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ORIGINAL PAPER

Regulation of spirocyst discharge in the model sea anemone, *Nematostella vectensis*

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Abstract Test probes were touched to tentacles to investigate whether discharge of spirocysts likely is regulated by hair bundle mechanoreceptors. Significantly more spirocysts discharge onto test probes in the presence of vibrations at 11–15 Hz as compared to 0 Hz. Adding *N*-acetylneuraminic acid, NANA, shifts maximal discharge of spirocysts upwards to 36–40 Hz, and possibly to 21–25 Hz. In contrast, NANA shifts maximal discharge of basitrichous isorhiza nematocysts downwards to 1–20 Hz. Thus, discharge of cnidae ('stinging capsules') is differentially regulated according to the type of cnida. Furthermore, it appears that chemodetection of *N*-acetylated sugars is not a prerequisite to capturing prey because, in seawater alone, maximal discharge of cnidae occurs at frequencies overlapping movements of calmly swimming prey. Nevertheless, chemodetection of *N*-acetylated sugars broadens the range of frequencies stimulating maximal discharge of cnidae and, therefore, likely enhances prey capture.

Introduction

In recent years, our understanding of prey capture in anemones has improved. While it has long been appreciated that prey capture involves discharge of nematocysts, we now know that 'tunable' hair bundle mechanoreceptors participate in regulating discharge. In the anemones, *Haliplanella lineata* and *Nematostella vectensis*, discharge of microbasic p-mastigophore and basitrichous isorhiza nematocysts, respectively, varies according to the frequency of nearby vibrations (Watson and Hessinger 1989; Watson et al. 1998; Watson and Mire 2004; Watson et al. 2009). In the presence of vibrations, discharge occurs maximally into test probes touched to tentacles at specific, key frequencies. Interestingly, upon exposure to *N*-acetylated sugars including NANA, key frequencies (those that elicit maximal discharge) shift downward to lower frequencies overlapping those produced by calmly swimming prey (Watson and Hessinger 1989; Watson et al. 1998, 2009; Watson and Mire 2004). *N*-acetylated sugars occur in glycoproteins and glycolipids where they are exposed to the extracellular fluid. Furthermore, certain secreted proteins such as mucins are extensively glycosylated. Hence, *N*-acetylated sugars commonly occur on the surfaces of prey organisms (or their secreted coatings) and, as such, constitute a good chemical cue to signify that a nearby object is living, was living, or is derived from a living object. The combination of *N*-acetylated sugars and vibrations at low frequencies confirms that a nearby object is living and swimming in the vicinity of the tentacles. Such stimuli, detected by specific receptors, sensitize the anemone to maximally discharge nematocysts in the event of contact with the prey organism.

The purpose of this study was to test whether discharge of spirocysts also is regulated by hair bundle mechanoreceptors. The spirocysts are perhaps least well understood among the types of cnida of anthozoans (corals and anemones). Spirocysts are the most abundant cnida in tentacles specialized for capturing prey (Hand 1955; Mariscal 1974; Watson and Mariscal 1983; Sebens 1998). For most anthozoans, the tentacle cnidom (complement of cnidae) consists of three types of cnida: spirocysts;

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microbasic p-mastigophore nematocysts; and basitrichous isorhiza nematocysts. In the sea anemone *Haliplanella*, the tentacle cnidom consists of 66% spirocysts, 26% microbasic p-mastigophore nematocysts, and 8% basitrichous isorhiza nematocysts (Watson and Mariscal 1983). The tentacle cnidom of the model sea anemone, *Nematostella* is unusual insofar as the microbasic p-mastigophore type nematocyst is absent (Sebens 1998). Despite the abundance of spirocysts in tentacles, the regulation of spirocyst discharge has received relatively little attention as compared to the regulation of nematocyst discharge. Whereas the capsules of discharged microbasic p-mastigophore and basitrichous isorhiza nematocysts are readily observed on test probes using phase contrast optics, spirocyst capsules are poorly contrasted and thus cannot be accurately counted. Thorington and Hessinger (1990) circumvented this problem by exploiting the ability of spirocyst tubules to bind glycoproteins. Test probes touched to tentacles (in the absence of vibrations) were incubated in asialomucin followed by a lectin conjugated to an enzyme. Because the lectin was conjugated to an enzyme that formed a colored product when provided with the appropriate substrates, the intensity of the color varied in relation to the number of spirocysts discharged. A spectrophotometer was used to quantify the intensity of the colored product in solution. Thorington and Hessinger found that spirocysts of the anemone, *Aiptasia pallida* adhere more efficiently to hard substrates than to soft substrates. Furthermore, dose response curves to known chemosensitizers were complex in shape commonly having more than one maximum (Thorington and Hessinger 1990). In this paper, we contrasted tubules of discharged spirocysts by allowing them to bind antibodies conjugated to alkaline phosphatase. After incubation in the appropriate substrate, spirocyst tubules were readily observed using bright field optics. The numbers of spirocysts attached to test probes were estimated by computer analysis of digital photomicrographs. We report that discharge of spirocysts varies according to the frequency of nearby vibrations. Interestingly, NANA shifts maximal discharge to higher frequencies (in the opposite direction that NANA shifts maximal discharge of basitrich nematocysts). Taken together, these observations indicate that discharge of cnidae is differentially regulated according to the type of cnida.

Materials and methods

Specimens of *Nematostella vectensis* were obtained from the Marine Biological Laboratory, Woods Hole, MA, USA. The animals were cultured in natural seawater diluted to 16‰ according to the methods described in Hand and Uhlinger (1992). The animals were fed twice weekly with freshly hatched brine shrimp nauplii. Feeding was followed by a change in seawater.

Spirocyst discharge bioassay—Specimens of *Nematostella* spawned in culture. The anemones were raised to a column length of approximately 2–4 cm. These anemones were tested for vibration dependent discharge of spirocysts according to a revision of methods previously described (Watson et al. 2009). Briefly, animals were touched with test probes constructed from 2 cm segments of nylon monofilament fishing line (4–6 lb test, Berkley, Trilene XL, Spirit Lake IA) without further treatment (i.e., they were not coated with gelatin as are test probes used to collect nematocysts). Test probes were immersed in seawater containing the anemones then touched to tentacles in the presence of vibrations at 1 Hz increments ranging from 1 to 60 Hz (and at 0 Hz for non-vibrating controls). A minimum of nine test probes was employed for each frequency in seawater alone and in seawater augmented with NANA to 10^{-7} M. Vibrations were produced by a digital function generator set to the sine-wave function. The output of the function generator was used to vibrate a piezo disc. A glass probe attached to the piezo disc was immersed into the seawater containing the anemones. The distance between the vibrating probe and animals was set at approximately 2 cm (probe to the tentacle tips). Each anemone was touched once. Test probes were immersed, moved into contact with tentacles and then withdrawn in a direction to minimize the possibility that additional tentacles would contact the test probe. The test probes were fixed, rinsed in phosphate buffered saline (PBS) and then transferred to a solution of goat anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase diluted in PBS 1/30,000 according to the manufacturer's instructions (Sigma). After a 30 min fixation in 4% paraformaldehyde in Millonigs' buffer at 22°C (Watson et al. 2009), probes were washed in PBS and then transferred to the solution containing the secondary antibody. After 60 min in the antibody solution, the probes were rinsed in PBS to remove unbound antibody and then transferred to the substrate (SigmaFast, BCIP/NBT). After 10 min in the substrate solution, test probes were moved to PBS where they were stored at 4°C. Wet mounts were prepared and the test probes were observed using an upright microscope at 200× and bright field optics (20× plan achromat, na = 0.40 objective lens and model RP011-T microscope, LOMO America, Prospect Heights, IL, USA). Images of darkly stained spirocysts on test probes were obtained using a STL-11000 SBIG cooled CCD camera (SBIG, Santa Barbara, CA) controlled by Maxim-DL software (Diffraction Limited, Ontario, Canada). Discharged spirocysts occurred in networks that we called 'patches.' Additional images were collected in the event that the patches of discharged spirocysts extended beyond the field of view of the camera.

The area of each patch was estimated by carefully tracing a line around it using Image-J software. Multiple patches were summed if necessary for each probe. To convert area to the number of spirocysts, the area obtained for each probe was divided by the 'standard' area for a single spirocyst based on the mean area obtained for 20, individual spirocysts. In the end, data were summed if necessary to obtain an estimated total number of spirocysts per probe. Noise in the original data set forced us to pool data over 5 Hz increments. Statistical analyses were performed using CSS Statistica software (Statsoft, Tulsa, OK, USA). An ANOVA was performed followed by LSD post hoc tests. In all cases, significance is reported at $P \leq 0.05$.

In addition, images of spirocysts in situ were obtained from tentacles fixed as described (Watson et al. 2009) using transmitted light and oblique contrast optics (Kachar 1985).

Scanning electron microscopy

Specimens of *Nematostella* were placed in glass dishes containing seawater diluted to 16‰ and allowed to recover from handling for 30 min. Freshly hatched *Artemia* nauplii were dropped into the *Nematostella* culture approximately 1–2 cm from the tentacle tips. Typically, anemones captured several shrimp within a minute of introducing the *Artemia* into the anemone culture. At this point, the anemones bent the tentacles toward the mouth. The tentacles and oral disc were dissected and dropped into a primary fixative consisting of 2.5% glutaraldehyde and 4% paraformaldehyde in Millonig's Buffer where they remained for 1 h at 22°C. The shrimp were gently teased free from the fixed anemones and washed in distilled water. Shrimp were post-fixed in 1% OsO_4 for 1 h at 22°C. The shrimp were rinsed in distilled water and then dehydrated in a graded series of acetone. The specimens were critical point dried, mounted on stubs and then examined using a JEOL 6300 field emission scanning electron microscope (FESEM).

Results

The tentacle cnidom of *Nematostella*

In tentacles of *Nematostella*, spirocysts are the dominant cnida followed by basitrichous isorhizas (Fig. 1). Our estimates indicate that spirocysts constitute 73% and basitrichs 27% of the tentacle cnidom.

Detecting spirocysts

Based on our experiences with immunocytochemistry for a variety of antigens on tentacles of the sea anemones *Haliplanella* and *Nematostella*, we observed that in fixed tissue, tubules of spirocysts readily bind secondary antibodies conjugated to fluorochromes. Although such binding has been an impediment to our progress, in the present study, we aimed to turn this phenomenon to our benefit. Thus, test probes (short segments of fishing line) were touched to tentacles and then incubated in dilute solutions of secondary antibody. The tubules of discharged spirocysts were readily visualized on test probes touched to tentacles and processed in secondary antibody conjugated to alkaline phosphatase using brightfield microscopy (Fig. 1b). As expected, tubules were dark purple but the capsules of discharged spirocysts were only faintly visible. Thus, the test probe featured 'patches', (i.e., complex networks) of darkly stained tubules (Fig. 1b). We generated a standard curve in which the images of stained spirocysts were carefully outlined using Image-J software and

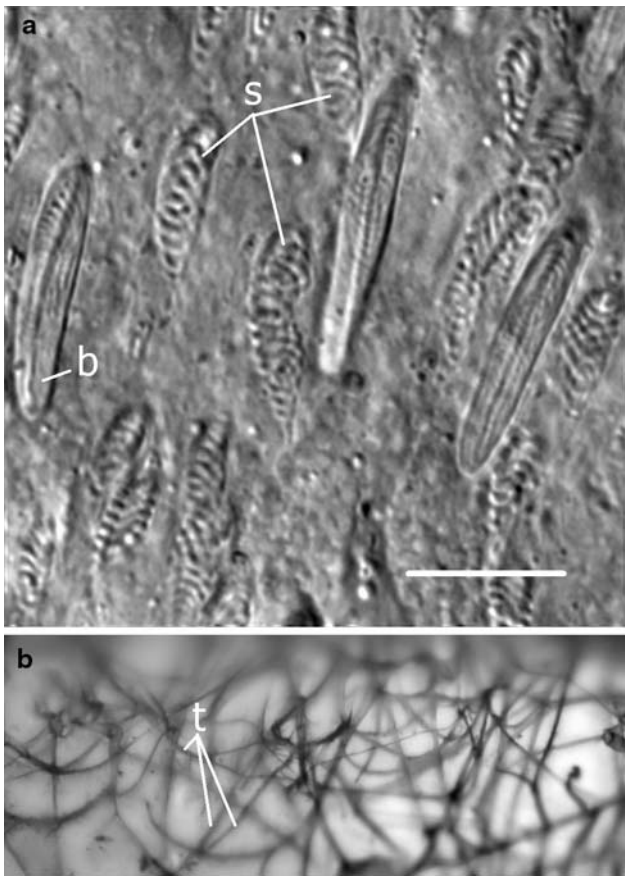

Fig. 1 Light micrographs of cnidae. **a** A fixed tentacle was imaged in top view with oblique-contrast light microscopy. Spriocysts (s) and basitrich (b) nematocysts are abundant. *Scale bar* = 10 μm. **b** Test probes, consisting of short segments of nylon fishing line, were touched to tentacles in the presence of nearby vibrations. Test probes were incubated in a dilute solution of an antibody conjugated to alkaline phosphatase followed by the appropriate substrate. *Darkly stained* tubules (t) of spirocysts are visible on the surface of a test probe. *Scale bar* = 52 μm

area computed (Fig. 2). Interestingly, the area increased as a linear function as the number of spirocysts was increased. The standard curve was limited at the upper end at $n = 13$ spirocysts because our confidence that counts were accurate fell sharply as patch size increased above 13. The slope of the line was 1.06 indicating that area might overestimate the number of spirocysts only by 6%.

Frequency response curves

In seawater alone and in the absence of vibrations, test probes had a mean of 211 ± 56 (mean ± SE based on $n = 5$ replicate experiments) spirocysts adhering to them. This response was set as the standard against which other responses were evaluated. In seawater alone and in the presence of vibrations, a significant maximum occurred at 11–15 Hz with a mean of 379 ± 90 spirocysts per probe (Fig. 3a). Interestingly, significant minima also were observed at 36–40 Hz (130 ± 34) and at 51–55 Hz (124 ± 22; Fig. 3a).

In the presence of 10^{-7} M NANA and in the absence of vibrations, discharge of spirocysts onto test probes was unaffected with a mean of 211 ± 30 spirocysts per probe. However, discharge maxima shifted to higher frequencies in the combined presence of vibrations and NANA. A significant maximum was detected at 36–40 Hz (267 ± 65 spirocysts; Fig. 3b). A modest peak at 21–25 Hz (263 ± 69) barely missed statistical significance ($P = 0.06$). Significant minima were detected at 1–5 Hz (132 ± 99),

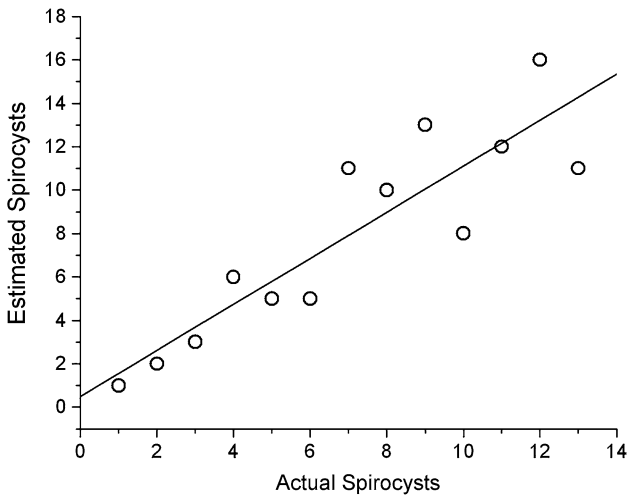

Fig. 2 Standard curve for enumerating discharged spirocysts on test probes. Test probes were examined using brightfield microscopy and micrographs collected. 'Patches' of *darkly stained* tubules of discharged spirocysts were analyzed as follows: The mean number of spirocysts counted in patches is plotted as a function of the number of spirocysts estimated by tracing the perimeter of the patch and computing its area. Patch area was divided by the mean area for a single spirocyst to give the estimated number of spirocysts per patch. A linear regression was fit to the data with $R = 0.89$

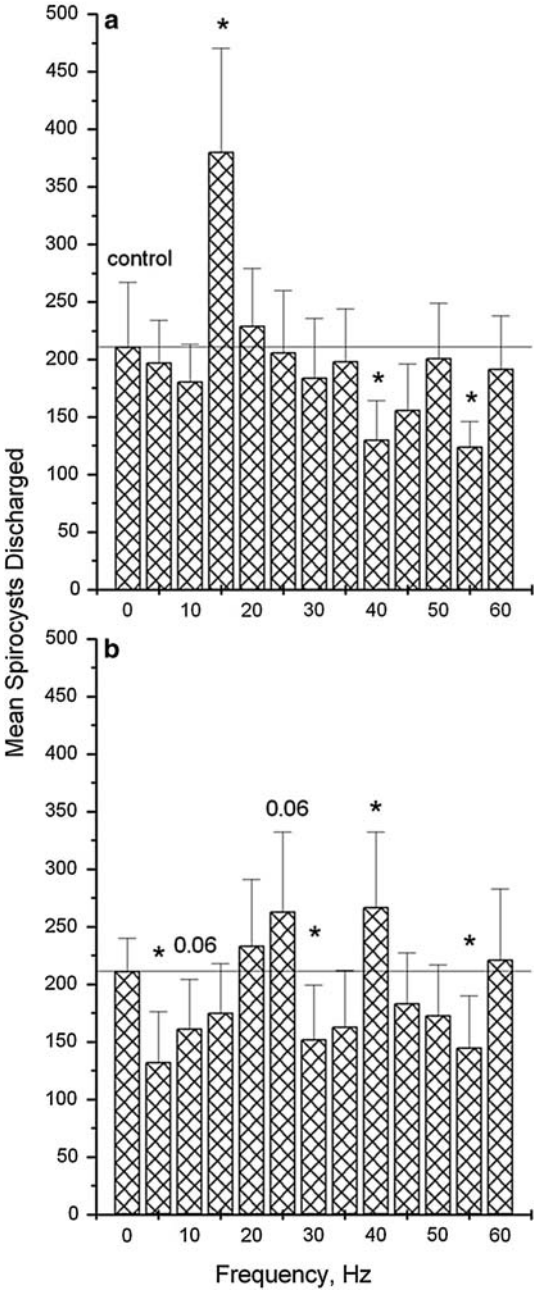

Fig. 3 Frequency response curves for spirocyst discharge. Anemones were touched with test probes in the presence of vibrations ranging from 1 to 60 Hz. The number of spirocyst tubules attached to the test probes was estimated for a total of nine test probes at each frequency. The data were pooled over 5 Hz increments (e.g., 1–5, 6–10, 11–15, and so on). The mean number of spirocyst discharged is plotted as a function of frequency for anemones tested (**a**) in seawater alone and (**b**) in seawater containing 10^{-7} M NANA. Statistical comparisons were performed for each frequency range versus non-vibrating controls. *Asterisks* indicate significant differences at the 0.05 level

26–30 Hz (152 ± 47) and at 51–55 Hz (145 ± 64; Fig. 3b). A modest minimum at 6–10 Hz (161 ± 43) barely missed statistical significance ($P = 0.06$). In order to get a better appreciation for the effects of NANA on the

frequency response curves, likewise comparisons (over the same range of frequencies, for example 1–5 Hz in seawater versus 1–5 Hz in NANA) were examined for significant differences. Exposure to NANA resulted in significant decreases in the mean number of spirocysts discharged at 1–5 Hz and at 11–15 Hz. Significant increases in discharge of spirocysts occurred at 36–40 Hz and nearly also at 21–25 Hz ($P = 0.053$).

We were interested in determining the function of spirocysts in prey capture. Accordingly, *Artemia* nauplii were released 1–2 cm away from the tips of tentacles and allowed to swim into contact with tentacles. The nauplii were captured and the tentacles bent toward the mouth. Prior to ingestion, the oral disc and tentacle crown were dissected and dropped into fixative. Fixed nauplii were teased free from the tentacles and processed for examination by FESEM. Extensive networks of spirocyst tubules were observed on the swimming appendages (antennae; Fig. 4a) or on the setae (Fig. 4b) that extend from the distal end of antennae. Networks of spirocyst tubules were never observed simply attached to the telson of the nauplius.

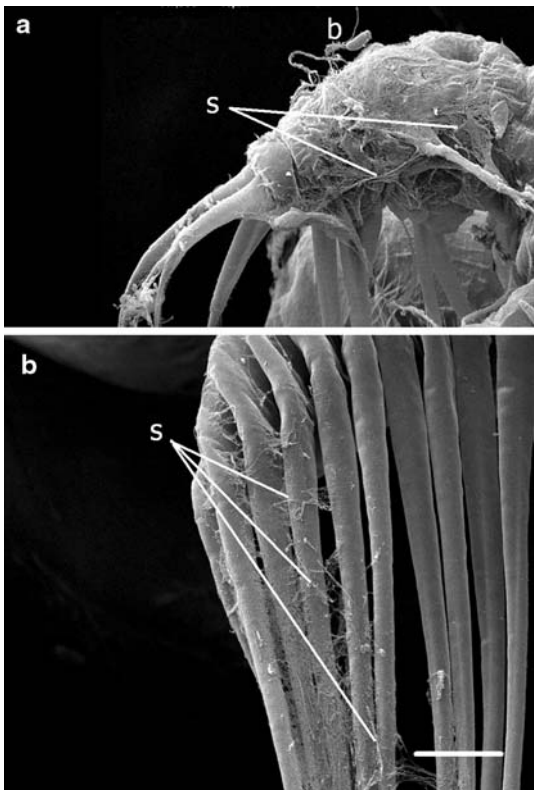

Fig. 4 Scanning electron micrographs of *Artemia* (sp) nauplii captured by *Nematostella vectensis* prior to fixation. The nauplii were teased from the surface of tentacles after fixation and further processed for examination by FESEM. **a** In the distal region of an antenna (swimming appendage), a discharged basitrich nematocyst (b) and a patch of spirocyst tubules (s) are visible. *Scale bar* = 23 μm. **b** Patches of spirocyst tubules (s) also were commonly observed on setae. *Scale bar* = 20 μm

Discussion

Spirocysts entangle swimming appendages

Our experiments using FESEM indicate that patches of spirocyst tubules commonly occur distally on antennae and setae of nauplii captured by anemones. Networks of spirocyst tubules were never observed on the telson of the nauplius although there were some instances in which bundles of spirocyst tubules tethered antennae to the telson. We conclude that spirocysts function to entangle swimming appendages of nauplii. It seems only logical that regulation of spirocyst discharge includes a component wherein maximal discharge occurs at frequencies overlapping those produced by calmly swimming prey. Nematocysts discharged from tentacles of calycophoran siphonophores likewise entangle appendages of crustacean prey (Purcell 1984).

Vibration dependent discharge of spirocysts

Spirocysts are discharged onto test probes differentially according to the frequency of nearby vibrations. Whereas in seawater alone, discharge maxima occur at 11–15 Hz, exposure to NANA induces tuning (a frequency shift) of discharge maxima to higher frequencies with a peak detected at 36–40 Hz, and possibly a second peak detected at 21–25 Hz. These data were surprising because NANA induces a shift in discharge maxima of nematocysts to lower frequencies (Watson et al. 2009). Thus, while NANA tunes maximal discharge of spirocysts, the direction of the frequency tuning is the opposite for that found to occur for nematocyst discharge. It also is interesting to consider discharge minima. Adding NANA induces the maximum of spirocyst discharge at 11–15 Hz to disappear while at the same time creating significant minima at 1–5 Hz and a nearly significant minimum at 6–10 Hz ($P = 0.06$). Taken together, NANA shifts discharge of spirocysts away from the lowest frequencies (1–15 Hz) while shifting maximal discharge of basitrich nematocysts to the lowest frequencies (1–20 Hz) (Watson et al., 2009).

Notice that the pattern of maximal discharge is complementary. In seawater, maximal discharge of spirocysts occurs at 11–15 Hz while maximal discharge of nematocysts is from 16 to 20 Hz (i.e., the discharge maximum for spirocysts occurs at lower frequencies than the discharge maximum for nematocysts). Adding NANA shifts the position of the discharge maxima such that maximal

discharge of nematocysts now occurs at the lowest frequencies (1–20 Hz) while maximal discharge of spirocysts occurs at higher frequencies (36–40 Hz and possibly also 21–25 Hz). However, under both conditions (seawater alone versus in the presence of NANA), discharge maxima of nematocysts and spirocysts do not overlap.

Exposure to NANA causes the total range of discharge maxima to broaden. Here, we are pooling the total range of discharge maxima for spirocysts and nematocysts. In seawater alone, the discharge maxima span 10 Hz (11–15 and 16–20 Hz). In NANA, discharge maxima broaden to span 25–30 Hz in NANA (1–20 and 36–40 Hz, and possibly also 21–25 Hz). How truly elegant is this system in which maximal discharge occurs in a complementary fashion across a range of frequencies.

Implications for prey capture

The results of our studies on *Nematostella* force a revision of our original model for prey capture developed using *Haliplanella* as the experimental organism. According to the original model, hair bundle mechanoreceptors on tentacles are tuned to unrealistically high frequencies in seawater alone (i.e., frequencies higher than those produced by swimming movements of potential prey) (Watson and Hessinger 1989; Watson et al. 1998; Watson and Mire 2004). Presumably, prey release *N*-acetylated sugars that bind to chemoreceptors located on hair cells of the tentacle epithelium. Such binding induces hair bundles to significantly elongate in a process that includes polymerization of actin (Mire-Thibodeaux and Watson 1994; Watson and Roberts 1995). The elongated hair bundles now resonate at frequencies that overlap the movements of calmly swimming prey. The membrane potential of hair cells (non-neuronal) depolarizes in response to prey movements (Mire and Watson 1997). Membrane depolarization of the hair cells is communicated to sensory neurons through gap-junctions (Mire et al. 2000). Presumably, neuronal communication sensitizes nematocytes to discharge nematocysts in the event of contact between the prey and tentacle. The possibility of a neuronal modulation of cnidocytes is supported by observations using TEM. Neuro-nematocyte synapses have been described in the sea anemone *Aiptasia* with differences noted according to the type of cnida (Westfall et al. 1998). In addition, neuro-spirocyte synapses also have been described in *Aiptasia* (Westfall et al. 1999).

Problems with the original model

Conceptual problems with the original model concern distance and time. First, given that tentacle chemoreceptors must bind prey-derived sugars, one wonders how close to the tentacle must the prey organism be located in order to allow frequency tuning of hair bundles. The chemoreceptors appear to be high affinity receptors insofar as they are activated at concentrations of NANA at 10^{-8} M (given 5 min exposure and based on the experimental observation of significant elongation of the hair bundles, Watson et al. 2009). Nevertheless, we presume that prey animals must be relatively close to the tentacles, no more than a few cm. Unfortunately, this important question has not yet been addressed experimentally. Second, because frequency tuning of hair bundles to lower frequencies involves morphological changes to the hair bundles (Mire and Nasse 2002) including polymerization of actin (Mire-Thibodeaux and Watson 1994; Watson and Roberts 1995), considerable time likely is required to achieve such tuning (perhaps minutes). Indeed, some experimental data suggest that such tuning requires minutes (Watson and Hessinger 1991; Mire-Thibodeaux and Watson 1995). Thus, under natural conditions how can the anemone frequency tune its hair bundles in time to maximally discharge its nematocysts into swimming prey? Given that planktonic prey commonly occur in swarms, we had long assumed that the biological significance of tuning occurred after the first several minutes of feeding had begun. At least for *Nematostella*, this assumption is incorrect.

Model for prey capture in *Nematostella*

Computer analysis of recordings of vibrations produced by individual nauplii swimming in a drop of seawater and detected by a sensitive microphone indicate maxima at 2, 7, 12, 19, 30 and 38 Hz (Watson and Hessinger 1989). Thus, a single, free-swimming nauplius produces vibrations at multiple frequencies. In seawater alone, maximal discharge of basitrich nematocysts is tuned to 16–20 Hz (Watson et al. 2009) while maximal discharge of spirocysts is tuned to 11–15 Hz. Evidently, chemodetection of *N*-acetylated sugars is not a prerequisite to capturing prey because, in seawater alone, maximal discharge of spirocysts and basitrich nematocysts occurs at frequencies overlapping swimming movements of potential prey. Interestingly, chemodetection of *N*-acetylated sugars frequency tunes maximal discharge of basitrich nematocysts to 1–20 Hz (Watson et al. 2009) and frequency tunes maximal discharge of spirocysts to 36–40 Hz and possibly also 21–25 Hz, frequencies that also are produced by swimming *Artemia* nauplii (Watson and Hessinger 1989). Thus, chemodetection of *N*-acetylated sugars broadens the range of frequencies stimulating maximal discharge and therefore, likely enhances prey capture.

Concluding remarks

The regulation of cnida discharge is a complex topic that was recently reviewed (Anderson and Bouchard 2009). We wonder to what extent do the results based on studying anemones, including those here reported, apply to other cnidarians? Studies of tentacles of hydrozoans indicate that chemoreceptors involved in regulating discharge can be located on non-cnidocytes. Whereas it is unclear whether such non-cnidocyte chemoreceptors are located on sensory neurons or supporting cells, it appears that chemosensory input is integrated by neurons that then communicate with cnidocytes (Purcell and Anderson 1995; Price and Anderson 2006). In *Physalia*, the specific ligand purified from fish mucus is uncertain although the compound is relatively small, having a mass less than 3,000 Da (Purcell and Anderson 1995). The presence of hair cells is known to occur in capitate bulbs at the ends of tentacles of the hydroid, *Coryne* (Holtmann and Thurm 2001), but it remains unclear whether other cnidarians respond to vibrations using hair bundle mechanoreceptors or whether vibration detection is involved in regulating discharge of cnidae. Finally, it is unknown whether vibration detection, if present, is modulated by chemoreceptors. From our perspective, it seems more likely that vibration detection participates in the regulation of cnida discharge in other cnidarians than the alternative hypothesis that such complex regulation of cnida discharge evolved only in anthozoans.

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