may also influence the life cycle of *C. finmarchicus,* leading to the early development of this exceptional mechanosensory system.

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