

The relationship between body size and evoked potentials from the statocysts of the prawn *Palaemon serratus*

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Summary

The organisation of the statocyst hair cells and auditory evoked potentials (AEPs) generated by the afferents in three body size classes of prawn (*Palaemon serratus*) have been studied using a combination of anatomical, electron microscopic and electrophysiological approaches. The statistical examination of the relationship between the sensory setae and body size showed an increase in both the length and number of statocyst hair cells as the animal grows. In view of this finding, the response of the statocyst organ to a 500 Hz tone burst was recorded from four specimens from each size class using two subcutaneous electrodes, positioned in the carapace close to the

supraoesophageal ganglion and statocyst. Neither body size nor the number of afferents in the statocyst has any significant impact on the amplitude of AEPs in response to the 500 Hz tone burst. The findings of this study show that *P. serratus* is capable of hearing a 500 Hz tone regardless of body size, a finding that is of ecological importance when considering the effect of anthropogenic sound on crustaceans.

Key words: crustacean, sensory system, hair cell, evoked potential, ontogeny, hearing, *Palaemon serratus*.

Introduction

It has been well established that the crustacean statocyst functions as an equilibrium organ by initiating corrective movements to maintain the animal's position in the water column (Cohen and Dijkgraaf, 1961; Fraser et al., 1987; Fraser, 2001), and more recently in auditory perception (Lovell et al., 2005a). The epithelium of the statocyst contains a number of mechanosensory hairs or setae (Hertwig et al., 1991; Sekiguchi and Terazawa, 1997) embedded in a highly innervated sensory epithelium or cushion (Prentis, 1901). The hairs support a dense statolith structure constructed from sand grains set in a gelatinous medium (Cohen and Dijkgraaf, 1961; Lovell et al., 2005a) and functions similarly to the otolith structures found in the vertebrate vestibule. In an investigation of statocyst structure and growth of the crayfish (*Cherax destructor*) Finley and Macmillan found that the total number of setae in samples from large animals was significantly higher than those in samples from small animals (Finley and Macmillan, 2000), implying the possibility of differences in the response of the organ during development.

In this work, the amplitude of the auditory evoked potentials (AEP) generated in the statocyst from three size classes of *P. serratus* when stimulated with a 500 Hz tone burst was measured, and the statocysts from each group were examined by scanning electron microscopy (SEM). The statocyst has been identified as being responsible for the mediation of acoustic

stimuli by Lovell et al., who demonstrated by both ablation of the organ and by cutting the innervating nerve that the AEP is absent post treatment (Lovell et al., 2005a). In this investigation, AEP recordings from the afferent neurones of the statocyst were recorded by averaging potentials arising from nuclei in the auditory pathway during acoustic stimulation (Lovell et al., 2005a). The AEP system used recorded the generation of neural waveforms over a user-defined time span and measures activity prior to, during and after stimulation with sound. Auditory information from diverse species is of importance because of rising concerns regarding the impact of anthropogenic sounds and other forms of marine pollution on aquatic animals. However, until recently, it has been generally assumed that crustaceans are only responsive to strong vibrations transmitted through a solid (e.g. Cohen and Dijkgraaf, 1961). This is contrary to Lovell et al.'s findings (Lovell et al., 2005a), which show that prawns hear with an acuity and frequency range similar to that of generalist fish and have the potential to be equally affected by loud anthropogenic noise sources generated in the marine environment.

Materials and methods

A total of 100 common prawns, *Palaemon serratus* (Pennant), of both sexes, and ranging in length from 27 mm to 71 mm were obtained from wild stock in the South West of

England (ordnance survey GB grid reading SX483539) using a dip net. Once captured, the prawns were dried by placing between towels, and carefully measured from the tip of the rostrum to the end of the uropods using a calliper whilst laid outstretched, then weighed on an electronic balance; the distribution of the length/mass of the captive population was normal. The prawns were transferred to a marine tank divided by a fine mesh screen into four equal sized compartments of 50 l each and the prawns divided into size class ranging from 27 mm to 40 mm (mean mass 0.3 g; group 1), 41 mm to 59 mm (mean mass 1.1 g; group 2) and 63 mm to 71 mm (mean mass 1.8 g; group 3). Prawns were placed in these categories, as the majority of the specimens appeared to belong in distinct groups composed of the previous year's cohort (group 3), the early spring cohort (group 2) and the most recent cohort (group 1). An Eheim type 2013 biological filter with a flow rate of 390 l h⁻¹ maintained water quality and provided aeration by spraying filtered seawater back into the tank *via* the filter outlet pipe located 60 mm above the water surface. In all of the experiments, and in the holding tank, the ambient water was kept at a temperature of 18°C and a salinity of 34 g l⁻¹. When not under experimental protocols, the prawns were provided with 14 h of light per day from a fluorescent tube controlled by a mains timer switch. Prior to any experimentation the prawns were divided by size into three populations; each group was fed on a granulated feed at a daily rate of 6 g for group 3 prawns, 4 g for group 2 and 2.5 g for group 1. Twelve specimens of *P. serratus* from each size class were dissected and the statocyst removed under seawater and placed in a watch glass containing 2.5 ml of 0.9% NaCl prior to microdissection. The capsules were opened by making a lateral incision around the statocyst chamber using a fine scalpel. Needlepoint tweezers were used to lift the upper section of the capsule, thus exposing the sand granules and ultrastructure. The NaCl solution was removed using a pipette and replaced with a solution of 2.5% S-carboxymethyl-L-cysteine in sodium chloride, which was used to hydrolyse the mucus surrounding the statolith receptors. The contents of the dish were gently agitated for 2 min, after which the solution was removed and replaced with chilled fixative (2.5% glutaraldehyde in 0.1 mol l⁻¹ cacodylate buffer with 3.5% sodium chloride). The statolith capsules were then dehydrated through a graded ethanol series ranging from 35% through 50%, 70% and 90% to absolute ethanol, prior to desiccation using the critical point drying method described elsewhere (Platt, 1977). Fully desiccated statolith capsules were subsequently mounted on a specimen stub using a carbon tab, and coated with approx. 8 nm of gold in an Emitech K 550 sputter coater. Finally, the processed specimens were investigated and imaged using a JEOL JSM 5600 scanning electron microscope operated at 15 kV, and a 15 mm working distance with the eucentric stage holding the specimen aligned for 'planar' image acquisition. All measurements were carried out on a PC using the analySIS® (Soft Imaging System, GmbH) program. The distance to the base of the closest neighbour was measured and recorded in micrometers, and each cell was measured from the

base to the point at which the delicate threads are first apparent at the tip; total sensory setae counts were all completed manually. Statistical calculations were carried out using the Statgraphics plus 5.1 professional edition program. Analysis of variance (ANOVA) was used to test whether or not hair cell length and distance between neighbouring bases or the amplitude of the electrophysiological response differed between each population of *Palaemon serratus*. The program constructs various tests and graphs to compare the mean values for the three size categories. The *F*-test in the ANOVA assists in determining whether any significant differences are present between the means. Multiple range tests were incorporated into the analysis as it applies a multiple comparison analysis to the data to determine which means are significantly different from one another.

AEP methodology

The procedure used to acquire the acoustically evoked potentials was approved by the United Kingdom Home Office. The test subjects were placed into a flexible cradle formed from a soft rectangular nylon mesh saturated with seawater. Oxygenated water kept at a temperature of 18°C was gravity fed at an adjustable flow rate of 3 ml s⁻¹ and directed toward the gills. The water was held in an aerated reservoir positioned in an adjacent room, and fed to the prawn through a 4 mm diameter plastic tube. The prawn was first placed lengthwise and centrally on an 80 mm×60 mm rectangle of fine nylon netting, which was wrapped firmly around the cephalothorax and pleon, and the two sides of the net were held together using a clip. A schematic of the stimulus-generating and AEP recording system along with details of the specimen holding equipment and electrode manipulators can be found elsewhere (Lovell et al., 2005a). The clip was placed in a retort stand clamp fitted with jointed electrode manipulator arms, and the aerated water pipe (for details, see Lovell et al., 2005a). During the procedure to position the electrodes, the specimen and clamp were suspended over a plastic tray, and aerated water was supplied to the prawn. A retort stand and the experimental tank (450 mm×300 mm×200 mm, L×W×D) were placed on an antivibration table, located in a concrete basement fitted with anechoic panelling 3 m×2 m×2 m (L×W×H). A selection of four prawns from each size group in the holding tank ranging from 27 mm (0.1 g) to 35 mm (0.3 g), 50 mm (1.1 g) to 55 mm (1.4 g), and 66 mm (1.8 g) to 71 mm (1.9 g), were tested with a 500 Hz, 4 cycle tone burst presented at a sound pressure level of 125 dB (re. 1 µPa) from an air mounted speaker. Working under a MEIJI binocular microscope, two small holes were made in the cuticle layer using a lancet, penetrating the carapace to a maximum depth of 0.3 mm to facilitate electrode positioning, in accordance with the published procedure (Lovell et al., 2005a). The reference electrode was located behind the supra-orbital spine, close to the neural complex associated with the antennule, and the record electrode was located in the peduncle close to the statocyst, at the junction between the lateral antennular and otic ganglia. The clamp assembly with the specimen and sited

electrodes were then suspended from the retort stand positioned over the experimental tank, and the prawn stationed 5 mm below the surface of the water. After the hearing assessment, the prawns were relocated to a holding tank for observation, prior to being returned to the divided aquarium. The evoked response was amplified and digitised to 12 bits resolution and recorded. This process was repeated 2000 times, and the response averaged to remove electrical interference caused by neural activities other than audition, and the myogenic noise generated by muscular activity. Each measurement was repeated twice, as this aids in separating the evoked response, which is the same from trace to trace, from the myogenic noise, which varies in two successive measurements. After the averaging process, the evoked potential could be detected, following the stimulus, by a short latency period of 5 ms. The latency is accounted for by the time it takes the sound in air to travel the 1 m to the prawn, plus 1–2 ms response latency. Each prawn was tested twice to show that the AEP was repeatable. In order to obtain a mean value for the EP, the μV value of the response with the largest peak-to-peak value was calculated using the root mean square method described in Eqn 1.

$$x_{\text{RMS}} = \sqrt{[1/T \int dx^2(t)]}, \quad (1)$$

where $x^2(t)$ is the amplitude of the evoked potential, and T is the duration.

The root mean square (RMS) values of both runs from the same animal were then averaged together and entered into a one way ANOVA to test the correlation between body size and receptor length on the AEP. The response of the prawn was measured using a proprietary control and analysis programme which both generated the stimulus signals and captured and analysed the response, and was installed onto the PC (for details, see Lovell et al., 2005a). Amplification of the sound was achieved using a Pioneer type SA-420 amplifier and a 200 mm Eagle L032 loudspeaker with a frequency response range of 40–18000 Hz. Additionally, the loudspeaker was placed inside a Faraday cage and connected to a centralised earth point located in an adjacent room where the PC, amplification, and analysis equipment was set up. Connecting wires were fed through a 100 mm port in the partitioning wall.

The sound field

The properties of the sound field are especially relevant when comparing the audio capabilities of both pressure-sensitive and motion-sensitive fish in the near field. In a small laboratory set-up, the complexities associated with independently measuring sound pressure and particle motion are compounded by the reflectivity of the tank sides and base. For this reason, a number of experiments have used air-mounted transducers to successfully generate sounds underwater (e.g. Fay and Popper, 1975; Yan et al., 2000; Akamatsu et al., 2002). The

principle advantage of such a system is that as the sound source is located at a distance of 1 m from the air/water interface, the moving part of the transducer does not contact the water and generate near-field displacements. In this situation the pressure and motion of the water adjacent to the receptor organ can be considered as being equal (Hawkins, 1981). The stimulus tone, presented from the loudspeaker to the prawn was calibrated using an insertion calibration. A calibrated Bruel and Kjaer (B&K, Stevenage, UK) Type 8106 hydrophone was placed in the experimental tank, and positioned adjacent to the shrimp cephalothorax region to record the intensity of the tone relative to the position of the statocyst. In addition, the intensity of the ambient noise spectrum within the holding tank was measured at a bandwidth centred around 500 Hz using the hydrophone and B&K sound analysis software. The sound pressure level was found to be 102 dB (re. 1 μPa) RMS at 500 Hz, with the Eheim filter pump active.

Results

Morphology of statocysts

The statocyst of *Palaemon serratus* is located in the basal segment of the left and right antennules (Fig. 1A,B), and is well developed and fluid filled in both male and female specimens from each of the size categories. The statocyst is oval in shape; being well rounded posteriorly and narrowing to a point anteriorly (Fig. 1C). The openings are protected by coarse setae and a thin layer of chitin that extends from the basal segment of the antennules, thus the statocyst is effectively closed to the external environment. The walls of the statocyst capsule curve symmetrically inward toward the base, where the setae are located on a mound rising $\sim 40 \mu\text{m}$ from the floor of the capsule. The number of setae in the statocyst ranged from 30 to 38 for group 1 prawns, 56 to 73

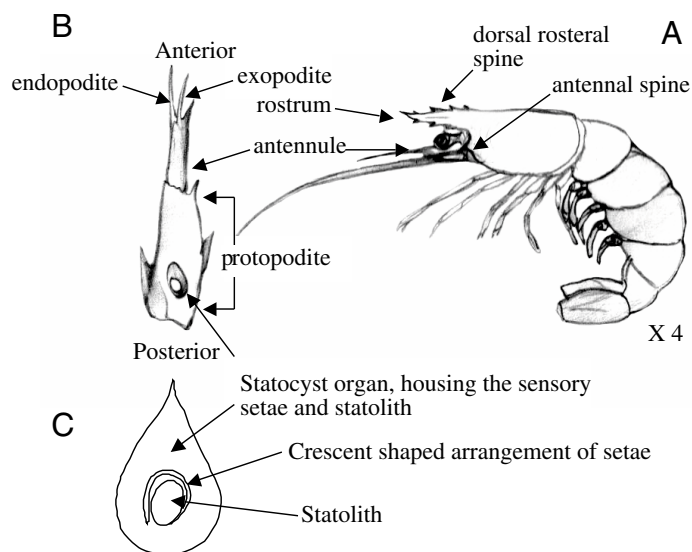


Fig. 1. (A) Diagrammatic representation of a prawn showing all body segments and location of statocyst (circled area). (B) Higher magnification of first antennule illustrating its segments and statocyst positioning. (C) The statocyst, showing the position of the sensory setae and statolith.

for the group 2 prawns, and 72 to 83 for group 3 prawns. The sensory setae in Fig. 2A are arranged in a 'horseshoe' constructed from opposing and adjacent structures. The statolithic material is primarily composed of sand grains; however other foreign substances were regularly observed in many of the specimens, as can be seen in Fig. 2A. The material is cemented to the projections from the tips of the surrounding setae by mucus, forming a dense elliptical mass. The setae are arranged in a regular row dividing further into two irregular rows at the base of the outer end of the crescent (oc), with a single sensory seta (sss) present just inside the aperture. The setae are slightly curved and orientate themselves towards the centre of the crescent (Fig. 2B), where thread-like projections from the tips of the setae interlace, forming a supportive basket. The projections (tlp) originate from the tip of the setae and are only found on the upper

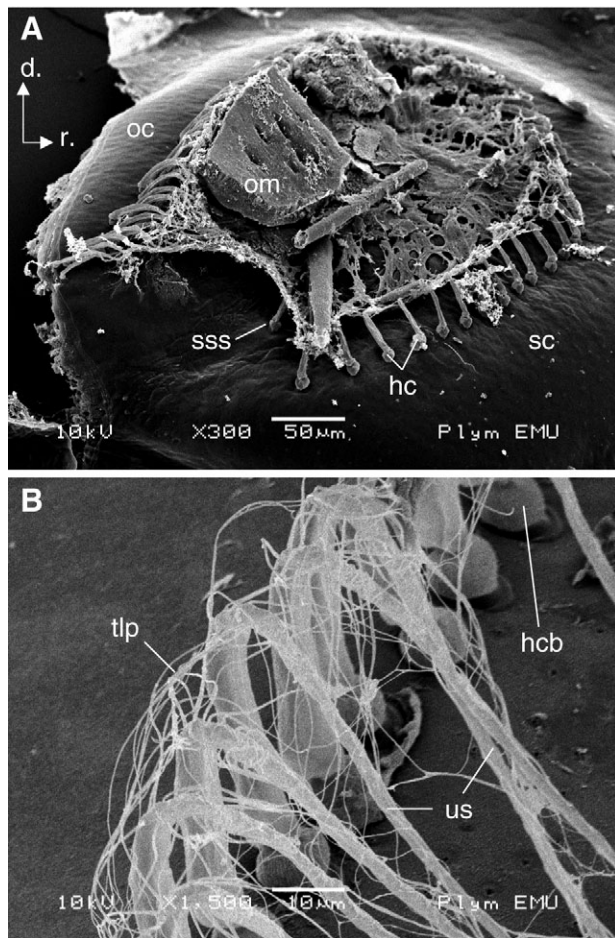


Fig. 2. (A) Scanning electron micrograph of the statocyst from a 43 mm *P. serratus* showing the solitary sensory seta (sss) positioned away from the organised row, a feature present on each specimen examined. A small amount of otolithic material (om) can be seen on the dorsal surface of the statocyst. hc, hair cell; oc, outer crescent; sc, sensory cushion. (B) Close-up of a typical row of sensory setae present on all statocyst samples, viewed from above with the binding mucous removed. hcb, hair cell base; us, upper tapering section of hair cell; tlp, thread-like projections.

portion of the cell tip, linking with adjacent and neighbouring sensory setae to create the statolith net, which works in conjunction with the mucous to hold the sand granules in position. Each seta is attached to the sensory cushion *via* a heavily ridged bulbous base, constructed of chitin resembling sclerotised cuticle. The shortest hairs (<120 µm) were found proliferating in a band running down the left side of the array, and the longest hairs (>170 µm) were found in the right caudal quadrant.

Relationship between body size and setae length

Setae lengths differed significantly from each other (one-way ANOVA: $F=207.91$, d.f., $P<0.00005$). *Post hoc* tests revealed that the setae in group 1 were significantly shorter than in the larger body groups, which did not differ from each other. The box and whisker plot (Fig. 3) shows similar mean setae lengths from both medium and large categories; the data from the small prawns were positively skewed with the largest setae outlier overlapping the mean of the medium and large data. Medium body length was also positively skewed, whereas the large body category was negatively skewed; however, the deviation from the mean in both cases was considerably less. Differences were significant at the 5% level, and to determine how significant each sample is from one another a multiple range test was completed. This revealed that the hair cell length from group 1 prawns was significantly different from that of groups 2 and 3, which showed very little significant difference.

Relationship between body size and distance between setae bases

The distance between setae bases differed significantly in the different groups (one-way ANOVA: $F=4.73$, d.f., $P<0.0095$). Group 1 prawns had the largest distance between neighbouring setae bases, with group 2 prawns having the smallest distance. All three body size categories had similar outlying data, though the group 1 data was negatively skewed whereas groups 2 and

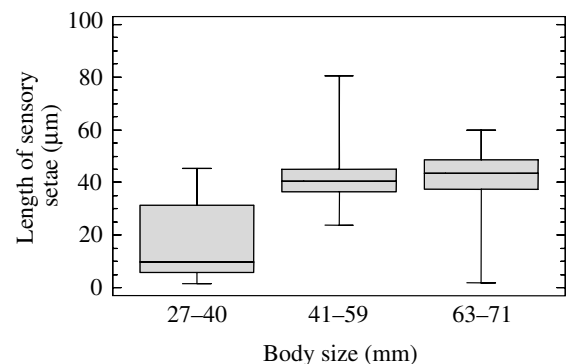


Fig. 3. Box and whisker plot displaying the relationship between setae length and body size in *P. serratus*. The grey boxes represent the lower and the upper quartile of the data, and the black line in the boxes specifies the median. The vertical bars represent the lower and upper extremes of the recorded setae lengths.

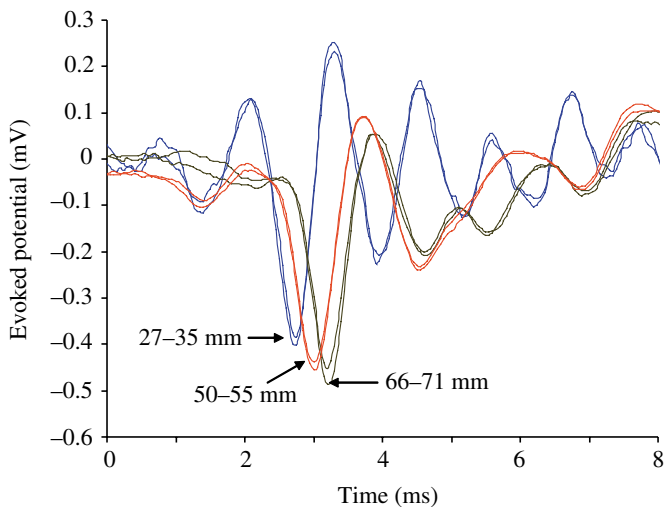


Fig. 4. Auditory evoked potentials from the 27–35 mm, the 50–55 mm and 66–71 mm body length *P. serratus* in response to a 500 Hz tone burst presented at 125 dB, and averaged from 1000 iterations of the stimulus sound. The arrows indicate the peak of the largest sinusoid of the response from the three sizes of prawn tested 27–40 mm, 41–59 mm and 63–71 mm.

3 were positively skewed. Comparing the medians allowed the differences to be accepted as being significant at the 5% limit. A multiple range test was conducted to determine which means were significantly different, resulting in one statistically significant difference, between that of group 1 and group 2 body size categories.

Electrophysiology

Twelve prawns (four from each size class) were stimulated with a 500 Hz tone burst, presented at 125 dB (re 1 µPa at 1 m). The potential difference of the largest complete sinusoid of the evoked response generated by the tone burst was calculated using the RMS equation. The evoked potentials from the three sizes of prawn, which are presented in Fig. 4, were averaged from 1000 repeat presentations of the stimulus sound. The data were grouped into the three size-related populations and the mean AEP amplitude (end column of Table 1) tested using a one-way ANOVA. This gave a result of ($F=0.08$, d.f., $P>0.925$), indicating that the AEP values are independent of body size.

Discussion

Detailed SEM observations of the statocyst reveal that *Palaemon serratus* has one very orderly row of setae arranged in a crescent, with the tips directed inwards towards a central locus. A solitary seta is located just inside the aperture of the crescent and may provide additional support to the overlying structure. The multiple range test of setae length versus body size revealed a significant difference between group 1 (27 mm to 35 mm body length) and group 2 (50 mm to 55 mm body length) categories, and between group 1 and group 3 (66 mm to 71 mm body length); however, no significant difference was found between group 2 and group 3 body sizes. This was an expected result as the size of structures increase relative to the overall growth of the organism. As *P. serratus* approaches its maximum size the growth rate slowly levels off, allowing less variation to occur within the data. Similarly, total setae number ranged from 30 to 38 in group 1 specimens, 56 to 73 in group 2, and 72 to 83 in group 3; indicating that more setae are required to support a larger statolith. In an investigation of statocyst structure and growth of the crayfish (*Cherax destructor*) Finley and Macmillan found that the total number of setae in samples from large animals was significantly higher than those in samples from small animals (Finley and Macmillan, 2000), supporting the results found in this study. The non-invasive AEP recording technique is a practicable substitute for thresholds obtained behaviourally, recording far-field synchronous activity from the neural complex associated with the organs of hearing and balance (Lovell et al., 2005b). The crab is known to code angular accelerations in three orthogonal planes through a highly developed semicircular canal system, essential for dynamically stable locomotion (Fraser, 2001). The physiological response of the system can be modulated by small changes in hydrostatic pressure (Fraser and Macdonald, 1994; Macdonald and Fraser, 1999) and relayed to the associated neural complex through rapid conduction interneurons (Fraser, 2001). However, the relatively small number of afferents in the crustacean statocyst compared to the number of afferents in the vertebrate vestibule may result in a comparatively lower signal to noise ratio (SNR) in the crustacean gravistatic system (Fraser et al., 2003). Auditory perception has as yet to be defined behaviourally, indicating a promising area for future psychoacoustic research. The use of behavioural paradigms to obtain audiological information from aquatic

Table 1. Comparison of the setae number, length and spacing along with the amplitude of auditory evoked potentials from three populations of *P. serratus*

Group	Body length (mm)	Mean number of setae	Mean seta length (µm)	Mean seta spacing (µm)	AEP amplitude (µV; RMS)
1	27–35	33	17.5	11.6	0.571
2	50–55	68	40.6	8.7	0.547
3	66–71	79	42.7	9.8	0.553

Prawn populations ranged in size from 27 mm to 71 mm.
AEP, auditory evoked potential; RMS, root mean square.

animals is a methodology favoured by a number of authors, that mostly involve classical conditioning of heart rate by administering electric shocks (e.g. McCormick and Popper, 1984; Mann et al., 1997). Also, non-invasive behavioural methodologies have been applied and require that animals are trained to react in a specified and measurable way, e.g. by seeking food when a tone at a given frequency is presented (e.g. Yan and Popper, 1992; Yan, 1995). Behavioural studies show that the spiny lobster (*Panulirus argus*) can be classically conditioned to olfactory stimuli (Livermore et al., 1997), the green crab to vibrational stimuli (Abramson and Feinman, 1988) and the estuarine crab (*Chasmagnathus spp*) to the repeated presentation of a visual danger stimulus (Merlo et al., 2005).

In conclusion, this study directly compares body size with the spatial arrangement and size of the sensory setae and amplitude of evoked potentials generated in the statocyst in response to a 500 Hz tone. The statocyst morphology of *P. serratus* is similar to closely related species, however, variation of the setae structure within the same genus was observed. Although only one frequency was tested in this work, the results show the AEP voltage averages between 0.55 μ V to 0.57 μ V from each of the experimental populations, when stimulated with a sound presented at 125 dB (re. 1 μ Pa at 1 m). As can be seen in Fig. 4, the onset latency of the response increases by nearly a millisecond in each successive size class, reflecting an increase in the length of the neuronal pathway as the prawn grows. In this experiment, the stimulus sound was generated in air, though the use of submerged transducers set up to generate a sound field dominated by particle motion can give different results compared to a pressure dominated sound field such as the one produced by the air mounted loudspeaker (see Lovell et al., 2005b). The information currently available shows that crustaceans are potentially affected by anthropogenic noise, a finding that should be considered when assessing the impact of environmental pollution on the ecology of marine animals. Given that a *P* value of 0.925 was obtained from the ANOVA, it is concluded that body size has no significant impact on the amplitude of the evoked potentials at 500 Hz, even though the length of setae altered with body size. Conversely, it has been shown (Kenyon, 1996; Wysocki and Ladich, 2001) that auditory sensitivity improves during ontogenetic development in teleost fish, though the latter found most sensitive frequency audible to the labyrinth fish (*Trichopsis vittata*) shifted from 2.5 kHz to 1.5 kHz during ontogeny. Although this work addresses the acquisition of AEPs from three body size classes of prawn, the statocyst response to frequency from a range of body size classes needs to be extended to incorporate the full audiograms defined (Lovell et al., 2005a).

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