Below the thunders of the upper deep;
Far, far beneath in the abysmal sea,
His ancient, dreamless, uninvaded sleep
The Kraken sleepeth: faintest sunlights flee...

The Kraken
Alfred Lord Tennyson, 1830

L'home no és només un problema per a si mateix, sinó també per a la biosfera en que li ha tocat viure.

Ramon Margalef

Laboratori d'Aplicacions Bioacústiques

Universitat Politècnica de Catalunya

Statocyst sensory epithelia ultrastructural analysis of Cephalopods exposed to noise

Thesis presented to obtain the Doctoral degree in **Environmental Engineering** by:

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ABSTRACT

Controlled Exposure Experiments revealed lesions in the statocysts of four cephalopod species of the Mediterranean Sea (*Sepia officinalis, Loligo vulgaris, Illex coindetii* and *Octopus vulgaris)*, when exposed to relatively low intensity low frequency sounds. The analysis was performed through: scanning (SEM) and transmission (TEM) electron microscopy techniques of the whole inner structure of the cephalopod statocysts, especially on macula and crista; SEM of the epidermal lines of cephalopod hatchlings; and proteomic studies (2DE/MALDI –MS) of the statocyst's endolymph. All exposed adult individuals presented the same lesions and the same incremental effects over time, consistent with a massive acoustic trauma observed in land species that were exposed to much higher intensities of sound. Immediately after exposure, the damage was observed in the *macula statica princeps* (*msp*) and in the *crista* sensory epithelium. Kinocilia on hair cells were either missing or were bent or flaccid. A number of hair cells showed protruding apical poles and ruptured lateral plasma membranes, most probably resulting from the extrusion of cytoplasmic material. Hair cells were also partially ejected from the sensory epithelium, and spherical holes corresponding to missing hair cells were visible in the epithelium. The cytoplasmic content of the damaged hair cells showed obvious changes, including the presence of numerous vacuoles and electron dense inclusions not seen in the control animals. The appearance of these lesions became gradually more pronounced in individuals after 12, 24, 48, 72, and 96 hours. Special attention was given to validate these findings with control animals that were caught, maintained and sequentially sacrificed following the same protocol as the exposed individuals. The statocyst ultrastructure was therefore revisited and a comparative analysis was carefully conducted to assess the lesions triggered by the exposure to noise

This study also presents preliminary results of the sound effects on epidermal lines of cephalopod hatchlings. The lesions, consistent with an acoustic trauma, were identic in the three species that were exposed, but their evolution over time, in opposition with what was observed in the statocysts, were different, suggesting that the animal size and metabolic response might play a role in a possible recovery process.

The analysis of noise effects in the statocyst endolymph by proteomic techniques was only conducted on *Sepia officinalis*. The presence of differential staining of gels from control and subjected to sound exposure individuals demonstrate that the injuries could be related to a possible physiological imbalance that would affect the protein levels of the endolymph.

The lesions and findings described here are new to cephalopod pathology. Given that low-frequency noise levels in the ocean are increasing (e.g. due to shipping, offshore industry, and naval maneuvers), that the role of cephalopods in marine ecosystems is only now beginning to be understood, and that reliable bioacoustic data on invertebrates are scarce, the present study and future investigations will bring an important contribution to the sustainable use of the marine environment.

Keywords: cephalopods, sensory systems, noise effects, electron microscopy, proteomic analysis.

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1. Introduction

1. Introduction

The massive and uncontrolled introduction of artificial sound sources in the ocean has become a threat to its balance. Ecosystems and species that occupy different trophic levels are strongly influenced by anthropogenic sound. These sources of marine noise pollution, a product of human activities, include amongst others: maritime transport, oil and gas exploration and exploitation, industrial and military sonar, experimental acoustic sources, undersea explosions, military and civilian engineering activities, supersonic aircraft noise and the construction and operation of sea-based wind farms.

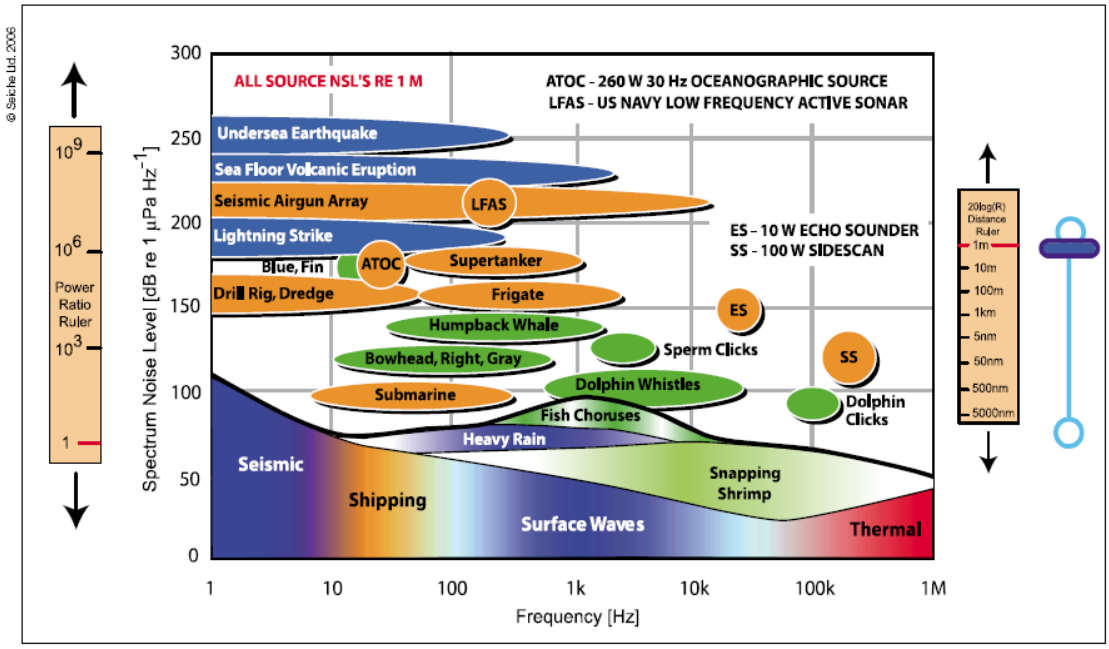


FIG. 1.1. Sound levels and frequencies from anthropogenic and natural sound sources in the marine environment (Boyd et al., 2008).

This artificial noise is introduced in the physical and acoustic space of the marine organisms and there are not yet threshold levels that would allow predicting the possible negative consequences of these interactions in a short, medium or long term on the balance of the sea. The control of these sources constitutes a scientific challenge and involves an important responsibility from society and the governments. Although the negative effects of loud sound sources such as industrial activities, seismic exploration, and vessel traffic have been demonstrated in terms of avoidance and other changes in behaviour, it has been very difficult to determine whether anthropogenic sounds actually lead to mortality.

However, it is becoming clear that anthropogenic ocean noise, at different intensity levels, can affect negatively cetacean populations, including displacement, avoidance reactions, collisions with ships, mass stranding and death. Evidence is particularly strong that high intensity active sonar, and other loud noise sources, like those from shipping, gas exploration or seismic surveys can cause lesions in acoustic organs which are severe enough to be lethal. The same sources may also produce behaviour reactions that may cause acute lesions that eventually may lead the animals to strand and die. The current scientific knowledge on the effect on noise on marine organisms and their habitat is insufficient to understand the relationships of frequencies, intensities, and duration of exposures in producing damage.

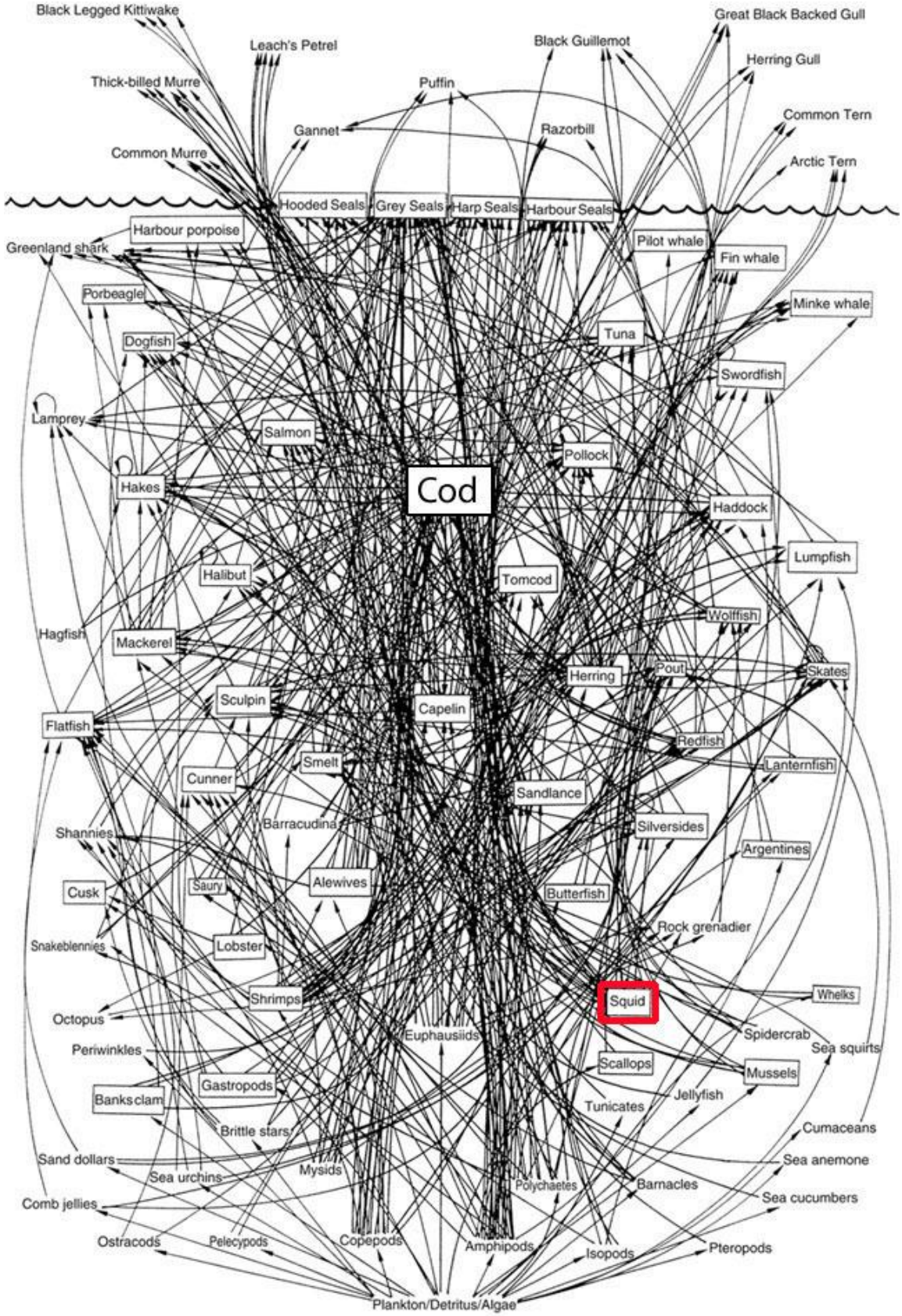
Many factors may be potentially involved in these processes: the level of sound source, transmission through the water, the position of the animal in the water column, their behavior and physiological state, as well as synergistic effects, including any chronic physical injury.

This situation requests a dynamic analysis which must go through the development and implementation of new technologies without slowing down human interests nor compromising the conservation of the marine habitat, if we want to avoid that human activities in the sea become in a short term a synonym of permanent loss of the natural balance of the oceans.

The role of cephalopods in marine ecosystems

Occupying roles as both predator and prey, cephalopods are integral components of marine food webs. Yet their role in the overall structure and functioning of marine ecosystems remains poorly understood due to their complicated life history patterns and uncertainty about their trophic position and interactions. Most cephalopod species have a short lifespan with rapid growth rates and corresponding physiological needs. They have highly plastic life history strategies meaning they are capable of responding quickly to disturbances induced both naturally and anthropogenically (Pecl and Jackson, 2008). Generally, coastal and shallow water cephalopods have short lives, about 1 year, and a life-history strategy with a single spawning followed by death soon. This framework has provided the foundation for understanding interrelationships between cephalopod life cycles, population dynamics, and environmental variability and change. Given the importance of cephalopods as prey for higher predators in the marine environment, and as a commercial fishery resource, this understanding has in turn shaped thinking more widely in marine ecosystem science. Owing to these traits, population abundances may fluctuate widely on an annual basis which can have wide effects on trophic structure throughout marine ecosystems. Most cephalopods are ecologically opportunistic, responding quickly to environmental change. Their populations are unstable owing to the absence of several year classes living at the same time. These facts and the presence of a pelagic larval phase in many species, added to their sensitivity to changes in the environment, such as temperature, make them good models to understand the impact of climate variability and change on physiological processes.

A short lifespan and spawning just once are evolutionary adaptations for ecological opportunism, enabling populations to expand rapidly when environmental conditions are favourable, but leading to rapid decline when the environment becomes unfavourable (Rocha et al., 2001; Boyle and Rodhouse, 2005) The size of cephalopod populations is therefore often variable at annual time-scales resulting from environmentally driven variability in recruitment. Exceptional recruitment events have given rise to plagues and range extensions, often with substantial impact on prey species, but such variability provides challenges for stock assessment and management of cephalopod fisheries.



A simplified food web for the Northwest Atlantic. © IMMA

FIG. 1.2. Scheme showing the position of cephalopods in the complex oceanic ecosystem occuping roles as both predator and prey on marine food webs.

Overfishing. Climate change. Ocean acidification

Overfishing is a global problem and its symptoms are well understood: overall decline in biodiversity; restructuration of food webs (trophic cascades and the proliferation of species with fast life histories at the expense of species with slow life histories); truncated age structure in populations; modified life history strategies (e.g. reduction in age/size at sexual maturity). **Climate change** also has the potential to induce changes in the structure of marine ecosystems (Hays et al., 2005). The rate of global climate change, rapid in geological terms, is slow in relation to the generation time of short-lived marine species such as cephalopods. As short-lived, ecological opportunists respond rapidly to environmental variability on annual time-scales, they are unlikely to be useful as indicators of underlying, long-term changes in the environment driven by human activity. Nevertheless, when tipping points arise in marine ecosystems, optimum conditions are created in which opportunists will thrive. Pioneer species will initially dominate, followed by an ecological succession and will lead to a new climax community. In light of these symptoms and the opportunist nature of cephalopods, there is increasing awareness that cephalopods hold a pivotal role in the structuring of marine ecosystems. Further evaluation is needed of the interactions between climate change and variability and the effects of fisheries on marine ecosystems (Scheffer et al., 2001). Cephalopods will undoubtedly play a part in the large-scale ecological changes that are predicted to occur as a consequence of global climate change. Short-term studies of cephalopod populations will contribute little into these larger-scale changes. Taken together they may reveal clearer trends. This has been demonstrated in empirical and theoretical studies noting marked increases in landing/biomass in response to fisheries pressure (Caddy and Rodhouse, 1998; Fulton et al., 2005). Due to their considerable commercial value, both directly and indirectly (Hunsicker et al., 2010), there is a need to examine how cephalopod responses to exploitation (direct and indirect) and climate change alter the structure and functioning of marine ecosystems.

The world's oceans naturally absorb carbon dioxide (CO_2) from the atmosphere. CO_2 levels in the oceans are expected to rise dramatically over the next century due to anthropogenic emissions into the atmosphere by burning fossil fuels (the ocean have absorved about 50 per cent of the amount of CO_2 released into the armosphere by the burning of them) and shift in land-use (deforestation, etc.). The increase in CO_2 entering the ocean has caused the ocean to become more acidic. **Ocean acidification** is the term that describes the increasing acidity of the ocean due to increased levels of carbon dioxide. Ocean acidification and associated changes in seawater carbonate chemistry negatively influence calcification processes and depress metabolism in many calcifying marine invertebrates. Carbonate chemistry will change as a result of increasing CO_2 and decreasing pH in seawater leading to an under-saturation of calcium carbonate. This in turn will affect marine organisms that build calcified structures. Along with reduced calcification rates found for molluscs, cnidarians and echinoderms, elevated CO_2 concentrations can disturb the acid-base regulation, blood circulation and respiration, as well as the nervous system of marine organisms, leading to long term effects such as reduced growth rates and reproduction. A growing number of studies show impacts from ocean acidification. The building block of many shelled organisms (skeletal structure of corals, phytoplankton, zooplankton…) decreases due to CO_2 increases; as a consequence, coral growth rates will be slowed. In the ocean ecosystems, changes in phytoplankton and zooplankton populations at the base of the marine food web may change ocean ecosystems equilibrium. CO_2 accumulation and lowered pH leading to a build-up of carbonic acid in the organism's body fluids can occur in invertebrates and some fish. This can lead to lowered immune response, metabolic depression, and death. Cephalopods (Gutowska et al., 2008) seem to be particularly sensitive to CO2 increases. Their energy-demanding means of moving through the water requires plentiful supplies of oxygen to the blood, which is impaired by lowered blood pH values.

The decrease in pH that results from an increase of dissolved CO_2 (The Royal Society, 2005; Solomon et al. 2007) will see CO2 partial pressure increasing leading to shifts in seawater carbonate chemistry. Cephalopods present sensory structures, the statocyts, which play an essential role as acceleration receptors and detectors of multidimensional movements (Arkhipkin and Bizikov, 2000). The statocysts are constituted by an aragonite form of $CaCO_3$ embedded in an organic matrix whose development could be challenged by the increasing acidification of the oceans. It was shown as well that ocean acidification from fossil fuel CO_2 invasion and reduced ventilation will result in significant decreases in ocean sound absorption for frequencies lower than about 10 kHz (Brewer et al., 2008) at lower pH. Ambient noise levels in the ocean within the auditory range critical for environmental, military, and economic interests are set to increase significantly due to the combined effects of decreased absorption and increasing sources from human activities at sea.

While concern about the effects on man-made noise on marine environment is increasing, there is an urgent need to define and quantify the added spatial-temporal variability of acoustic pollution from different sources and identify the resulting short, medium or long-term changes and effects on marine fauna. However, we face the relative lack of information on the sound processing and analysis mechanisms in marine organisms, particularly in cephalopods, and we still do not know enough about the important role they play in the balance and development of populations. In the context of noise pollution, the potential and direct impacts of climate change on cephalopods are in many cases speculative. However, climate change may potentially affect them at, at least, one important level: the change in water temperature has a direct effect on the sound propagation characteristics, thus probably altering sound received levels when these species are exposed to noise. In addition, ocean acidification that has a direct effect to the sensory cells of the statoliths (Guerra et al., 2011), the organs responsible for equilibrium, may alter the sensory information of these species and increase the problem.

Underwater acoustics

Sound is a physical phenomenon that resides in the mechanical oscillation of the particles in an elastic medium, produced by a vibrating element that is capable of provoking an auditory sensation, in function of the receptor's sensitivity. Sound travels at different velocity depending on the medium in which it propagates. In the case of air, its speed is around 350 meters per second while in water (a fluid of far greater density where the particles are grouped closer together) it travels at roughly 1450 meters per second. This demonstrates a significant change in the behaviour of sound waves in both scenarios, water being the medium where sound is transmitted with greater ease and therefore, over greater distances.

The oscillation of water particles, happens at a standstill, meaning that particles move themselves in relation to a position of equilibrium, transmitting this movement to their neighboring particles. This oscillation can be slow or fast producing what we differentiate between low pitch sounds (slow oscillation) or high pitch sounds (fast oscillation). The concept of frequency is used to put values on these oscillations which establish the oscillations per second that are produced in the particles from the medium with respect to their position of equilibrium. The magnitude for measuring said oscillations is Hertz (oscillations per second).

Sound propagates in the form of pressure waves. A wave is a physical magnitude that propagates in space and time. It is mathematically expressed as a "function" of space and time, analogous to magnitudes as disparate as the height of a wave of water, the electrical impulses that regulate heartbeat, or include the probability of finding a particle in quantum mechanics. Pressure waves corresponding to sound waves are thus, variations of pressure, which are transmitted through space and time resulting from movement of the particles moving themselves from their position of equilibrium, which in turn transmit this movement to neighboring particles and so on.

To understand the magnitude of Sound Pressure we must start from the concept of "atmospheric pressure", i.e. the pressure exerted by the ambient air in the absence of sound. This is measured in units of SI (Systéme International d'unités) called Pascal (1 Pascal is equal to the force of 1 Newton applied uniformly over the surface of 1 square meter and is abbreviated 1 Pa). The Sound Pressure Level, which is expressed in the abbreviation "Lp", is the expression of the magnitude of sound pressure in dB referred to a concrete magnitude (more on this later). Sound Pressure values are in general far lower than those in atmospheric pressure. For example, the most intense sounds one can hear without experiencing severe auditory pain are around 20Pa, while those hardly audible at all are nearer 20μPa (μ is the symbol for micro-Pascal, i.e. a millionth part of one Pascal).

The Decibel (dB) is the unit measure of Sound Pressure Level. It is not an absolute value but relative to a reference measure. Decibels are used since, in mammals, perception on an auditory level in pressure variations is not linear, but rather, closer to a logarithmicscale from where decibels are derived. Decibel measurements are not absolute but are calculated in comparison to a reference that is different for measurements in air and measurements in water for which both cannot be directly compared. For all of this, it is fundamental to include in all measurements the reference with respect to which levels have been calculated. Any measure is useless without specifying this reference. Typical references are 20μPa in air and 1μPa in water.

Underwater sound detection

Sound energy propagates through the water in terms of motion of the fluid particles that induce longitudinal pressure changes. Underwater hearing may involve the detection of the pressure component, the particle motion component, or the detection of either of these two sound field components (Chapman1974, Webster 1992). When a sound signal is emitted in water, water particle motion attenuates faster than pressure waves because pressure decreases linearly while displacement decreases with the square of the distance from the source. Consequently, in the field, it is difficult to propagate particle motion to fish from a distant sound source (Kojima 2010). Fishes detect the kinetic sound components by lateral lines and otolith system. When the fish is further away from the sound source, the particle motion encompasses the whole fish and causes it to move with the same phase and amplitude, whithout stimulating the lateral line system. In contrast, the otholit organs are stimulated by whole-body displacements. The otholit organs are inertial detectors in which a calcareous otolith is attached to the sensory hair cells. When a fish accelerates, the otolith moves, bending the sensory hair cells. Thus, the fish inner ear is a receptor of kinetic sound components. The working distance of the lateral lines is restricted to l body length (Coombs 1992), whereas the otolith system, as an inertial sound detector, would allow an animal to obtain sound information from a distant source.

Little is known about sound perception in cephalopods. There is indeed a considerable lack of information concerning the cephalopod reception of the sounds process (Packard et al. 1990; Bleckmann et al. 1991; Bullock and Budelmann 1991; Budelmann et al. 1995; Hu et al. 2009). Although to date there is no definitive scientific evidence for it, statocysts may play an important additional role in low frequency sound reception (Hu et al., 2009). In addition, ciliated sensory cells which behave as mechanoreceptors have been found in epidermal lines on the head and arms of different species of cephalopods (Hanlon and budelmann 1987; Sundermann 1983). It has been demonstrated that local water movements evoke microphonic potentials in the head lines of these animals (Budelmann and Blecmann 1988b). Indeed, there is controversy regarding the possibility that the statocyst would work as a low-frequency accelerometer-like detector (Packard et al., 1990) and would be able capable of detecting the component of the sound pressure field (Hu et al., 2009). While there is uncertainty regarding the biological significance of particle motion sensitivity versus acoustic pressure, recent electrophysiological methods confirmed the species' sensitivity to frequencies under 400Hz (Kaifu et al. 2008; Hu et al. 2009; Mooney et al. 2010).

Sound pressure and particle motion must be measured to be able to describe the sound field in the water. Close to a sound source ("near field") and in shallow water, there is no analitycal relation between acoustic pressure and particle motion due to the complexity in the acoustic field affected by the impedance and interference. Particle motion will therefore dominate close to the sound source. Further away ('far-field'), the effect of excess particle motion is negligible. In small water volumes, such as most experimental tanks, the acoustic impedance will be affected by factors such as source wavelength and tank dimensions (Au and Hastings, 2009). Standing waves in a small tank can generate complex pressure and particle velocity patterns compared with the free field.

The cephalopods as bioindicators of marine noise pollution

The posible effects of an acoustic impact on cephalopods and consequently, their repercussion on the whole marine ecosystem due to the fundamental role of these species on the marine food webs, are also unknown. Owing to a lack of previous works concerning acoustic trauma in cephalopods, a comprehensive study was needed to assess the direct effects of acoustic impact on these species.

The assessment of the possible noise impact of artificial sound sources in the marine environment is not a trivial question, for several reasons. The first is the relative lack of information on the mechanism of processing and analysis of sound by marine organisms. Although we are able to record and classify most of these signs, their role and importance in the balance and development of populations is unknown. The second is that the possible impact of noise not only concerns hearing systems but can also affect other sensory or systemic levels that could be equally lethal for the animal concerned. On the other hand, a prolonged exposure to certain noise can have negative consequences in the medium and long term. Sometimes, these effects are not immediately apreciated. These facts allows to understand, without excusing the lack of means provision to investigate, the great difficulty the scientific community experiences to obtain objective data to effectively control the introduction of anthropogenic noise in the sea.

Cetaceans have been the most studied group in terms of the effects of artificial sound sources. Some species of cetaceans such as sperm whale (*Physeter macrocephalus*) have been approached from a biological point of view due to the specific nature of their acoustic signals (Leaper et al., 1992; Mellinger et al., 2004; Thode, 2004). More recently, the effort has focused on more applied aspects of research such as the need to avoid ship collisions (Andre et al. 1997; Andre and Kamminga, 2000; Delory et al., 2007). The auditory system of cetaceans is characterized by a series of unique morphological adaptations such as the capacity (species-specific and related to the environment where they evolved) to select frequencies in order to distinguish acoustic images across auditory channels which act as frequency filters, the absence of vocal chords, and not using of the ear canal for hearing. These features have placed this group in a privileged position with regard to the species used as bioindicators of noise pollution.

The effects of acoustic pollution on cetaceans and the potential devastating consequences of any interference in their intra and interspecific communication system have a decisive consequence on the whole marine ecosystem, because of their role as the dominant predators in the food chain. This fact does not exclude that noise effects on other trophic levels of the food chain do not imply severe consequence to the ecosystems.

Any ecosystem is totally dependent on all the individuals who occupy different ecological niches. The ocean, as any natural ecosystem, is governed on the basis of the balance of the organisms that inhabit it. Each specific trophic level allows the development and maintains higher levels. An alteration of any of these levels can indirectly unbalance the chain in both directions.

Cephalopods have a major importance in the trophic structure of the marine ecosystems –as predators of different species of fish, bivalves and other cephalopods and as prey themselves for the larger fish, birds and mammals that are at the top of the ocean foodweb - and represent good **bio-indicators** of the natural balance of the oceans. Any harmful effect infringed on this *class* of the ancient *phyllum Mollusca* caused by the impact of acoustic sound sources, necessarily would indeed affect other trophic levels of the marine ecosystem.

The context and objectives of the study

Owing to a lack of previous reports concerning acoustic trauma in cephalopods, a comprehensive study was therefore needed to assess the direct effects of acoustic impact on cephalopods. This study was designed to conduct preliminary controlled exposure experiments on commercial species of cephalopods to assess how low frequency sounds, generally associated to human activity at sea would trigger lesions commonly found in land animals when exposed to anthropogenic sound sources. The main objective was to contribute to identifying potential injuries at different structural levels (organs, tissues and proteins) in adult individuals, hatchlings of common cuttlefish (*Sepia officinalis*), European squid (*Loligo vulgaris*), broadtail squid (*Illex coindetii*) and common octopus (*Octopus vulgaris*) of the Mediterranean Sea by imaging techniques (light (LM), scanning (SEM) and transmission electron (TEM) microscopy) and proteomic analysis.

Structure of the dissertation

The organisation of this dissertation is articulatd in chapters that reflect the technical approaches followed during the evolution of the study. Because several of these chapters were prepared to be published in peer-reviewed journals, we chose in these cases to maintain the format of a manuscript to ease the reading as self-contained information chapters, that can be read separately from the rest of the dissertation.

For that reason, the reader may found some similar paragraphs along the dissertation.

2. Literature Review

2.0 Noise pollution on marine organisms

Natural sources of ocean noise can be categorized into biological and non-biological sources. Biological sources can be produced by fishes, invertebrates and marine mammals while non-biological sources include wind, earthquakes and rain.

The introduction of artificial sound sources in the marine environment has shown to have negative effects on marine organisms. Noise pollution can cause direct effects at different structural levels, on the auditory and sensory systems and as well as on other funcional systems. Chronic exposure to noise can induce long-term effects. Noise may also have indirect effects on animals such as at the survival level, development, behaviour and population balance due to changes in access to their prey in areas used for migration routes, breeding and feeding. While marine mammals (Au and Nachtigall, 1993; Andre et al., 1997; Scheifele, 1997; Au and Green, 2000; Schlundt et al., 2000; Finneran et al., 2002; André et al., 2003; Tougaard et al., 2003; Nachtigall et al., 2004; Schlundt et al., 2006; Finneran et al., 2007; André, 2009; Lucke et al., 2009; Edren and Andersen, 2010) and fishes (Wilkins, 1972; Banner and Hyatt, 1973; Dancer et al., 1973; Rucker, 1973; Popper and Clarke, 1976; Konagaya, 1980a; Konagaya, 1980b; Blaxter et al., 1981; Schwarz and Greer, 1984; Ha, 1985; Schwarz, 1985; Hastings et al., 1996; Scholik and Yan, 2002; McCauley et al., 2003; Smith et al., 2004; Hastings and Popper, 2005; Jørgensen et al., 2005; Popper et al., 2007; Popper and Hastings, 2009; Popper and Hastings, 2009b; Kane et al., 2010) have attracted most of the attention of the research conducted in that area, fishes and invertebrates were also suspected of being negatively affected by noise exposure.

This literature survey is aimed to framing our study by synthetically describing the current knowledge of acoustic trauma triggered by noise exposure, not only on marine organisms but also on land animals for comparison.

2.1 Noise effects on cetaceans

The concept of noise pollution does not necessarily imply a pathology that leads to acoustic trauma. Noise pollution can affect cetaceans in different ways. It can hide vital information, a phenomenon called **signal masking**. Any noise source at a given level and frequency can prevent a good and vital reception of sonar echoes or communication signals. Indeed, the ambient noise derived from anthropogenic activities affect all the seas 24 hours a day and overlaps the range of frequencies used by cetaceans (below 1000 Hz, see Fig. 1.1).

The **behavioural change responses** to noise are complex and still not fully known (Richardson et al., 1995). Short term reactions to man-made sounds on cetaceans include sudden dives, fleeing from sound sources, vocal behavioural change, shorter surfacing intervals with increased respiration, attempts to protect the young, increased swim speed and abandonment of the polluted area. Little is known with respect to the long term effects on behavioural changes in individuals or populations. Nevertheless, it is suspected that the disruption of feeding activity, reproduction, migration or caring for the young induced by noise, can precipitate a reduction in successful reproduction, chance of survival in the young and a reduction in food intake.

Cetaceans can present **non-auditory alterations or injuries** as a consequence of sound exposure. In necropsies performed on beaked whales that had atypically stranded after naval

maneuvers in the Bahamas (NOAA & U.S. Navy., 2001) and in the Canary Islands (Fernández, 2006) multiple focal hemorrhages were found, particularly in the kidneys, lungs, eyes, oral cavities, peribular tissues and in the inner ear cranial cavities, tissue surrounding inter-cranial membranes and along the length of the acoustic fatty tissue (mandibles and peribular sinuses).
Another possible noise effect on cetaceans is **stress**. In this context, the term stress is used to describe physiological changes that transpire in immune (and neuroendocrine) systems following exposure to sound. The effects of intense, chronic noise in humans and other terrestrial animals is well documented (Myrberg, 1978; Richardson et al., 1995). Research has shown that noise affects some aspects of animal physiology, and can induce a stress response. Stress indicators in marine mammals have been recorded but physiological responses to stress are still not completely known. For example, dolphins undergo changes in heartbeat rhythm in response to sound exposure (Miksis et al., 2001). A beluga showed a higher hormonal stress level (norepinephrine, epinephrine and dopamine) with an increase in exposure level (Romano et al., 2004). Prolonged stress brought about by noise may weaken resistance to illnesses and endocrine imbalances that could affect an animal's ability to reproduce (Geraci and St. Aubin, 1980).

Hearing loss constitutes today one of the most important threats that Cetaceans face and would be a consequence of a repeated exposure to certain levels and frequencies. The pollution levels of a particular sound and its morphological and physiological impact depends on exposure time and intensity of the received signal. As it has been demonstrated in humans and other land mammals (Lindquist et al., 1954; Ward and Duvall, 1971; Hamernik et al., 1984) this exposure to noise can cause injuries resulting in hearing loss.

Exposure to high noise levels that are artificially introduced into the habitat of cetaceans for extended periods of time may affect them at individual and population level (Ketten, 1998).

2.1.1 Acoustic trauma in cetaceans

As it has been reported above, sound exposure can result in hearing loss (**acoustic trauma**) on marine mammals (Scheifele, 1997; Schlundt et al., 2000; Finneran et al., 2002; André et al., 2003; Nachtigall et al., 2004; Schlundt et al., 2006; Finneran et al., 2007; Lucke et al., 2009)

There are two types of trauma related to noise: lethal and sublethal impacts. The lethal impacts are those that cause the immediate death of the subject directly exposed to intense sound emission. In the case of exposure to an explosion, for example, there is a very sudden increase followed by a decrease in pressure. This process increases the cerebellar-spinal fluid pressure, which in turn causes the membranes of the oval and round windows to burst. In terms of land mammalian inner ears, data showed fracturesin the bones of the middle ear and ruptures of the tympanic membrane (Kerr and Byrne, 1975; Phillips et al., 1989; Richmond et al., 1989; Bruins and Cawood, 1991; Patterson and Hamermik, 1997; Henderson et al. 2008).

Even though there is no conclusive data on the implication of medium and low frequency acoustic pollution on the disorientation and death of these animals, it is widely accepted that the negative effects of noise can permanently affect their hearing capability.

Sublethal impacts happen when hearing loss is induced by the exposition to a perceptible sound, and are called acoustic trauma. In these cases, the exposure exceeds the tolerance limit of hearing. Basically, if any animal can hear a sound, the prolongated exposure to this sound at a certain level can damage the ear causing a reduction in sensitivity. The minimum level at which sound can be perceived is called the hearing threshold level.

If an individual needs a significantly higher intensity than usual for its species to perceive a particular frequency, there is a hearing deficit translated to a shift of the threshold level. This is

called *threshold shift*. A particular noise exposure at a sufficient level may change the hearing threshold, while different noises produced at the same level, may not cause similar changes. The question is whether a received broadcast produces a temporary loss (*temporary threshold shift*, TTS) or a permanent threshold (*permanent threshold shift*, PTS).

The mechanism of temporary hearing loss implies damage to the ciliated cells of the inner ear. The recovery periods can vary from several hours to several weeks depending on individuals. But repeated exposures to TTS levels without adequate recovery period, can cause permanent and acute threshold changes (PTS). The duration of a change in hearing threshold is directly related to the duration and intensity of exposure.

Continuous sounds may cause more severe lesions than intermittent sounds of the same intensity. In addition, the lesions that can be induced to the cochlea are not proportional to the total energy of the sound (Pourbakht and Yamasoba, 2003). In relation to the received frequency it has been shown that high frequencies are more harmful than low frequencies for the same level of intensity.

Examination of the cells of the organ of Corti (Fig. 2.2) in the cochlea (inner ear) allows the determination of the hearing threshold shift (TTS or PTS) and the corresponding frequencies affected, previous knowing the citocloclear map of the species (Schuknecht, 1994).

In terrestrial mammal studies, especially in chinchillas, rats and mice it was shown that, after minutes of sound exposure, vascular oedema appeared, usually persisting for several days. The external ciliated cells are usually more susceptible to noise exposure to internal ciliated cells. Therefore, TTS is anatomically correlated with the rigidity of estereocilia of external ciliated cells, becoming disorganized and soft.

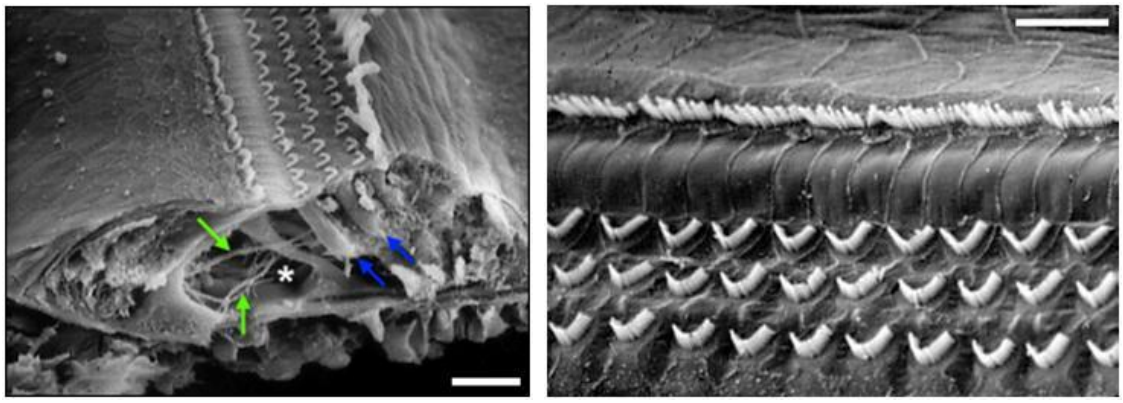


FIG. 2.1. SEM. Images of a rat cochlea. Left: The cross section shows three rows of external ciliated cells (OHC) and a row of internal ciliated cells (IHC). To the right of OHC it can be observed traces of the OHC extracted tectorial membrane. The blue arrows point to the bodies of OHC and green indicate the efferent fibers. Bar: 50 µm. **Right**: View of the apical part of rat ciliated cells. Bar: 10 µm (Source: Lenoir et al. 1987)

However, PTS is at least associated with the fusion of adjacent estereocilia and estereocilia loss. From a histopathological perspective, the first occurrence of an injury seems to be at the basis of estereocilia (in contact with the cuticular plate). The lost of estereocilia causes the death of the cell. When a ciliated cell dies, this cannot be regenerated, but it is replaced instead by a supporting cell in order to prevent the contact of the endolimfa (rich in potassium ions) with neurons. This would indeed provoke its despolarization and would lead to a walleriana degeneration or a progressive axonal degeneration and cause the loss of the primary auditory nerve fibers (Raphael, 2002). When a cell is replaced by a supporting cell, the process leaves a scar. There is a SCR-ptoteina tyrosine kinase (PTK-Mr) that might be involved in the metabolically and mechanically induced apoptosis of ciliated cells. Maybe there is an activation of this protein in the ciliated cells after exposure to external noise (Hu et al., 2000). If the

exposure is more severe, injuries can range from loss of adjacent supporting cells to the complete disruption of the entire organ of Corti.

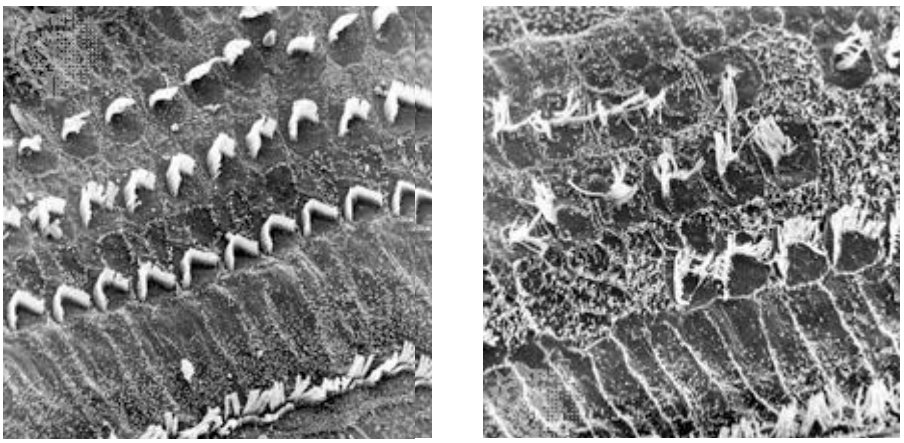


FIG. 2.2. SEM. Chinchilla cochlea. Left: Healthy chinchilla cochlea. Three rows of external ciliated cells (OHC) and a row of internal ciliated cells (IHC) are visible. **Right:** Chinchilla cochlea after sound exposure. (© Robert Preston and Joseph E. Hawkins)

For marine mammals, (Ketten, 1998) found that all species were likely to be potentially affected by sound sources of 300 Hz or higher frequencies. It was suggested that a received level of 140 dB re 1 µPa at 1m, for any narrowband source would induce a TTS.

The levels are very difficult to establish experimentally, because for ethical reasons cetaceans can not be subjected to tests of extreme noise tolerance. Also no data is available for most species of cetaceans due to the difficulty of keeping them in captivity or access them in their natural environment. The access of stranded cetaceans belonging to different species, certainly represents a promising source of information on the audiometry of these marine mammals.

2.1.2 Acoustic trauma in terrestial vertebrates

Given that, for the moment, it has not yet been possible to demonstrate the effects of acoustic trauma on marine mammals, a literature survey on terrestrial vertebrates is included in this section. Some previous studies reported indeed that sound exposure caused lesions that can result in hearing loss on terrestrial mammals (Lindquist et al. 1954; Ward and Duvall, 1971; Hamernik et al., 1984).

Research and experiments on amphibians (Hodichok and Steyger, 2007), mammals (Robertson, 1981; Lenoir et al. 1987; Schuknecht, 1994; Leonova and Raphael, 1997; Raphael, 2002; Pourbakht and Yamasoba, 2003) and avians (Nakagawa et al., 1997) allowed to determine inner ear sensory epithelia damage after acoustic trauma or ototoxic treatment (Nakagawa et al., 1997b). The lesions were similar in both cases (Leonova and Raphael, 1997; Hodichok and Steyger, 2007). Some authors reported that sound overstimulation induced sensory hair cell loss (Slepecky et al., 1982; Nakagawa et al., 1997; Hawkins and Schacht, 2005) or alterations on stereocilia of hair cells including losing, buckling, bending, breaking or fusion in giant stereocilia, wrinkled membranes of flaccid stereocilia, floppy stereocilia and reduction of stereocilia size (Bredberg et al. 1972; Slepecky et al., 1982; Hamernik et al., 1984; Pye and Ulehlova, 1989; Raphael, 2002; Pourbakht and Yamasoba, 2003). The supporting cells responded to auditory overstimulation by an increase in the number and size of microvilli (Bredberget al, 1972). The hair cell degeneration is usually produced by two types of typical processes; *necrosis* (swelling of the cell body and rupture of the plasma membrane) and

apoptosis (chromatin compaction and fragmentation of the cell body) (Li et al., 1995). Hair cells which do not progress into typical cell death process can be deleted by *extrusion* from the epithelium after sound exposure. In a mammal's organ of Corti cell death results in degeneration by necrosis or apoptosis (Theopold and Scheler, 1981; Slepecky et al., 1982; Fredelius, 1988; Fredelius and Rask-Andersen, 1990) and is followed by a removal of damaged cells debris through phagocytes (Hirose et al., 2005; Ladrech et al., 2007). Apoptosis is thought to play an important role on maintaining tissue homeostasis (Nakagawa et al., 1997b); (Oyadomari and Mori, 2004). Necrosis induces inflammatory responses and secondary damage to tissue structure, which can result in irreversible destruction. In the basilar papilla of avian's inner ear the cells can be deleted by extrusion (Cotanche, 1987) just after sound exposure or deteriorated within the epithelium and contribute to the following repair process (Nakagawa et al., 1997; Hu et al., 2000b). Disintegration of hair cells increases with time after the exposure, and in mammals this can result in the entire disappearance of the organ of Corti during the course of several weeks (Spoendlin, 1971). In avians experiencing acoustic trauma, hair cells which are affected by apoptosis within the basilar papilla continue degenerating after the beginning of the cell regeneration process. After acoustic trauma, lost hair cells are replaced by expansion of adjacent supporting cells which form a scar (Corwin and Cotanche, 1988).

Several factors can contribute to blistering and cell extrusion: changes in the osmotic pressure of the extracellular fluids resulting in an increase in cell volume, changes in cell mechanical, elastic or tensile properties. In noise trauma, the mixing of cochlear fluids cause changes in the osmotic pressure and cell swelling (Hamernik et al., 1984). Direct mechanical destruction as well as metabolic exhaustion are competing factors in acoustic traumatic damage to the cochlea. Direct mechanical damage (the organ of Corti is disintegrated with ruptures on pillar heads or reticular membrane, or totally missing, the sensory cells are dislocated or expulsed) is usually irreversible and appears immediately after short exposures at high intensities, whereas metabolically induced damage (swelling of sensory cells, vacuolization of cytoplasm, mitochondrial degeneration, swelling of dendrites) is partly reversible, occurs after long exposures with moderate intensities and develops over a longer period after sound exposure (Spoendlin and Brun, 1973). Early changes in hair cells consist basically of hair cells stereocillia abnormalities and protusion and loss of microvilli on supporting cells. Subsequently there is a progressive hair cell loss by protusion and posterior expulsion whereas supporting cells repair the lamina reticular (Lim and Melnick, 1971b; Thorne, Gavin, Herdson, 1984). The susceptibility to loss of the affected cells depends on their position within the lesion area (Thorne, Gavin, Herdson, 1984). In the cochlea of mammals, the first row of outer hair cells and the inner hair cells are generally more affected than row two and row three of outer hair cells.

Two mechanisms were proposed to explain the noise-induced hearing loss in mammals, mechanical injuries to the receptor cells induced by excessive movement of the cochlear partition, and damage due to metabolic exhaustion resulting in distortion of the homeostasis of the organ of Corti. It is probable that both mechanisms contribute to the process. Noise trauma causes alterations to the permeability of the hair cell stereocilia membrane, as well as changes in the surface and arrangement of actin (Slepecky et al., 1982).

In vertebrates, intense sound can also induce excitotoxicity, which is the excessive release of the neurotransmitter glutamate from the inner hair cells to the underlying post-synaptic element, the afferent dendrites of the type I spiral ganglion neurons (Cappaert et al., 2000; Mumtaz et al., 1999; Pujol and Puel, 1999; Bledsoe et al., 1980; Coyle and Puttfarcken, 1993). The excitotoxic effects of glutamate in response to noise could be the result of increased release or inadequate removal of glutamate, primarily due to the breakdown of recycling mechanisms (Rebillard et al., 2003). This may eventually result in toxic cellular events leading to neuronal degeneration and cochlear damage.

2.2 Noise effect on fishes

2.2.1. Sound emissions

Despite fishes do not possess a larynx, some fish species produce sounds by rubbing serrated surface components on their skeletal structure. Many species produce very sharp sounds by grinding their teeth but it is the vibrations of the swim bladder wall, using specialized muscles, which emits the greatest range of sounds or repertoire of calls.

Some fish species produce a variety of sounds, for example, herring (*Clupea harrengus*) produces "chirp" (pulses that vary in a range between 1800 and 3200 Hz) and "whistles" (continuous sounds with frequencies between 1600 and 2000 Hz) apart from the noise caused by hydrodynamic and feeding (Schwarz and Greer, 1984). Regarding the importance of acoustic signals in the social behaviour of fishes, there are observations that support the hypothesis that many fishes produce sounds deliberatelly (Schwarz, 1985), besides having the ability to ear them. This, added to the complexity of the teleost auditory system, suggests that marine environments affected by acoustic stimuli must necessarily affect the ability of fish survival.

2.2.2 Perception of sounds in fish

The ear processes in terms of a sound capture organ is well-known in some species of fishes. There are also some works about acoustic impact effects, from the oldest which prioritized ethological or behavioural response to the most recent which examined the impact of acoustic trauma.

For small vertebrates and invertebrates, the perception of sounds and pressure respond to similar mechanisms due to the fact that sound propagation through water requires a variation in pressure, as well as in a slow or fast displacement of its particles. In teleostean (bony) fishes, the swim bladder is clearly a potential pressure receiver, for it is flexible, gasified and reacts to pressure fluctuations by changing its volume. Furthermore, the swim bladder of many fish species has been discovered to have a direct or indirect relation to the perilymph of the inner ear.

Fish lacking swim or gas bladders perceive nearby acoustic pressures, since these are transmitted by bone conduction, vibration of the otolith or by lateral linear reaction, and being insensitive to distant sounds that surpass 400 Hz. Fishes with swim or gas bladders without inner-ear connections posses excellent reflexes conditioned with frequencies inferior to 520 Hz. Some species have a direct connection which allows them to pick up varying frequency levels from 13 to 4000 Hz. If a fish remains at depths without varying, for compensation, the volume of the swim bladder, not only will it jeopardize its flotation system but also its ability to perceive sounds.

With regard to pressure, fish possessing a swim bladder can perceive equivalent variations of less than 0.5% of the hydrostatic environment, whilst those without can only perceive changes that vary between 2.5-10%. The distinction between frequencies in the case of ‘bony’ fish is similar to the process that takes place in the cochlea (the coiled part of the inner ear) of vertebrates, although the precise mechanism in the case of bony fish is unknown.

The regionalization of the frequencies is similar to the mechanism that takes place in the cochlea of vertebrates, in which different frequencies stimulate different areas of the macula,

however, the exact mechanism of frequency discrimination in the case of fish bone is unknown (Cox et al., 1986). Fishes have a high variability regarding the frequency range that they can detect and in their sensitivity to these frequencies, however some species may have similar hearing capabilities if they live in similar acoustic environments (Schwarz, 1985). Some marine fishes can detect very high frequencies (herring from 5 to 10 kHz), but most are only sensitive to frequencies below 2000 Hz (Myrberg, 1978).

Some work on the hearing and the structure of the inner ear of marine catfish (*Arius felis*) showed that it can detect sounds with frequencies between 50 and 1000 Hz, but presents an optimal capacity of hearing between 100 and 200 Hz (Popper and Tavolga, 1981). These results contrast with the hearing capabilities of other ostariophysans that can detect sounds above 3000 Hz, and present great sensitivity between 500 and 1000 Hz. The utricle of the marine catfish is longer and has a pattern different from other ostariophysans sensory epithelia, it is probably an adaptation to capture low-frequency sound to detect objects in their environment through 'echolocation.

Popper and Clarke (1976) studied the hearing ability of the salmon (*Salmo salar*) in sea and in laboratory conditions. The fish responded only to tones of low frequency (below 380 Hz); stimuli caused by the movement of particles were found to be more important than the sound pressure. The fish sensitivity to the sound was not affected by the noise of the sea under natural conditions, but the hearing ability is affected by the noise of a turbulent river. Sound measurements made on the river Dee, near Aberdeen in Scotland, concluded that the salmon can not detect sounds originating in air unless the source is very close and directly above it. Compared with carp and cod, salmon hearing is poor and similar to the European perch (*Perca fluviatilis*) and flounder (*Pleuronectes platessa*).

2.2.3 Underwater noise effects on fishes

There are three main ways in which underwater noise may affect marine fish: physiologically, pathologically and behaviourally. Several studies have been conducted to measure the effect on **behavioural and abundance changes** in populations in their natural enviromement (Wilkins, 1972; Banner and Hyatt, 1973; Dancer et al., 1973; Rucker, 1973; Popper and Clarke, 1976; Konagaya, 1980a; Konagaya, 1980b; Blaxter et al., 1981; Ha, 1985; Schwarz, 1985; Skalski 1992; Wardle et al., 2001). Adult and larval fish have been used to identify **pathological effects**, such as hemorrhaging, ruptures and damaged acoustic receptor organs -inner ear sensory epithelia, lateral line system and other tissues damaged by acoustic impact (Hastings et al., 1996; Scholik and Yan, 2002; McCauley et al., 2003; Smith et al. 2004; Jørgensen et al., 2005)- and death induced by underwater explosives (Pearson et al. 1992; Santulli et al., 1999). Other studies (Santulli et al., 1999) suggested that underwater noise could lead to a **stress response** in exposed fish. Some works have shown that signals with a rise time have a more notable impact on behaviour (McCauley et al., 2000a) and signals with a short pulse length are not perceived as intense as sounds of longer duration (Popper and Fay, 1993).

2.2.3.1Behavioural effects

Many of the behavioural changes are due to a pathological damage and/or the stress induced by physiologycal and biochemical changes on exposed fishes (Schreck, 1990). These behavioural alterations can have a significant effect on the survival of the animal. Food foraging, predator evasion, reproduction, habitat selection and intra and extra interactions will be affected (Schreck, 1990; McCormick, 1998).

Recent research has determined that sound can be used to control fish behaviour (Popper, 2002); (Kuwada et al., 2000). Observations on the behavior of rainbow trout (*Salmo gairdneri*) exposed to acoustic pulses characteristic of shock wave indicated quite mild reactions (Rucker,

1973). The levels of glucose and cortisol in the blood of rainbow trout exposed to simulations of acoustic pulses were similar to those of control individuals.

There are very few works which refer to the effect of sound on sharks, but this is a group absolutely depending on sound to locate their prey. Beulig (1982) showed that sharks were attracted by irregular pulses of broadband and low frequency sound, between 20 and 100 Hz. To investigate whether the sharks were attracted by significant biological sounds (such acceleration of the masses of fish, fighting or wounded fish, and eating animals) that produce sounds with frequencies below 20 Hz, Beulig (1982) measured responses of juvenile shark *Negaprion brevirostris* at low frequencies (12.5 Hz), with irregular pulses of sound. Sharks were born in captivity and neither had experience in catching their natural prey, nor had interaction with wild sharks. Initially, individually tested juvenile sharks, were not attracted by low frequency sounds, even when the capture of live preys was conditioned to auditory stimulation associated with wounded or struggling fish. When sharks were tested in groups of three, the level of response indicated some attractive approach for low frequency sounds and the results were comparable to those of juvenile shark that had experience in capturing their natural prey. Therefore it proves the existence of a social factor in terms of responsiveness to the sounds of the shark (Beulig, 1982).

The effect of the shock wave in fish eggs during critical stages of their development was studied in some fishfarms in Nevada, Oregon and Washington (Rucker, 1973). During its development the fish larves suffer a critical period in which they become susceptible to vibrations or turbulence (approximately the first 24 hours after hatchling and before the development stage in which it appears the eyes). The eggs of the trout *Salmo clarkii* and the salmon *Oncorhynchus tshawytscha* were exposed to the shock wave produced by military jets (F-111 or F-101) or simulations of shock wave of variable presure. Different experiments were made though a unic exposure to shock wave, and repeated exposures over several days. The comparison with control groups showed that there was an increase in mortality of eggs.

Schwarz and Greer (1984) described the behavioural responses of Pacific herring (*Clupea harengus pallasi*) subjected to different sounds: moving and stopped ships, sonar, echo sunder and deck mechanical noise. Herring did not show visible response to sounds produced by sonar or echo sunder. But it showed avoidance response when they were exposed to sounds of large vessels approaching at a constant speed, to sounds produced by small boats approaching with acceleration and to 11 different electronically synthesized sounds. They showed reaction of alarm, or less frequently panic when they were subjected to those electronic sounds that produced an increase in the instantaneous amplitude. The herring also showed a characteristic panic response when they were subjected to vibratory stimuli obtained by sounds at pressures between 2 and 18 Pa caused by a diaphragm in the wall of the tank (Blaxter et al. 1981).

Growth rates of *Cyprinodon variegatus* and *Fundulus similis* (sheepshead minnow / longnose killifish) were significantly reduced when the tanks were exposed to high levels of noise by over 20 dB re 1 μPa at 1m (Banner and Hyatt, 1973). The viability of eggs of sheepshead minnow also decreased significantly. The lethal effects of sound are apparently restricted to the embryonic stages of the species.

The effects due to acoustic pulses produced by shock waves in guppies, showed that fish subjected to the effects of the shock wave produced by a generator only showed reactions of short duration (0.5 sec.), when they were subjected to intensities above 1 mbar (Dancer et al., 1973). The images of fish in small tanks when a bullet had passed at a speed of 1200 m/sec a few cm above the tank, indicated that the fish could feel the dynamics wave, but this didn't produce any trauma on them (Wilkins, 1972). The increase in pressure due to the dynamic wave was 0.26 atm, 275 times the pressure associated with the sound of a shock wave of a supersonic transport.

While ascending streams from the sea to the upper river the Asian species *Plecoglossus altivelis* (asiatic "Ayu") have a strong anadromous character and presented a reaction that involved jumping, not only when hearing a sound produced by the falls, but also the underwater sound (Konagaya, 1980b). This response of jump when they heard the sound underwater was studied and showed a greater sensitivity to frequencies of approximately 200 Hz, however it was not possible to determine the sensitivity of the species above 600 Hz. The lowest hearing threshold for underwater sound that created a response of jump was 72 dB re 1 μPa at 1m at 200 Hz. The number of fishes that respond to sound pressure was distributed as a normal curve in the range between 70 and 80 dB re 1 μPa at 1m.

Changes in the acoustic environment were analysed to determine the effects of noise produced by dredging in Lake Biwa, Japan on the fish stocks (Konagaya, 1980a). By acoustic biotelemetry, this work analyzed the response of fishes to dredging noise as well astheir swimming direction close to the area where works were taking place. The level of background noise from Lake Biwa was within the limits of prevalent noise at sea. The pressure level of underwater sound produced by a drainage vessel at a distance of 150 m was approximately 38 dB re 1 μPa at 1m, and a submerged pipe at a distance of 2 m was 75 dB re 1 μPa at 1m. Fishes showed no response to noise but avoided the acoustic field produced by dredging.

SPECIES	NOISE TYPE	EFFECT
Rainbow trout	Shock wave (Rucker, 1973)	Slight behavioural reaction
Herring	Sounds of recorded fishing boats (Schwarz and Greer 1984)	Alarm and panic reaction avoiding the sound source
	Sound produced by pressure waves between 2 and 18 Pa in the walls of the tank (Blaxter and Hoss 1981)	Panic reaction
Sheepshead minnow/ Longnose killifish	Tank exposed to high noise levels (above 30 dB re 1 μPa at 1m) (Banner and Hyatt 1973)	Reduction on growth rate and eggs viability
Guppy	Simulation of shock waves (Dancer, 1973)	Short duration reactions
Asiatic "ayu"	Underwater sounds (200-600 Hz, 72-80 dB re 1 μPa a 1m) (Konagaya 1980a)	Jumping
Undetermined species	Underwater dredging sounds (38-75 dB re 1 μPa a 1m) (Konagaya 1980b)	Avoiding the acoustic field produced by dredging
Finfish	Seismic survey noise (Engas, 1993)	Behavioural and abundance changes
Rockfish	Airgun (200-205 dB re1 μPa a 1m) (Pearson 1992, Wardle 2001)	Startle response

TABLE 2.1. Some examples of negative effects on fish behaviour caused by sound exposure

Behavioural responses that were observed in marine finfish in response to noise, include: changes in schooling behaviour (Pearsonet al., 1992), changes in positioning in the water column (Dalen and Raknes, 1985; Greene, 1985; Pearson et al., 1992), reluctance to take baited hooks (Skalski et al., 1992), changes in swimming speeds (Engas and Løkkeborg, 2002) , migration (Lokkeborg and Soldal, 1993; Engas et al., 1996) and startle responses (Blaxter et al., 1981; Wardle et al., 2001).

Some studies reported behavioural and abundance changes in finfish induced by seismic survey noise (Engas et al., 1993). Pearson et al. (1992) exposed captive rockfish (*Sebastes spp*.) to noise from 1639 cm^3 airgun; at 200-205dB re 1 μPa a startle response was observed. A **startle response** is an involuntary reflex behaviour induced by an advers estimuli –visual or acoustic-. An unilateral muscular contraction bends the fish into a "C" or "S" shape. This stage is followed by a propulsive phase where the tail bends in the oposite direction, turning the body of the fish, which is accelerated forward. This state involves a period of sustained swimming (Godin, 1997). In natural conditions, startled response has evolved as an effective mechanism used for evading predators. Other authors have also reported startled responses to air-gun noise (Pearson et al. 1992; Wardle et al., 2001).

As shown in a previous section (**Sound emissions),** many fishes are known to produce sounds. These sounds are used in social interactions between individuals of the same species -courtship behaviour- and amongst different species -warning signals, calls for help, to elicit an escape response, etc. (Bone et al., 1995; Godin, 1997; Hawkins and Schacht, 2005). **Interference with acoustic communication** could alter the behaviour of both the animals generating the signal and the receivers. Interference of these acoustic signals by anthropogenic underwater sounds could also lead to a masking of the communicative signal.

2.2.3.2 Effects of noise on stress response

In addition to these previous works which prioritized ethological or behavioral effects of fishes exposed to sound, both in their natural environment and in laboratory conditions, some recent works analyzed the effects of underwater noise on stress levels. Noise exposure has been reported indeed to induce stress response in fishes (Smith et al., 2004). **Stress** is defined as the response reaction by an animal to a stimulus that may alter the animal's homeostatic state (Barton and Iwama, 1991).

The physiologycal reponse of fish to environmental stressors has been widely studied (Barton and Iwama, 1991). Exposure to a stressor results in a cascade of events that can be organised into primary, secundary and tertiary effects, depending on the level of the organization of the reponse (Barton et al., 1986). Primary response involves increased levels of circulating cortico-steroids and catchcolamines (Reid et al., 1998). The increased level of these hormones induces secundary effects (metabolic, hydromineral, hematological ans structural changes (Barton and Iwama, 1991). If stress is severe or prolonged, it could induce tertiary effects which involve whole animals and population level reponses. At an individual level, these include decreased growth, reduced reproductive success, reduced immunocompetence and death (Weytes et al., 1999). At the population level, they include reduced growth rate and altered species abundance and diversity (Rusby, 1995).

Smith (2004) analyzed the effects at short and long term, caused by anthropogenic sounds (ships, seismic experiments, sonar systems and water pumping for aquaculture facilities) in the stress level and on the hearing capabilities (*Carrasius auratus*, a hearing specialist). The individuals were kept in conditions of high noise exposure (white noise, 160-170 dB re 1 μPa) during different duration periods, and were compared with other animals kept at 110-125 dB re 1μPa. The work showed that noise induced physiological stress by measuring plasma cortisol and glucose levels. By the technique of ABR (Auditory Brainstem Response), which measures

neural activity in response to an auditory stimulus, a technique that is used routinely to measure the hearing of fish and other vertebrates (Corwin et al., 1982; Kenyon et al., 1988; Higgs et al., 2001; Scholik and Yan, 2001), it was shown that sound exposure also produced effects on hearing.

Air-gun noise was also reported to induce stress response in captive fish (Santulli et al., 1999).

2.2.3.3 Pathological effects and acoustic trauma on fishes

Diverse species of fishes have been used to identify pathological effects, such as hemorrhaging, ruptures and damaged acoustic receptor organs and death.

Evidence now exists that shows intense sound can damage fish auditory system. The variation in auditory systems amongst fish species contributes to the unpredictability of determing which will be the effects of noiseon a particular species. Generally speaking, the fish ear includes three organs (saccule, lagena and utricle). Each contains a dense calcareus otolith. In a sound field, the differential motion between the otolith and the sensory epithelim deflects the hair cells, thus resulting in an electrical impulse, which in turn induces a neurotransmitter release that stimulates the neurons innervting the sensory cells. The signal is then interpreted by the brain as a sound.

Intense stimulation above the threshold of hearing in goldfish (*Carassius auratus*) indicated that a level stimulation of 300 and 500 Hz caused a decrease in hearing threshold between 800 and 1000 Hz (Popper and Clarke, 1976). This species is sensitive to frequencies ranging between 70 Hz and 4600 Hz (Sawa, 1976). The inner ear of teleost fish responds in a complex way to acoustic stimuli of different frequencies, which could indicate a gradation of the spatial analysis of the acoustic signal in the inner ear.

During a study of the effects on the striped snapper hearing (*Lutjanus synagris*), hearing thresholds of individuals kept in an aquarium were significantly higher than normal when they were tested, *a posteriori*, on devices with a low noise level (Ha, 1985). The only difference in these tanks (the tanks containing the fish less sensitive to sound) was that they had conventional porous stones for the dissemination of air to oxygenate the water. Hearing tests in two groups of fishes proved that fish kept in aquaria without porous stones showed significant differences in auditory sensitivity.

Other researchers (Scholik and Yan, 2002) referenced, using the ABR technique, that a small boat with an engine of 55 horsepower produced a clear increase in the threshold of hearing in *Pimephales promelas*. The study concluded that if a short exposure to a not overly powerful noise source had produced effects on the hearing threshold, it was extrapolable that the engines of the boats had to produce a great environmental impact on fish.

Hastings et al. (1996) studied the effects of low frequency underwater sound on ciliated cells of the inner ear and lateral line of teleost fish. McCauley et al. (2003) analyzed the effects on hearing in fish caused by high intensity sound of anthropogenic origin. Other lesions that fish can present as a result of these detonations are breakage on the swim bladder, hemorrhage and rupture of muscles and viscera.

Some studies indicated that air-gun noise was not normally lethal for adult fishes (Rusby, 1995) but it could induce some pathological effects such as swim bladder collapse or damage, broken blood vessels in the liver and gonads (Rusby, 1995). Holliday recorded a significant decrease in the survival of anchovy (*Engraulis mordax*) eggs after air-gun exposure (Holliday et al., 1987).

The lateral line of fish is a sensory organ that provides information regarding the fish position within its school. Externally, it has a prominence that covers the sides of the fish from tail to head, where it forms several branches around the eye and tail. Internally, it comprises some channels filled with a gelatinous substance, which are accessible from the outside by tiny pores. The channels are covered with thousands of cells sensitive to vibration, similar to the sound receptor cells that cover the human ear. With these mechanisms, a fish can sense vibrations produced by the neighbour swimming in the water, and thus control the speed and direction of its neighbors within the school of fishes. The information coming from the lateral line and eyes, allows the perfect coordination of movements of schools of fishes. The ear also provides information on proprioception and balance.

The detonations in the marine environment can temporarily damage the lateral line, eyes and ears, or be incompatible with fish life. It can cause the dispersion of fish stocks, which significantly reduces their chances of survival. On the other hand, a quick escape produces the use of energy reserves, stress and inmunodepression, making them more vulnerable and can also cause infertility and decrease in growth.

A thorough and complete work was performed by Jørgensen et al. (2005), who analyzed the effects of low frequency sonar signals on survival, development and behaviour of larval and juvenile herring (*Clupea harengus*), cod (*Gadus morhua*), coal (*Pollachius virens*) and spotted wolf fish (*Anarhichas minor*). They used for analysis acoustic pulses (from 4 to 100) with frequencies ranging from 1 to 6.5 kHz and a sound pressure of 150 to 190 dB re 1μPa at 1m . The fish behaviour was observed immediately after exposure to sound; some individuals were kept in tanks up to 34 days to see traumatic effects and long-term mortality. The possible damage to internal organs and tissues were analyzed by histological examination of the eyes, heart, ears, liver and swim bladder; and by SEM, the external neuromastocists of the head (present kinocilia) and cells of the lateral line of the herring larvae. Mortality was significantly higher in juvenile herrings. Some panic reactions or confused or irregular swimming behaviour were observed, especially when juvenile herrings were exposed to lower frequencies (1.5 kHz).

Codfish (Cox et al., 1986) exposed to intense sound presented damage in the sensory epithelium of the inner ear. *Astronotus ocellatus* showed damage to the inner ear when exposed to 180 dB re 1μPa at 300Hz (Hastings et al., 1996). It was suggested that the damage to hair cells caused by intense acoustic stimulation was a result of over stimulation of the otholit (Hastings et al., 1996). Like other vertebrates, exposure to intense noise can cause a temporary decrease in sensitivity of the fish ear to sounds of certain frequency and amplitude (TTS) (Popper and Clarke, 1976). Anthropogenic underwater noise can produce the death of the fishes or, as in cetaceans, induce temporal (TTS) or permanent (PPT) hearing threshold shift. High intensity sounds can damage internal organ leading to the death of the exposed animal or to a damage of the sensory hair cells in the otolith organs (Popper and Hastings, 2009b). If the hearing loss is only temporal, the fish will recover within hours or days. Physical damage ocurr only close to airguns in a seismic survey (Popper and Hastings, 2009; Popper and Hastings, 2009b), but during the recovery time of the TTS the fish could be exposed to higher predation or be disabled to perform biological activities.

Fishes are known to be sensitive to fluctuations of sound pressure as well as to particle motion. Some recent works (Peter Sigray and Andersson, 2011) measured particle motion *in-situ* using a bottom-anchored sensor in a field trial at a wind farm located in the Baltic Sea. The wind turbines generate particle motion that fishes can sense. The area where the environment is affected by anthropogenic generated particle motion is restricted to the close vicinity of the wind turbine. But the complementary component of the particle motion (e.g. acoustic pressure) could still have an influence at longer distances.

Although sonar, pile-driving and explosions typically attract most of the attention, it is reasonable to argue that the greater impact on fish will be from less intense sounds that are of longer duration, such as those produced by vessels, and that can potencially affect whole ecosystems (Slabbekoorn et al., 2010).

2.3 Noise effects on cephalopods

2.3.1 Introduction. Sensorial perception in cephalopods

The nervous system of cephalopods is the most developed of the invertebrates and complex patterns of behaviour are associated to it (Young, 1977). The type of active life and the high level of organization of cephalopods is necessarily accompanied by a high degree development of the sensory systems, in which it is especially relevant the role of the mecanoreceptors, and a centralized nervous system. The giant neurons of several species of the *Teuthoidea* order, especially of the squids of genus *Loligo*, are an excellent material for neurophysiology studies. They were discovered in the early thirties by Young, and many works have provided important results for understanding the transmission of nerve impulses.

Eyes are the most developed sensing organs of cephalopods. They are able to form a good image, can detect the plane of polarized reflected light but cannot distinguish colors. Most species have monocular vision, even while for hunting and eggs laying most species of the Sepioidea order have binocular vision. The visual field of the species that have well developed eyes is approaching 360º.

Extraocular receptors are also exclusive organs of cephalopods, whose function is still uncertain at present, but it seems that they provide the animal with a general idea of the lighting of the surrounding water and its seasonal changes, information that is related to processes of sexual maturation and seasonal migrations.

The olfactory organ is supposed to help perceiving the prey's smell, predators and sex pheromones. There are also very abundant tactile cells located in the arms and tentacles, and areas of high sensitivity. A body that acts similarly to the lateral line of teleost fish has recently been discovered. A lot of propioceptors localized on the skin, muscles, suckers, gut, gills and cornea allow them to receive information about their position, the relative position of body parts and the operation of the muscles.

The mecanoreceptors of cephalopods are a diverse group of receivers. Basically, all of them respond to mechanical stress (deformation of the membrane) caused by relative movement between the ciliated receptor cells and the surrounding medium (e.g. in the case of **statocysts** - receptor organs of equilibrium-, the analogous system to fish lateral line and the propioceptor organ of the neck) or by mechanical deformation of the membrane of the uni-or multipolar neurons (as in the case of the mecanoreceptors of suckers, skin and muscles).

2.3.1.1 Acoustic perception on Cephalopods

Very little information about hearing ability of marine invertebrates is available. However, all cephalopod species have **statocysts**, which are highly sophisticated structures, analogous to the vestibular system of the vertebrate ear (Offutt, 1970; Budelmann, 1988a; Budelmann and Bleckmann, 1988b; Budelmann, 1992; Williamson, 1995)

The cephalopods statocysts are responsible of determining the position of the individual in the water column, thus they operatre in a similar way as the vestibular system does in the vertebrate ear. It is also possible that the cephalopod statocysts are also used for the perception of low frequency sounds.

In some species of crustaceans, such as crabs, there are also structures similar to those of cephalopods statocysts, but this system in these two groups have evolved separately. Although not much data is available regarding the hearing in marine crabs, many species of ghost and violon crabs are suspected not only to be able to detect sounds, but also to use special sounds for communication. Indeed, a large number of physiological studies on marine crabs suggested that some of them would be potentially able to detect sound (Popper et al. 2001).

2.3.1.2 Cephalopod Statocysts

Statocyst of cephalopods, mentioned in the literature for the first time by Hunter (1782), has become common models in comparative research (Hunter, 1782). They have a complex structure, the main parts of which are receptor epithelium (macula, crista) and ciliated mecanosensorials cells, the attached superstructures (statolith, statoconia and cupula), the cartilaginous lobes of the anticrista, the non-sensory ciliated cells and the Kölliker's channel, involved in regulating the volume and chemical composition of the endolinfàtic fluid (Boycott, 1960; Budelmann, 1990; Williamson, 1991)

All cephalopods have a couple of statocysts located within the cephalic cartilage. The brain of cephalopods is surrounded by a cartilaginous capsule that serves as a skull. In the occipital region of the capsule there are housed a pair of organs of balance, the statocysts. The statocysts are sophisticated balloon-shape bodies filled with endolymph that contain the sensory hair cells which lie on the inside wall of the inner sac and are grouped into two main areas of sensory epithelium. The structure of these bodies is quite complex and differs between the groups according to their way of life. Inside the statocysts are housed the estatoliths, two hard calcareous structures that have taxonomic value, and show periodic deposition growth increments, from which it is possible determining the age of individuals. A statocyst is a body that allows to perceive the position of the animal with respect to gravity (acting as an organ of balance), and linear and angular accelerations caused by the mouvement, as well as low frequency sounds (less than 100Hz). It is a system similar to the semicircular canals of vertebrates. The statocyst morphology and its functions have been extensively described (Budelmann, 1988a; Budelmann, 1990; Williamson, 1991; Bigelow, 1992; Budelmann, 1992; Williamson, 1995; Williamson and Chrachri, 2007).

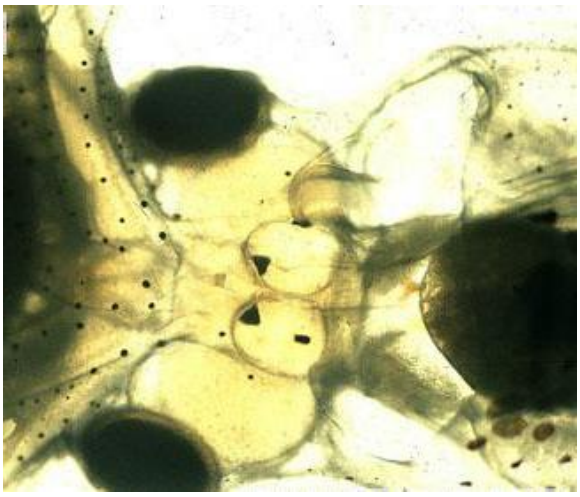


FIG. 2.3. Statocysts during the primary stages of development of *Octopus digueti*. Statocysts are the circular globules containing triangular structures. (http://www.cephbase.utmb.edu/)

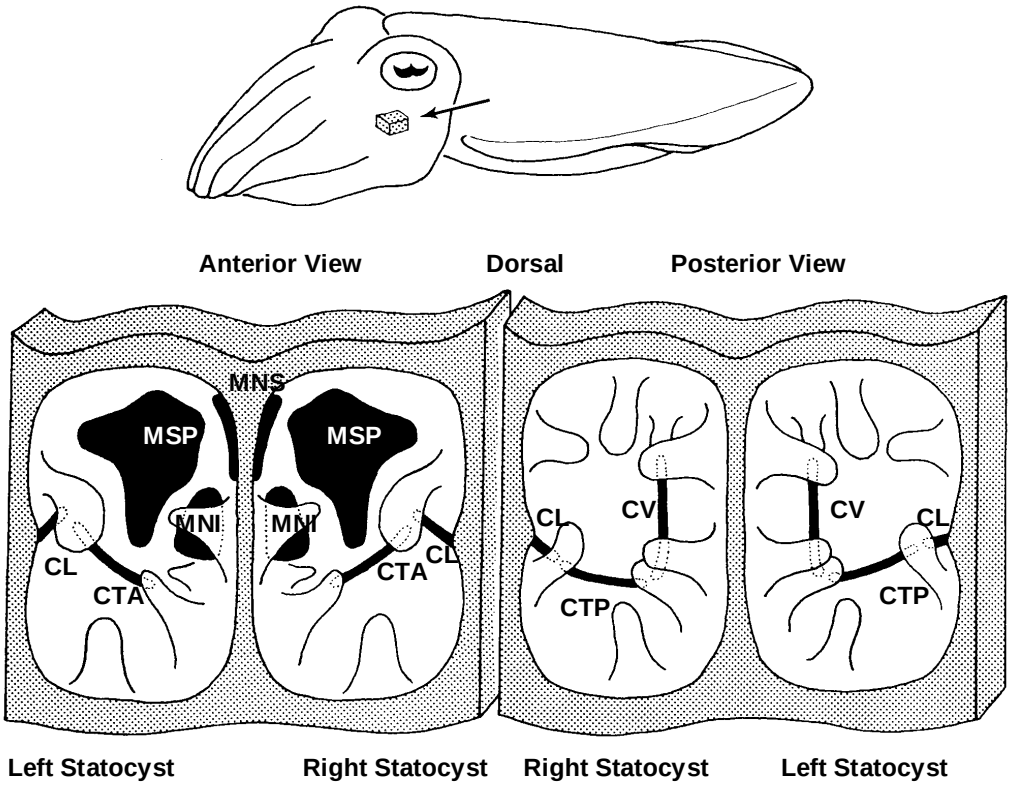


FIG. 2.4. The statocyst of *Sepia officcinalis* is hosted in the cartilage that protects the brain (area indicated in the figure above). The diagrams below show the **front and rear cavities of statocysts according to a vertical cross section.** Each cavity contains three systems of gravity receptors (MNI, inferior macula neglecta; MNS, superior macula neglecta; MSP, macula statica princeps) and a system of angular acceleration reception, which is divided into four segments (CL, *crista longitudinalis*; CTA, *crista transversalis anterior*; CTP, *crista transversalis posterior*; CV, crista verticalis) (Ferguson, 1994).

Cephalopod statocysts show a variety of forms but there are three main types of statocysts (Budelmann, 1988a). The simplest are found in the nautiloids and consist of a pair of oval sacs completely inner covered of hair cells. The sacs are embedded in the central nervous system and open to the exterior via Kölliker's canal (Young, 1965; Neumeister and Budelmann, 1997).

Besides the simplest statocyst of the *Nautilus* which is an oval-shaped cavity completely lined with hair cells (Young, 1965), cephalopod statocyst includes two types of receptor systems: the *macula*-statolith system and the *crista-cupula* system. The *macula*-statolith system indicates the changes in the position according to the gravity and the linear acceleration, while the *crista-cupula* system indicates changes in the angular acceleration. These systems are analogous to the vestibular system of the inner ear of vertebrates (Williamson and Chrachri, 2007). However, unlike ciliated cells of the latter species, the cephalopods' statocyst sensory cells carry kinocilia. Octopods and decapods have 50-200 kinocilia of about 10µm in length and 0,24 in diameter elongated from a basal body, with an internal 9 x 2 + 2 tubules content - this is the most motile cilia structure involved in cell motility and movement of extracellular fluid (Dustin, 1984; Vincensini et al., 2011) - per hair cell (Barber, 1966; Budelmann et al., , 1973). Surrounding the base of the kinocilium, are microvilli of 0,1µm in diameter and 2µm of length. Kinocilia and microvilli form elongated bundles. Each bundle represents a single hair cell. Every hair cell is arranged in line with an adjacent hair cell both in the *crista* and *macula* (Budelmann et al., 1973) and is unidirectional morphologically and physiologically polarized. Adjacent accessory structures (statolith, statoconia, *cupula*) are responsible for the sensory perception. When there is a stimulus, tiny deflations occur in the hair bundles, resulting in cell body depolarization and subsequent transmission of the information to the sensory nervous system. Within the central nervous system, the sensory input of the statocysts is used to regulate a wide range of

behaviours, including locomotion, posture, control of eye movement and of the pattern of the body coloration, and are suspected to be responsible for the reception of the low frequency sound waves (Packard et al., 1990; Budelmann et al., 1997; Hu et al., 2009). The sensory epithelia of the gravity receptor system, in resemblance to the vertebrate auditory apparatus (Puel et al., 2002) have secondary sensory hair cells which are unidirectional morphologically and physiologically polarized, first-order afferent neurons, and efferent nerve fibres. The synaptic arrangements are as complex as those in the vestibular *macula*e (among others: Sans et al., 2001; Desai et al. 2005): the outputs of several hair cells converge onto an afferent neuron and the output of a single hair cell diverges onto several afferent neurons. The efferent fibres of the statocyst terminate both on hair cells and the axons of afferent neurons (Colmers et al., 1984; Budelmann et al. 1987). For a better understanding of the neural network of the cephalopod vestibular system, see Colmers 1977 or Williamson 2007 (Colmers, 1977; Williamson and Chrachri, 2007).

The second type is found in the octopods. In that case, it is a spherical inner sac, suspended in the cartilage cavity by fibrous strands, which presents a gravity receptor system and an angular acceleration receptor system divided in nine segments (Young, 1960). The differential sensitivity from the individual segments divides the response range of the *crista-cupula* system (Budelmann and Young 1985). Octopods have one sac within each of the two cavities of the cranial cartilage, below and to the side of thebrain. Octopods present only one oval shaped *macula* underlying a calcareous stone, the statolith (Budelmann et al. 1973). The *macula*/statolith system is vertically oriented and is responsible for the detection of gravity and linear accelerations. The *crista*/*cupula* system detects the angular acceleration and is linked to a cartilaginous anti*crista* lobe. There is a single cartilaginous projection between 6 and 7 *crista* segments, an anti*crista* that influences the flow within the statocyst sac. The octopod *crista* is divided into nine segments consisting of up to nine rows of sensory hair cells with up to 100 hair cells per row. Every *crista* has an overlaying gelatinous *cupula*, which has a dense structure made of long fibrils. The nine octopod *cupula*e differ in form and size. There are two types of *cupula*, a small and a large *cupula* that alternate regularly in every *crista* segment. This division leads to the idea that there are two *crista* sub-systems differing in their sensitivity ranges (Budelmann et al., 1987). This has been interpreted as an adaptation to the cephalopod forms of locomotion, slowly crawling and fast swimming by jet propulsion (Williamson and Budelmann, 1985a; Williamson and Budelmann, 1985b).

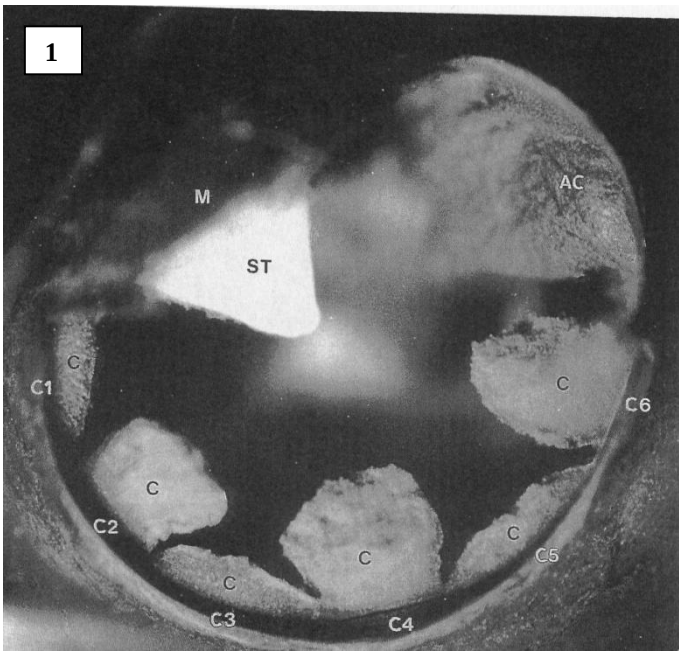
The third type of cephalopod statocyst is found in decapod squids and cuttlefishes, and presents a three gravity receptor system (Budelmann et al., 1973; Budelmann, 1976; Stephens and Young, 1982; Budelmann, 1990) and an angular acceleration receptor system divided in four segments (Young, 1971; Budelmann et al., 1973; Stephens and Young, 1982; Budelmann et al., 1997). In decapod cephalopods the two statocysts are in separate cavities within the cranial cartilage, in the same position as in octopods. There is an inner sac filled with endolymph that, unlike the octopod statocyst, remains in contact with the cartilage wall and there is no perilymphatic space. The walls of the statocyst bulge outwards to make sac-like projections into the cavity. The sacs together with the two types of projections of the statocyst wall, anti*crista* and hamuli (the last are situated at the point where the rows of hair cells of the *crista* are interrupted), direct the movement of the endolymph across the sections of the *crista*. These structures and the Kölliker's channel modify the pattern of flow of endolymph and its chemistry composition, to change the response of the statocyst receptor system (Boycott, 1960; Young, 1989; Budelmann, 1990; Williamson, 1991). This system is comparable to the semicircular canals of vertebrates. Decapods present three *macula*e (Budelmann et al., 1973; Budelmann, 1976; Stephens and Young, 1982; Budelmann, 1990) which are oriented at right angles each other to cover the three axes of movement. The *principal macula statica* is an inverse 3 plate shaped, attached to the frontal wall and overlaid by a dense calcareous statolith, which is attached to the *macula* with a layer of mucus and presses down on the cilia to stimulate the underlaying sensory hair cells. The two additional *macula*e are smaller and both of them are overlaid by less compact structure, statoconia. The *superior macula neglecta* is attached to the

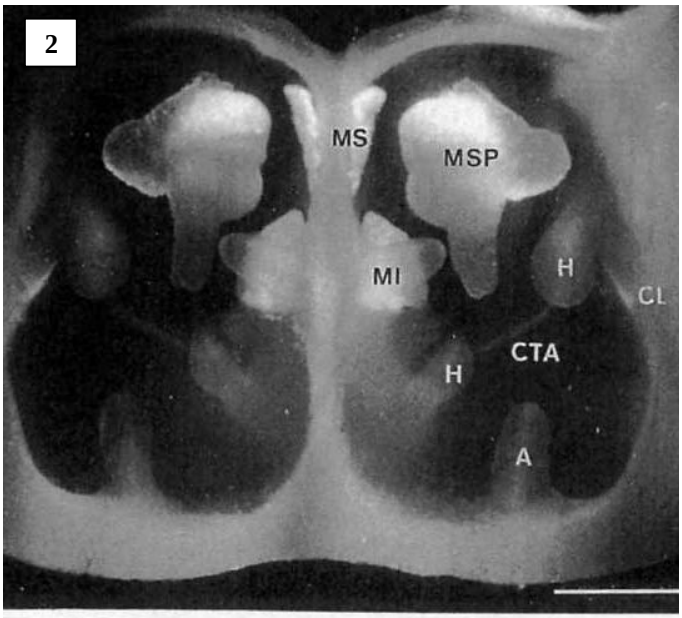
medial wall and the *inferior macula neglecta* is attached to the fronto-ventral wall (Williamson, 1995). In decapods, the *crista* is a ridge of cells lying in the transverse, longitudinal and vertical plane and is divided into four sections (Budelmann et al., 1973); Stephens and Young 1982 (Budelmann et al., 1997): anterior transverse, posterior transverse, longitudinal and vertical which are oriented at right angles to each other (Young, 1971). Every section of hair cells is overlaid by a gelatinous *cupula*, which is deflected by the flow of endolymph during movements of the animal.

In decapods, the *macula*e and *crista* segments are innervated by one *macula* nerve and two *crista* nerves (Stephens and Young, 1982). In octopods, the *macula* and *crista* segments are innervated by one *macula* nerve and three *crista* nerves (Budelmann et al., 1987). In both groups the nerves are composed of afferent and efferent fibers. Glial cells surround the larger individual axons and small axons are enveloped in bundles. The primary afferent neurons of the secondary ciliated sensory cells are part of the neuronal soma of the sensory epithelium of the statocyst. The sensory epithelium of the statocyst receives great innervation of the brain that can excite or inhibit afferent activity. It is thought that these two efferent effects are regulated by two different neurotransmitters, acetillcolina for inhibition and catecholamine for excitement

Some epithelial cells of the cephalopods' *macula* are thought to expel the organic and inorganic material to the statocyst cavity to form the statolith and statoconia in situ. The composition (organic matrix and saline structure) and process of formation of the statoconia and statolith are not well known.

The sensory applications related to control balance and orientation in the movement of cephalopods and vertebrates have evolved in a similar way. The comparison of these two systems can help building a better understanding of the basic functional mechanisms of sensory systems (Williamson, 1995). In vertebrates, the sensory organs that are located in the inner ear are the utricle, the sacul and the semicircular canals. The ciliated cells of the utricle and sacul continually transmit signals to the brain through nerve fibers, indicating the position, the acceleration and gravitational changes.





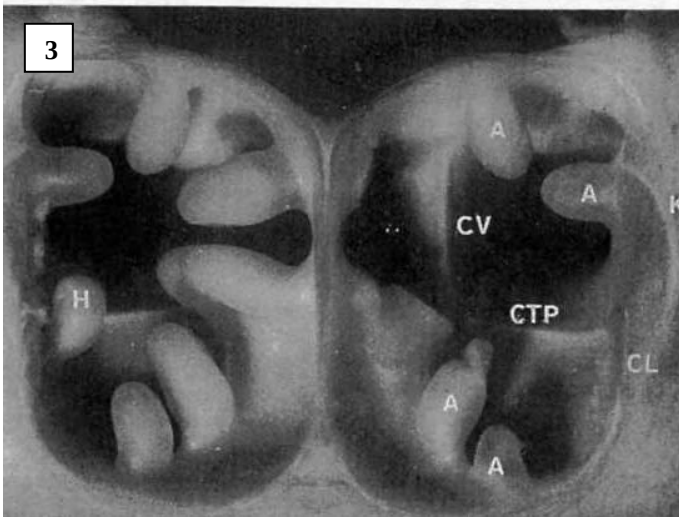


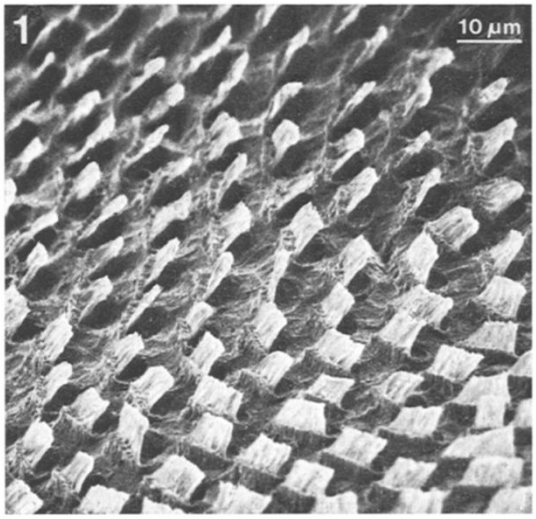
FIG. 2.5. Opened *Octopus vulgaris* and *Sepia officinalis* statocysts

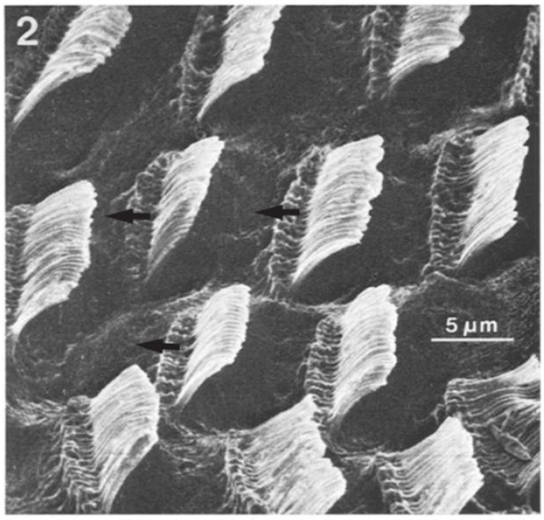
1: Ventral view into an opened statocyst sac of *Octopus vulgaris (Octopoda).* (Osmium-fixed preparation). Photomicrograph shows the cupulae (C) of the six horizontally arranged crista segments (C1-C6). Note the alternating heights of the cupulae. AC catilaginous anticrista; M, cartilaginous macula plate; ST, statolyth attached to the macula. (Budelmann, 1987).

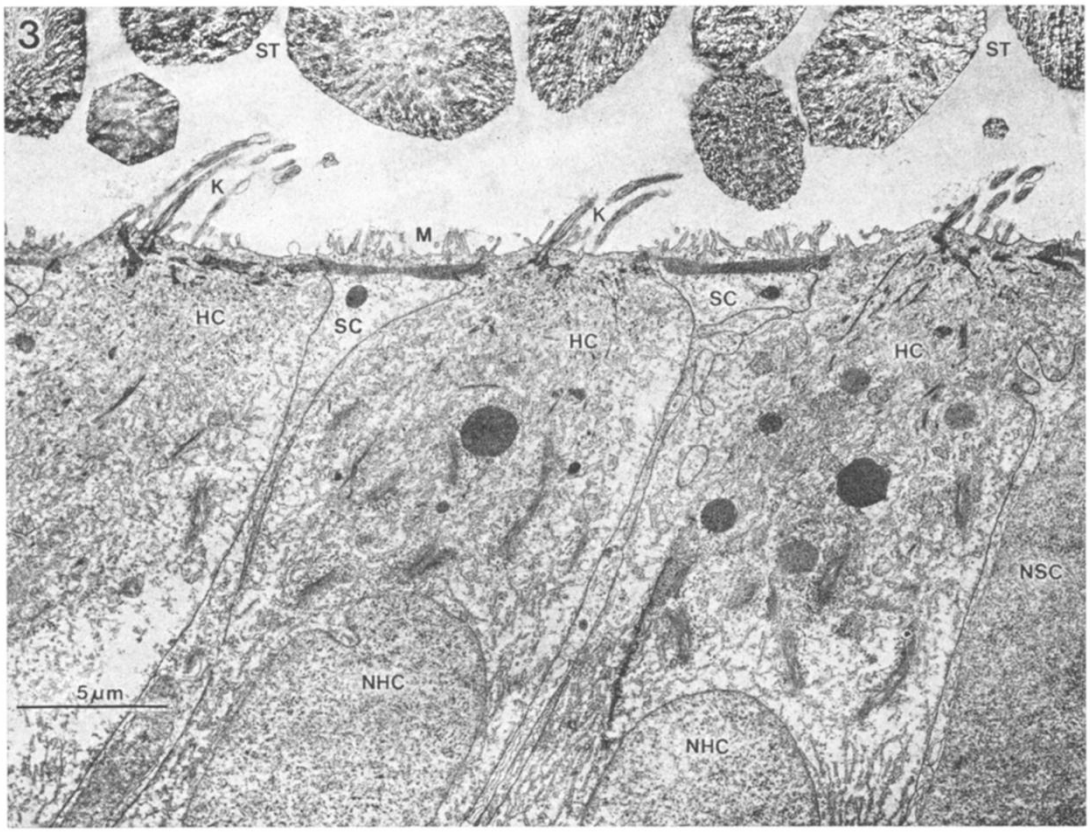
2, 3. *Statocyst of Sepia officinalis (Decapoda).* The two statocyst cavities have been cut open transversally. Bar = 2 mm. (Budelmann, 1976).

2: Anterior view showing, into both cavities, the three systems macula/statoliht (for the perception of gravity and other linear accelerations), and two of the four systems crista / cupula (for the perception of angular acceleration); the cupulae attached to the crista segments are not visible. A, cartilaginous anticrista lobes; CTA, anterior transverse crista; CL, longitudinal crista (frontal part); H, cartilaginous hamuli; MI, inferior macula neglecta; MS: superior macula neglecta; MSP, principal macula statica.

3: Posterior view, showing the remaining two posterior crista segments. In the cavity on the right, some of the cartilaginous hamuli (H) and the anticrista lobes (A) have been removed to show the course of the crista segments. CL, longitudinal crista (posterior part); CTP, posterior transverse crista; CV, vertical crista; K, Kölliker's canal.


1
10 µm


2
5 µm


3
ST
ST
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5µm
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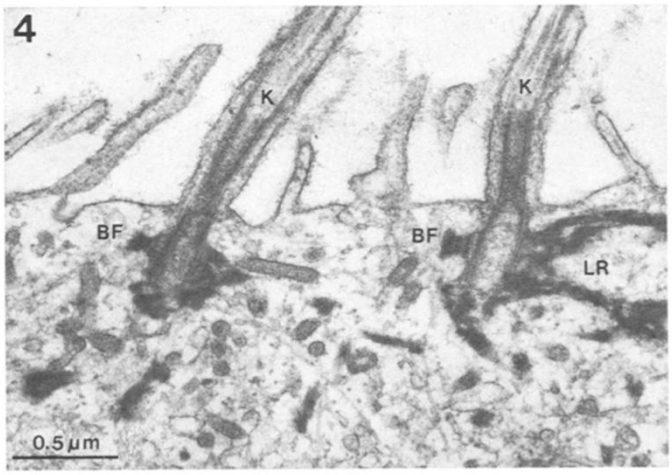
4
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BF
LR
0.5µm

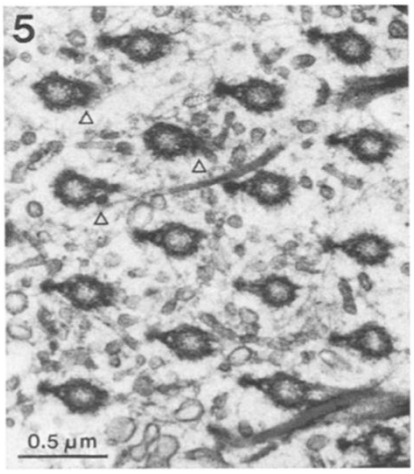

5
0.5 µm

FIG. 2.6. (Pag 43) SEM (1, 2). TEM (3-5). Cellular organization of the macula. 1: Groups of kinocilia of the hair cells in the macula statica princeps of *Sepia officinalis*. **2:** Detail from 1. Each kinocilliary group represents one hair cell, arrows indicate the hair cells'direction of polarization. **3**: Section through the distal ends of the hair cells in the superior macula neglecta of the statocyst of *Loligo vulgaris*. **4**: Longitudinal section through the basal body of a kinocillium of a macula hair cell in the statocyst of Loligo vulgaris. **5**: Transverse section through some of the basal bodies of the kinocilia of a single hair cell, showing the uniform orientation of the basal feet on the macula neglecta superior of Sepia officinalis. In the upper left, some lateral roots (△) can be seen emerging from the basal bodies. **HC**: hair cell, k: bundle of kinocillia, M: microvilli, N: nucleus, P: electrodense plate, SC: supporting cell, ST: statoconia. LR: lateral roots, BF: basal foot. (Budelmann, 1979)

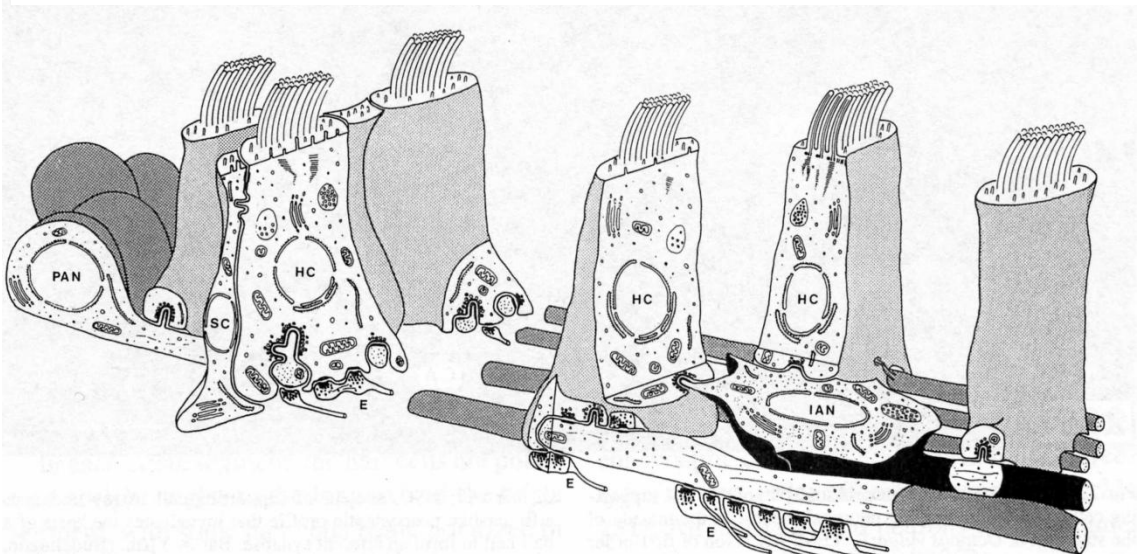


FIG. 2.7. Diagram of the celular, neuronal and synaptic organization of the macula of the statocyst of *Octopus vulgaris*. The secondary sensory hair cells (HC) are in afferent synaptic contact with two types of first-order afferent neurons (IAN, intramacular afferent neuron; PAN, perimacular afferent neuron). Hair cells and neurons receive many efferent endings (E). SC, supporting cells. (Colmers, 1982)

2.3.1.2.1 Endolymph composition. Two-dimensional difference gel electrophoresis (2D-DIGE)

Although several studies were conducted on the structural and biochemical composition of cephalopod statoliths, there are very few works that focused on the endolymph composition. Statoliths, which are calcified biomineral structures composed of calcium carbonate crystallised as aragonite with a small percentage of organic material that has been ascertained to be protein (Radtke, 1983), are found in the gravity receptor system ofcephalopods. Statoliths had becomed a useful tool to provide information on aging (Villanueva, 1992; Lipinski, 1993; Arkhipkin, 2005), food (Zumholz et al., 2006a), environmental conditions (Durholtz and Lipinski, 2000; Zumholz et al., 2007), timing of exposure to pollutants, or timing of migrations (Ikeda et al., 2003). They grow throughout the lifetime of the individuals and deposit microscopically visible periodic growth increments. Since the two theoretical models (Morris, 1988, 1991; Lipinski, 1993) had been hypothesized to establish the physiological mechanisms by which cephalopods form statoliths, some works have contributed with new data on mineralization process (Zumholz, 2006b; Durholtz et al., 1997). Morris proposed that the low concentration of Mg^{2+} ions in the statocyst lymph allows the $CaCO_3$ precipitation in the form of aragonite, this process being controlled by the pH of the endolymph and organic matrix. Lipinski hypothesised that strontium is responsible for the definitions of growth layers and increments in the statoliths. Only a few studiess focused on the characterization of the organic matrix proteins of the statolith (Durholtz et al., 1999) as well as on the quantification of protein concentration on statolyth and statocyst endolymph (Bettencourt and Guerra, 2000).

The most common application of proteomics involves the use of electrophoresis for comparative mapping of expression of proteins from populations of cells or tissues under

conditions of treatment as opposed to those that have not been subjected to treatment. Difference gel electrophoresis (DIGE) is a form of gel electrophoresis where up to three different protein samples can be labeled with fluorescent dyes, prior to two-dimensional electrophoresis.. The important aspect of this technique is its ability to label two or more samples with different dyes and separate them on the same gel, eliminating gel-to-gel variability making the analysis more accurate. Following the separation and statistical analysis by DIGE, the identification of the differentially expressed proteins is performed usually by a combination of enzymatic digestion and peptide analysis by mass spectrometry. The combination of two techniques like 2-DE followed by MALDI-MS allows the identification and sequencing of proteins before and after undergoing traumatic situations. There are several papers published in this regard like those engaged in the study of several diseases (Haas et al., 2006; Nedelkov et al., 2006).

2.3.1.3 Vibration receptors and hearing in cephalopods

Cephalopods are sensitive to vibrations. They respond to water movement by sensing particle motion with the **statocyst receptor systems** (macula/ statolith and crista/cupula) and the **superficial receptor systems** (system analogous to the fish lateral line) (Budelmann, 1976). The hearing ability of marine invertebrates, especially in cephalopods, is a controversial topic (Hanlon and Budelmann, 1987; Packard et al., 1990; Popper et al., 2001). It should be said that there is no evidence that cephalopods can sense the pressure component of sound. Cephalopods species possesing gas filled cavities associated with sound receptors, or with reception capacity of frequencies above 100Hz (Packard et al., 1990) are not known.

The **statocyst perception systems** of cephalopods have experienced an increasing interest (Young, 1960, 1984; Stephens and Young, 1982; Messenger, 1983; Budelmann and Young, 1984, Budelmann, 1990; Williamson, 1995; Neumeister and Budelmann, 1997; Quast et al., 2001). Many authors studied in depth the gravity receptor system (Young, 1960; Budelmann et al., 1973; Budelmann, 1979; Colmers, 1982, 2004; Colmers et al., 1984;). Other authors have almost completely described the angular acceleration receptor system (Young, 1960; Barber, 1966; Budelmann, 1977b; Budelmann et al., 1987). More recent works showed a good approach of the study of neuronal and synaptic organization (Williamson, 1988, 1989, 1992; Williamson and Chrachri, 2004, 2007).

However, little is known about sound perception in invertebrates, but evidence points out to the notion that cephalopods may be sensitive to low frequency sounds (Hanlon and Budelmann, 1987). There is indeed a considerable lack of information concerning the cephalopod sound processing (Packard et al., 1990; Bleckmann et al., 1991; Bullock, 1991; Budelmann et al., 1995; Hu et al., 2009). Although to date there is no definitive scientific evidence for it, statocysts may play an important additional role in low frequency sound reception (Hu et al., 2009). While there is uncertainty regarding the biological significance of particle motion sensitivity versus acoustic pressure, recent electrophysiological methods confirmed the species' sensitivity to frequencies under 400Hz (Kaifu et al., 2008; Hu et al., 2009; Mooney et al., 2010).

Cephalopods have well developed **superficial receptors systems**. Each receptor system has an hair-like projections with a flexible base. When exposed to water motion the hairs bend and send a signal to the sensory cells. Cephalopods present lines of ciliated sensory cells, which run parallel to each other in a longitudinal direction over the head and arms. Cilliated sensory cells are also present over the cephalopods body (Hanlon, 1990; Packard et al., 1990; Budelmann, 1992).

2.3.2 Noise effects on invertebrates and cephalopods

2.3.2.1 Sound emissions

Sound produced by marine invertebrates has not been investigated to the same extent as that of fish or marine mammals. Nevertheless, the sounds produced by some 40 species of marine crustaceans (*Palinuridae*) and some shrimps (*Alpheus*) have been documented. Other mollusks such as *percebes* (goose barnacle) do emit sounds but the mechanisms involved have not been studied in great detail.

The majority of marine invertebrates are known to produce sound by rubbing parts of their bodies. Some species of decapod crustaceans make loud chirping noises by rubbing their legs against their body (Demski et al., 1973). Shrimps, on the other hand, are an exception; by closing a specialized claw they produce a clicking sound creating 'cavitation' and making a bubble that generates acoustic pressure of 80 kPa at a distance of 4 cm from the claw when the bubble collapses. This pressure is sufficiently strong enough to be able to kill small fish (Versluis et al., 2000).

Marine crayfish do not posses claws but produce harsh sounds through antennae friction, which is believed to be a method of repelling predators. Mussels (*Mytilus edilus*) make sound with the *byssus*, the 'beard' that is used for adherence to hard surfaces. In temperatures above 10ºC mussels can produce clicking noises by stretching and breaking the byssus. It is uncertain whether these sounds are produced intentionally or not.

Fiddle or violin crabs make up 97% of the genus *Uca* whose males are renowned for their asymmetric claws. The larger of the claws is used to produce sound by hitting parts of its own body or the surface of the area where it is located. A great variety of sounds produced in this manner have been described as harsh noises, drumming, whistling or rapping. Sounds particular to each species have been identified based on different frequencies and time intervals. For example, the species *Uca pugilator* (fiddler crab) produces rapping sounds between 600 and 2400 Hz while the *Uca rapax's* sounds are between 300 and 600 Hz.

Tropical sea urchins (*Diadema setosum)* produce 'sparking' sounds with the rubbing of the spines as they move. These sounds can also be made from the chaffing between the 'Aristotle's Lantern' (a specialized masticating structure) and its exoskeleton during feeding and reproduction.

These noises are used in social interations between individuals of the same and the different species (Popper and Fay, 1993; Bone et al., 1995). **Interference with these acoustic signals** could alter the behavior of the emisor and receiver animal, avoiding their acoustic communication.

2.3.2.2 Acoustic impact

The specific literature on noise impact on invertebrates in general is scarce and its possible effects on cephalopods are also unknown.

Land invertebrates
At the end of the 60s some works were performed (Frings, 1969; Frings and Frings, 1971), which determined the volume of sound that produced lesions in some species of insects. Many studies on the effects of sound in insects have emerged from the need to protect the crops of grain of their destruction (Fletcher et al., 1971). Tsao (1969) showed that the Indian meal moth (*Plodia interpuctella*) stopped their movement when it was stimulated by speakers, bells and whistles (Tsao, 1969). Kirkpatrick and Hare (1965) referenced that the hatching of their larvae

was reduced on 75% when they were exposed to frequencies of 120 to 2000 Hz for 4 days of their larval state (Kirkpatrick and Harein, 1965). Lindgren (1969) used a variety of frequencies and intensities to study the effects of sound in pupae and adults of the same species, as well as from flour beetles (*Tribolium spp.*). Some effects were observed in reproduction, except in the case of flour beetles exposed to a continuous frequency of 40 kHz. Although a large number of insects were exposed to the sound in different replicas of the experiments, the effects of sound exposure are difficult to prove because of the variability in egg production (Lindgren, 1969). The discrepancy between the data of Kirkpatrick and Hare (1965) and Lindgren (1969) could possibly be explained because the stimulation was done during different stages of the life cycle of insects (larva, pupa and adult, respectively), as well as differences in the sound exposure (Fletcher et al., 1971).

Cutkomp (1969) referenced that 72 hours of exposure to acoustic pulses (50 kHz), 25 pulses per second to 65 dB, reduced the longevity of the corn worm (*Heliothis zea*) and the Mediterranean flour moth (*Ephestia kuehniella*) from 20 to 10 days. In addition, the number of eggs per female was reduced by 59% in the group of individuals exposed to sound (Cutkomp, 1969).

The bee (*Apis mellifera*) stop their movement for more than 20 minutes under exposure to frequencies between 200 and 2000 Hz with intensities ranging from 107 to 119 dB (Frings and Little, 1957; Little, 1959) and don't habituate to sound. It has been an increase in the movement of lobsters (*Lucustidae*) exposed to frequencies of 1, 4, and 10 kHz at 80 dB (Shulov, 1969) and quironòmids (*Chironomidae*) exposed to 125 Hz and 13-18 dB above the ambient noise (Frings and Frings, 1959).

Marine invertebrates

The literature of the impact noise on marine invertebrates is even more scarce. Some marine invertebrates are able to detect and react to natural sounds of the marine environment (especially to prey capturing or escape from predators). In many species these capabilities are based on statocysts hearing systems. Lovell (2005) studied the mechanism of the reception of sound and hearing abilities of the prawn *Palaemon serratus* using a combination of anatomical techniques, electron microscopy and electrophysiology. He concluded that *P. serratus* is sensitive to sounds with frequencies ranging between 100 and 3000 Hz. It was the first time that the ABR technique (Auditory Bransteim Response) was used in crustaceans, in order to acquire auditory evoked potentials for this species. Two subcutaneous electrodes were placed on the suboesophageal ganglion and the statocyst, located in the antenula's peduncle. The statocyst presents sensory cells, which are contained in a cavity filled with lymph. This work intended to demonstrate that statocyst responds to sounds propagated through water produced from a transducer. The Audiogram (fundamental measure of the hearing capacity from a species which shows the lowest level that can capture) of *Palaemon* statocyst showed that this species is sensitive to the movement of particles displaced by low frequency sounds (between 100 and 3000 Hz) with acuity similar to that of fish in general. Also neural waves are similar in amplitude and shape to those obtained in fish and higher vertebrates when stimulated with low frequency sounds. Also not electrophysiological response was obtained if the statocysts were extracted from *Palaemon* (Lovell et al., 2005).

The same authors (Lovell et al., 2006) working with *Palaemon serratus* (100 individuals with sizes between 27 and 71mm) and with the same techniques referenced in the previous paragraph, demonstrated that all individuals were able to hear sound with a frequency of 500Hz, regardless of their size. This fact is very important when it refers to harmful effects of anthropogenic sound on crustaceans.

The physiological effects of noise on marine invertebrates are not well documented. Lagardère (1983) conducted studies on the effect of sound on the growth and reproduction of the brown shrimp (*Crangon crangon*). The animals used were bred in captivity on deposits, which

reproduced the acoustic conditions typical of the natural ecosystem of shrimp. The growth and reproduction of these individuals were compared with animals from the same place which were kept in thermoregulated aquariums and subjected to sound level of 30 dB re 1 µPa at 1m on intervals that ranged between 25 and 400 Hz. This permanent high level exposure to sound caused a significant reduction in the rate of growth and reproduction of shrimps. Other consequences, though not so obvious, was that exposure to the sound seemed to cause an increase in the level of aggressiveness (cannibalism) and the mortality rate, and a reduction in feed intake(Lagardère and Regnault, 1983). These symptoms are very similar to those caused by adaptation to stress. Reduced growth and reproductive rates are kwoun tertiary effects on the stress response (Wedemeyer and McLeay, 1981).

There is very little information about the effect of seismic survey noise on the behaviour of invertebrates. Wardle 2001 esposed an small reef system to 195-218 dB re 1µPa from 2.5 L air-gun and observed the resident invertebrates (crabs, starfish ans sea urchins) 14 days before, during and after exposure. No significant changes in behaviour and no signs of the invertebrates migrating were observed (Wardle et al., 2001). Rusby 1995 suggest that shellfish and crustacenas are relative immune to air-gun noise.

A part from **behaviour responses** (startle response) to sound documented by McCauley (2000), there are no reports in the literature on the effect of intense noise on the acoustic receptors of cephalopods. McCauley reported that squids (*Loligo* spp.) enclosed in cages were exposed to experimental seismic pulses. It was observed that squids showed some avoidance response on hearing the noise pulses.

However, vibrational and directional sensitivity of the hair cells of the cephalopods statocyst have been reported (Williamson, 1988; Williamson, 1989; Packard et al., 1990; Budelmann and Williamson, 1994). Because of the high degree of development of their nervous system, characterized by the large size of its neurons, cephalopods are often used to neurophysiology studies, and it's possible to find some papers about evoked potentials (ABR) in cephalopods (Bleckmann et al., 1991; Bullock, 1991; Budelmann et al., 1995) to determine their hearing threshold.

Between September and October 2001 and in October 2003 the natural rhythm of annual records of giant squids (*Architeuthis dux*) stranded in the area of the West coast of Asturias experienced a significant increase (Guerra et al., 2004a, b). In these cases, the stranding and collection of the bodies were related to the proximity of vessels using compressed air guns for geophysical prospecting, producing sound waves of low frequency (below 100 Hz) and high intensity (200 dB re 1 µPa at 1m per airgun). Some of the individuals had lesions in different tissues and organs, but all presented pathologies in the gills and the receptor of equilibrium or statocysts. Because none of these lesions could be related to known causes of death, the presence of geophysical prospecting vessels suggested that the death of these animals could be related to effects produced by sound waves. However, no further study addressed this problem and doubt remained as to whether high intensity low frequency pulses could negatively affect cephalopods.

3. Objectives

3. Objectives. Research lines

The literature on the direct (injury at different structural levels in sensory reception systems or other anatomical apparatus) or indirect (level of survival, development, behavior and equilibrium populations) effects of noise pollution in marine invertebrates is virtually nonexistent. For this reason the lines of research that suggests this project are quite innovative and offer a wide range of possibilities, both in terms of investigating the effect of noise on non-yet-studied species and for the interest in the application of innovative techniques in the study of acoustic trauma in marine organisms.

Given the impossibility of working with giant squids (e.g., *Architeuthis dux*), we chose four commercial species of cephalopods,-common cuttlefish (*Sepia officinalis*), European squid (*Loligo vulgaris*), broadtail squid (*Illex coindetii*) and common octopus (*Octopus vulgaris*) - of the Mediterranean Sea to reproduce in the laboratory the possible acoustic impact conditions of Asturias, described in the previous section.

Research lines of the thesis

- To analyze the possible effect at cytological levels on the statocysts of adult cephalopods - common cuttlefish (*Sepia officinalis*), European squid (*Loligo vulgaris*), broadtail squid (*Illex coindetii*) and common octopus (*Octopus vulgaris*) - after exposure to low frequency sounds, by scanning electron microscopy (SEM).

- To analyze the effect at cytological level on the statocysts of adult cephalopods - common cuttlefish (*Sepia officinalis*) and common octopus (*Octopus vulgaris*) - after exposure low frequency sounds by transmission electron microscopy (TEM).

- To analyze the effect at cytological level on the superficial receptors systems **(**analogous system to fish lateral line) of hatchlings belonging to three species of cephalopods - common cuttlefish (*Sepia officinalis*), European squid (*Loligo vulgaris*) and broadtail squid (*Illex coindetii*)- after exposure to low frequency sounds, by scanning electron microscopy (SEM).

- To estimate the effects of noise on specific protein (biomarkers), which are part of the statocyst's endolymph of adult individuals of *Sepia officinalis*, after noise exposure using 2-DE/MALDI-MS techniques.

4. General Material and Methods

4. Gener*al Material an*d Methods

The specific material and methods for each objective analysis are contained in respective chapters. This section summarizes the characteristics of the facilities and gives an overview of the material and preliminary tests that will not further described in the specific chapters.

4.1 Experimental animals

The individuals used in this study belong to four native cephalopod species from the Iberian Peninsula and Balearic Islands with high commercial value: common cuttlefish (*Sepia officinalis*), European squid (*Loligo vulgaris*), broadtail squid (*Illex coindetii*) and common octopus (*Octopus vulgaris*).

Sepia officinalis Linnaeus, 1758

FIG. 4.1. ***Sepia officinalis*** male showing the characteristic skin pigmentation pattern as a zebra striped. (**Photo**: M. Solé)

Species of the *Sepiidae* family that can reach up to 400 mm of dorsal lenght. The suckers are arranged in four transverse rows on the arms. It has an oval-shaped and well defined cuttlebone without fins. The IV left arm of males is hectocotylus.

It is present throughout the Mediterranean and northwest Atlantic from the Baltic to about 17 º N. Very common on the coasts of the Iberian Peninsula from the coastline up to 200 m depth. It is a nektobenthic species, which lives in the continental shelf waters, mainly on sandy and muddy bottom covered with algae and plants. This species makes a migration to the coast to breed throughout the year but especially between February and September. Their fertility can vary between 150 and 1000 eggs per female. The hatching of eggs occurs between 30 and 90 days after the egg laying and depends on water temperature. Growth is rapid and longevity of two years. Cuttlefish feed on crustaceans and fishes and have a great commercial interest (Guerra, 1992).

Loligo vulgaris Lamarck, 1798

FIG. 4.2 ***Loligo vulgaris*** showing iridescent pigmentation due to the distribution of its epidermal cells. (**Photo:** http:www.mba.ac.ukfellowswilliamsonanimals.htm)

Species of the *Loliginidae* family that can reach between 550 mm (males) and 340 mm (females) of dorsal mantle length. Adult fins form a rhomboidal figure and reach between 65-70% of mantle length. They present four transverse rows of suckers on the arms and hectocotylized arm in males is the ventral left.

Present throughout the Mediterranean and Eastern Atlantic from approximately 55 ° N to 20 º S. It is common in the Iberian Peninsula. It is a nektobenthic species which lives from the surface to 550 m depth, but is very abundant between 20 and 200 m, forming more or less dense masses. It performs egg layings at 20 to 40 m depth, by adhering digitiformes capsules of 60 to 160 mm in length containing 90 eggs each on the average to different substrates. Egg size is approximately 2.2 to 1.6 mm. The time of eggs laying is understood to happen throughout the year, but the busiest periods are between March and August. The duration of embryonic development depends on water temperature, being about 25 days at 22 ° C. Paralarvae measured between 2 and 3 mm dorsal mantle length; spend a period of about two months as plankton organisms. They have a lifespan of 12 to 16 months. They feed on fishes, crustaceans and other cephalopods. Their fertility varies between 3500 and 6000 eggs per animal. Vertical and horizontal migrations are conducted together with feeding and breeding purposes. This species lives in temperate waters with a salinity range between 30 and 60 ‰ and has again a great commercial value (Guerra, 1992).

Illex coindetii Vérany, 1839

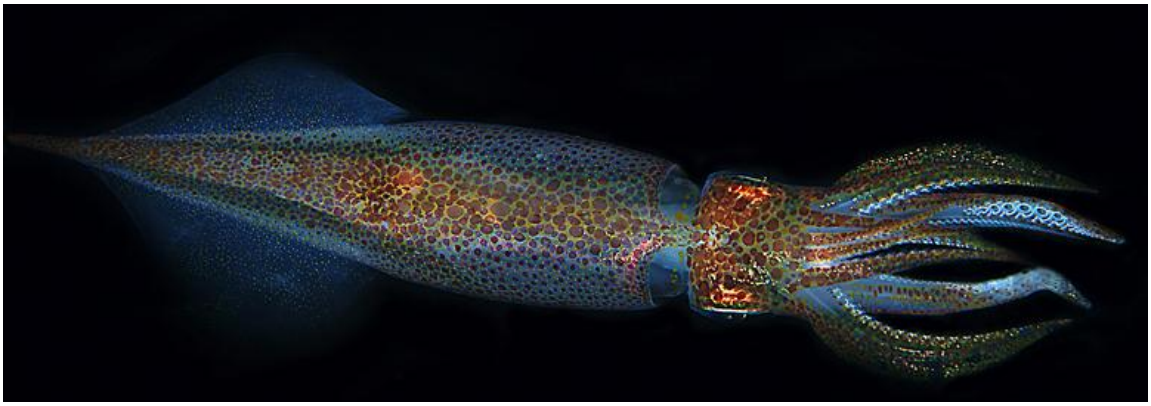


FIG. 4.3.*Illex coindetii* showing iridescent pigmentation due to the distribution of its epidermal cells. (**Photo:** S. Guerrieri**.** http://www.amimalakos.com/clio/MTE/suppl003/pg138.htm)

Species of the family *Ommastrephidae*, which can reach 370 mm (males) and 320 mm (females) of dorsal mantle length. Adult fins form a rhomboidal figure and reach Between 45-60% of mantle length. Tentacular end with 8 rows of suckers. The hectocotylization equally often affects the right or left ventral arm.

Illex coindetii is widely distributed in the Mediterranean, from the South of Britain to Namibia in the Eastern Atlantic, and in the Caribbean, Gulf of Mexico and Straits of Florida in the Western Atlantic. Data from putative daily growth increments in the statolith indicate an average life span of one year. Squid hatched in spring/summer grow slightly faster than those hatched in autumn/winter. *I. coindetii* preys on fishes, crustaceans and cephalopods. Mature squids are present yearround, but peaks in spawning appear to occur in the spring/summer in the Sicilian Channel and Northwest Atlantic off Spain, in summer off West Africa and in autumn in the Catalan Sea. The squid remain near the seabed during daytime and migrate into the water column at night. Seasonal migrations are not well understood but there appears to be a movement from shallow to deep water between summer and winter in the Catalan Sea. The life cycle of the species is poorly understood. In the Mediterranean, there are a number of cohorts each year which overlap, making analysis difficult. The species is mostly taken as a by-catch in other fisheries (Guerra, 1992).

Octopus vulgaris Cuvier, 1797

FIG 4.4. ***Octopus vulgaris*** showing its dermic flaps and the iridescent pigmentation due to the distribution of its epidermal cells. (**Photo**: J. Regàs. http://www.cibsub.com/bioespecie-octopus_vulgaris-27539)

Species of the *Octopodidae* family that can reach up to 400 mm of dorsal mantle lenght and 1600 mm of total lenght. The suckers are arranged in two transverse rows on the arms. The III right arm of males is hectocotylized.

It is a cosmopolitan species, from tropical, subtropical and temperate waters. Present throughout the Mediterranean and Eastern Atlantic from Scotland (60º N) to White Cape (21º N). *Octopus vulgaris* is very common on the coasts of the Iberian Peninsula from the coastline up to 200 m depth. This specielives in the continental shelf waters, mainly in waters from 7ºC to 33ºC of temperature, and from 32 to 40‰ of salinity. Their fertility can vary between 100000 and 400000 eggs per female. The hatching of eggs depends on water temperature and hatchlings are 2mm of lenght. On the first 30-40 days are planktonic and later they will become benthonic. Growth is rapid and their longevity is of two years. This species makes a migration to the coast to breed and, to the depth where it grows and matures. Common octopuses feed on polychaetes, crustaceans, molluscs and fishes and have also a great commercial interest (Guerra, 1992).

4.2 Facilities

As specified before, the exposure of individuals to noise was conducted on individuals at different development stages to assess the effects of noise impact throughout their life cycle. We therefore used different structures to maintain the animals that we describe here:

All experiments were performed under the rules of Law 5/1995 ("Act for the protection of animals used for experimentation and for other scientific purposes") approved by the Parliament of Catalonia on 21 June 1995 and developed on the Order of july 30, 1997, that governs scientific testing on animals.

4.2.1 Tanks for rearing of hatchlings

Due to the extremely small size of the larval stages of cephalopods an installation that allows to easily collect the samples was necessary. For this reason, and to check the feasibility of obtaining hatchlings of the eggs some preliminary tests were conducted in 30L capacity tanks, which had a closed circuit water circulation with a flow rate of 900L / h, with natural seawater of salinity 35 ‰ and a temperature between 18 and 24°C. The installation consisted of two 30L capacity tanks, where eggs of squids and cuttlefishes were hang (LAB - UPC, Vilanova i la Geltrú).

We mainly wanted here to assess the feasibility of hatclings from cephalopods before exposing them under the same protocol as the adult individuals (see below).

4.2.2 Tanks for maintenance of adult and subadult individuals

Cephalopod specimes that were obtained from the Catalan Coast (NW Mediterranean Sea) over a period of 2 years, between February of 2008 and August of 2010, were kept in a closed system of recirculating natural seawater (at 18-20ºC, salinity 35‰ and natural oxygen pressure) consisting of 2 mechanically filtered fiberglass reinforced plastic tanks of 2000L capacity, that were connected to each other (LAB - UPC, Vilanova i la Geltrú). This included a physicochemical self-filtration system with activated carbon and sand, driven by a circulation pump (see chapter 4.3).

Individuals were supplied with live crab (*Carcinus maenas*) food ad libitum and were maintained in the tank system until the exposure. Part of these animals were used as controls and were kept in the same conditions as the experimental animals until being exposed to noise, sacrificing them respecting the same sequential process.

4.3 General Protocol

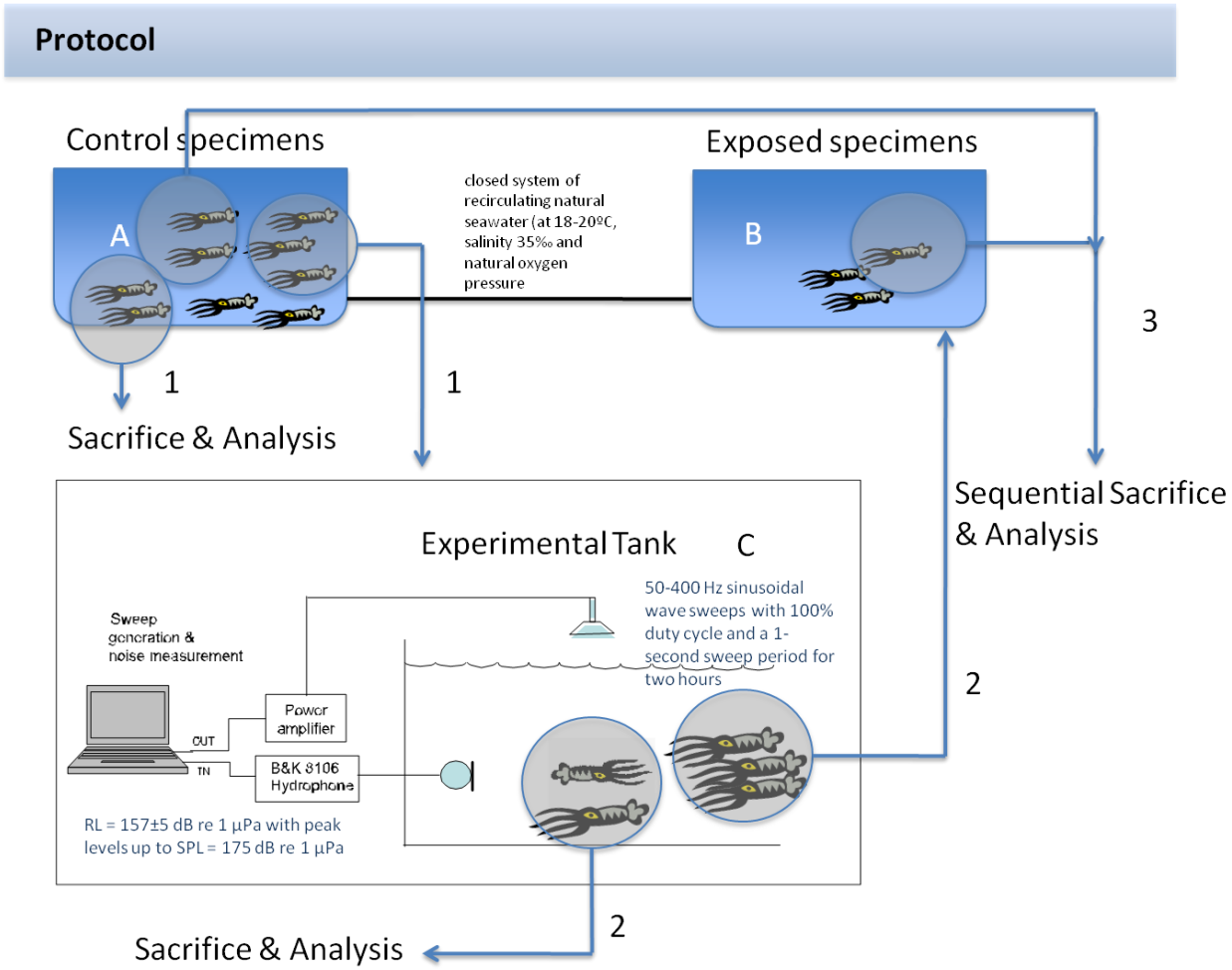


FIG. 4.5. Scheme of the general protocol of the exposure to sound and posterior analyses.

Cephalopod individuals

Adult and young cephalopod individuals were kept in a closed system of recirculating natural seawater (at 18-20ºC, salinity 35‰ and natural oxygen pressure) consisting of 2 mechanically filtered fiberglass reinforced plastic tanks (A and B) of 2000L capacity, that were connected to each other. This included a physicochemical self-filtration system with activated carbon and sand, driven by a circulation pump. Individuals were supplied with live crab (*Carcinus maenas*) food *ad libitum* and were maintained in the tank system (tank A) until the exposure**.** Part of these animals were used as controls and were kept in the same conditions as the experimental animals until we exposed the latter to noise, in an independent tank (C), sacrificing them respecting the same sequential process. After the exposure, the individuals that were not immediately sacrificed were placed in tank B (see FIG. 4.5 and sequence of sacrifices below).

The independent experimental tank (C) was located in a separate location, acoustically isolated from tanks A and B.

Sound Exposure Protocol

Sequential (at different times and seasons) Controlled Exposure Experiments (CEE) were conducted over a period of two years on subadult and adult individuals. An additional set of live adult individuals was used as a control and sequentially processed (same procedure as with noise-exposed cephalopods right after being caught, before and after the CEE. All the animals were caught by local fishermen following the same protocol (use of basquet traps and ceramic pots) and transferred to our laboratory a few minutes after capture (our facilities are located in the Vilanova i la Geltrú fishing harbour).

After keeping the animals for some time in tank A (ranging from a few hours to a few days) the protocol included immediate exposure of the individuals, which were put in tank C, to 50-400 Hz sinusoidal wave sweeps with 100% duty cycle and a 1-second sweep period for two hours. The sweep was produced and amplified through an in-air loudspeaker while the level received was measured by a calibrated B&K 8106 hydrophone (RL = 157±5 dB re 1 µPa with peak levels up to SPL = 175 dB re 1 µPa) (André et al., 2011).

Following exposure, the samples were obtained from the non-anesthetized individuals (exposed and controls) at different intervals. Except for the animals sacrificed immediately after exposure, the rest of the individuals were put in tank B. An underwater video camera recorded the sound exposure experiments in order to register behaviour reaction.

FIG. 4.6. (Pag 58) C**ollection of cephalopod individuals. A**: Gears for cephalopod collection. **B**: A gear showing a bush branch (*Illex aquifolium*) to attract the cuttlefish female to lay eggs. **C**: Fisherman installing a gear on the sea. **D**: Fisherman collecting a cuttlefish. **E**: A cuttlefish collected for the experiments. **F**: *Octopus vulgaris* collected for the experiments. (**Photos**: M. Solé)

FIG. 4.7. (Pag 59) **Cephalopod hatchlings**. **A**: Installation for preliminary tests to check the feasibility of obtaining hatchlings of *S. officinalis* and *L.vulgaris*. The tank at the bottom of the photograph shows the sound transducer to monitor background noise. The tank on the upper part of the photo contains the control hatchlings. **B**: Eggs of cephalopods. The black eggs are from cuttlefish. The white ones are from squid. **C:** Eggs from cuttlefish (*S. officinalis*). Some of them are black because of the ink cover. On the transparent eggs, cuttlefish attached to the external yolk sac are visibles. **D**: Eggs from squid (*L. vulgaris*). In the center of the image a hatchling squid is visible. **E, F, G**: Different views of 1 day old cuttlefish. **H, I**: (Light microscopy) Different views of hatchling common squids (*L. vulgaris*) showing iridescent pigmentation due to the distribution of its epidermal cells. **J**, **K, L:** (Light microscopy). Diferent views of hatchling broadtail squids (*Illex coindetii*) from "*in vitro"*provided by Dr. Roger Villanueva, (Villanueva et al., 2011). **Scale bars**: **E - G** = 0,5 cm. **H, I** = 1 mm. **J, K, L** = 0,5 mm.
(**Photos**: **A-I**: M. Solé; **J-L**: R. Villanueva).

FIG. 4.8. (Pag 60) **Cephalopod subadult and adult individuals**. **A**: Tanks for maintenance of subadult and adult individuals. **B**: Detail of the tanks for maintenance of subadult and adult individuals. In the center of the image the physicochemical self-filtration system with activated carbon and sand and the circulation pump are visibles. **C, D, E**: Different views of cuttlefish in the maintenance tank. In C a cuttlefish is laying eggs on a hanging bracket. **F**: Eggs layed by the female cuttlefish in the maintenance tank. (**Photos**: **A**: M. André, **B-F**: M. Solé)

FIG. 4.9. (Pag 61). C**ollection of samples**. **A**: Dissected female of *Sepia officinalis* shows its major internal organs (ang: accesory nidamental gland, bv: branquial vein, g: gill, gc: genital cavity, go: genital orifice, is: ink sac, l: digestive gland, m: mantle, ng: nidamental gland, o: ovary, og: oviducal gland, r: rectum, s: stomach). **B**: Cephalic cartilage extraction. Arrows sign to the two statocysts. **C**: Dorsal view of cephalic cartilage of *S. officinalis* containing the two statocysts -after fixation-. **D**: Ventral view of cephalic cartilage of *S. officinalis* containing the two statocysts -after fixation-. **D**: Cephalic cartilage of *S. officinalis* (ventral view) shows the two statocyst open cavities –previous metalization-. **F**: Endolymph extraction from the statocysts of the cephalic cartilage of a *S. officinalis*.
G: Detail from F. The needle introduced in the statocyst cavity to extract the endolymph is visible. (**Photos**: **A-E**: M. Solé, **F, G**: M. Morell)

FIG. 4.6. Collection of cephalopod individuals**.**

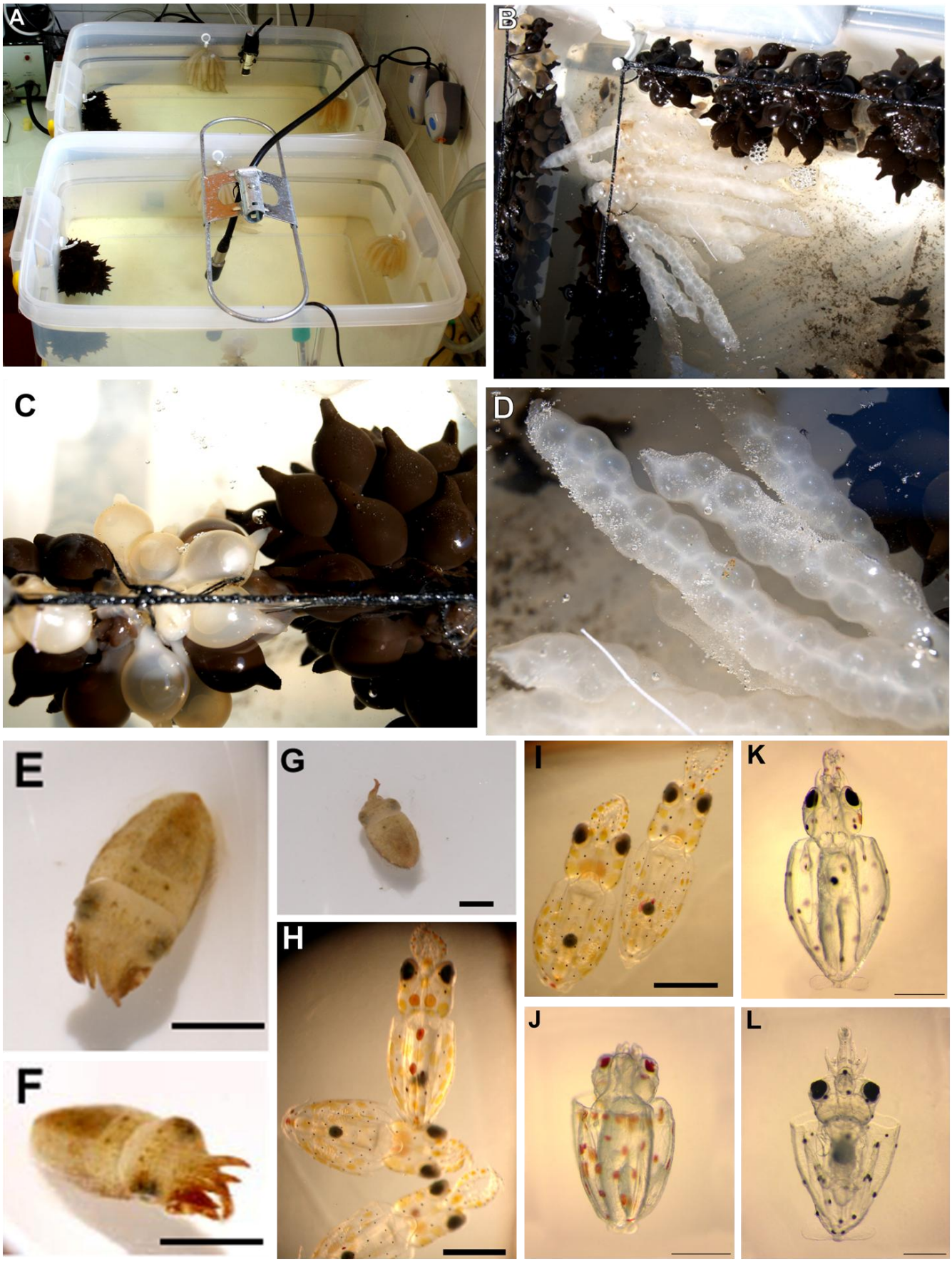


FIG. 4.7. Cephalopod hatchlings.

FIG. 4.8. Cephalopod adult individuals**.**

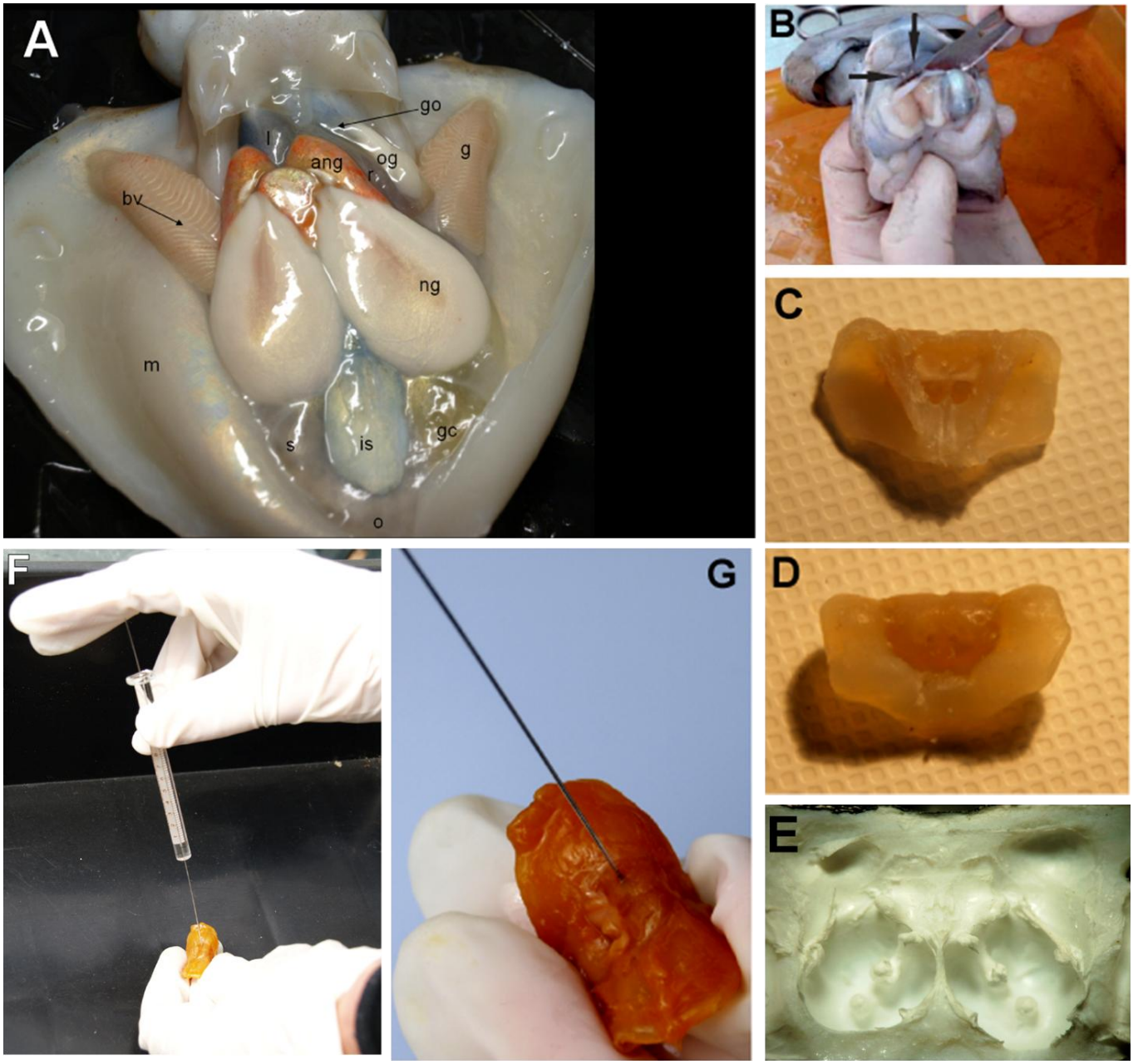


FIG. 4.9. Collection of samples**.**

5. **Revisiting the statocyst ultrastructure of *Sepia officinalis, Loligo vulgaris, Illex coindetii and Octopus vulgaris* to assess acoustic trauma induced by controlled exposure experiments**

5. Revisiting the statocyst ultrastructure of *Sepia officinalis, Loligo vulgaris, Illex coindetii and Octopus vulgaris* to assess acoustic trauma induced by controlled exposure experiments

5.1 Introduction

All cephalopods have a couple of statocysts located within the cephalic cartilage. The statocysts are sophisticated balloon-shape bodies filled with endolymph that contain the sensory hair cells which lie on the inside wall of the inner sac and are grouped into two main areas of sensory epithelium. The statocyst morphology and its functions have been extensively described (Budelmann, 1988a, 1990, 1992; Bigelow, 1992; Williamson, 1991, 1995; Williamson and Chrachri, 2007). Besides the simplest statocyst of the *Nautilus* which is an oval-shaped cavity completely lined with hair cells (Young, 1965), cephalopod statocyst includes two types of receptor systems: the *macula*-statolith system and the *crista-cupula* system. The *macula*-statolith system indicates the changes in the position according to the gravity and the linear acceleration, while the *crista-cupula* system indicates changes in the angular acceleration. These systems are analogous to the vestibular system of the inner ear of vertebrates (Williamson and Chrachri, 2007). However, unlike ciliated cells of the latter species, the cephalopods' statocyst sensory cells carry kinocilia. Octopods and decapods have 50-200 kinocilia of about 10µm in length and 0,24 in diameter elongated from a basal body, with an internal 9 x 2 + 2 tubules content - this is the most motile cilia structure involved in cell motility and movement of extracellular fluid (Dustin, 1984; Vincensini et al., 2011) - per hair cell (Barber, 1966; Budelmann et al., 1973). Surrounding the base of the kinocilium are microvilli of 0,1µm in diameter and 2µm of length. Kinocilia and microvilli form elongated bundles. Each bundle represents a single hair cell. There are some several rows of kinocillia that have all the same height. This gives the bundle a rush like appearance. Every hair cell is arranged in line with adjacent hair cell both in the *crista* and *macula* (Budelmann et al., 1973). Adjacent accessory structures (statolith, statoconia, *cupula*) are responsible for the sensory perception. When there is a stimulus, tiny deflations occur in the hair bundles, resulting in cell body depolarization and subsequent transmission of the information to the sensory nervous system. Within the central nervous system, the sensory input of the statocysts is used to regulate a wide range of behaviours, including locomotion, posture, control of eye movement and of the pattern of the body coloration, and are suspected to be responsible for the reception of the low frequency sound waves (Packard et al. 1990; Budelmann et al., 1997; Hu et al., 2009). The sensory epithelia of the gravity receptor system, in resemblance to the vertebrate auditory apparatus (Puel et al., 2002) have, in addition to primary hair cells, secondary sensory hair cell swhich are unidirectional morphologically and physiologically polarized, first-order afferent neurons, and efferent nerve fibres. The cells respond in an excitatory way for only one axis of stimulation. The basal bodies structure can be used to determine this polarity. The synaptic arrangements are as complex as those in the vestibular *macula*e (among others: (Sans et al., 2001; Desai et al., 2005): the outputs of several hair cells converge onto an afferent neuron and the output of a single hair cell diverges onto several afferent neurons. The efferent fibres of the statocyst terminate both on hair cells and the axons of afferent neurons (Colmers et al., 1984; Budelmann et al., 1987). For a better understanding of the neural network of the cephalopod vestibular system, see Colmers 1977 or Williamson 2007 (Colmers, 1977; Williamson and Chrachri, 2007).

Cephalopods statocysts show a variety of forms but there are three main types of statocysts (Budelmann, 1988a). The simplest are found in the nautiloids and consist of a pair of oval sacs completely inner covered of hair cells. The sacs are embedded in the central nervous system and open to the exterior via Kölliker's canal (Young, 1965); (Neumeister and Budelmann, 1997).

The second type is found in the octopods, which is a spherical inner sac suspended in the cartilage cavity by fibrous strands, which presents a gravity receptor system and an angular

acceleration receptor system divided in nine segments (Young, 1960). The differential sensitivity from the individual segments divides the response range of the *crista-cupula* system (Budelmann and Young, 1985). They have one sac within each of the two cavities of the cranial cartilage, below and to the side of the brain. Octopods present only one oval shaped *macula* underlying a calcareous stone, the statolith (Budelmann et al., 1973). The *macula*/statolith system is vertically oriented and is responsible for the detection of gravity and linear accelerations. The *crista*/*cupula* system detects the angular acceleration and is linked to a cartilaginous anti*crista* lobe. There is a single cartilaginous projection between 6 and 7 *crista* segments, an anti*crista* that influences the flow within the statocyst sac. The octopod *crista* is divided into nine segments consisting of up to nine rows of sensory hair cells with up to 100 hair cells per row. Every *crista* has an overlaying gelatinous *cupula* which has a dense structure made of long fibrils. The nine octopod *cupula*e differ in form and size. There are two types of *cupula*, a small and a large *cupula* that alternate regularly in every *crista* segment. This division leads to the idea that there are two *crista* sub-systems differing in their sensitivity ranges (Budelmann et al., 1987) This has been interpreted as an adaptation to the cephalopod forms of locomotion, slowly crawling and fast swimming by jet propulsion (Williamson and Budelmann, 1985a, b).

The third type of cephalopod statocyst is found in decapods squid and cuttlefish, and presents three gravity receptor system (Budelmann et al., 1973; Budelmann, 1976, 1990; Stephens and Young, 1982) and an angular acceleration receptor system divided in four segments (Stephens and Young, 1982; Budelmann et al., 1973, 1997; Young, 1971). In decapod cephalopods the two statocysts are in separate cavities within the cranial cartilage, in the same position as in octopods. There is an inner sac filled with endolymph that, unlike the octopod statocyst, remains in contact with the cartilage wall and there is no perilymphatic space. The walls of the statocyst bulge outwards to make sac-like projections into the cavity. The sacs together with the two types of projections of the statocyst wall, anti*crista* and hamuli (the last are situated at the point where the rows of hair cells of the *crista* are interrupted), direct the movement of the endolymph across the sections of the *crista*. These structures and the Kölliker's channel modify the pattern of flow of endolymph and its chemistry composition, to change the response of the statocyst receptor system (Boycott, 1960; Young, 1989; Budelmann, 1990; Williamson, 1991). This system is comparable to the semicircular canals of vertebrates. Decapods present three *macula*e (Budelmann et al., 1973; Budelmann, 1976, 1990; Stephens and Young, 1982) which are oriented at right angles each other to cover the three axes of movement. The *principal macula statica* is an inverse 3 plate shaped, attached to the frontal wall and overlaid by a dense calcareous statolith which is attached to the *macula* with a layer of mucus and presses down on the cilia to stimulate the underlaying sensory hair cells. The two additional *macula*e are smaller and both of them are overlaid by less compact structure, statoconia. The *superior macula neglecta* is attached to the medial wall and the *inferior macula neglecta* is attached to the fronto-ventral wall (Williamson, 1995). In decapods, the *crista* is a ridge of cells lying in the transverse, longitudinal and vertical plane and is divided into four sections (Budelmann et al., 1973, 1997; Stephens and Young 1982): anterior transverse, posterior transverse, longitudinal and vertical which are oriented at right angles to each other (Young, 1971). Every section of hair cells is overlaid by a gelatinous *cupula*, which is deflected by the flow of endolymph during movements of the animal.

In decapods, the *macula*e and *crista* segments are innervated by one *macula* nerve and two *crista* nerves (Stephens and Young, 1982). In octopods, the *macula* and *crista* segments are innervated by one *macula* nerve and three *crista* nerves (Budelmann et al., 1987). On both groups the nerves are composed of afferent and efferent fibers. Glial cells surround the larger individual axons and small axons are enveloped in bundles. Some epithelial cells of the cephalopods' *macula* are thought to expel the organic and inorganic material to the statocyst cavity to form the statolith and statoconia in situ. The composition (organic matrix and saline structure) and process of formation of the statoconia and statolith are not well known.

Sound perception

The statocyst perception systems of cephalopods have raised an increasing interest (Young, 1960, 1984; Stephens and Young, 1982; Messenger, 1983; Budelmann and Young, 1984; Budelmann, 1990; Williamson, 1995; Neumeister and Budelmann, 1997; Quast et al., 2001). Many authors studied in depth the gravity receptor system (Young, 1960; Budelmann, et al. 1973; Budelmann, 1979; Colmers, 1982, 2004; Colmers et al., 1984). Other authors have almost completely described the angular acceleration receptor system (Young, 1960; Barber, 1966; Budelmann and Thies, 1977a; Budelmannet al., 1987). More recent works show a nice approach of the study of neuronal and synaptic organization (Williamson, 1988, 1989, 1991, 1992; Williamson and Chrachri, 2004, 2007).

However, little is known about sound perception in invertebrates, but evidence points out to the notion that cephalopods may be sensitive to low frequency sounds (Hanlon and Budelmann, 1987). There is indeed a considerable lack of information concerning the cephalopod reception of the sounds process (Packard et al., 1990; Bleckmann et al., 1991; Bullock, 1991; Budelmann et al., 1995; Hu et al., 2009). Although to date there is no definitive scientific evidence for it, statocysts may play an important additional role in low frequency sound reception (Hu et al., 2009). While there is uncertainty regarding the biological significance of particle motion sensitivity versus acoustic pressure, recent electrophysiological methods confirmed the species' sensitivity to frequencies under 400Hz (Kaifu et al., 2008; Hu et al., 2009; Mooney et al., 2010).

Controlled Noise Exposure Experiments were conducted on four species of cephalopods, *Sepia officinalis, Loligo vulgaris, Illex coindetii* and *Octopus vulgaris* to assess their sensitivity to low frequency sounds. During the experiments, the protocol included the use of control animals to monitor the influence of noise over time on the species sensory structures, in particular on the statocysts. The careful examination of the control animal statocyst ultrastructures was of primary importance to discard any possible artefact due to the cephalopod capture and handling in a captive environment. Here, we revisit these structures and compare them to previous published descriptions that processed the samples with the same imaging techniques: SEM and TEM, to validate their use as controls.

5.2 Material and Methods

5.2.1 Cephalopod individuals

Fifty-eight (58) individuals of *Sepia officinalis* (mantle length 10-18cm), four (4) *Loligo vulgaris* (mantle length 15-23cm), two (2) of *Illex coindetii* (mantle length 10cm) and six (6) of *Octopus vulgaris* (weight 200-400g),were obtained from the Catalan Coast (NW Mediterranean, Spain) over a period of 2 years, between February 2008 and August 2010, and kept in a closed system of re-circulating natural seawater (at 18-20ºC, salinity 35‰ and natural oxygen pressure) consisting of 2 mechanically filtered tanks PRFV of 2000L capacity, connected to each other. This included a physicochemical self-filtration with activated carbon and sand, driven by a circulation pump and filtration. (LAB-UPC, Vilanova I la Geltrú). The adult and subadult individuals were supplied with live crab (*Carcinus maenas*) food *ad libitum* and were maintained in the tank system until the CEE started. These animals were used as control individuals during Controlled Noise Exposure Experiments (see the complete protocol in chapter 6 and 7). Note that *L. vulgaris* and *I. coindetii* are species very sensitive to pressure changes thus very difficult to collect alive from the wild and to keep under captive conditions. This explains the small sample sizes included in this study.

5.2.2 Magnetic Resonance Image

Previous to the dissection and removal of statocysts, some individuals were analysed by Magnetic Resonance Image (MRI) for a better understanding of the statocyst morphology. Four *Sepia officinalis* cephalic cartilages (fixated in glutaraldehyde 2,5% for 24-48h at 4ºC), one alive individual of *Loligo vulgaris* and one of *Octopus vulgaris*, which were previously anaesthetized with 1% $MgCl_2$ solution (Messenger, Nixon, Ryan, 1985), were observed with a 7 Teslas Bruker Biospec USR 70/30 spectrometer at the Autonomous University of Barcelona (UAB, Barcelona) facilities.

5.2.3 Electron microscopy

After extraction, statocysts were processed for TEM and SEM.

5.2.3.1 Removal of statocysts

In all experiments, isolated head preparations, obtained by decapitation without prior anaesthesia, were used. The statocysts with the surrounding cartilage were extracted and fixed for posterior observation and analysis. For fixation, statocyst cavity was opened and the maximum care was taken to minimize damage on the inner tissues. The tissues were obtained and analyzed from the left and the right statocysts, but no difference was observed between sides. For the specific fixation of the different imaging techniques, see below.

5.2.3.2 Scanning Electron Microscopy (SEM)

Thirty-eight (38) statocysts from 20 *Sepia, 4 Octopus, 4 Loligo* and *2 Illex were* used for this study. Fixation was performed in glutaraldehyde 2,5% for 24-48h at 4ºC. The statocysts were dehydrated in graded alcohol solutions and critical-point dried with liquid carbon dioxide in a Leica EmCPD030 unit (Leica Mycrosystems, Austria). The dried statocysts were cut open and flattened out to expose the statocyst structures and then mounted on specimens stubs with a double-sided tape. The mounted tissues were gold-paladium coated with a Polaron SC500 sputter coated unit (Quorum Technologies, Ltd.) and viewed with a variable pressure Hitachi S3500N scanning electron microscope (Hitachi High-Technologies Co., Ltd, Japan) at an accelerating voltage of 5kv at the Institute of Marine Sciences of the Spanish Research Council (CSIC) facilities (Barcelona).

5.2.3.3 Transmission Electron Microscopy (TEM)

Twelve (12) statocysts from 8 *Sepia* and 2 *Octopus* were used for this study. Statocysts were extracted and fixed glutaraldehyde 2,5% for 24-48h at 4ºC or glutaraldehyde 2,5%-paraformaldehid 2% on filtered sea water for 24h at 4ºC. Macula and *crista* sections were dissected from the statocysts and subsequently osmicated in 1% osmium tetroxide, dehydrated in acetone and embedded in Spurr. Semithin sections (1µm) of the *macula* area and *crista* ridge were cut transversally or tangentially with a glass knife, stained with methylen blue covered with Durcupan and observed on Olympus CX41. Then, ultrathin (around 100 nm) sections of the *macula* and *crista* were obtained by using a diamond knife (Diatome) with an Ultracut E ultramicrotome from Reichert-Jung. Sections were double-stained with uranyl acetate and lead citrate and viewed with a Jeol JEM1010 at 80 Kv. The images were obtained with a Bioscan camera model 792 (Gatan) at the University of Barcelona technical service.

5.3. Results

Statocyst gross morphology

The use of RMI techniques presents the advantage of the absence of irradiation and artefacts by bone tissues and gas provides a good contrast for soft tissue and allows to construct models. Therefore, it is an optimal technique for locating statocysts into the cranial cavity of cephalopods. The statocyst location is shown on axial view of *O. vulgaris* (Fig. 5.1A) and coronal views of *L. vulgaris* (Fig. 5.1 B, C) heads as well as cranial cartilage of *S. officinalis* (5.1D).

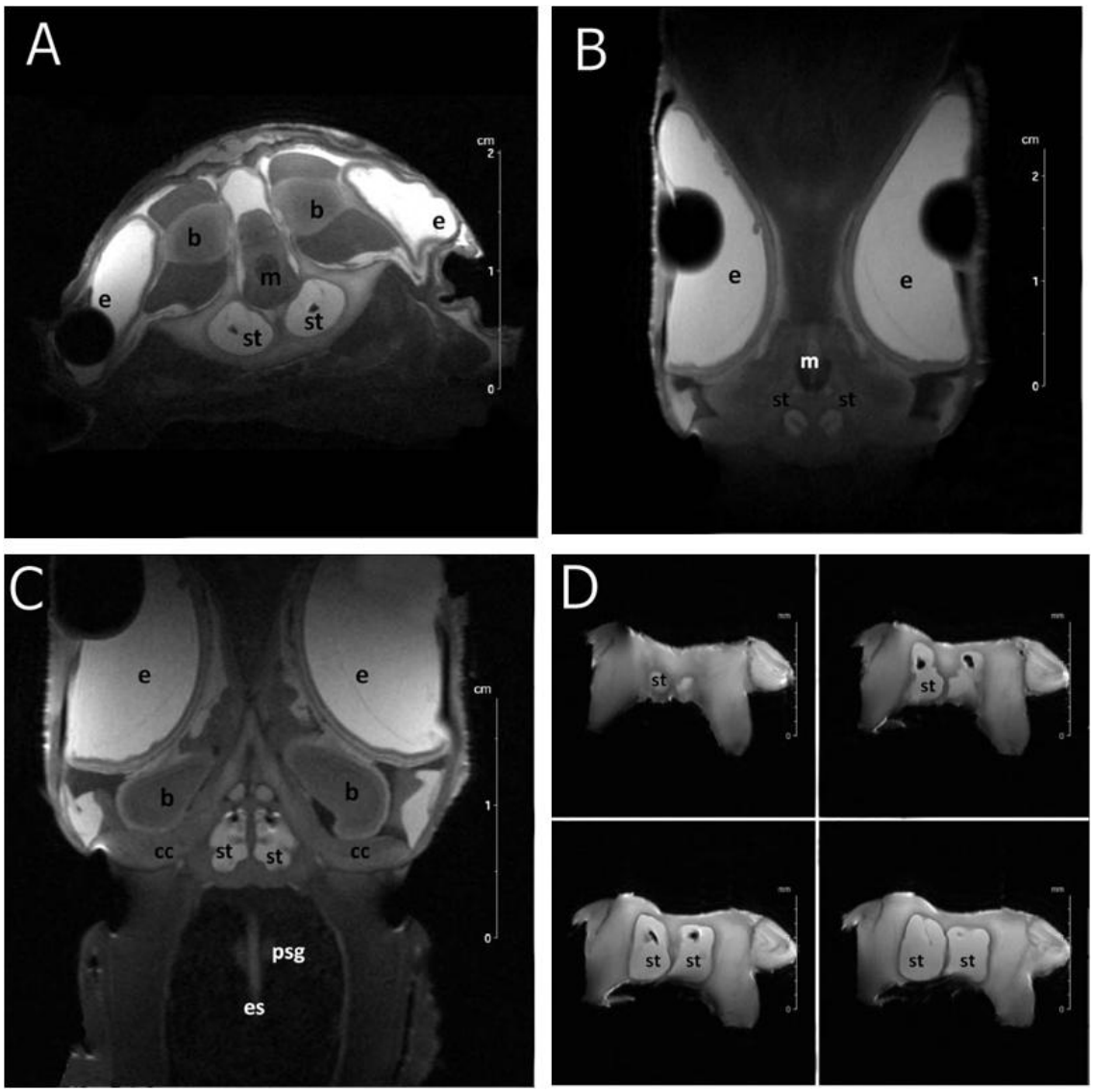


FIG. 5.1 RMI. Octopods and decapods statocyst location into the cephalic cartilage. A: Axial view of octopus head. **B, C**: Two coronal views –anterior section- of squid (Loligo vulgaris) head. **D**: Four views of cuttelfish cartilage showing the statocyst cavities at different levels. The statocyst cavities correspond to the white masses in the centre of the images. **B**: Brain, cc: cranial cartilage, e: eye, es: oesophagus, m: mouth, psg: posteror salivary gland, st: statocyst. **Scale bars**: **A, B, C** = 2 cm. **D** = 8 mm.

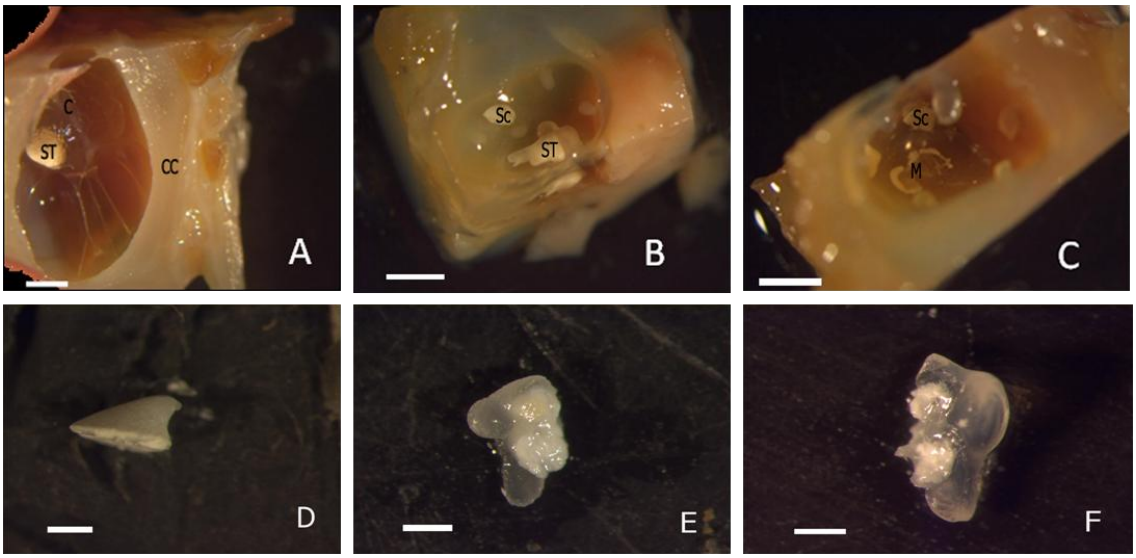


FIG. 5.2 Photomicrograps of octopod and decapod statocyst structure viewed in light microscopy. A: Lateral view into an opened *Octopus vulgaris* statocyst. Photomicrograph shows the spherical inner sac suspended in the cartilage cavity by fibrous strands. The *crista* and the statolith attached to the *macula are* visible. **B, C:** Upper view into the opened *Sepia officinalis* statocyst. **B** shows the statocyt attached to the *macula statica princeps* and a *statoconia* attached to the superior *macula neglecta*. In **C** the statocyst was removed and the *macula statica princeps* is visible. **D:** *O. vulgaris* statolith, **E:** *S. officinalis* statolith. **F:** *L. vulgaris* statolith. *St: Statolith. Sc: statoconia. M: Macula statica princeps, c: crista, CC: cephalic cartilage*. **Scale bars**: **A, E, F** = 1 mm. **B, C** = 2 mm. **D** =0, 2 mm.

5.3.1 Scanning Electron Microscopy (SEM)

5.3.1.1 Decapod inner statocyst morphology

The walls of the statocyst bulge outwards to make sac-like projections (anti*crista* and hamuli) into the cavity (Fig.5.3). Each anti*crista* consists of a core of cartilage covered by the lining epithelium of the statocyst. They differ in shape and in function. Some of them, called hamuli, (Fig. 5.3E, F) are situated where the rows of the *crista* hair cells are interrupted to limit the flow of endolymph at the sides of the *cupula*.

The lining epithelium of the cavity consists of flat hexagonal cells with oval nucleus. In some parts of the cavity, cilia emerge between the epithelial cells (Fig. 5.4). They carry a basal network of fibres in the outer statocyst wall. The cells of the lining membrane carry cilia on the outer side, which project into the cavity and with hamuli, anti*crista* and the Kölliker's channel modify the pattern of flow of endolymph and its chemistry composition to change the response of the statocyst receptor system. In decapods they are especially abundant ventrally from the three horizontal *crista* segments and medially from the vertical *crista*. Ciliated cells also occur on the base of anti*crista* lobes (Fig. 5.3D).

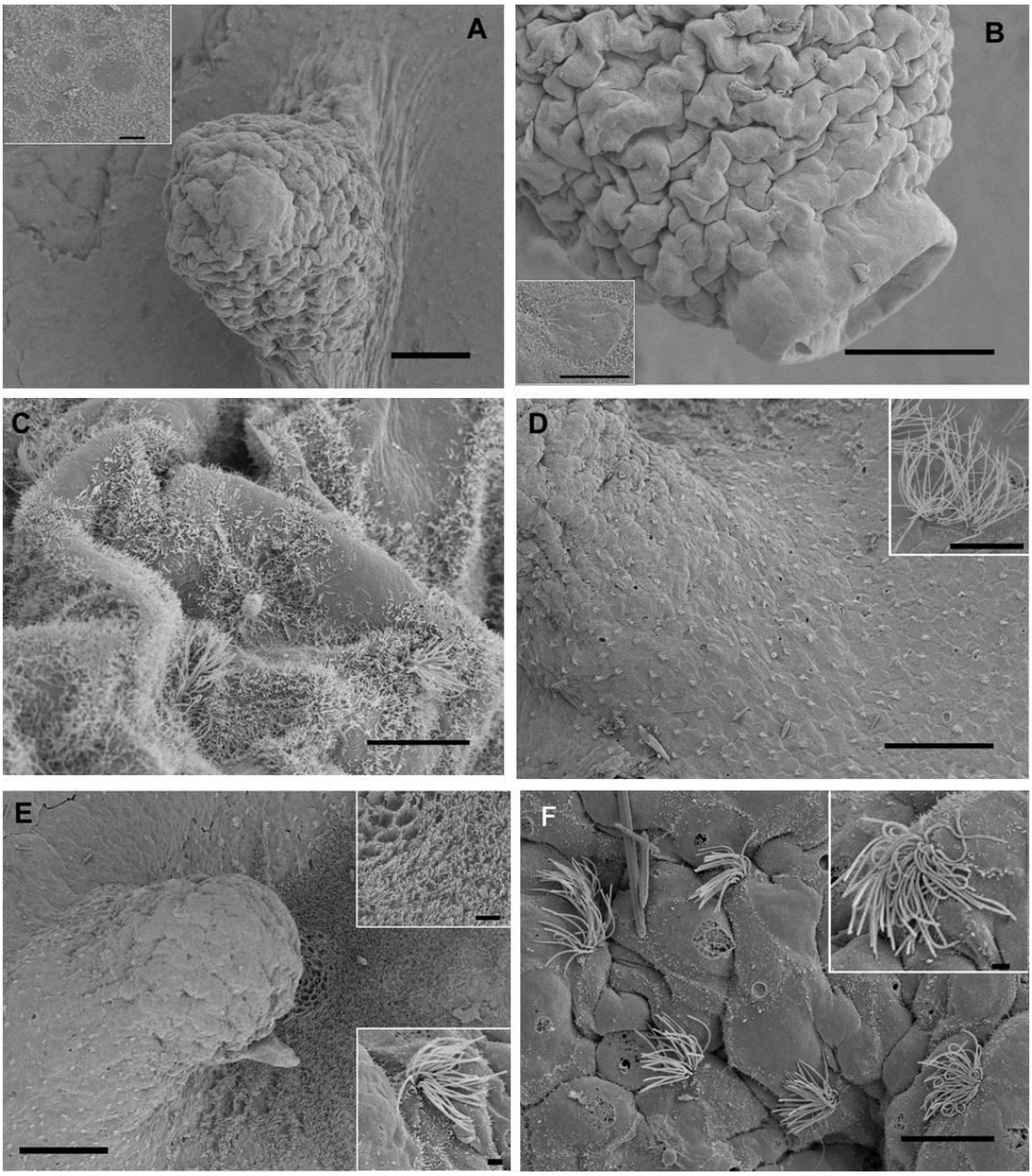


FIG. 5.3 SEM *Sepia officinalis* inner statocyst structure. A-D: Anti*crista* lobes. E, F: Hamuli lobes. A, B: Superior (A) and lateral (B) views of anti*crista* lobes into an opened statocyst show the folded surface. **Insert in A, insert in B:** Detail from the liner epithelium of anti*crista* lobes. In some parts microvilli cover near completely the lining epithelium, or surround its flat hexagonal cells. **C:** In other zones anti*crista* lobes show a different model of liner epithelium. The cells of the lining membrane carry cilia on the outer side, which project into the cavity and microvilli surround them. **D:** At the base of the anti*crista*, the cells of the lining membrane carry cilia on the outer side, which project into the cavity and play part in the circulation of the endolymph. **Insert in D**: Detail of a bundle of cilia. **E:** Superior view of hamuli lobes into an opened statocyst that shows the folded surface. **Upper insert in E**: At the base of the hamuli, the *crista* ends on a densely ciliated area which lies between two *crista* segments. **Lower insert in E**: Liner epithelium of hamuli lobes. The cells of the lining membrane carry bundles of cilia on the outer side, which project into the cavity. Microvilli are present with less density on the hamuli than on the anti*crista* lobes and surrounds the flat hexagonal cells. **F**: Detail from the liner epithelium of hamuli lobes. **Insert in F**: Detail of a bundle of cilia. **Scale bars**: **A, B, D**: 100 µm. **C, F** = 10 µm. **E** =50 µm. **Insert in A, B, D**: Upper **insert in E** =10 µm. **Lower insert in E**, **insert in F**: 1 µm.

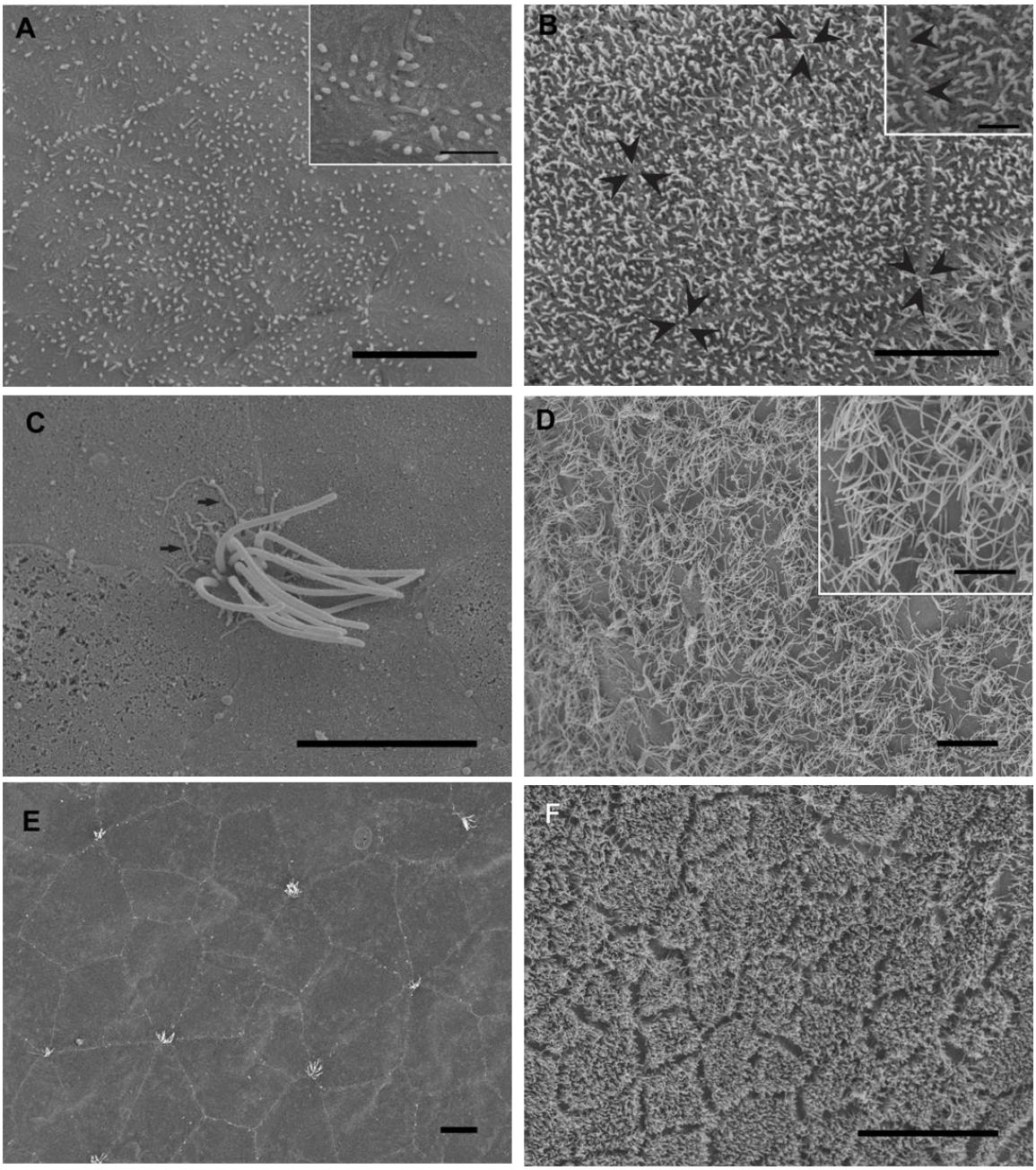


FIG. 5.4 SEM. *Loligo vulgaris* (A-D) and *Illex coindetii* (E, F) inner statocyst structure. The inner surface of decapods statocyst is lined by an epithelium that shows different models. **A:** The flat hexagonal cells of the lining membrane are visible. **Insert in A:** Detail from A. The growing microvilli are visible. **B:** In other area the microvilli are present at high density and cover all the flat hexagonal cells. In A and B itis possible to appreciate the cellular limit and the growing microvilli (arrowheads signs the vertex of the flat hexagonal cells). **Insert in B**: Detail from B. Arrowheads sign the limit of one flat hexagonal cell. **C** shows an individual bundle of cilia (note the root-like structures at the base -arrows-). **D**: A very high density long cilia area is shown. **Insert in D**: Detail from D. The cilia are clearly visible. **E**: In *Illex coindetii* the flat hexagonal cells carry cilia on the outer side, which project into the cavity. Microvilli are present with less density than in *Loligo vulgaris* and surround the epithelium cells. **F:** Another inner statocyst area near of the *macula* from *Illex coindetii* shows very high density of cilia covering all the surface of the flat hexagonal cells of the lining epithelium. **Scale bars**: **A, B, C** = 5 µm. **D, E, F** = 10 µm. **Insert in A, B** = 1 µm. **Insert in D** = 5 µm.

5.3.1.2 Decapod macula morphology

Macula Statica Princeps

In decapods, the *macula statica princeps (msp)* is shaped like an inverse 3. The ciliary groups of these hair cells are arranged in regular lines, which follow the shape of the epithelium (Fig. 5.5A, C). The microvilli surround the base of the kinocilia. Cilia and microvilli form elongated groups (Fig. 5.5B). Each group represents a single hair cell. Every hair cell is arranged in line with an adjacent hair cell (Fig. 5.5A, C).

The basal foot structure on the basal body is an important feature of the kinocilium because it defines the direction of their morphological polarization as well as that of the hair cells (Fig. 5.5). This results in a specific pattern of morphological polarizations for the receptor epithelia.

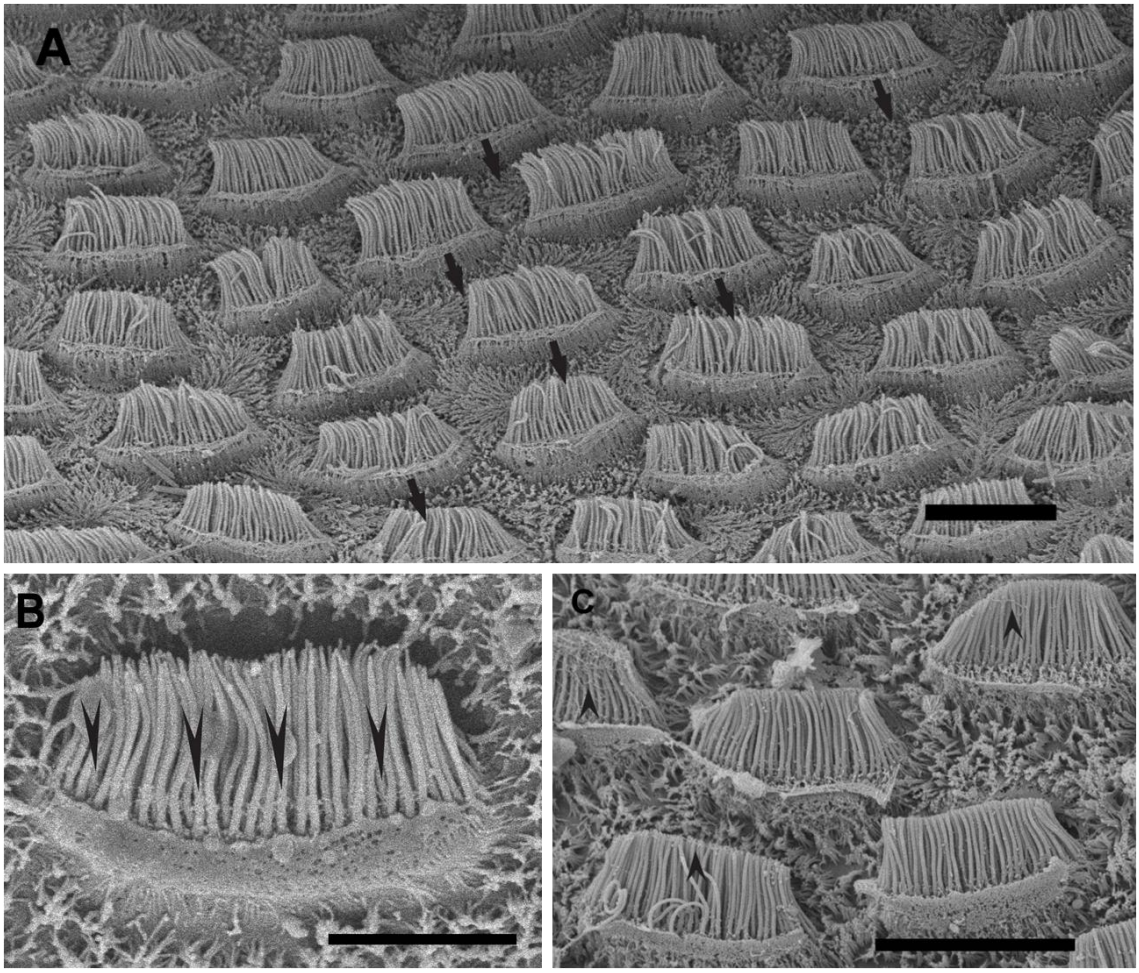


FIG. 5.5. SEM. *Sepia officinalis* (A, B) and *Loligo vulgaris* (C) macula statica princeps (msp). **A**: View of the arrangements of the kiociliary groups of the hair cells in regular lines following the epithelium shape. Because of their uniform orientation per cell, each hair cell is morphologically polarized in just one direction. Arrows indicate hair cells' direction of polarization. Note the high density of microvilli. **B**: Detail of a hair cell. Cilia and microvilli form elongated groups. Each kinociliary group represents a single hair cell. Note the space under the hair cell which permits its polarized movement. Arrowheads sign the longitudinal strand that allows the polarized movement of each kinocilliary group of a hair cell. **C**: Detail of the arrangements of the kinociliary groups of the hair cells in regular lines on a *macula* of *Loligo vulgaris*. Note the organization of the *macula* epithelium is very similar in the two species of decapods. Arrowheads show links between the kinocillia that allow polarized movement of the hair cell. **Scale bars**: **A, C** = 10 µm. **B** = 5 µm.

Superior macula neglecta

The *superior macula neglecta (smn)* is shaped nearly as a half circle. The diameter of this half-circle runs from the dorso-frontal to the ventro-caudal orientation (Fig. 5.6B). The ciliary groups of the SMN are more sparsely distributed (Fig. 5.6C) than those of the MSP, and have fewer kinocilia per ciliary group. The groups are arranged in half-circular rings around a centre, which lies near the perifery (Fig. 5.6B).

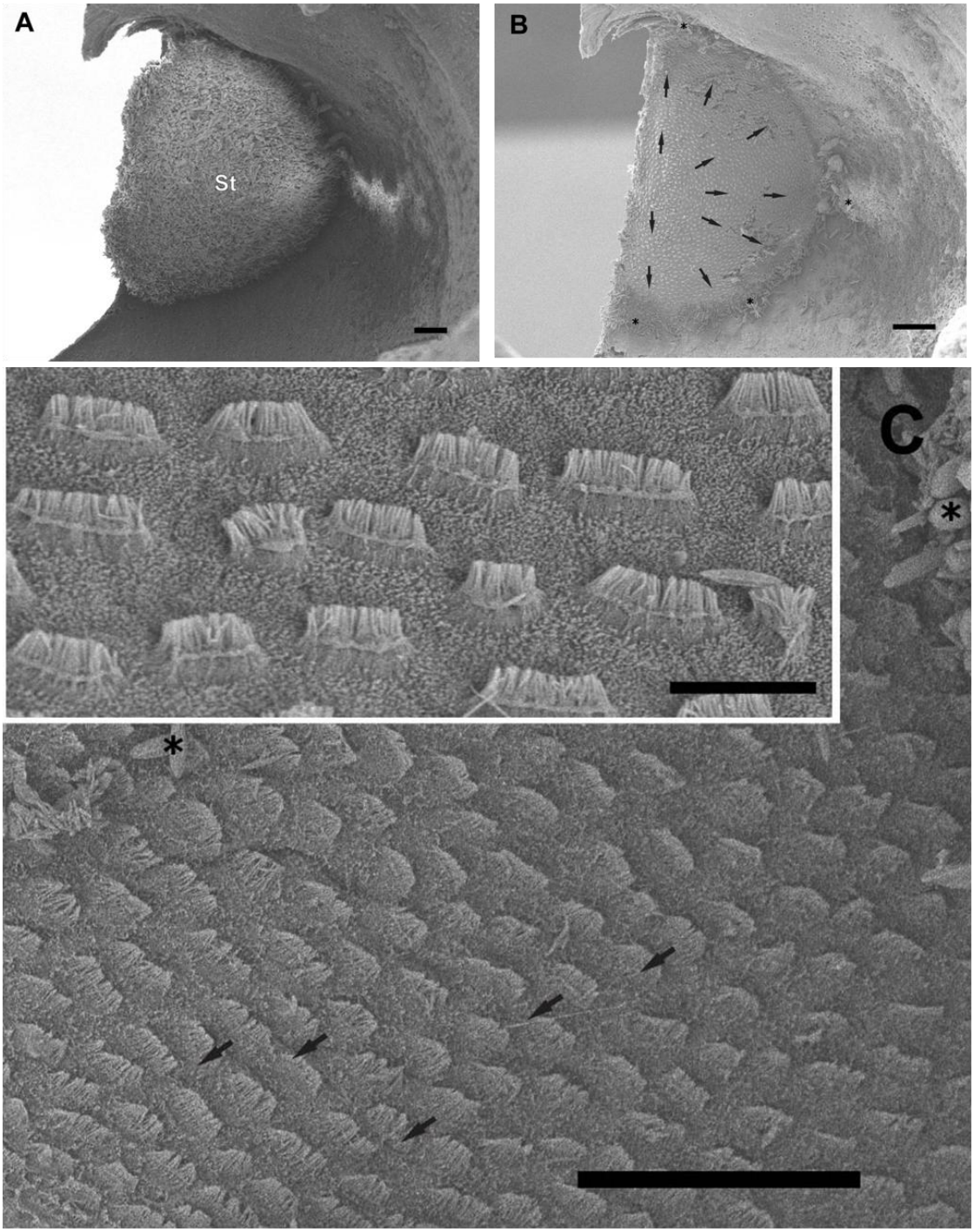


FIG. 5.6. SEM. *Sepia officinalis* superior macula neglecta (smn). A: Lateral view into an opened *Sepia officinalis* statocyst. The statoconia is attached to the *macula* and does not allow its vision. **B:** The statoconia was removed and the nearly half-circular shaped superior *macula* neglecta is now visible. The polarization pattern of *smn* is shown. The groups are arranged in half-circular rings around a centre, which lies near the diameter. **C:** The ciliary groups of the *smn* are more sparsely distributed than those of the *msp*. The arrows indicate the hair cells' direction of polarization. Some crystals of the statolyth are visible (asterisk). **Insert in C**: Detail from C. The high density of microvilli that surround the hair cells is visible. **Scale bars**: **A, B** = 100 µm. **C** = 50 µm. **Insert in C** = 10 µm.

Inferior macula neglecta

The *inferior macula neglecta* (*imn*) is mostly circular shaped (Fig. 5.7A, B). The hair cells are arranged in nearly concentric rings (Fig. 5.7B), but in the periphery the rings follow the irregular outline of the epithelium. The ciliary groups, in a similar way as the *smn*, are more sparsely distributed than those of the *msp* and with fewer kinocilia per ciliary group (Fig. 5.7C, D). A longitudinal strand on the hair cells acts as a link between kinocillia and allows the polarized movement of each kinocilliary group of a hair cell (Fig. 5.7C).

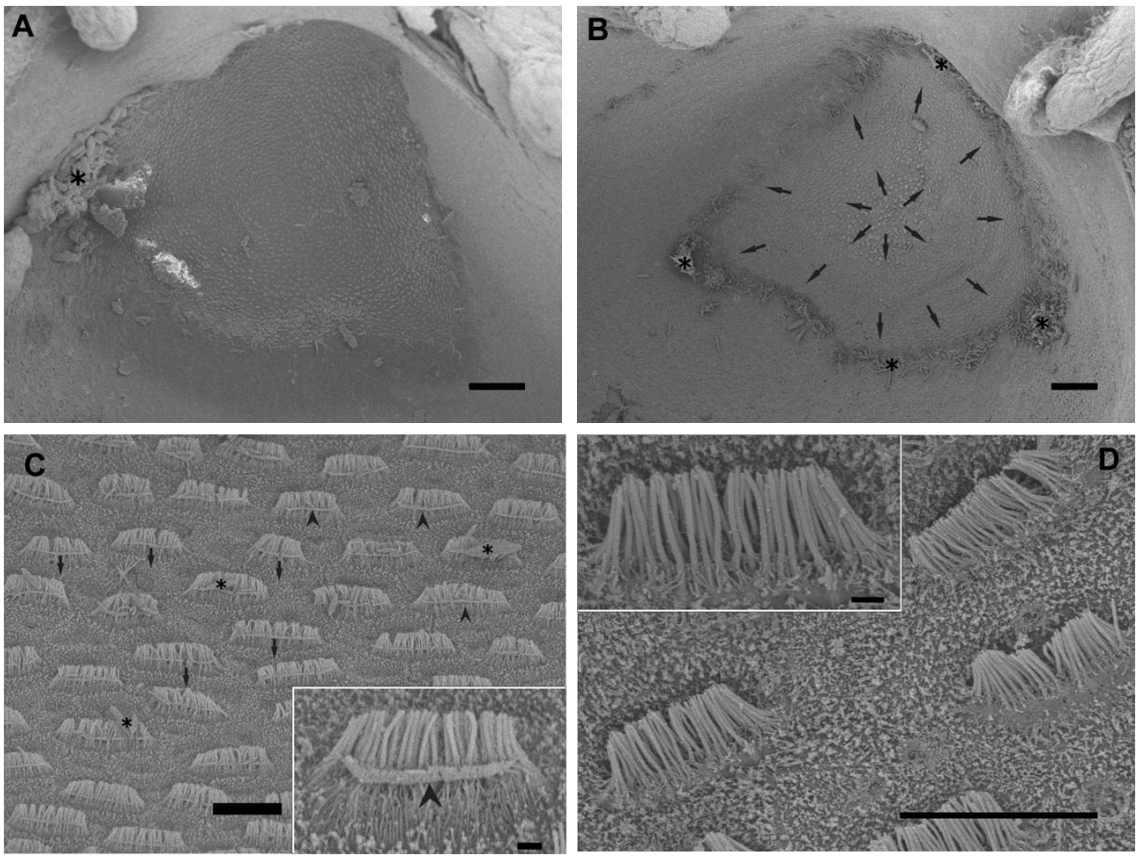


FIG. 5.7 SEM. *Sepia officinalis* inferior macula neglecta (imn). A: Upper view of i*mn* into an opened *Sepia officinalis* statocyst. Some crystals of the statolyth are visible (asterisk). **B**: Polarization pattern of IMN. The hair cells are arranged in nearly concentric rings around a centre. Some crystals of the statolyth are visible (asterisk). **C:** The ciliary groups of the *inm* are more sparsely distributed than those of the *msp*. The arrows indicate the hair cells' direction of polarization. Some crystals of statolyth are visible (asterisks). A longitudinal strand is visible on hair cell (arrowheads). **Insert in C**: Detail of one hair cell of C. Note the high density of microvilli surrounding the hair cells. Arrowhead signs a longitudinal strand. **D**: Distribution of hair cells on the IMN of another animal. The longitudinal strands are not visible on it. **Insert in D**: Detail of one hair cell of D. **Scale bars**: **A, B** = 100 µm**. C, D** = 10 µm. **Insert in C, D** = 1 µm.

5.3.1.3 Decapod crista morphology

The decapod's *crista* is divided into four (4) sections, each oriented at a right angle from one to another. Anterior *crista transversalis* (CTA) runs along the frontal surface of the statocyst cavity. The *crista longitudinalis* (CL) lays in the horizontal plane on the lateral wall of the statocyst. The *crista transversalis* posterior (CTP) lays in the horizontal plane from the lateral to the caudal wall. Finally, the *crista verticalis* (CV) runs upwards on the caudal statocyst wall. The hair cells of the *crista* are arranged in four main rows (Fig. 5.9, 5.10), two of them – i.e. the two ventral ones of the CTA, CL and CTP, and the two medial ones of the CV - are composed of larger hair cells than the two other rows. Surrounding the *crista*, other hair cells that are similar to the smaller ones of the main rows, can be found.

The hair cells of each *crista* segment are polarized at right angles from the long axis of the *crista*, and some hair cells are polarized in the opposite direction from the others (Fig. 5.9E, insert in 5.10B).

Every section of 4 hair cell rows is overlaid by a thin gelatinous *cupula*, which is composed of fibrous strands and of an amorphous ground substance that is attached to the kinocilia of the ciliary groups (Fig. 5.8, 5.10).

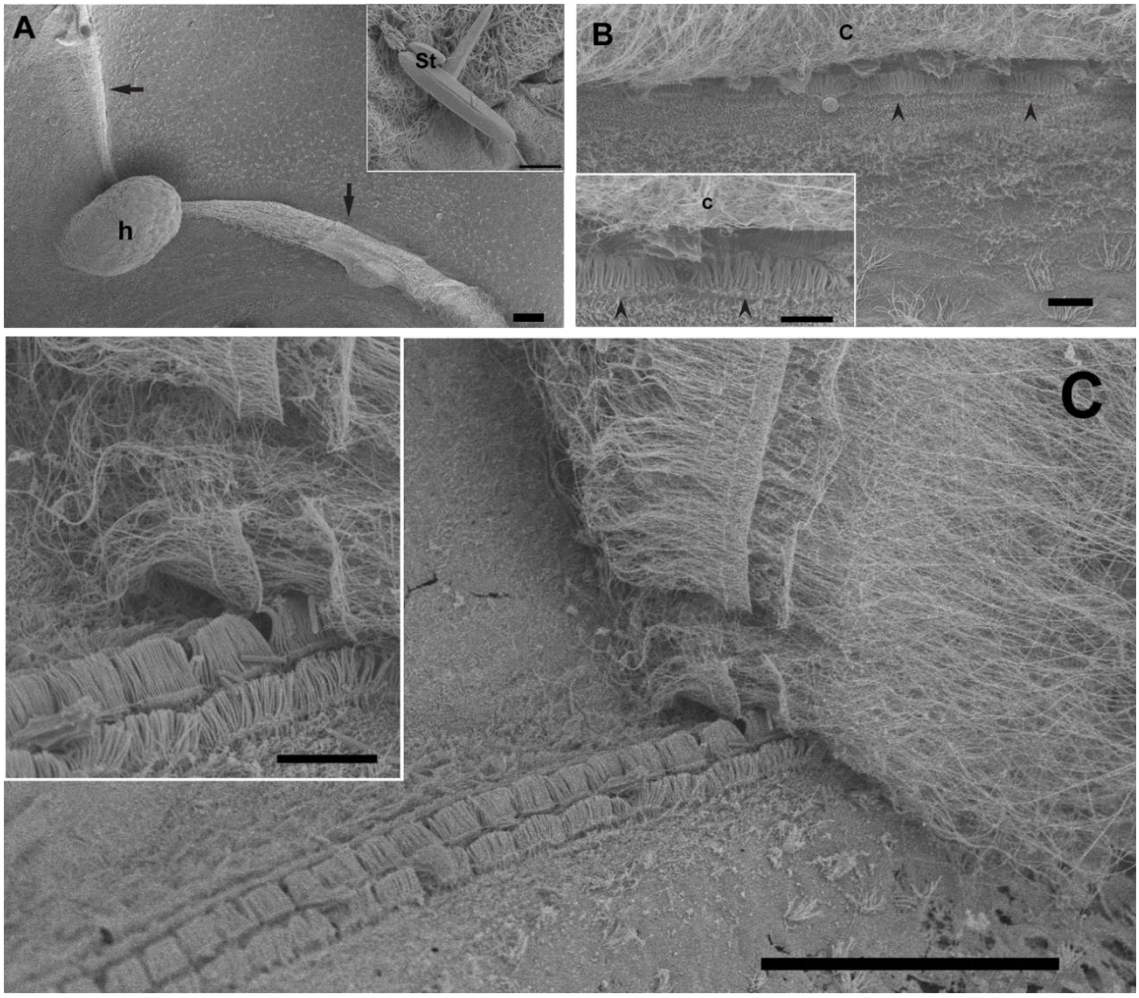


FIG. 5.8. ***SEM. Sepia officinalis crista-cupula system***. **A**: Upper view into an opened Sepia officinalis statocyst. The cupulae that are attached to crista segments are visible (arrows). The crista rows with their attached cupulae end on the base of the hamuli (h). **Insert in A** shows the filamentous texture of the cupula. Different sized crystals of statocyst (st) are visible. **B**: Cupula (c) attached to the hair cells of the crista (arrowheads). **Insert in B**: Detail of kinocilliary groups of hair cells (arrowheads). **C**: The marks of the kinocilia of the hair cells of the crista on the partially removed cupula are visible. **Insert in C**: Detail of the kinocilliary groups of crista hair cells and their marks on cupula. Scale bars: **A** = 100 µm. **B** = 10 µm. **C** = 50 µm. **Insert in A, C** = 10 µm. **Insert in B** = 5 µm.

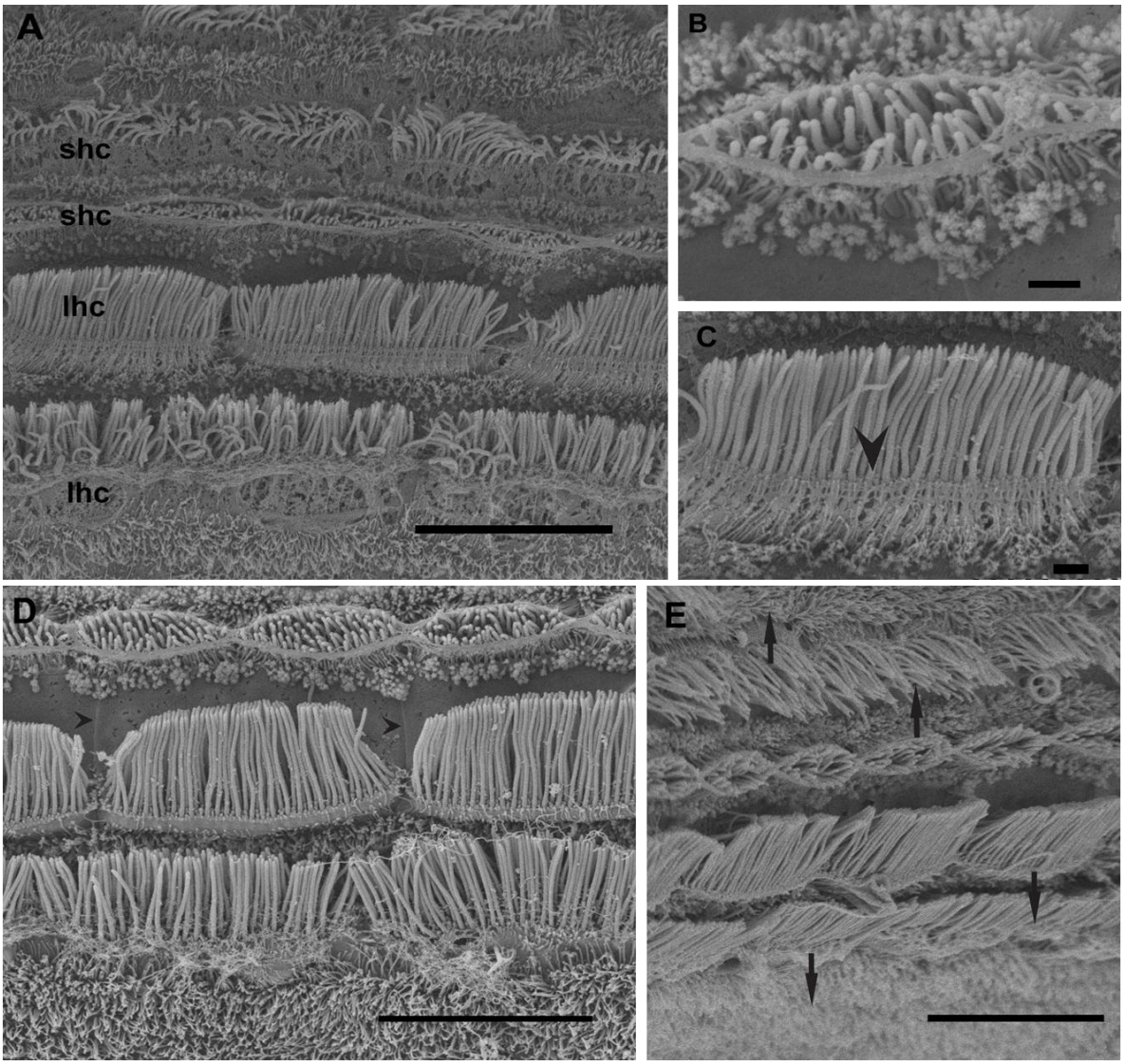


FIG. 5.9. SEM. ***Sepia officinalis crista*****. A:** The four rows that form the hair cells of the *crista* are visible. The hair cells from two of them are larger (large hair cell row, lhc) than those of the other two rows (small hair cell row, shc). Surrounding the *crista*, other hair cells similar to the shorter ones of the main rows are found. **B:** Detail of one cell of one shorter row of the *crista*. Note the high density of the surrounding microvilli. **C:** Detail of one cell of one larger hair cell rows. A longitudinal strand on the hair cells act as a link between kinocillia and allows the polarized movement of each kinocilliary group of a hair cell. **D:** View of three rows of hair cell in the *crista*. The contacts on the base of the hair cells are visible behind the kinocilia and near the microvilli on the base of the shorter row (arrowheads). **E**: Lateral view of the four rows of the *crista*. Note the dense microvilli that surround the hair cell rows and the rows similar to the smaller ones of the main rows that are found surrounding the *crista*. The arrows indicate the hair cells' direction of polarization. **Scale bars**: **A, D, E =** 10 µm**. B, C** = 1 µm.

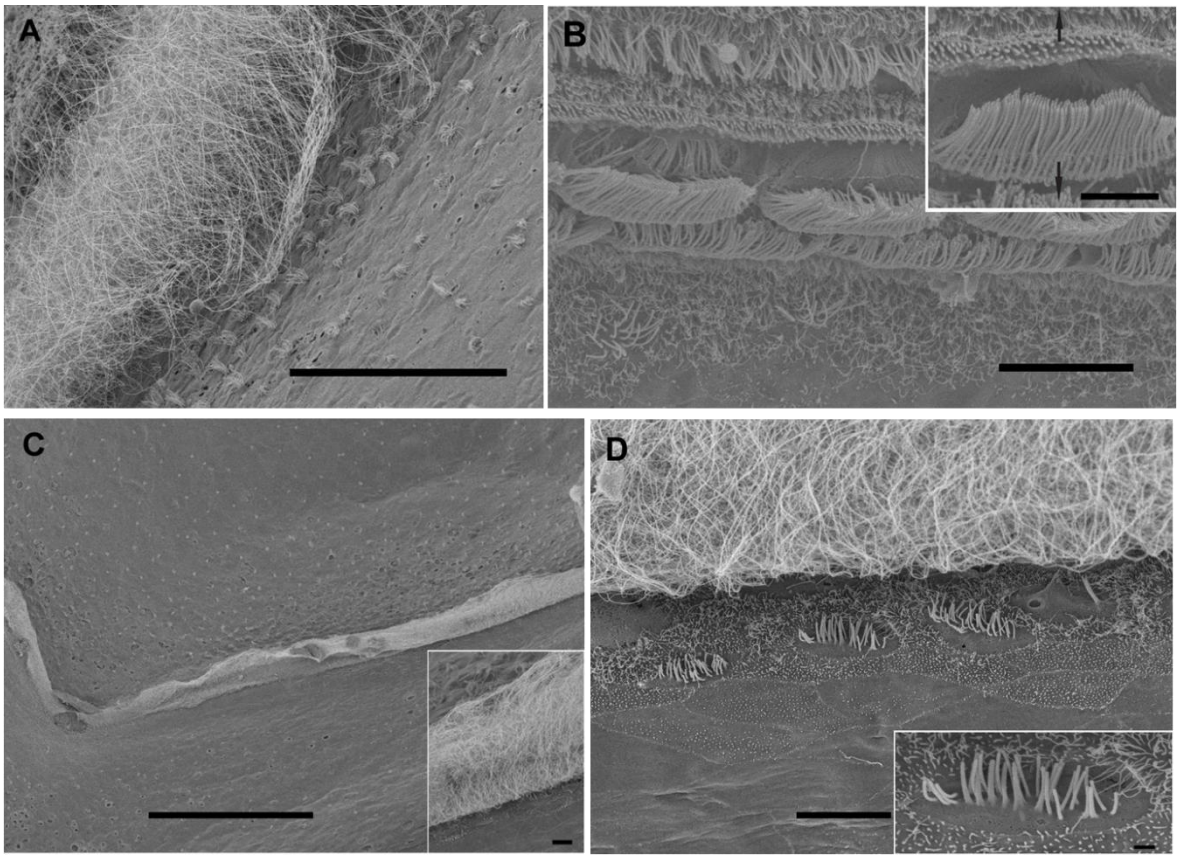


FIG. 5.10. SEM. *Loligo vulgaris* (A, B) and *Illex coindetii* (C, D) crista-cupula system. A: Fibrous *cupula* attached to the *crista*. **B:** *Crista* of *Loligo vulgaris*. The four rows of hair cells are visible. The *cupula* has been partially removed and the kinocilia of the hair cells is now visible. **Insert in B**: Detail of one cell of the two central rows of the *crista*. Arrows indicate the hair cells' direction of polarization. **C:** Upper view of two *Illex coindetii cupula*. **Insert in C**: Detail of fibrous *cupula* of *Illex coindetii* attached to the *crista*. **D:** The contacts of the kinocilia with the fibrous *cupula* are visible. Model of the *Illex coindetii* statocyst liner epithelium is shown next to the hair cell rows that surround the main rows of *crista*. *Microvilli* grow in some of the liner epithelium cells. **Insert in D:** Detail of the kinociliary group of a hair cells that surround the main rows of the *crista*. The ground microvilli completely surround the hair cells. **Scale bars**: **A, C =** 100 µm**. B, D** = 10 µm. **Insert in B** = 5 µm. **Insert in C** = 10 µm. **Insert in D** = 1 µm.

5.3.1.4 Octopod inner statocyst sac morphology

The inner lining epithelium of the sac (Fig. 5.11A) consists of flat hexagonal cells with an oval nucleus. In some parts of the sac, bundles of cilia emerge between the epithelial cells. Microvilli grow on the flat cells of the lining epithelium. In octopods, ciliated cells also occur on a distinct posterior sac of unknown function.

5.3.1.5 Octopod macula morphology

Octopods present only one oval shaped *macula* underlined by a calcareous stone, the statolith. The hair cells are arranged in concentric rings, although none are present in the region of the *macula* nerve. The hair groups are more closely packed at the periphery than in the centre of the plate. As in decapods, each hair cell is morphologically polarized in just one direction (Fig. 5.11B). This results in a specific pattern of morphological polarizations for the receptor epithelia.

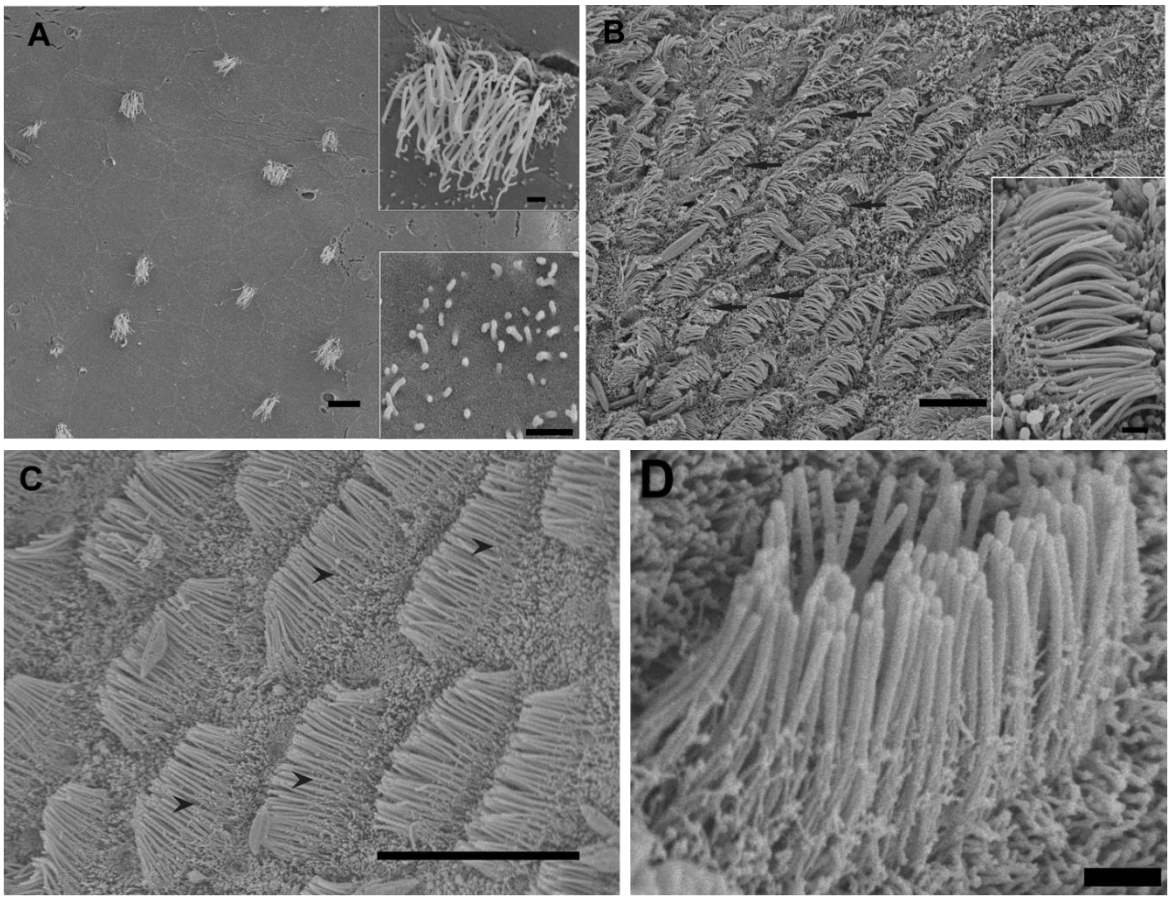


FIG. 5.11. SEM. *Octopus vulgaris* inner sac statocyst morphology (A) and ***Octopus vulgaris macula* (B-D). A**: The inner lining epithelium of the statocyst sac shows different structures. The cells of the lining membrane carry very dense bundles of cilia on the inner side, which project into the cavity (**upper insert in A).** In some parts, microvilli cover the lining epithelium, or surround its flat hexagonal cells (**lower insert in A**). **B**: Arrangements of the kinociliary groups of the hair cells in regular lines following the circular shape of the epithelium. Cilia and microvilli form elongated groups. Each group represents a single hair cell. Arrows indicate the hair cells' direction of polarization **C**: Note the very high density of the microvilli that surround the kinocilliary groups of the hair cells. The longitudinal strand is visible on some hair cells (arrowheads). **D**: Detail of individual hair cells surrounded at their base by microvilli. **Scale bars**: **A, B, C, D =** 10 µm**. Inserts in A, B, D** = 1 µm.

5.3.1.6 Octopod crista morphology

Octopod *crista* is divided into 9 sections which are alternated between those with a double row of large hair cells (odd-numbered *crista* sections) (Fig. 5.12B) and those with a single row (even-numbered *crista* sections) (Fig. 5.12C). On the ventral side of the double and single row of large hair cells are several rows of fairly regularly arranged ciliary groups (Fig. 5.12). On the dorsal side are irregularly arranged smaller ciliary groups.

The hair cells of each *crista* segment are polarized at right angles to the long axis of the *crista*, while some hair cells are polarized in the opposite direction (Insert in 5.12A).

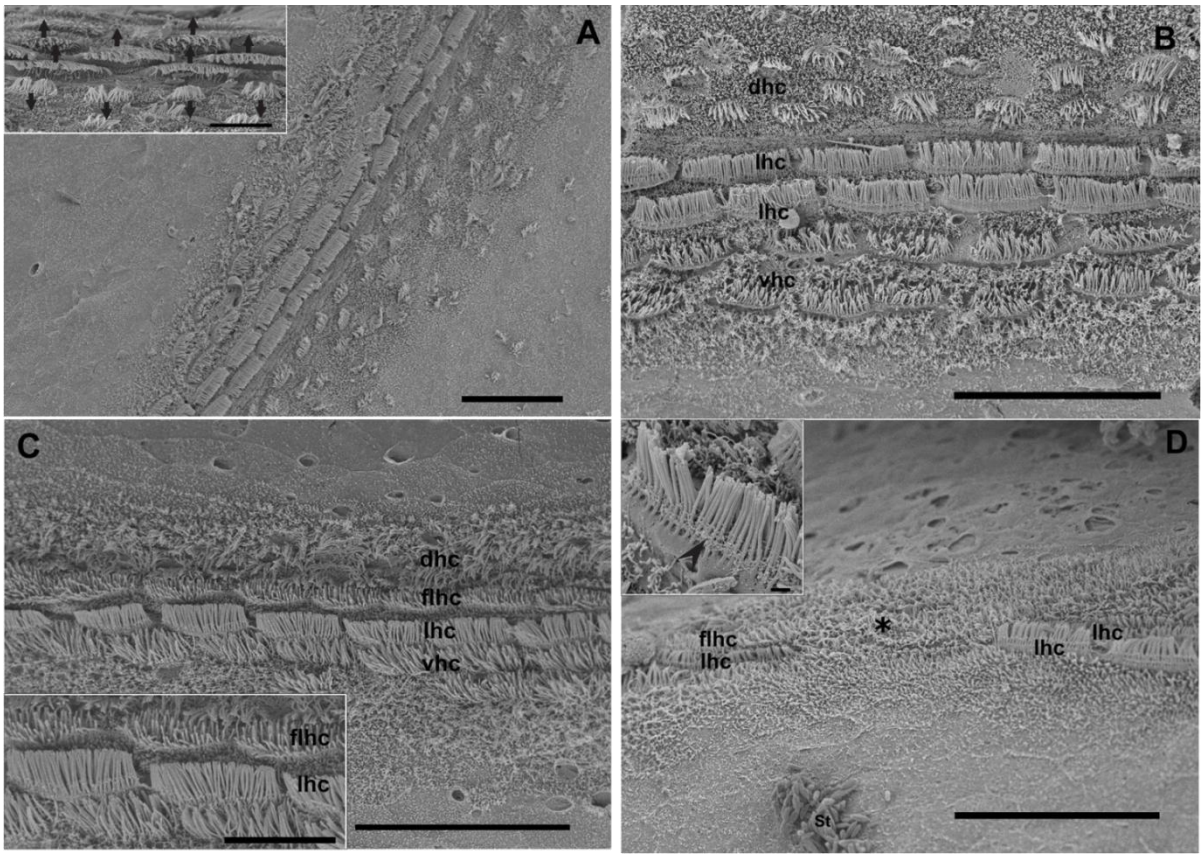


FIG. 5.12. SEM. *Octopus vulgaris* crista morphology. A: Upper view of a *crista* in an opened *Octopus vulgaris* statocyst inner sac. This image shows the ridge that runs along the inner sac statocyst and the hair cell rows of the *crista* epithelium on it. The *cupula* has been removed. Note the two regular rows of large secondary hair cells that are characteristic of odd-numbered *crista* sections. **Insert in A**: Lateral view of a fragment of odd-numbered *crista* segments. The two regular rows of large secondary hair cells are visible. The inclination of the image allows to appreciate the orientation (arrows) of the main and peripheral *crista* hair cell rows. **B, C:** Different distribution of hair cell rows on odd-numbered and even-numbered *crista* sections. **B:** Part of an odd-numbered *crista* segment. **C:** Part of an even-numbered *crista* segment (**dhc**: small dorsal primary hair cell; **lhc**: regular row of large secondary hair cells; **flhc**: regular row of fairy large hair cells; **vhc**: small ventral secundari hair cell). **D:** Limit between two *crista* segments. On the left part of the image the lhc and flhc of an even-numbered *crista* section finish on a very high density ciliated zone (asterisk) that acts as a junction to the two lhc rows of the odd-numbered *crista* section located on the right part of the image. Statocyst crystals are visible (St). **Insert in D:** Detail of a large secondary hair cell. A longitudinal strand is shown on the hair cell (arrowhead). **Scale bars**: **A, B, C, D =** 30 µm**. Insert in A, C =** 10 µm. **Insert in D** = 1 µm.

5.3.2 Transmission electron microscopy (TEM)

5.3.2.1 Decapods macula

In decapods, the simple prismatic *macula* epithelium is formed by hair cells with oval shaped nuclei located on the central-basal portion of the cell (see Fig.5.13). Each *macula* hair cell has an elongated group of kinocilia at its distal ends. Numerous microvilli occur on the luminal surface of each hair cell, surrounding kinocilia. Between the hair cells there are supporting cells that exhibit microvilli on their distal ends, a very electrondense plate at the apical part, and have an irregular elongated nucleus. The hair cell epithelium lays above the cartilaginous plate and the nerve plexus runs underneath the hair cells.

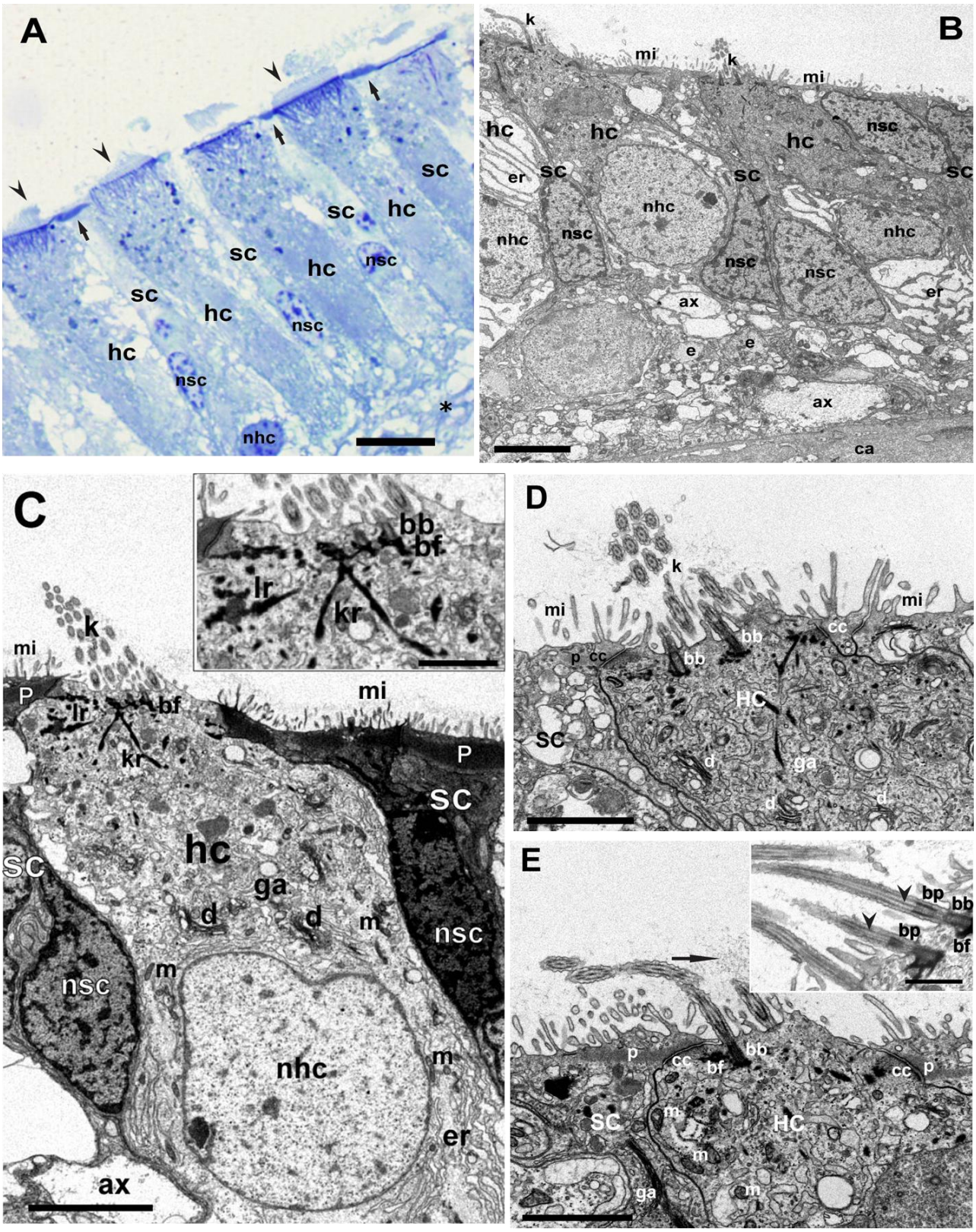
The epithelial hair cells of the decapod's *macula* contain an abundance of swollen vesicles, as well as dictyosomes of Golgi apparatus, rough endoplasmic reticulum, mitochondria and

lsosomes. The voluminous hair cells have a round or oval shaped nucleus surrounded by a prominent rough endoplasmatic reticulum and located on the central-basal portion of the cell. Mitochondria are abundant on the proximal part of the hair cells near the contact point with the underlying neuronal plexus, (Fig. 5.13C).

Each *macula* hair cells has an elongated group of kinocilia at its distal ends. The internal structure of kinocilia is 9x2 + 2 tubuli structure (Fig. 5.13C-E). The location of the basal foot structure and the orientation of its internal 9x 2+2 tubuli structure define the morphologically polarization of each kinocilium. (Fig. 5.13C-E). The basal foot is attached to the basal body bellow the cell surface and is orientated in the direction of tubuli 5 and 6. The basal feet of all kinocilia of a hair cell point in the same direction (at right angles to the axis of the kinociliary group. Numerous microvilli occur on the luminal surface of each hair cell, surrounding kinocilia.

There are supporting cells between the hair cells (Fig. 5.13A-C) that exhibit microvilli on their distal ends and have irregular (triangular or oval shaped) elongated nuclei. The interdigitations between hair and supporting cells include pronounced septate junctions. A very electrondense plate is shown through the apical region of the supporting cells, between the desmosomes. This electrodense plate is composed by bundles of tonofilaments, proteinaceous fibers, associated with desmosomes to which anchor to cytoskeleton. Tonofilaments have a supporting function. The hair cells are all secondary sensory cells and are in afferent synapses with intra and peri*macula*r first order afferent neurons. Efferent endings are present either on neurons and hair cells (Fig. 5.14A-D).

FIG. 5.13. (Pag 80). LM (A) and TEM (B-E). Cellular organization of *Sepia officinalis msp*. **A:**View of the simple prismatic epithelium of the macula. The oval shaped nucleus located in the central-basal portion of the hair cells and its elongated group of kinocilia (arrowheads) at distal ends are visible. The supporting cell nucleus is more electrodense. The very electrodense plate (arrows) at the distal part of the supporting cells is visible too. At the base of the hair and suppoting cells the nervous estructures are visible (asterisk). **B**: Ageneral view of the macula epithelium shows its structure of simple prismatic epithelium. In the distal part of the cells, kinocilia of hair cells and microvilli of supporting cells are visible. At the base of the hair and supporting cells, efferent and afferent nervous structures are shown. **C:** Detail of the macula epithelia in the statocyst of Sepia officinalis. Section through the distal ends of the hair and supporting cells. **Insert in C**: Detail of **C**. The structure of kinocilliary roots are shown. **D**: Other view of the distal end of hair and supporting cells. Note the dark zones around the desmosomes (cellular contacts) which suggest the presence of tonofilaments. **E**. Detail of the distal end of an hair cell that shows the 9x2+2 structure of the kinocillia. The arrow points the direction of polarization of the cell. **Insert in E**: A longitudinal section of kinocillia shows their internal structure. Arrowheads show the internal and external pairs of microtubules. **Hc:** hair cell, **sc:** supporting cell, **nhc:** nucleus of hair cell, **nsc:** nucleus of supporting cell, **k:** bundle of kinocilia, **mi:** microvilli, **p:** electrondense plate, **er:** rough endoplasmatic reticulum, **d (ga):** dictyosomes of Golgi apparatus, **ax:** axon of first-order afferent neuron, **e**: efferent terminal, **m:** mitochondrion, **kr:** striated ciliary rootlet (kinocilia root), **lr:** lateral root, **bf:** basal foot, **bb:** basal body, **bp**: basal plate, **cc:** cellular contact (desmosoma). **Scale bars**: **A =** 100 µm**. B** = 5 µm**. C, Insert in C =** 0,25 µm. **D, E**: 2 µm. **Insert in E =** 1 µm.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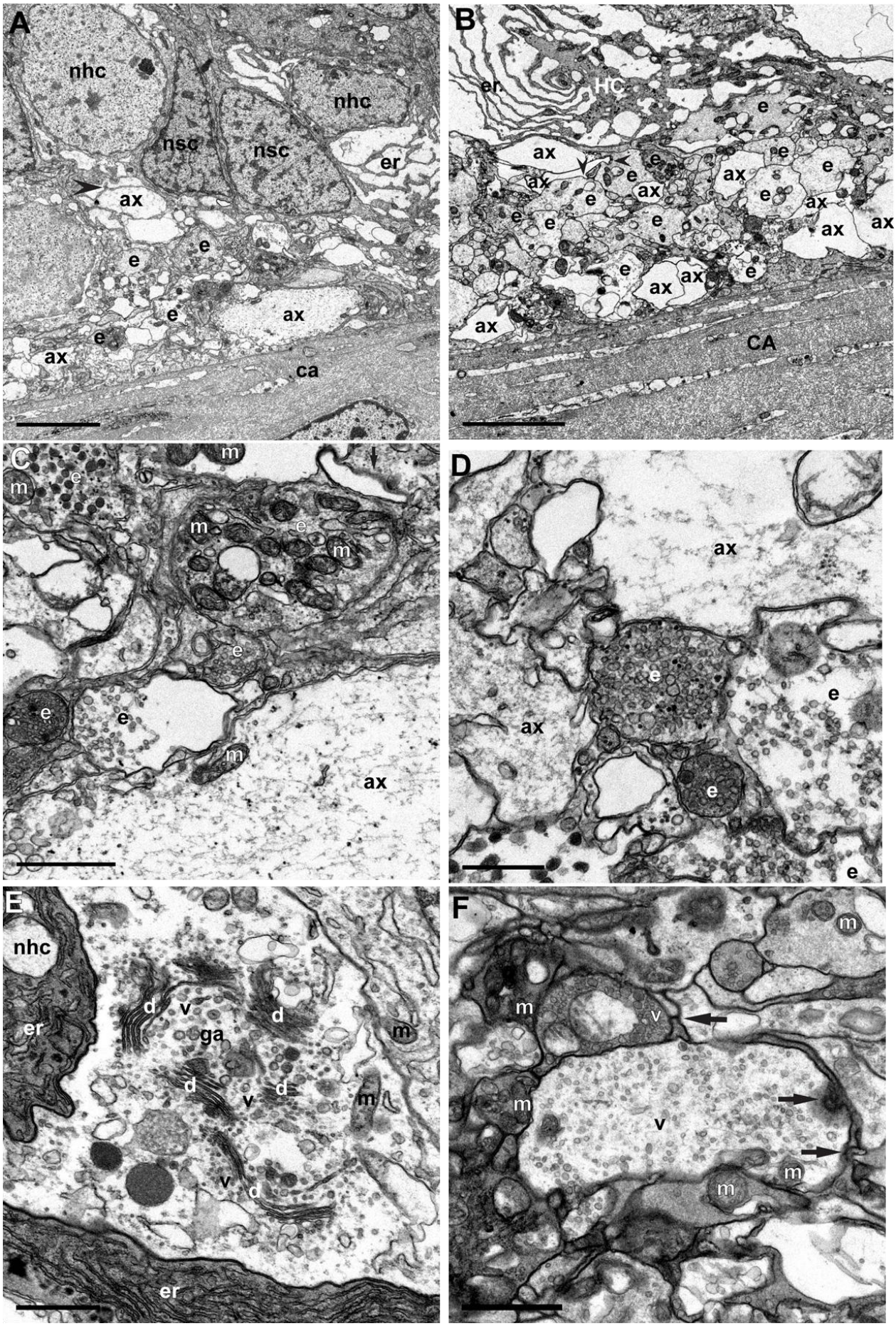
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FIG. 5.14. (Pag 81) SEM. Macula statica princeps (A-E) and crista (F) of *Sepia officinalis*. A-D: **General nervous organization of *macula statica princeps.* A**: Proximal part of *macula* epithelia in contact with cartilaginous plate. The arrowhead shows a finger-like postsynaptic profile that invaginates the base of a hair cell to form an afferent synapse **B:** Detail from A**.** Section through the base of a sensory hair cell of the *macula* epithelia of the statocyst**.** Numerous efferent terminals (e) synapse into a single hair cell (hc). The general nervous organization of *macula* epithelia at its base is shown. **Arrowheads** show two finger-like synaptic profiles between an axon of an afferent neuron and two efferent terminals. **C:** Detail of afferent and efferent innervations of the *macula* epithelium**.** Numerous efferent terminals synapse in the axon. **Arrow**: Flat synaptic contact. **D:** Detail of efferent innervations of the *macula* epithelium. Efferent terminals are filled with small synaptic vesicles. **E**: Detail of Dictyosomes of Golgi apparatus of a hair cell of the *macula statica princeps* epithelium. **F**: Detail of afferent and efferent innervations of the *crista* epithelium. Arrows indicate finger-like synaptic profiles. **Hc:** hair cell**; sc: s**upporting cell, **nhc:** nucleus of hair cell, **nsc:** nucleus of supporting cell, **k:** bundle of kinocilia**, mi:** microvilli**, p:** electrondense plate**, er:** rough endoplasmatic reticulum**, ax:** axon of first-order afferent neuron, m**:** mitochondrion**, e:** efferent terminals, filled with small synaptic vesicles, ca**:** cartilaginous plate, **d**: dictyosomes of Golgi apparatus (ga) **v**: vesicules. **Scale bars**: **A, B** = 5 µm**. C-F**: = 1 µm.

5.3.2.2 Decapods crista

The decapods *crista* epithelium (not shown) is formed by four regular rows of larger hair cells in the middle of the *crista* and other less regularly arranged smaller hair cells on either side. Each *crista* hair cell has an elongated group of kinocilia at its distal part. Between the hair cells, we can appreciate supporting cells that exhibit microvilli on their distal ends, with opposing polarization to the *crista* hair cells, and a very electrondense plate at the apical part. The *crista* epithelium rests on a cartilaginous plate and the nerve plexus runs underneath the epithelium.
The neuronal organization of the *crista* segments is very complex, even unusual for invertebrate standards. On each *crista* segment primary sensory cells are present, each of them with its own axon underneath them, and two types of secondary sensory cells, without axon, that make afferent synaptic contacts with two types of first-order afferent neurons. The synapses are of two types, flat or finger-like post-synaptic profile (Fig. 5.14F). Efferent endings are present on both neurons and hair cells. The presence of both primary and secondary sensory hair cells together on *crista* epithelium is an unusual feature on invertebrates**.**

5.3.2.3 Octopods macula

The structure of *macula*'s epithelium of octopods (Fig. 5.15) is very similar to the simple prismatic epithelium of the decapods' *macula*, formed by voluminous hair cells containing large round nucleus, located on the central-basal portion of the cell, surrounded by prominent rough endoplasmatic reticulum, abundant mitochondrion and swollen vesicules, lisosomes and Golgi complexes. Each *macula* hair cell has an elongated group of kinocilia at its distal ends. High density of microvilli occurs on the luminal surface of each hair cell, surrounding kinocilia. Between the hair cells, there are supporting cells that exhibit microvilli on their distal ends as well as a very electrondense plate at the apical part and that have irregular elongated nucleus. The hair cell epithelium lays above the membranous sac and the nerve plexus runs underneath the hair cells. At the proximal part of the hair cells near the contact point with the underlying neuronal plexus, mitochondria are found to be abundant.

Each *macula* hair cell has an elongated group of kinocilia at its distal end with internal 9x2 + 2 tubuli structure (Fig. 5.15D, E, F). The morphological polarization of each kinocilium (Fig.5.15F) is defined by the location of the basal foot structure and the orientation of its internal 9x 2+2 tubuli structure. The basal foot of all kinocilia of a hair cell is attached to the basal body bellow the cell surface and points in the same direction (at right angles compared to the axis of the kinociliary group. In cephalopods lateral roots can be seen originating from the basal body of the kinocilium, opposite to the basal foot (Fig. 5.15F).

Between the hair cells there are supporting cells (Fig 5.15C, D,G) that exhibit microvilli on their distal ends and that have an irregular shaped nucleus. In octopods, tonofilaments do not form an electrodense plate between desmosomes as thick as in decapods. The interdigitations between hair and supporting cells include pronounced septate junctions (Fig. 5.15G) .In octopods, the *macula* hair cells are arranged in concentric rings. The hair cells are all secondary sensory and are in afferent synapses with intra and peri*macula*r first order afferent neurons. Efferent endings are present either on neurons and hair cells (Fig. 5.16). The synapses show all the morphological criteria that are characteristic in chemical synapses: presynaptic aggregation of synaptic vesicles, parallel electron-dense pre and postsynaptic membranes, and cleft of dense cleft material (Fig. 5.16D).

Several vesicles can be appreciated at the centre of the cytoplasm as well as at the contact point between two axons (fig 5.16C, D). Their presence is probably due to an exocitocis process, although no definitive conclusion can be drawn here since these structures also resemble clathrin-like electrodense covers that would not be compatible with the above hypothesized exocitosis mechanism.

FIG. 5.15. (Pag 84) LM (A, B) and TEM (C-G). *Octopus vulgaris macula*. Cellular organization of the macula. **A:** View of a whole *macula* embedded in epoxy resin, previously to process it for TEM. Statolith (st) attached in the centre of *macula* (m) and some *macula* nervous fibers attached to the statocyst sac are visible. On the low part of the image the *macula* nerve (n) emerge from it. **B**: View of the simple prismatic epithelium of the *macula* where h Hair cells and supporting cells alternate *macula*. The oval shaped nucleus (arrowheads) located at the central-basal portion of the hair cells are visible. The very electrodense plate at the distal part of the supporting cells is visible too. **C**: General view of *macula* ephitelium. The oval shaped nucleus (nhc) located on the central-basal portion of the hair cells and its elongated group of kinocilia at distal ends are visible. At the base of the hair and supporting cells the neuronal and synaptic structures are visible. **D:** Section through the distal ends of the hair and supporting cells**.** The basal bodies of the kinocillia are visible**. E**: Detail of a distal end of a hair cell. Kinocilia show the 9x2 +2 microtubule structure. Basal bodies and striated ciliary rootlets (kinocilia roots) are visible. **F**: Detail of a distal end of a hair cell. Internal kinocilia microtubules are visible. The basal foot of kinocilia are attached to the basal body bellow the cell surface and point at right angles to the axis of the kinociliary group (arrow). Lateral root point in the same direction. **G:** Detail of a distal end of a supporting cell. Note the interdigitations between hair and supporting cells include pronounced septate junctions (arrowhead). **Hc:** Hair cell; **sc:** supporting cell**; nhc:** nucleus of hair cell**; nsc:** nucleus of supporting cell**, k:** bundle of kinocilia**, mi:** microvilli, **ax:** axon of first-order afferent neuron **ca:** cartilaginous plate, **cc**: cellular contact (desmosoma), **bb**: basal body, **bf**: basal foot, **kr**: striated ciliary rootlet, **lr**: lateral root. **Scale bars**: **A =** 1mm**. B** = 100 µm**. C, D, E =** 5 µm. **F, G =** 1 µm.

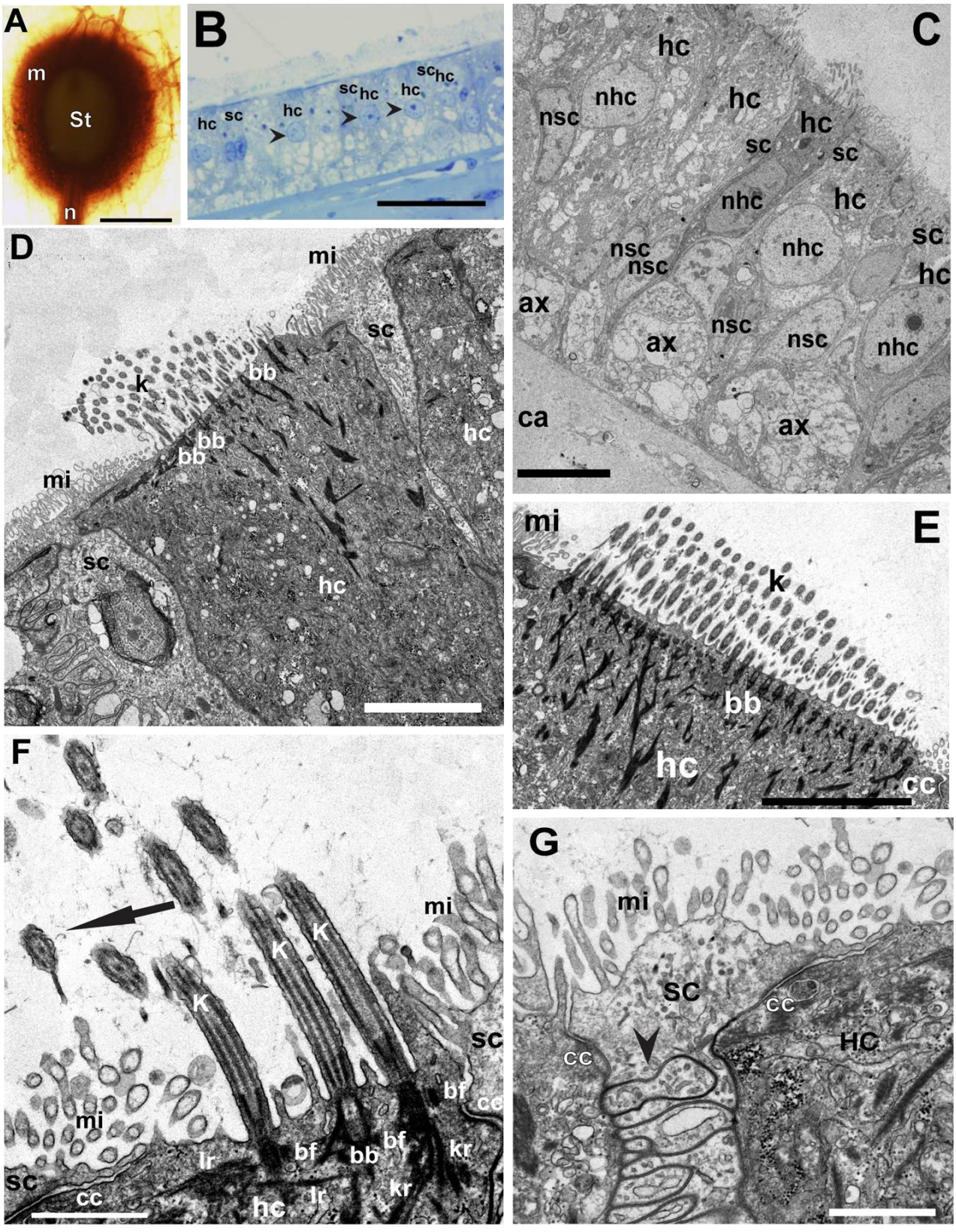
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FIG. 5.15

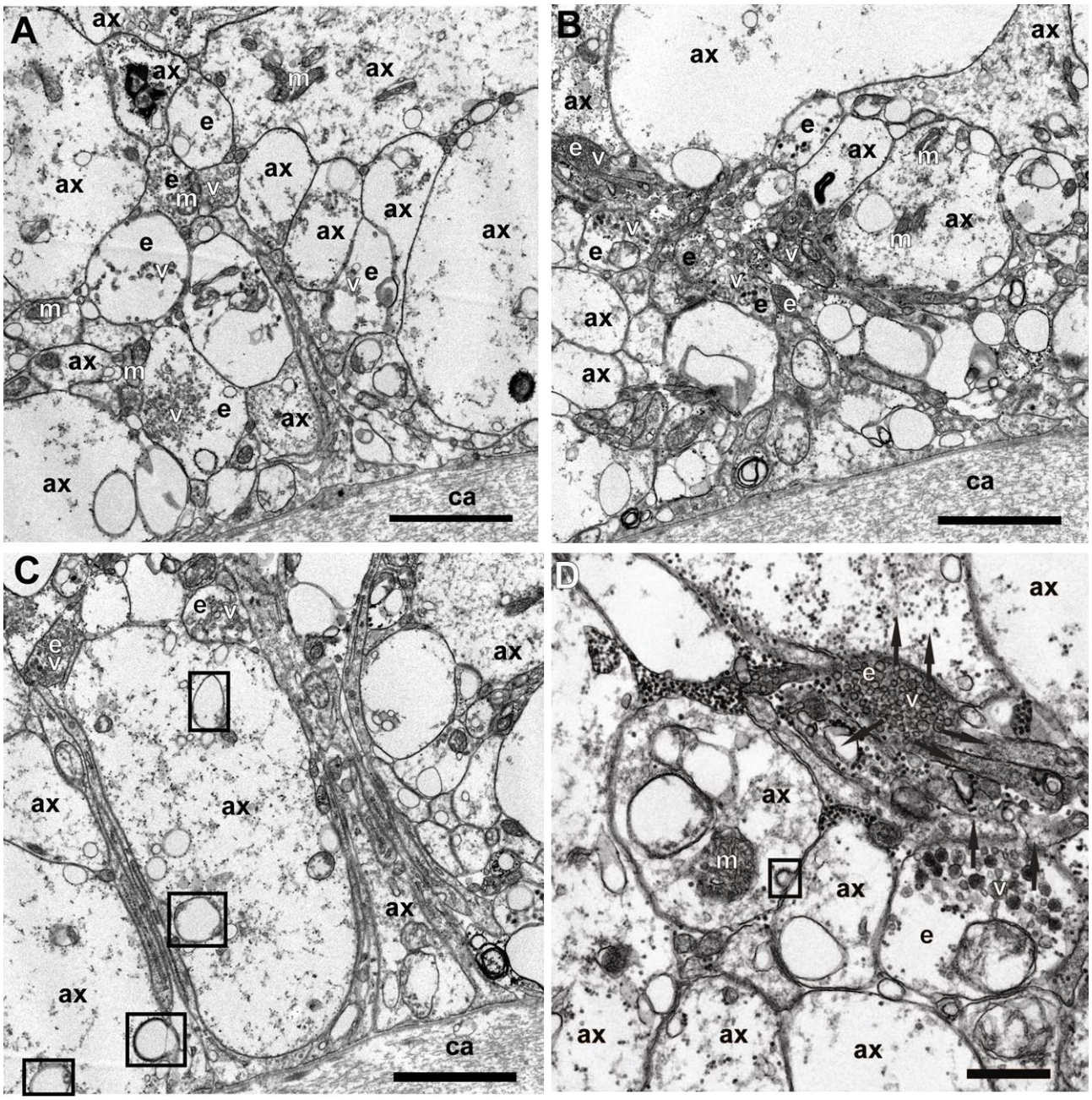

FIG. 5.16. TEM. *Octopus vulgaris* macula. Afferent and efferent innervations of the macula epithelium in the statocyst of Octopus vulgaris. **A**: At the base of hair and supporting cells numerous afferent and efferent terminals synapse between them and into the hair cells. **B**: Other view of the nervous area at the base of sensitive epithelium. In the centre of the image the vesicles synaptic accumulation in the afferent terminals is more electrodense. **C:** squares show exocitosi vesicles or clathrin-like electrodense covering the centre of cytoplasm and on the contact between two axons. **D**: Small arrows indicate the direction of information flow across the flat synapses. The synapses show all the morphological criteria that are characteristic of chemical synapses: presynaptic aggregation of synaptic vesicles, parallel electron-dense pre- and postsynaptic membranes, and cleft of dense material. Square shows exocitosi vesicles or clathrin-like electrodense covering on the contact between two axons. **Ax**: axon of first-order afferent neuron, **e**: efferent terminals, filled with small synaptic vesicles, **m**: mitochondrion, **v**: vesicles. **Scale bars**: **A, B, C** = 2 µm**. D = 0,**5 µm.

5.3.2.4 Octopods crista

As in the decapods, the octopods *crista* epithelium (Fig.17, 18) is formed by four regular rows of larger hair cells in the middle of the *crista* (with different features depending on whether it is an odd-numbered or even-numbered *crista* segment) and other less regularly arranged smaller hair cells on either side. The large hair cells at the centre of the *crista* are voluminous with a prominent rough endoplasmatic reticulum, large round nucleus and abundant mitochondria. Each *crista* hair cell has an elongated group of kinocilia at its distal part. Between the hair cells there are supporting cells that exhibit microvilli at their distal ends, with opposing polarization compared to the *crista* hair cells and a very electrondense plate at the apical part. The *crista* epithelium rests on a cartilaginous plate and the nerve plexus runs underneath the epithelium.

The cellular organization of *Octopus crista* is the most differentiated angular system described, even more than in mammals. There are four types of hair cells (dhc, lhc, flhc, vdc) which have kinocilia that are inclinated towards the apical cell surface (Fig.5.17, 5.18), except the flhc of the even-numbered *crista* sections. This feature is one of the factors responsible for the morphological and cell's physiological polarization. There are close membrane contacts at the tips of kinocilia and filamentous interconnections between them (Fig 5.18C, D).

As in decapods, the presence of both primary and secondary sensory hair cells in the *Octopus' crista* is an unusual feature amongst invertebrates. The presence of different types of neurons (first-order afferent neurons to secondary hair cells) denotes a very complex synaptic organization too. The synapses are of two types, flat or finger-like post-synaptic profile. Efferent endings are present either on neurons and hair cells. The efferent profiles are vesicle-filled and their contacts with primary, secondary hair cells or neurons are mostly flat, containing a large number of agranular vesicles (Fig. 5.19).

The large hair cells in the middle of the *crista* ridge are voluminous with abundant mitochondrion, large round nuclei and prominent rough endoplasmatic reticulum (Fig. 5.17, 5.19). The supporting cells, that exhibit microvilli on its distal ends, have an irregular (triangular or oval shaped) elongated nucleus. The interdigitations between hair and supporting cells include pronounced septate junctions. As in decapods, each *macula* hair cell has an elongated group of kinocilia (9x2 + 2 tubuli structure) at its distal ends. For all the bundles, except the shorter ones in the crista, the axis of sensitivity appears to be clear because the bundles are inclined towards the epithelium, and the excitatory direction is away from this inclined direction. The basal feet of all kinocilia of a hair cell point in the same direction (at right angles to the axis of the kinociliary group) (Fig.5.17, 5.18). Surrounding kinocilia numerous microvilli occur on the luminal surface of each.

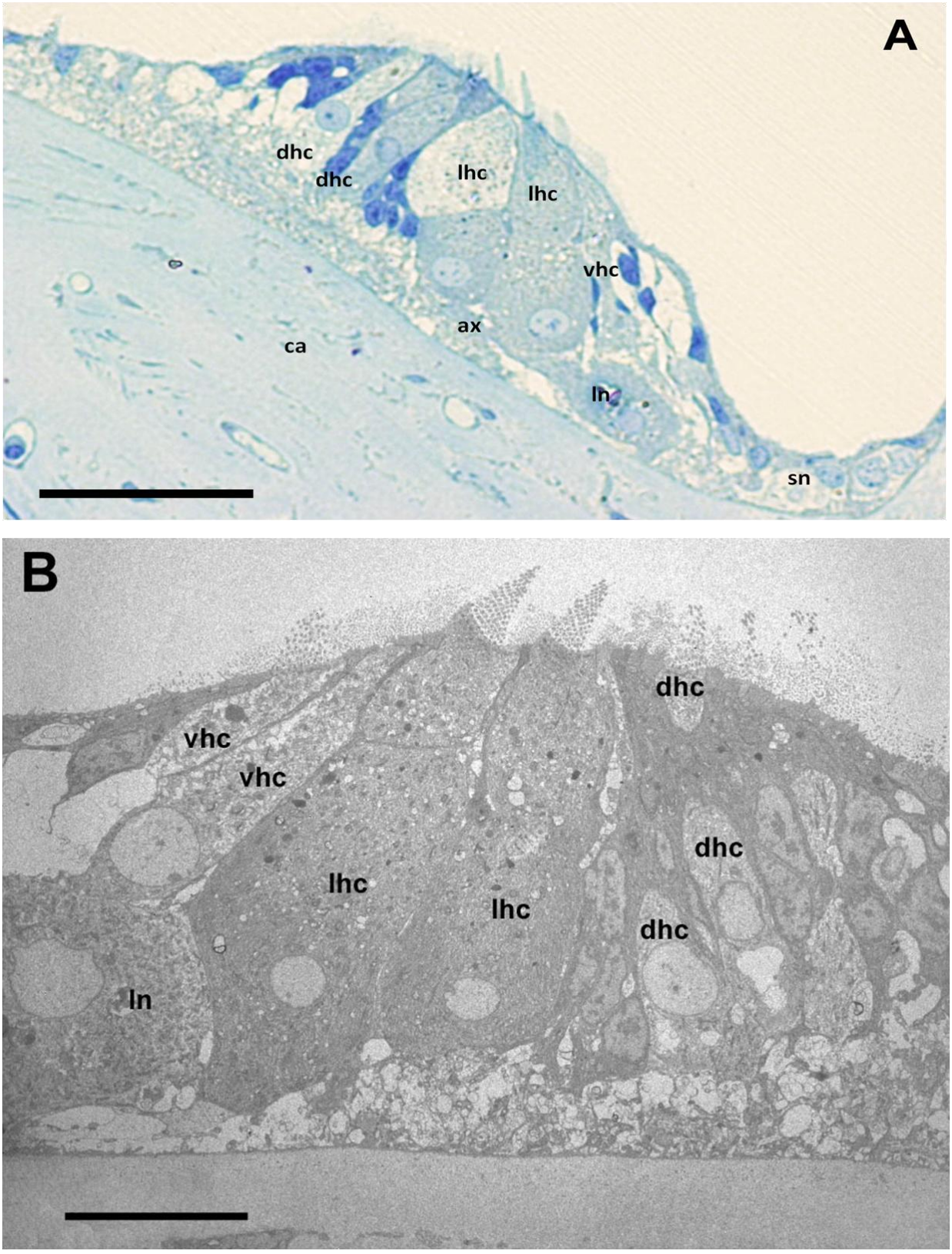

FIG. 5.17. LM (A) and TEM (B). Cellular organization of *Octopus vulgaris* crista. Cross section of an odd-numbered *crista* segment (**dhc**: small dorsal primary hair cell; **lhc**: regular row of large secondary hair cells; **vhc**: small ventral secondary hair cell; **ln**: large first-order afferent neuron; **sn**: small first order afferent neurons). Dhc and lhc are receptor cells that are in afferent synapses with large first-order afferent neurons (ln). Vhc are in afferent synapses with small first-order afferent neurons (**sn**). All hair cells and neurons receive multiple efferent endings. **Scale bars**: **A =** 100 µm**. B** = 10 µm**.**

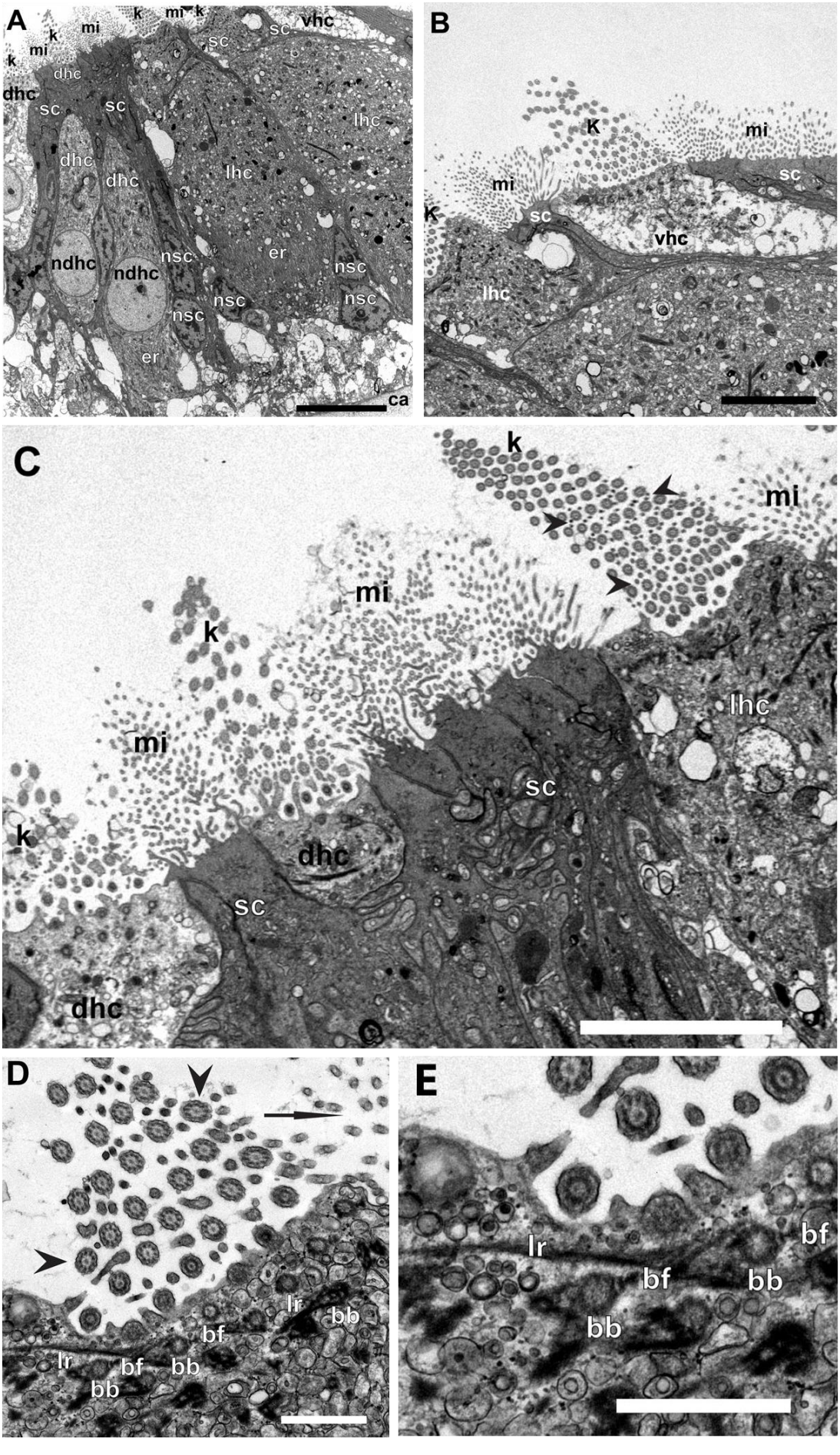
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FIG. 5.18. (Pag 88) TEM. *Octopus vulgaris* crista. A: A general view of *crista* ephitelium shows the sensitive epithelium and the nervous plexus at the base. **B:** Detail of the distal end of a vhc and a lhc of an odd-numbered *crista* segment. **C:** Section through the distal ends of the hair and supporting cells of an odd-numbered *crista* segment. The alternance between kinocillia of hair cells and microvilli of supporting cells are clearly visible. Note how the interdigitations between hair and supporting cells include pronounced septate junctions (arrow). The arrowheads indicate the links between kinocilia while the regular arrows indicate the direction of polarization of hair cells. **D**: The basal feet of kinocilia are attached to the basal body bellow the cell surface and point at right angles to the axis of the kinociliary group (arrow). Lateral roots point in the same direction. Kinocilia show the 9x2 +2 microtubule structure. Basal bodies and striated ciliary rootlets are visible. **E**: Detail from D shows basal structure of kinocillia. **Dhc**: small dorsal primary hair cell; **lhc**: large secondary hair cell; **vhc**: small ventral secundary hair cell; **sc**: supporting cell; **ndhc:** nucleus of small dorsal hair cell**; nsc:** nucleus of supporting cell**, k:** bundle of kinocilia**, mi:** microvilli, **ax:** axon of first-order afferent neuron **ca:** cartilaginous plate, **cc**: cellular contact (desmosoma), **bb**: basal body, **bf**: basal foot, **kr**: striated ciliary rootlet (kinocilia roots), **lr**: lateral root. **Scale bars**: **A** = 10 µm, **B, C** = 5 µm,**. D, E = 1** µm.

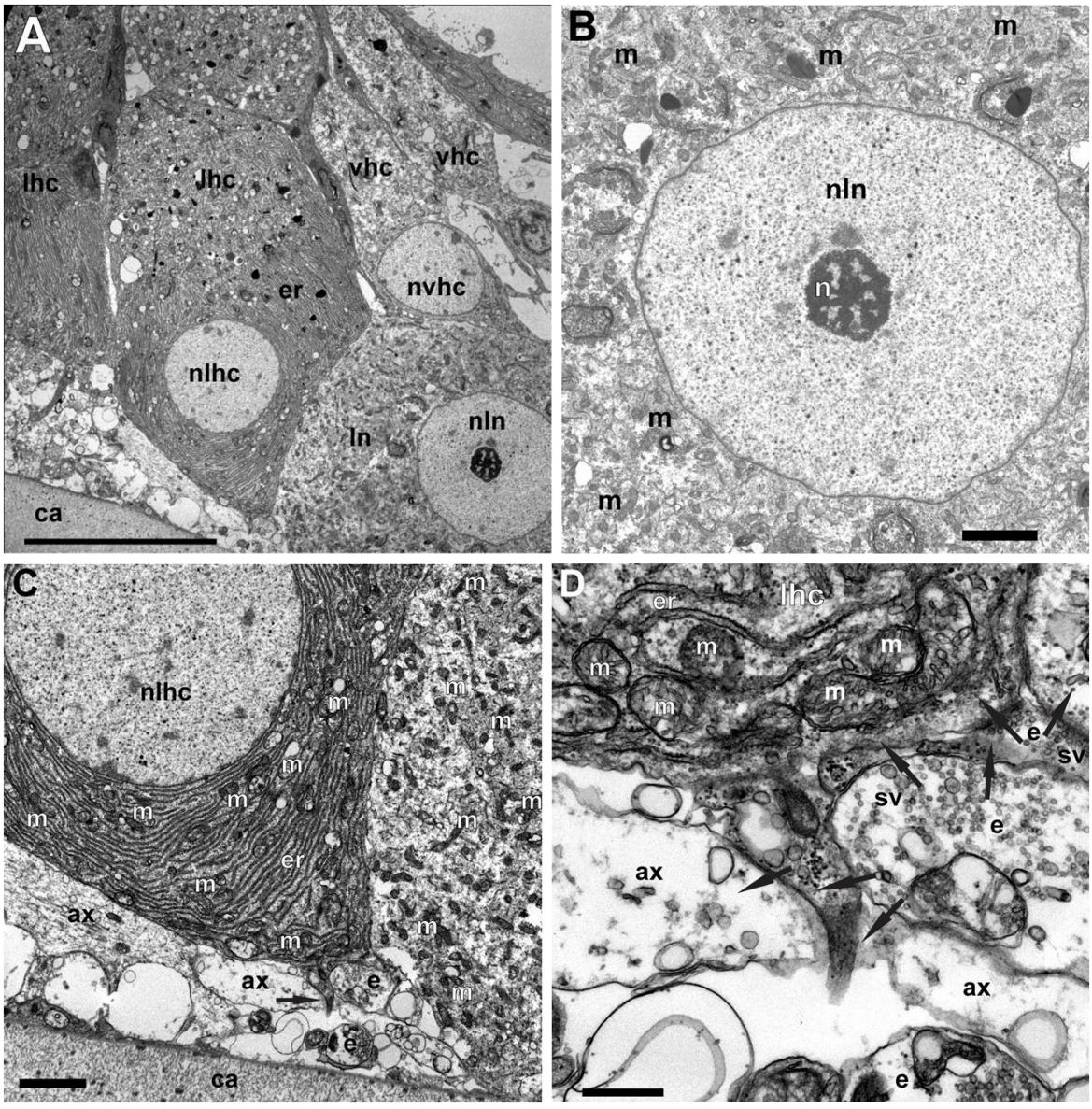

FIG. 5.19. TEM. *Octopus vulgaris* crista nervous organization. A: Section through the base of hair cells of the *crista* epithelia of the statocyst**.** The general nervous organization of *macula* epithelia at its base is shown. Numerous nervous terminals at the base of two secondary hair cells are visible. A secondary hair cell and a ventral hair cell are in contact with a large neuron. **B:** Detail from A**.** A nucleolus is visible within the nucleus of the large neuron. **C**: Detail from A. Afferent and efferent terminals synapse into a single large hair cell are visible. Numerous mitochondria at the base of the hair cell and in the large neuron are visible. The arrow points to a finger-like synaptic contact between a hair cell and an afferent and an efferent terminal. **D:** Detail from C. Small arrows show the direction of information flow across the flat synapses. The synapses exhibit all the morphological criteria that are characteristic of chemical synapses: presynaptic aggregation of synaptic vesicles, parallel electron-dense pre- and postsynaptic membranes, and cleft of

dense material. **Lhc**: large secondary hair cell; **vhc**: small ventral secondary hair cell; **nlhc:** nucleus of large secondary hair cell**; nvhc:** nucleus of ventral hair cell; **n:** nucleolus; **ax:** axon of first-order afferent neuron, **ln**: large neuron; **nln**: nucleus of large neuron; **m**: mitochondrion, **er:** rough endoplasmatic reticulum, **ca:** cartilaginous plate; **sv**: synaptic vesicles. **Scale bars**: **A =** 10 µm. **B, C** = 2 µm**. D** = 0,5 µm.

5.4 Discussion

New images of described and non-previously-described images of cephalopod species

This work contributes with new light of cephalopod inner statocyst structures to the current knowledge of these sensory organs. In addition, although *macula* and *crista* of *Sepia officinalis*, *Loligo vulgaris* and *Octopus vulgaris* have been described in previous excellent works, this paper presents new structural and ultrastructural aspects using SEM and TEM. For instance, we show non-previously-described images of different ciliated structures located in the lining epithelium of the cavity which consists of flat hexagonal cells with oval nucleus. In some parts of the cavity, cilia emerge between the epithelial cells. The cells of the lining membrane carry cilia on the outer side, which project into the cavity and play part in the circulation of the endolymph. In decapods, they are especially abundant ventrally from the three horizontal *crista* segments and medially from the vertical *crista*. Ciliated cells also occur on the base of anti*crista* lobes.

Images of *Illex coindetii*

This study shows the first published images of *crista-cupula* system and inner statocyst cavity of *Illex coindetii*. No previous studies have been carried out on this species. As in other decapods, in some parts of its inner cavity the flat hexagonal cells carry cilia on the outer side, which project into the cavity, microvilli are present with less density than in *Loligo vulgaris* and surround the epithelium cells. Inner statocyst area near of the *macula* shows very high density of microvilli covering all the surface of the flat hexagonal cells of the lining epithelium. The *cupula* of *Illex coindetii* attached to the *crista* presents a filamentous structure similar to the other decapods. Model of the *Illex coindetii* statocyst liner epithelium is shown next to the hair cell rows that surround the main rows of *crista*. Microvilli grow in some of the liner epithelium cells.

Unidirectional polarization and orientation of lateral roots

On cephalopods the morphological polarization of each kinocilium is defined by the location of its basal foot structure and the orientation of its internal 9x 2+2 tubuli structure. The basal foot of all kinocilia of a hair cell is attached to the basal body bellow the cell surface and points in the same direction (at right angles compared to the axis of the kinociliary group (Budelmann, 1979). The presence of close membrane contacts at the tips of kinocilia and filamentous interconnections between them (Fig. 5.18C, D) contribute to the unidirectional polarization of each kinocilliary group. Unidirectional polarization from each hair cell of all receptor systems is characteristic of cephalopods. This feature was also described in vertebrates and in a few species of gastropods (*Pomacea)* (Stahlschmidt and Wolff, 1972) and bivalves (*Pecten)*(Barber and Dilly, 1969). Hovewer, all other molluscs have morphologically non-polarized hair cells. In addition, in cephalopods, lateral roots can be seen originating from the basal body of the kinocilium, opposite to the basal foot. (Fig. 5.18D, E). This orientation of lateral roots is very unusual and has only been described on cephalopod species. We confirm this feature and the idea that it could play a fundamental role on the morphological polarization of the kinociliary groups and as a consequence on the transduction mechanism in hair cells. In cephalopods, a passive deflection of the kinocilia in the direction of their basal feet causes a depolarization (maximal excitation) of the hair cells and a hyperpolarization (maximal inhibition) when

deflection occurs in the opposite direction. Mechanical deformation is thought to be the first step of the transduction mechanism.

Basal plate

A very electrondense plate, probably of a proteinical nature, is shown through the apical region of the supporting cells, between the desmosomes. We hypothesize that this electrodense plate is composed by bundles of tonofilaments, proteinaceous fibers, associated with desmosomes (*macula* adherens) to which they anchor to the cytoskeleton. Tonofilaments are filamentous structures that form part of the cytoskeleton of cells. These filaments, which are abundantly present in keratinocytes and that are found at desmosomal junctions, have a supporting function. Several epithelial cells, which are capable of continuous division in culture, continuously produce large, balanced amounts of prekeratin-like material, which is assembled in tonofilament-like structures (Werner et al., 1978). The strong assembly of tonofilaments that anchor the supporting cells to the cytoskeleton enables that the sensory epithelium has a mesh-like structure, the basal plate, capable of strong maintaining hair cells in the precise position to capture the sensory information.

Exocytosi vesicles on nervous plexus

This study shows several vesicles at the centre of the cytoplasm as well as at the contact point between two axons on the nervous plexus of *Octopus vulgaris macula*. No previous studies have describedthis type of synapse between two afferent processes**.** Their presence is probably due to exocytosis, although no definitive conclusion can be drawn here since these structures also resemble clathrins that would not be compatible with the above hypothesized exocytotic mechanism. The presence of these vesicles both in the cellular cytoplasm and opened on the extracellular medium suggests a possible function related to intercellular communication, or a possible influence on extracellular medium at least.

A basis for studying the effects of noise exposure

The characterization of the cephalopod statocyst structures in control animals represents a fundamental approach to determine the presence of pathologies in individuals under external pressure from human activities. The detailed description of the four cephalopod species normal statocyst structures presented here appeared to be identical as previously published (e.g. Budelmann 1973, 1977, 1979, 1987, 1990, 1995; Stephens 1982; Young 1960, 1965). This, therefore, confirms that these animals can be used as a control set to contrast and define lesions compatible with acoustic trauma after noise exposure experiments conducted in parallel on another set of individuals caught, handled and maintained in the exact same conditions (André et al, 2011). The results presented here allow the authors to discard the possibility that the trauma observed in exposed individuals could constitute artefacts derived from the capture or the handling of the cephalopods in a captive environment.

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6. Imaging techniques reveal the effects of low frequency sound exposure on common cuttlefish *(Sepia officinalis)*

6. Imaging techniques reveal the effects of low frequency sound exposure on common cuttlefish *(Sepia officinalis)*

6.1 Introduction

The introduction of artificial sound sources in the marine environment has shown to have negative effects on marine organisms. While marine mammals (Au and Nachtigall, 1993; Andre et al., 1997; Scheifele, 1997; Au and Green, 2000; Schlundt et al., 2000; Finneran et al., 2002; André et al., 2003; Tougaard et al., 2003; Nachtigall et al., 2004; Schlundt et al., 2006; Finneran et al., 2007; André, 2009; Lucke et al., 2009; Edren and Andersen, 2010) and fishes (Wilkins, 1972; Banner and Hyatt, 1973; Dancer et al., 1973; Rucker, 1973; Popper and Clarke, 1976; Konagaya, 1980a, b; Blaxter et al., 1981; Schwarz and Greer, 1984; Ha, 1985; Schwarz, 1985; Hastings et al., 1996; Scholik and Yan, 2002; McCauley et al. 2003; Smith et al., 2004; Hastings and Popper, 2005; Jørgensen et al., 2005; Popper et al., 2007; Popper and Hastings, 2009, 2009b; Kane et al., 2010) have attracted most of the attention of the research conducted in that area, invertebrates were also suspected of being negatively affected by noise exposure. Little is actually known about sound perception in invertebrates, but evidence points to the notion that cephalopods may be sensitive to low frequency sounds (Hanlon and Budelmann, 1987).

Between September and October 2001 and in October 2003 the natural rhythm of annual records of giant squids (*Architeuthis dux*) stranded in the area of NW coast of Asturias (Spain) experienced a significant increase (Guerra et al., 2004a, b). In both cases, the stranding and collection of the bodies were coincident with the proximity of vessels using compressed air guns for geophysical prospecting, producing sound waves of low frequency (below 100 Hz) and high intensity (200 dB re 1 μPa at 1m). Some of the individuals had lesions in different tissues and organs, but all presented pathologies in the gills and the receptor of equilibrium or statocysts. Because none of these lesions could be related to known causes of death, the presence of geophysical prospecting vessels suggested that the death of these animals could be related to (direct or indirect) effects produced by sound waves. However, no further study addressed this problem and doubt remained as to whether high intensity low frequency pulses could negatively affect cephalopods. A comprehensive study was therefore needed to assess the direct effects of acoustic impact on these species. The first step was to choose which organ, found in all cephalopods could be an indicator of noise-induced damage. Amongst other less sensitive-to-noise tissues, the statocysts were presumably the best candidates to injury if exposed to loud sources. All cephalopods have a couple of statocysts generally located within the cephalic cartilage. The statocysts are sophisticated balloon-shape bodies filled with endolymph that contain the sensory hair cells which lie on the inside wall of the inner sac and are grouped into two main areas of sensory epithelium. In chapter 5, the inner statocyst structures of the individuals that were used as control are detailed and documented with SEM and TEM images of four Mediterranean Sea cephalopod species (*Sepia officinalis, Loligo vulgaris, Illex coindetii* and *Octopus vulgaris).* The aim of this ongoing project was therefore to conduct controlled noise exposure experiments (under laboratory conditions) following the sound exposure scenario that took place in Asturias and make a thorough analysis of possible lesions associated to low frequency sources in individuals from common cuttlefish (*Sepia officinalis*) of the Mediterranean Sea by imaging techniques: light (LM), scanning (SEM) and transmission electron (TEM) microscopy.

Acoustic impact

The possible effects of an acoustic impact on cephalopods are also unknown. In fact, there is limited knowledge on the effect of noise in invertebrates in general. Indeed, although startle responses were observed in caged cephalopods exposed to airguns (McCauley et al., 2000), no

further studies addressed noise-induced morphological changes in these species and doubts remained on the possible negative impact of high intensity low frequency sounds on cephalopods. Some works reported these effects on terrestrial (Frings and Little, 1957; Frings and Frings, 1959; Fletcher et al. 1971); (Kirkpatrick and Harein, 1965; Cutkomp, 1969; Lindgren, 1969) and marine invertebrates (Frings and Frings, 1967; Lagardère, 1982; Lagardère and Regnault, 1983; McCauley et al., 2000; Lovell et al., 2005, 2006, 2007). Several studies were conducted to measure the behavioural effect on fishes (Wilkins, 1972; Banner and Hyatt, 1973; Dancer et al., 1973; Rucker, 1973; Popper and Clarke, 1976; Konagaya, 1980a, b; Blaxter et al. 1981; Ha, 1985; Schwarz, 1985) and inner ear sensory epithelia, lateral line system and other tissues damaged by acoustic impact (Hastings et al., 1996; Scholik and Yan, 2002; McCauley et al. 2003; Smith et al., 2004; Jørgensen et al., 2005). Other works reported that sound exposure caused lesions that could result in hearing loss on terrestrial (Lindquist et al., 1954; Ward and Duvall, 1971; Hamernik et al., 1984) and marine mammals (Scheifele, 1997; Schlundt et al., 2000; Finneran et al., 2002; André et al., 2003; Nachtigall et al., 2004; Schlundt et al., 2006; Finneran et al., 2007; Lucke et al., 2009)

Previous studies can be found in the literature on amphibians (Hodichok and Steyger, 2007), mammals (Robertson, 1981; Lenoir et al., 1987; Schuknecht, 1994; Leonova and Raphael, 1997; Raphael, 2002; Pourbakht and Yamasoba, 2003b) and avians (Nakagawa et al., 1997) to determine inner ear sensory epithelia damage after acoustic trauma or ototoxic treatment (Nakagawa et al., 1997b). The lesions were similar in all cases (Leonova and Raphael, 1997; Hodichok and Steyger, 2007). Several authors reported that sound overstimulation induced sensory hair cell loss (Slepecky et al., 1982; Nakagawa et al., 1997; Hawkins and Schacht, 2005) or alterations on stereocilia of hair cells including losing, buckling, bending, breaking or fusion in giant stereocilia, wrinkled membranes of flaccid stereocilia, floppy stereocilia, reduction of stereocilia size (Bredberg et al., 1972; Slepecky et al., 1982; Hamernik et al., 1984; Pye and Ulehlova, 1989; Raphael, 2002; Pourbakht and Yamasoba, 2003a). The supporting cells responded to auditory overstimulation by an increase in the number and size of microvilli (Bredberg et al., 1972). The hair cell degeneration is usually produced by two types of typical processes; *necrosis* (swelling of the cell body and rupture of the plasma membrane) and *apoptosis* (chromatin compaction and fragmentation of the cell body) (Li et al. 1995). Hair cells which do not progress into typical cell death process can be deleted by *extrusion* from the epithelium after sound exposure. In a mammal's organ of Corti cell death results in degeneration by necrosis or apoptosis (Theopold and Scheler, 1981; Slepecky et al., 1982; Fredelius, 1988; Fredelius and Rask-Andersen, 1990) and is followed by a removal of damaged cells debris through phagocytes (Hirose et al. 2005; Ladrech et al. 2007). Apoptosis is thought to play an important role on maintaining tissue homeostasis (Nakagawa et al., 1997b); (Oyadomari and Mori, 2004). Necrosis induces inflammatory responses and secondary damage to tissue structure, which can result in irreversible tissue destruction. In the basilar papilla of avian's inner ear the cells can be deleted by extrusion (Cotanche, 1987) just after sound exposure or deteriorated within the epithelium and contribute to the following repair process (Nakagawa et al., 1997; Hu et al., 2000b). Disintegration of hair cells increases with time after the exposure, and in mammals this can result in the entire disappearance of the organ of Corti during the course of several weeks (Spoendlin, 1971). In avians experiencing acoustic trauma, hair cells which are affected by apoptosis within the basilar papilla continue degenerating after the beginning of the cell regeneration process. After acoustic trauma, lost hair cells are replaced by expansion of adjacent supporting cells which form a scar (Corwin and Cotanche, 1988).

Several factors can contribute to blistering and cell extrusion: changes in the osmotic pressure of the extracellular fluids resulting in an increase in cell volume, changes in cell mechanical, elastic or tensile properties. In noise trauma, the mixing of cochlear fluids cause changes in the osmotic pressure and cell swelling (Hamernik et al., 1984). Direct mechanical destruction as well as metabolic exhaustion are competing factors in acoustic traumatic damage to the cochlea. Direct mechanical damage (organ of Corti is disintegrated with ruptures on pillar heads or reticular membrane, or totally missing, the sensory cells are dislocated or expulsed) is usually

irreversible and appears immediately after short exposures at high intensities, whereas metabolically induced damage (swelling of sensory cells, vacuolization of cytoplasm, mitochondrial degeneration, swelling of dendrites) is partly reversible, occurs after long exposures with moderate intensities and develops over a longer period after sound exposure (Spoendlin and Brun, 1973). Early changes in hair cells consist basically of hair cells stereocillia abnormalities and protusion and loss of microvilli on supporting cells. Subsequently there is a progressive hair cell loss by protusion and posterior expulsion whereas supporting cells repair the lamina reticular (Lim and Melnick, 1971b; Thorne et al., 1984). The susceptibility to loss of the affected cells depends on their position within the lesion area (Thorne et al. 1984). In the cochlea of mammals, the first row of outer hair cells and the inner hair cells are generally more affected than row two and row three of outer hair cells.

Two mechanisms were proposed to explain the noise-induced hearing loss in mammals: mechanical injuries to the receptor cells induced by excessive movement of the cochlear partition, and damage due to metabolic exhaustion resulting in distortion of the homeostasis of the organ of Corti. It is probable that both mechanisms contribute to the process. Noise trauma causes alterations to the permeability of the hair cell stereocilia membrane, as well as changes in the surface and arrangement of actin (Slepecky et al., 1982).

In vertebrates, intense sound can also induce excitotoxicity, which is the excessive release of the neurotransmitter glutamate from the inner hair cells to the underlying post-synaptic element, the afferent dendrites of the type I spiral ganglion neurons (Cappaert et al., 2000). The excitotoxic effects of glutamate in response to noise could be the result of increased release or inadequate removal of glutamate, primarily due to the breakdown of recycling mechanisms (Rebillard et al. 2003). This may eventually result in toxic cellular events leading to neuronal degeneration and cochlear damage. Interestingly, glutamate has been also described as a neurotransmitter in cephalopod species (Tu and Budelmann, 1994; Di Cosmo et al., 2006).

Owing to a lack of previous reports concerning acoustic trauma in cephalopods, a comprehensive study was therefore needed to assess the direct effects of acoustic impact on these species. In this study, we set out Controled Experiments to determine the lesions which would occur on the sensory epithelia of the statocysts' inner structures of the Mediterranean common cuttlefish *Sepia officinalis* when exposed to sound overstimulation by low frequency sounds. Since no data was available on the effects of acoustic overstimulation in these species, the main objective of this study was to determine if the exposure to sounds would trigger lesions in the sensory cells of the statocysts.

6.2. Material and Methods

6.2.1 Cuttlefish individuals

As a preliminary step before starting with the noise exposure experiments' protocol, we caught from the wild and kept in the same tank conditions (see below), a first set of 30 individuals of *Sepia officinalis* for several weeks to observe and analyse their adaptation to captivity. These animals were swimming, eating, mating, laying eggs and behaving normally over the entire observation period. The analysis of their inner sensory statocysts epitheliums (see protocol below) was used as a first control and did not reveal any lesion. Following this preliminary observation that showed the adaptability of these animals to our facilities, we started the experiments.

One hundred and sixty four adult and young individuals from *Sepia officinalis* (mantle length 11-18 cm) were obtained from the Catalan Coast (NW Mediterranean Sea) over a period of 2 years, between February of 2008 and August of 2010, and kept in a closed system of recirculating natural seawater (at 18-20ºC, salinity 35‰ and natural oxygen pressure) consisting of 2 mechanically filtered fiberglass reinforced plastic tanks (A and B) of 2000L capacity, that were connected to each other (see Fig. 4.8) (LAB - UPC, Vilanova i la Geltrú). This included a physicochemical self-filtration system with activated carbon and sand, driven by a circulation pump. Individuals were supplied with live crab (*Carcinus maenas*) food *ad libitum* and were maintained in the tank system (tank A) until the exposure**.** Part of these animals were used as controls and were kept in the same conditions as the experimental animals until we exposed the latter to noise, in an independent tank (C), sacrificing them respecting the same sequential process. After the exposure, the individuals that were not immediately sacrificed were placed in tank B (see sequence of sacrifices below).

The independent experimental tank (C) was located in a separate location, acoustically isolated from tanks A and B.

6.2.2 Sound Exposure Protocol

Sequential (at different times and seasons) Controlled Exposure Experiments (CEE) were conducted over a period of two years on adult and subadult individuals (n=76) of *Sepia officinalis*. An additional set of live adult and subadult individuals (n=88) was used as a control and sequentially processed (same procedure as with noise-exposed cephalopods, see below) right after being caught, before and after the CEE. All the animals were caught by local fishermen following the same protocol (use of basquet traps and ceramic pots) and transferred to our laboratory a few minutes after capture (our facilities are located in the Vilanova i la Geltrú fishing harbour).

After keeping the animals for some time in tank A (ranging from a few hours to a few days) the protocol included immediate exposure of the individuals, which were put in tank C, to 50-400 Hz sinusoidal wave sweeps with 100% duty cycle and a 1-second sweep period for two hours. The sweep was produced and amplified through an in-air loudspeaker (see Fig. 6.1) while the level received was measured by a calibrated B&K 8106 hydrophone (RL = 157±5 dB re 1 μPa with peak levels up to SPL = 175 dB re 1 μPa) (André et al. 2011).

Following exposure, the non-anesthetized individuals (exposed cuttlefish and controls) were decapitated at different intervals, ranging from immediately afterwards to 12, 24, 48, 72, and 96 hours after exposure, respectively. Except for the animals sacrificed immediately after exposure, the rest of the individuals were put in tank B. An underwater video camera recorded the sound exposure experiments in order to register behaviour reaction. (See Fig. 4.5 Scheme of the general protocol of the exposure to sound and posterior analyses).

It must be emphasized here that the experiment was not set up to find specific threshold levels, but designed to investigate if cephalopods are subject to acoustic trauma when exposed to low frequency sounds, commonly encountered associated to human activities in all oceans. The transducer did not have a constant (or linear) response over the sweep which means that the levels of individual frequencies covered a wide range. The acoustic characteristics of the tank also added to differences in levels. The level measurements presented in this study are meant to provide a global characterisation of the received levels from frequencies within the sweep (157 dB re 1 μPa was the median received SPL with 50% of the peaks falling within +- 5 dB. The maximum received SPL was 175 dB re 1 μPa), and cannot therefore be taken as references to

assess thresholds levels. For the same reasons, no quantitative data are presented.

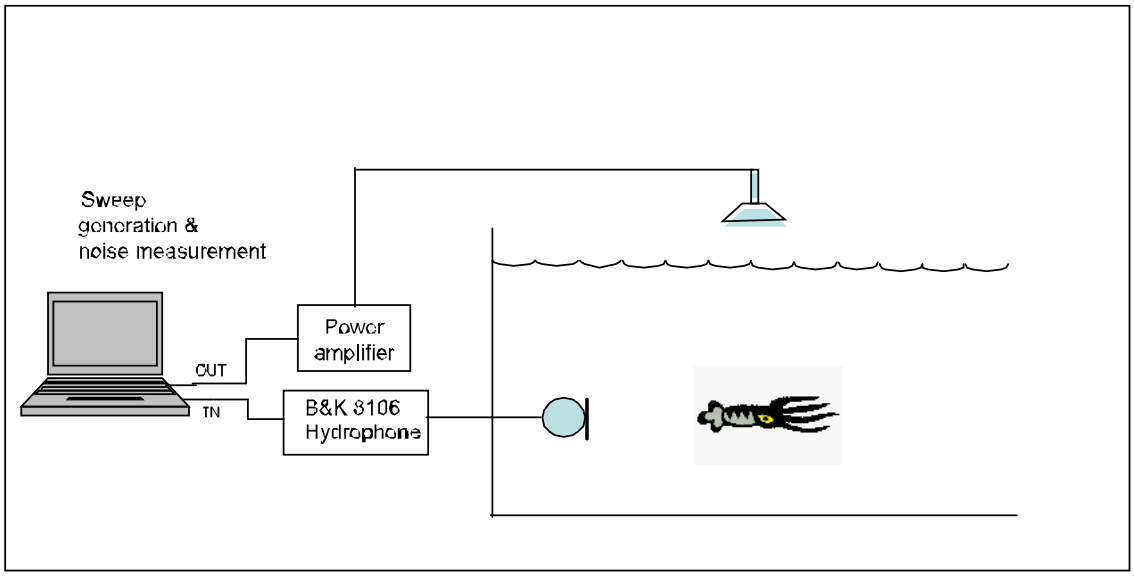

FIG. 6.1. Scheme of the setup of the CCE.

6.2.3 Imaging Techniques

The extraction of the statocysts was performed immediately following decapitation and the tissue was fixed for scanning electron microscopy (SEM), for Light microscopy (LM), and for transmission electron microscopy (TEM). Individuals were then processed according to classical SEM, LM, and TEM procedures. In addition, the endolymph was extracted from a further set of individuals and immediately frozen at –70°C for proteomic analysis. No quantification of the lesions was attempted for the same reasons as mentioned in Section 2.2. Because the transducer did not have a constant response over the sweep, threshold levels triggering the onset of the lesions could not be determined and quantifying the lesions (e.g. fraction of hair cells that were damaged, number of kinocilia that were lost, number of cells exhibiting swollen endoplasmic reticula) could not be related to reference levels. The results section will thus concentrate on a qualitative description through the analysis of the tissues with the following techniques

6.2.3.1 Light microscopy (LM)

In addition to the statocysts extraction, a routine necropsy was conducted, to collect samples of different tissues from controls and exposed individuals, which were further fixed in formalin 10%, sectioned, stained with methylen blue, covered with Durcupan and observed on Olympus CX41 light croscope. This analysis was conducted to determine the presence of lesions in mantle surface epithelia, inner muscular fibres of collagen, various organs of the digestive tract, the circulatory, nervous, sensory, respiratory, reproductive and excretory systems, sepion and the ink gland complex.

6.2.3.2 Electron microscopy of the statocysts

After extraction, statocysts were processed for SEM and TEM.

6.2.3.2.1 Removal of statocysts

In all experiments, isolated head preparations, obtained by decapitation without prior anaesthesia, were used. The statocysts with their surrounding cartilage were extracted and fixed for posterior observation and analysis. For fixation, the statocyst cavity was opened and special care was taken to prevent mechanical damage to the inner tissues. The analysis was performed on tissues obtained from left and right statocysts, but no difference was observed between sides. Specific fixation was applied depending on the imaging technique (see below).

6.2.3.2.2 Scanning electron microscopy

Thirty-six statocysts from 18 cuttlefish were used for this study. Fixation was performed in glutaraldehyde 2,5 % for 24-48h at 4ºC. Statocysts were dehydrated in graded alcohol solutions and critical-point dried with liquid carbon dioxide in a Leica EmCPD030 unit (Leica Mycrosystems, Austria). The dried statocysts were cut open and flattened out to expose the statocyst structures and then mounted on specimen stubs with double-sided tape. The mounted tissues were gold-paladium coated with a Polaron SC500 sputter coated unit (Quorum Technologies, Ltd.) and viewed with a variable pressure Hitachi S3500N scanning electron microscope (Hitachi High-Technologies Co., Ltd, Japan) at an accelerating voltage of 5kV in the Institute of Marine Sciences of the Spanish Research Council (CSIC) facilities.

6.2.3.2.3 Transmission electron microscopy

Statocysts were extracted and fixed in Glutaraldehyde 2,5% for 24-48h at 4ºC or Glutaraldehyde 2,5%- paraformaldehid 2% on filtered sea water for 24h at 4ºC. Macula and crista sections were dissected from 12 statocysts belonging to 6 sepias and subsequently osmicated in 1% osmium tetroxide, dehydrated in acetone and embedded in Spurr. In order to orientate propely the specimens, semithin sections (1µm) of the macula area and crista ridge were cut transversally or tangentially with a glass knife, stained with methylen blue covered with Durcupan and observed on Olympus CX41.

Ultrathin (around 100 nm) sections of the macula and crista were then obtained by using a diamond knife (Diatome) with an Ultracut E ultramicrotome from Reichert-Jung. Sections were double-stained with uranyl acetate and lead citrate and viewed with a Jeol JEM1010 at 80 Kv. The images were obtained with a Bioscan camera model 792 (Gatan) at the University of Barcelona technical service.

6.3. Results

6.3.1 Behaviour responses to noise

The behaviour reaction of the experimental animals showed a light startle response (inking in some occasions) immediately after the start of the sound exposure, before remaining motionless at the bottom of the tank during the rest of the exposition. Immediately after the exposure stopped and the animals were put in tank B, all remained motionless, though breathing regularly, in the middle of the water column or close to the surface, showing no activity (none ate anymore, nor mated or layed eggs) until they were sacrificed up to 96 hours later.

6.3.2 Light microscopy (LM)

The samples neither showed any post-mortem artefacts nor any specific lesion in any organ (see 2.3.1) except light haemorrhaging in some individuals, at mantle level, probably due to impacts

against the tank walls. Apart from statocysts (see data below), the systematic comparison of the histological preparations between exposed individuals and controls did not allow determining the presence of any pathology associated with sound exposure in any of the tissues analyzed.

6.3.3 Structural and ultrastructural investigations of the statocyst sensory epithelium

6.3.3.1 Sepia officinalis macula statica princeps

Just after sound exposure (Fig. 6.2 A-C) damage was observed on the *macula statica princeps* (*msp)* sensory epithelium by SEM analysis (Fig. 6.2, 6.3, 6.4). The hair cells were partially ejected from the sensory epithelium. There were appreciable spherical holes on the base of the hair cells and a rupture of the plasma membrane, probably due to the extrusion of the internal cellular material. Some hair cells had lost a number of kinocilia or showed bent and flaccid kinocilia

Animals sacrificed 24 (not shown) and 48h after sound exposure (Fig. 6.2 D, 6.3A-C) presented a wide range of lesions. The sensory epithelium of the msp presented hair cells partially or totally ejected from the sensory epithelium. The apical ciliated apex and part of the cellular body were extruded above the sensory epithelium into the statocyst cavity. Some hair cells had totally, or in a considerable number, lost the kinocilia and rests of their roots were visible within the damaged epithelium or exhibited bent and flaccid kinocilia. Large extensions of msp epithelium presented rupture of the plasma membrane on the base of the kinocillia probably due to the swelling and extrusion of the cellular body. The spherical holes observed in animals sacrificed right after the exposure were more pronounced here, confirming the extrusion of the internal cellular material. In some areas a scar has been completed by supporting cells after the loss of hair cells.

At 72h after sound exposure (Fig. 6.3 D, E) the lesions were clearly visible on msp. The extrusion of hair cells was present even on the statolith area, where the extruded cellular material went through its calcareous material. The neighbouring areas presented spherical holes on the base of the hair cells caused by the extrusion of the internal cellular material and, rupture of the plasma membrane on the base of the kinocillia following the swelling and extrusion of the cellular body. Some hair cells exhibited bent and flaccid kinocilia. Kinocilia were disorganized and did not follow the typical polarized pattern of the msp epithelium.

Both LM and TEM images (Fig. 6.5, 6.6) confirmed the lesions revealed by SEM. Some damages were recognized on the *macula statica princeps* sensory epithelium. Cuttlefish affected by sound exposure (Fig. 6.5) exhibited a destructured epithelium, which lost its typical simple prismatic structure, showing translucient hair cell bodies, due to the extrusion of internal material. Extruded inner cellular material was visible above the sensory epithelium. The nuclei of hair cells nucleous had lost their central-basal location and were sited near the cellular apex or were simply missing. Chromatin aggregations were visible inside the nuclei which adopted irregular shapes. The cilliated apex of hair cells was partially or totally ejected from the sensory epithelium and in some cases the supporting cells had extended to fill the space left by them (Fig. 6.5, 6.6C). Translucient and smaller hair cell bodies were visible as a consequence of the expulsion of its inner cellular material. Some hair cells had lost kinocilia or presented bent and flaccid kinocilia. In other cases, kinocillia were found to adhere to each other. Disorganization of endoplasmatic reticulum cisternae surface and damage on mitochondrion including lysis of the cristae and dilution of the matrix, were visible. An increase of lysosomes and multivesicular bodies were found. Nerve endings showed vacuolitzaction and mitochondrial damage as a sign of metabolic exhaustion. In addition, afferent dendrites appeared to be hypertrophied probably due to an excessive amount of glutamate released during noise exposure which induced excitotoxicity (Fig. 6.6D, E, F).

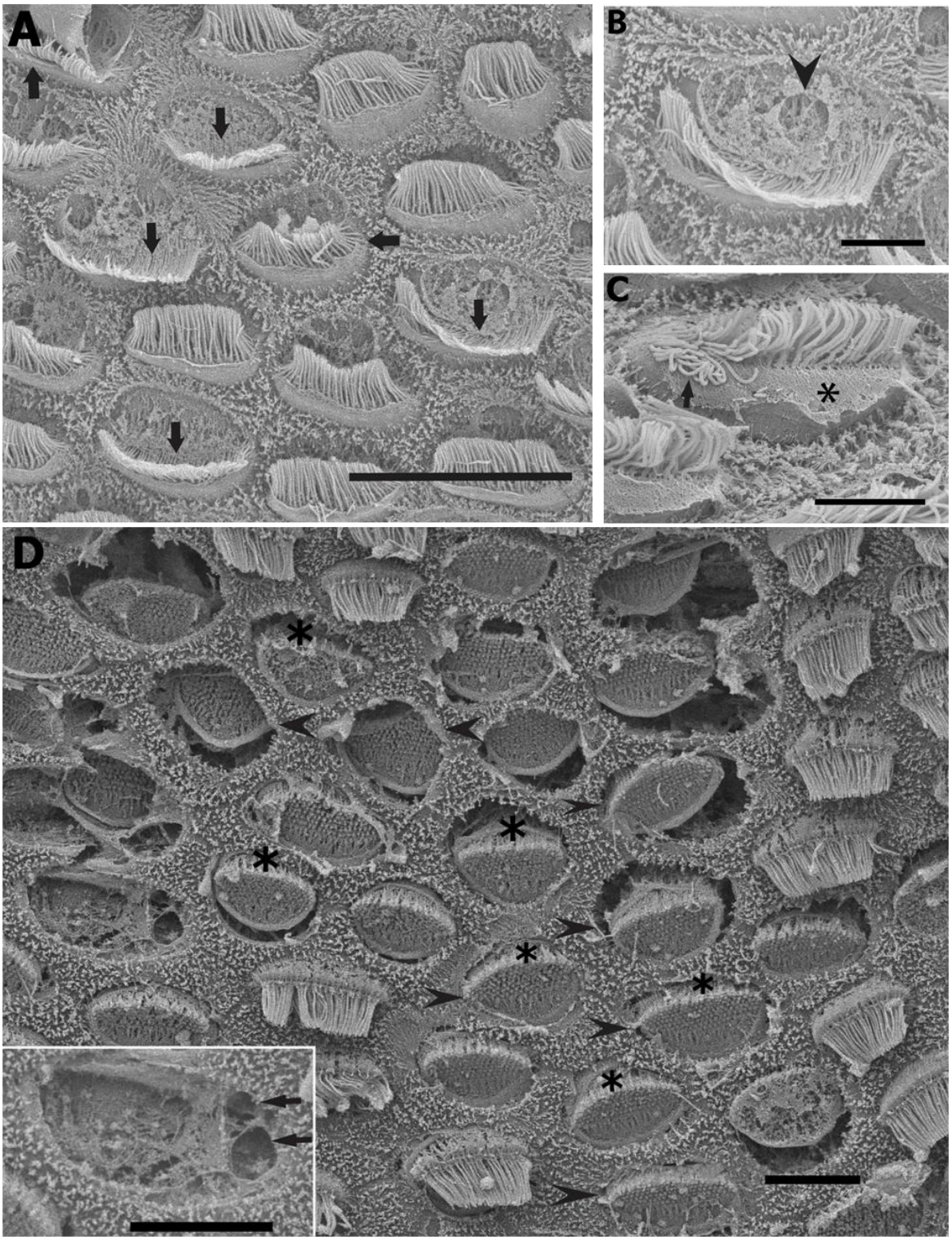

FIG. 6.2. SEM. *Sepia officinalis* msp, presents different levels of lesions. A-C: sacrificed immediately after sound exposure. D: sacrificed 48h after sound exposure. A: The surface of the epithelium presents deformation of numerous bundles of kinocilia (arrows). **B**: Some hair cells show spherical holes (arrowhead) on their apical pole. **C**: other hair cells show flacid kinocilia (arrow) and signs of cell extrusion from the sensory epithelium (asterisk). **D:** The hair cells show severe damage. Most hair cells are partially (asterisks) ejected from the sensory epithelium. A number of them have lost their kinocilia (arrowheads). The insert shows a hair cell with missing kinocillia and two spherical holes at its periphery (arrows). **Scale bars: A** = 20 µm. **B, C** = 5 µm. **D**, **Insert in D** = 10 µm.

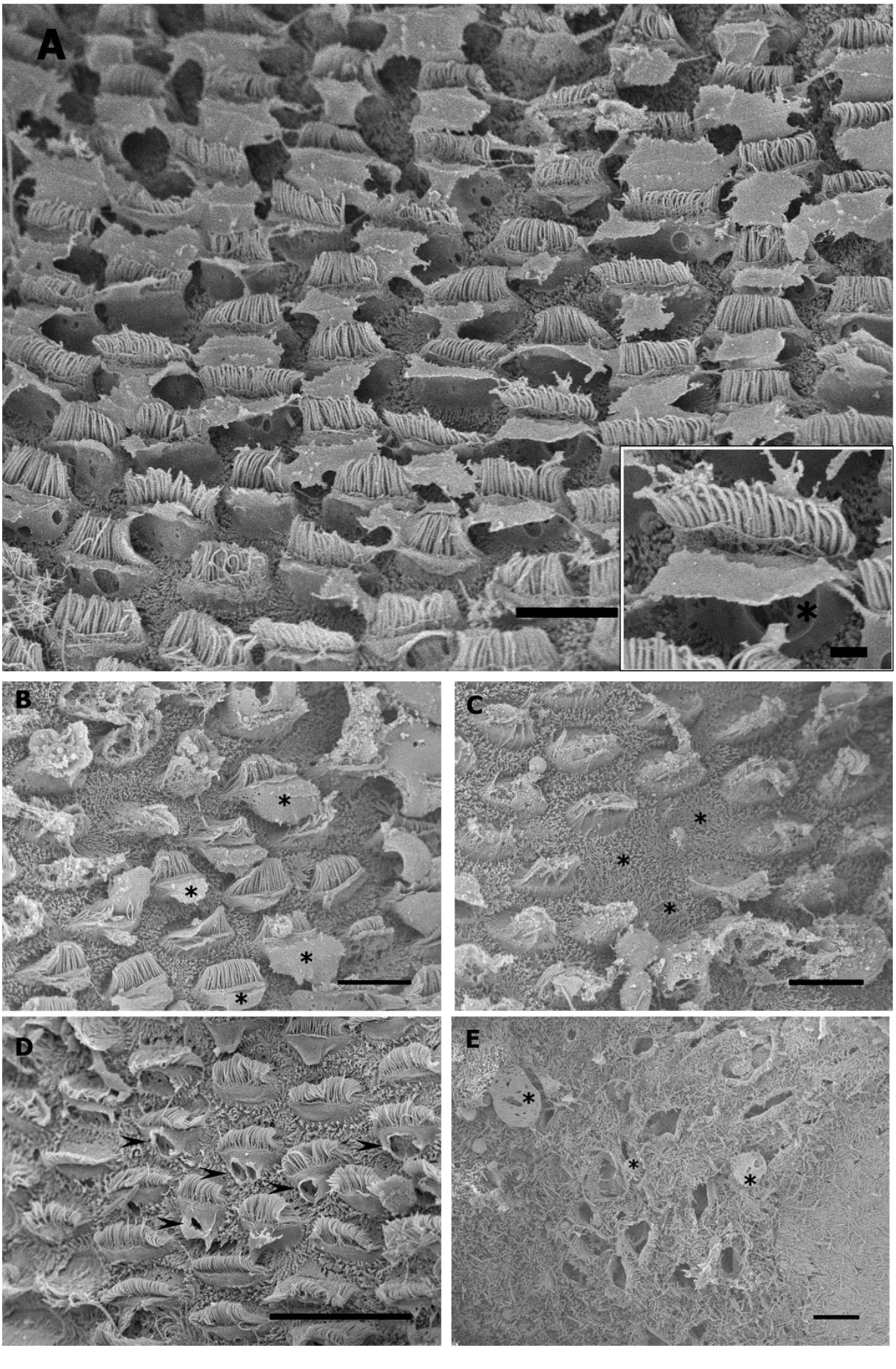
A
B
C
D
E

FIG. 6.3. (Pag 104) SEM. *Sepia officinalis* msp, A-C: sacrificed 48h after sound exposure. D, E: sacrificed 72h after sound exposure. A: Large area of another individual different from the one shown in Fig. 6.2. All hair cells are partially extruded. **Insert**: a partially extruded hair cell. Note the rupture of the plasma membrane (asterisk). **B**: In this area of the *msp*, the cell bodies of most of the hair cells are protruding into the statocyst cavity (asterisk)**. C:** In this area, a scar has been completed by supporting cells after the loss of three hair cells (asterisks). **D:** Statolith neighbouring areas present spherical holes (arrowheads) on the base of the kinocilia bundles. Kinocilia are bent and disorganized. **E:** Extruded cellular material (asterisks) is visible through statolith calcareous material. **Scale bars: A, B, C** = 10 µm. **Insert in A** = 1 µm. **D, E:** 20 µm.

The greatest damaged msp epithelium were present on individuals sacrificed 96h after sound exposure (Fig. 6.4). In all cases the individuals presented damages on the *msp*. Some individuals showed the sensory epithelium completely devastated, practically without hair cells, most of which had been expulsed by extrusion. Only the supporting cells made a holed mosaic with some residual hair cells, which showed no kinocilia or very few bent, flaccid or fused kinocillia. In other cases, the sensory epithelium of the msp presented hair cells partially or totally ejected from the sensory epithelium. The damaged (lost or fusion of most of the kinocillia) apical ciliated apex and part of the cellular body were extruded above the sensory epithelium into the statocyst cavity. Some individuals showed this extruded apex supported on its base by the epithelium, probably due to the recovery process, as reported on mammals an avian species, in which lost hair cells are replaced by expansion of adjacent supporting cells.

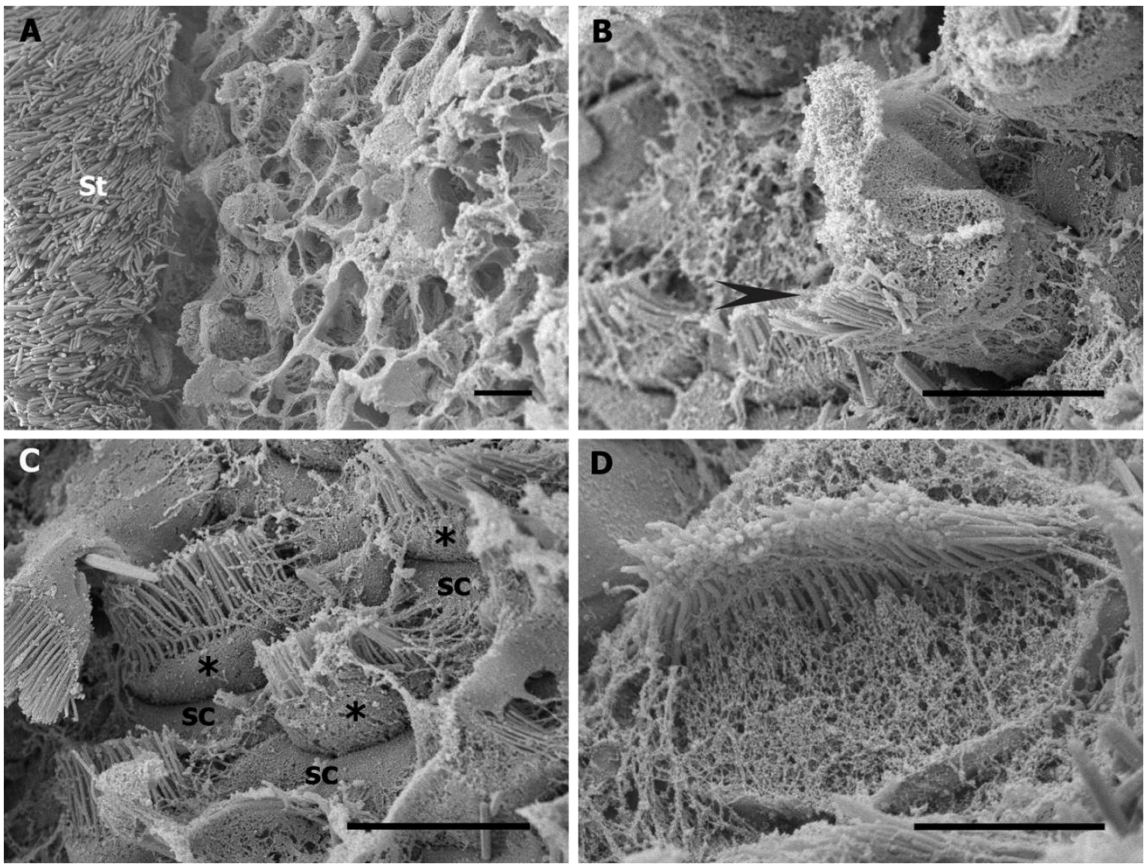

FIG. 6.4. SEM. *Sepia officinalis* msp, sacrificed 96h after sound exposure. A: The sensory epithelium is completely devastated, practically without hair cells, and supporting cells make a holed mosaic. The statolith attached to the *msp* is visible on the left of image (St). **B:** Detail of a partially extruded hair cell. Only a few kinocillia remain (arrowhead). **C:** Some partially extruded apexs (asterisks) of individuals seem to be supported by expansion of adjacent supporting cells (sc) which have started the recovery process. **D:** Some hair cells still retained a more preserved appearance. **Scale bars**: **A-C** =10 µm. **D** =5 µm.

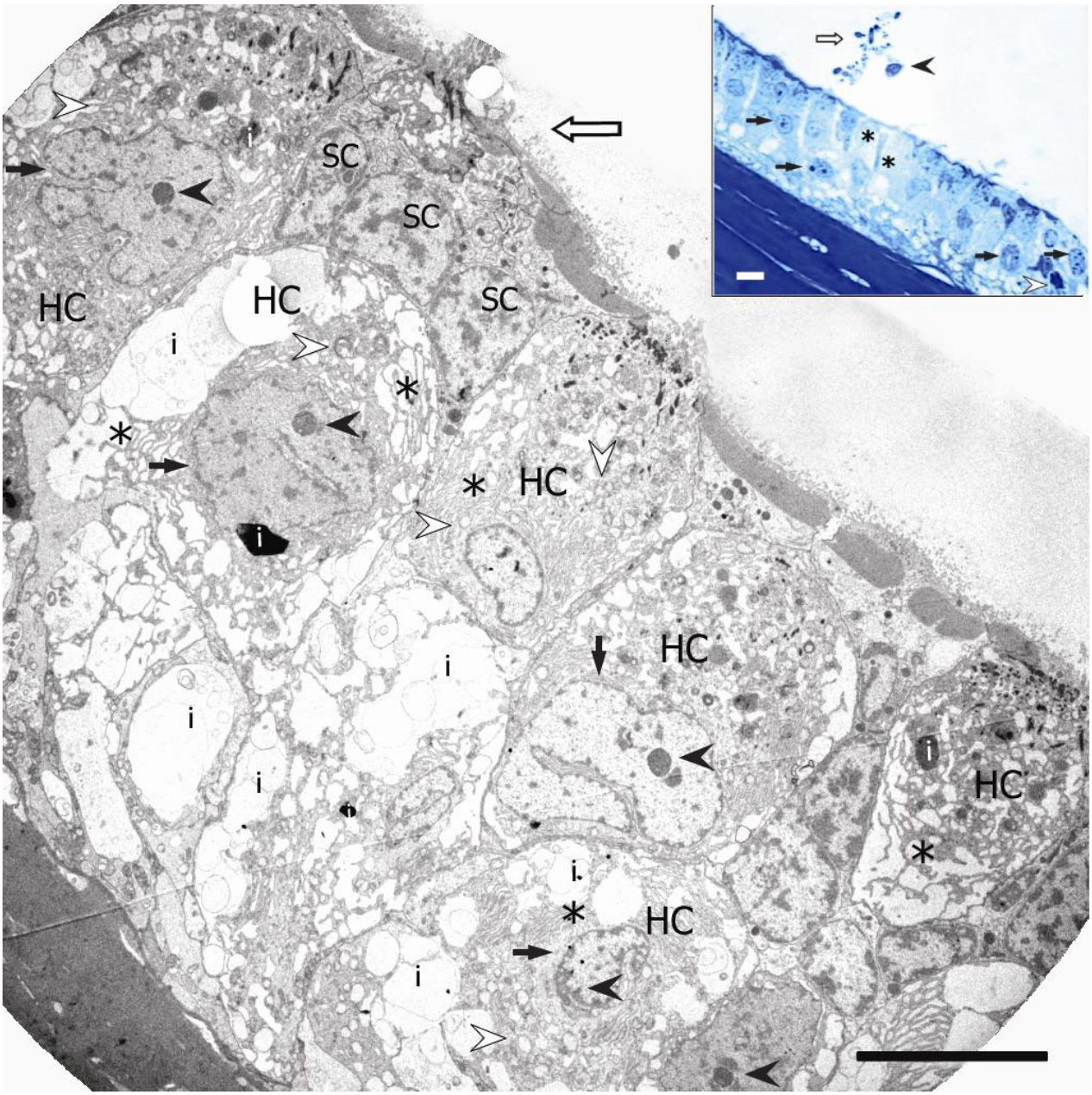

FIG. 6.5. TEM and LM (insert). *Sepia officinalis*, sacrificed 48h after sound exposure. Drastic ultrastructural changes are seen into the hair cell (HC) bodies including swelling of rough endoplasmatic reticulum cisternae (asterisks), deformation of the nucleus (full arrow) and chromatin compaction (full arrowheads), empty mitochondria (empty arrowheads), and presence of dark cytoplasmic inclusions (white i) and large vacuoles (i). In the upper part of the epithelium, three supporting cells (SC) make a continuous scar formation isolating the plate (large empty arrow) of one hair cell from its fully degenerated cell body. Note that the other hair cells have lost their kinocilia. **Insert**: In the acoustically damaged epithelium, the arrangement of hair cells and supporting cells is destructured. Some hair cells have translucient hair cell bodies (asterisks). Most of the hair cell nuclei show irregular shape, chromatin aggregations (full arrows), and they have lost their typical mid-basal position in the cell. One apoptotic-like nucleus (empty arrowhead) is seen on the right side of the picture. The electrondense plate of the supporting cells lines the apical part of the epithelium. Note the extruded cytoplasmic material (empty arrow) and nucleus (full arrowhead) in the extracellular space above the sensory epithelium. **Scale bars** = 10 µm.

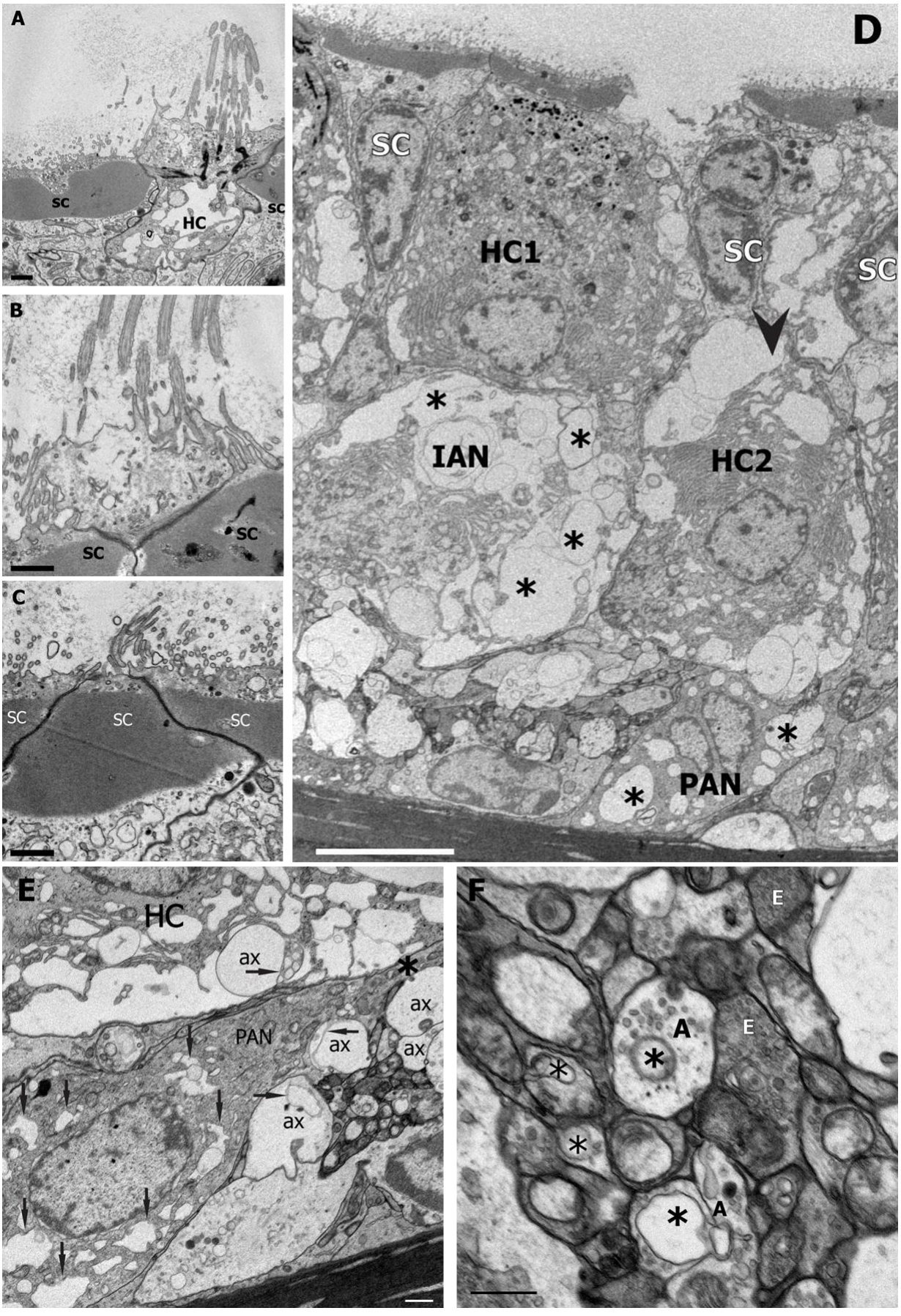
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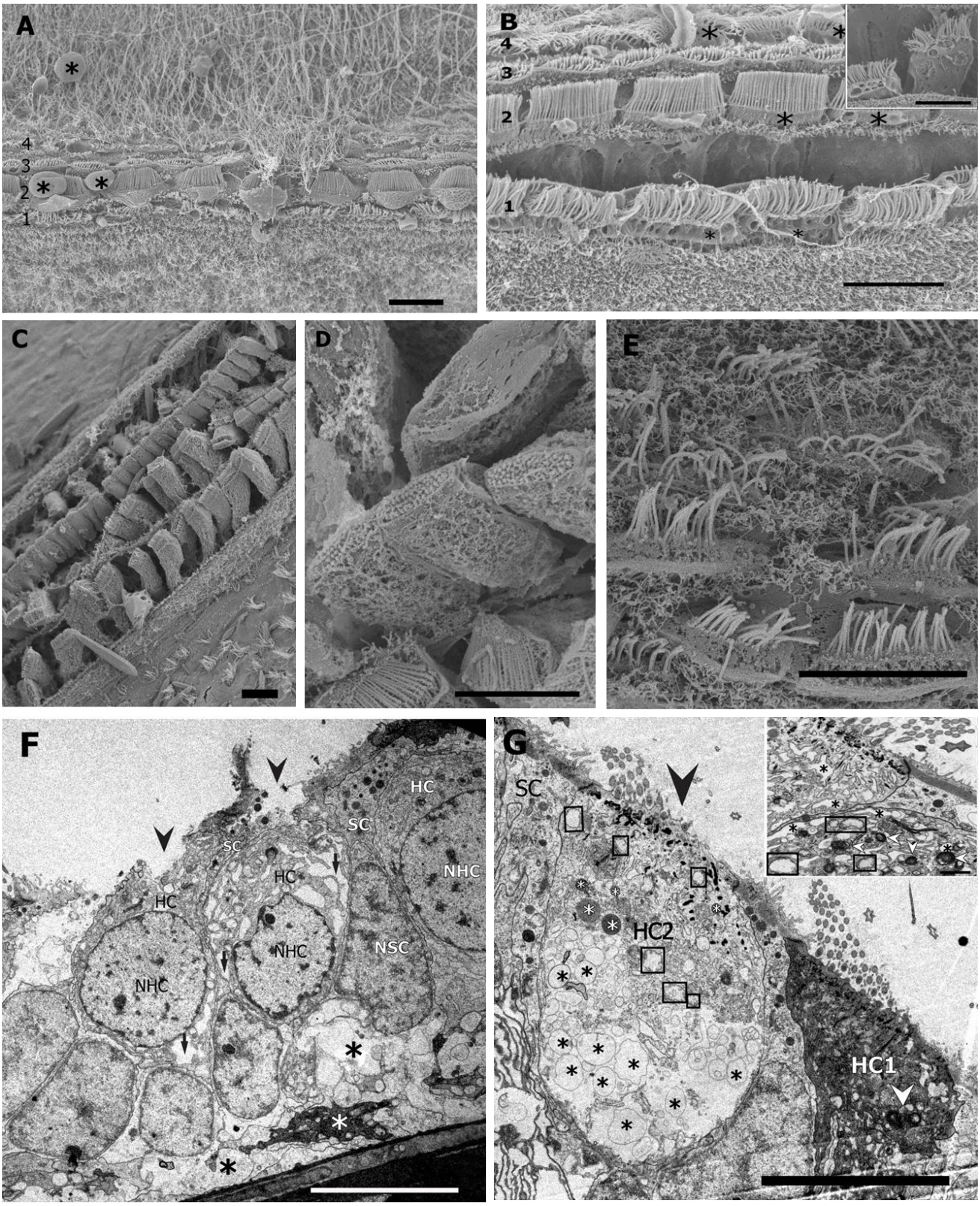
FIG. 6.6. TEM. (Pag 107) *S. officinalis* msp, sacrificed 48h after sound exposure. A-C: sequential extrusion of the apical part of hair cells. **A**: the hair cell (HC) apex is constricted by two supporting cells (SC). **B**: The hair cell apex is isolated from its cell body by extensions of the supporting cells. **C:** the apex of the hair cells has been extruded and a scar is made by the supporting cells. **D**: ultrastrucural changes at the level of the two types of first order afferent neurons, intramacular (IAN) and perimacular (PAN), contacting two sound damaged secondary hair cells (HC1 and HC2). HC1 occupies a normal upper position in the epithelium whereas HC2 body has been deplaced towards the bottom of the epithelium by supporting cells (SC) undergoing a healing process (arrowhead). Underneath HC1, the IAN is swollen and shows large vacuoles (asterisk). Underneath HC2, the PAN is vacuolized (asterisk) but is not swollen. **E**: a perimacular afferent neuron (PAN) and axons from intramacular afferent neurons (ax) are seen under a hair cell (HC). The IAN axons contain large vacuoles (arrows) and seem to be swollen. The cell body of the PAN also contains vacuoles (arrows) but its axon (asterisk) has a normal appearance. **F**: Afferent and efferent axons. Afferents are vacuolized (asterisks) while efferents show a dense cytoplasm with many cytoplasmic small vesicles. **Scale bars**: **A, B, C =** 1µm. **D** = 10 µm. **F**= 0,5 µm.

6.3.3.2 Sepia officinalis crista

From immediately after (Fig. 6.7) until 72h after sound exposure, damage was recognized on the crista sensory epithelium. Spherical holes could be noticed at the base of the hair cells arranged in rows, as well as rupture of the plasma membrane, due to the extrusion of the internal cellular material. As a consequence, apical ciliated hair cells were partially ejected from the sensory epithelium. The damage on kinocilia was not extensive to all the individuals but some individuals showed bent and flaccid kinocilia in their hair cell rows. *Crista* exhibited its two central regular rows of large hair cells totally or partially split by a sensory epithelium fracture.
The individuals sacrificed 96h after sound exposure (Fig. 6.7 C-E) showed the most severe damages. Hair cells were completely extruded into the statocyst cavity independently of the neigborouging cells. Hair cells had totally lost the kinocilia and remains of their roots were visible on the apex. The crista dorsal small hair cell rows showed loss of kinocilia or bent, flaccid or fused kinocillia.

TEM images confirmed the lesions shown in SEM. Some damage appeared on the crista sensory epithelium. Cuttlefish affected by sound exposure (Fig. 6.7F, G) showed a destructured epithelium, losing its typical simple prismatic structure and showing translucient hair cell bodies, due to the extrusion of internal material. Some chromatin aggregations could be seen in the nucleus, which had adopted irregular shapes. The cilliated apex of hair cells was partially or totally ejected from the sensory epithelium. Translucient and smaller hair cell bodies were visible as a consequence of the expulsion of its inner cellular material. Some hair cells had lost kinocilia or showed bent and flaccid kinocilia. Disorganization of the endoplasmatic reticulum cisternae surface and damage on mitochondrion (lysis of the cristae and dilution of the matrix) were visible. There was a significant increase in the number of lysosomes and multivesicular body. Underneath the hair cells, the nerve endings showed signs of metabolic exhaustion such as mitochondrial damage, and afferent dendrites were hypertrophied probably due to an excitoxic process. Macrophages were present at the bottom of the epithelium.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NHC
G
SC
HC2
HC1

FIG. 6.7. (Pag 109). SEM (A-E) and TEM (F, G). *Sepia officinalis* crista. A,B, insert in B: sacrificed immediately after sound exposure. C-E: sacrificed 96h after sound exposure. F, G: sacrificed 24h after sound exposure. Insert in G: sacrificed 48h after sound exposure. A: amongst the four rows of primary hair cells, three rows (row 1, 2 and 4) show obvious signs of damage including bending kinocilia (row 1 and 4) and cytoplasmic blebs (row 2) while row 3 seems to be more preserved. Note the cellular material extruding (asterisk) **B**: in other region, the epithelium is fractured between row 1 and row 2 of hair cells. Note that hair cells in row 1, 2 and 4 are partially extruded (asterisks). By contrast, kinicilia on hair cell of row 3 show a healthy aspect. **Insert in B**: the more severe alterations are seen in hair cells of row 1. **C, D:** Hair cells are completely extruded into the statocyst cavity independently of the neigborouging cells. **E**: crista dorsal small hair cell rows. Hair cells show loss of kinocilia or bent, flaccid or fused kinocillia. **F:** dramatic changes are seen in the epithelium including lost of the apical pole of hair cells (arrowheads) and swelling of their endoplasmic reticulum (small arrows). Underneath the hair cells, the nerve fibers are swollen (black asterisks). Note the macrophage (white asterisk) at the bottom of the epithelium. **G:** Apex of two damaged hair cell, HC1 and HC2. HC1 has a condensed cytoplasm with dark inclusions (white arrowhead) like an apoptotic cell. HC2 shows high number of multivesicular bodies (black asterisk), damaged mitocondria (squares) and lysosomes (white asterisks). Note the loss of kinocillia on the left apical part of the HC2 (black arrowhead**). Insert**: details of the apical part of a damaged hair cell showing mutivesicular bodies (black asterisk), dark inclusions (white arrowhead) and empty mitochondria (squares). **Scale bars**: **A-B, insert in B, D, E, F, G** = 10 µm. **C** = 20 µm. **Insert in G** = 1 µm.

6.3.3.3 Lining ephitelium of Sepia officinalis statocyst

Apart from the specific sensory areas (*msp* and *crista*), the individuals showed acoustic trauma, affecting a wide range of statocyst inner ciliated areas (Fig. 6.8), even on individuals immediately sacrificed after exposure but particularly on the individuals sacrificed 96h after exposure. All tested groups showed lesions in some areas of the lining epithelium of the cavity which consists of flat hexagonal cells with oval nuclei. In some areas of the statocyst, bundles of cilia emerge between the epithelial cells. The lesions on this epithelium consisted basically in the extrusion of the cellullar material into the statocyst cavity. In some cases, the epithelium showed the damaged nucleus expulsed or almost extruded. In individuals sacrificed at 48, 72 and 96 hours after sound exposure there was a massive extension of holes folowing the extrusion of inner cellular material. The cilia and microvilli were bent flaccid and disorganized in almost the totally samples. In individuals sacrificed 96h some individuals presented some cilia fused on giant cilia.

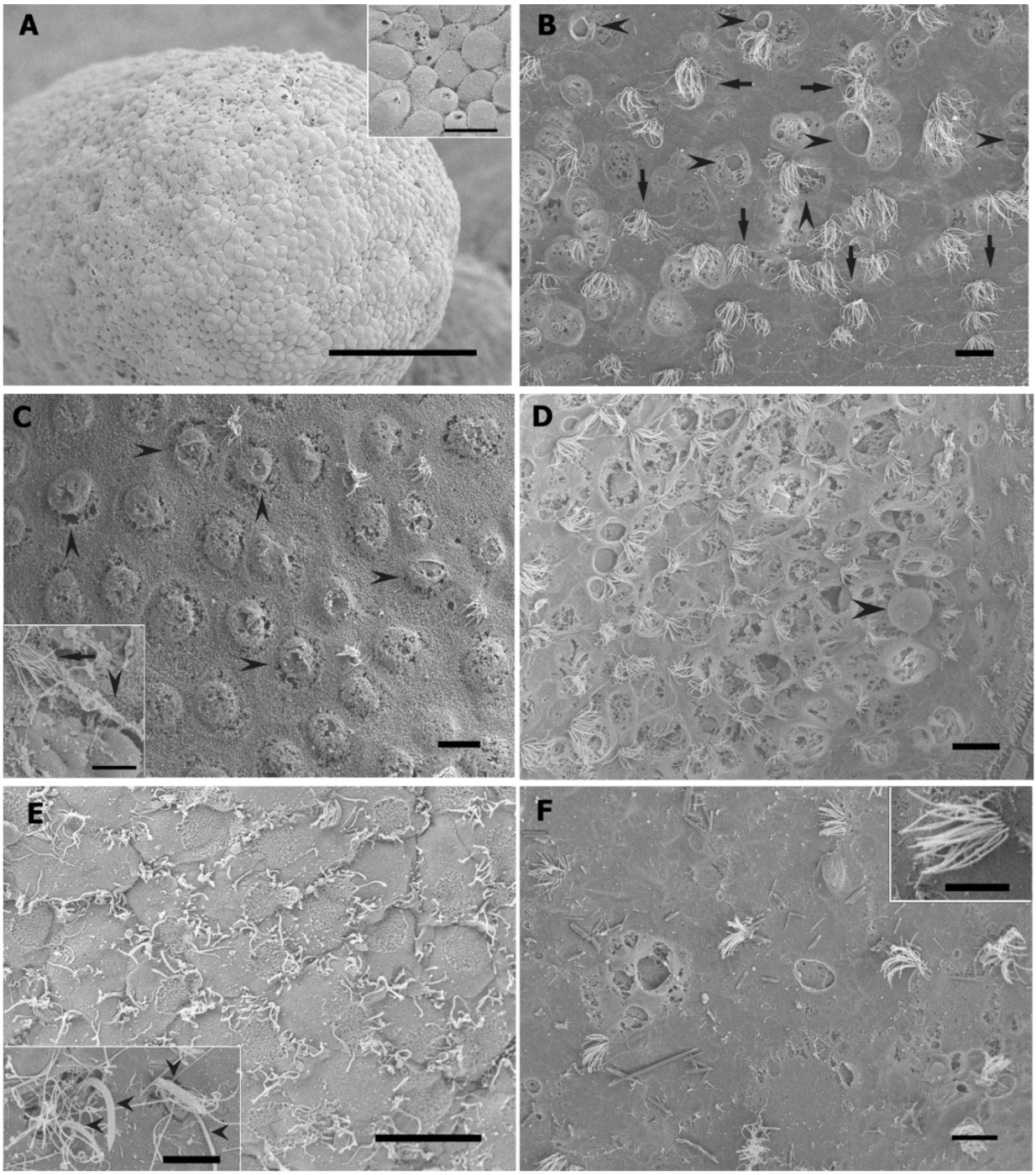

FIG. 6.8. SEM. *Sepia officinalis*. A: Hamuli lobule; **B-F**: lining epithelium of the statocyst cavity. **A**: sacrificed **immediately after sound exposure.** The cilia are missing and some cells exhibit holes (**Insert**). **B**: sacrificed **immediately after sound exposure.** Note the holes (arrowheads) in the epithelial cells and the bending and flaccid cilia (arrows). **C: sacrificed 24h after sound exposure.** Nuclei are expulsed or almost extruded (arrowheads) from the epithelial cells, and the cilia are bending (**Insert** (arrow)). **D: sacrificed 72h after sound exposure.** Lining epithelium of the statocyst cavity shows massive extension of holes**.** On the left a nucleus is extruding (arrowhead). **E-F: sacrificed 96h after sound exposure**. **E:** damaged microvilli form a perimeter surrounding the hexagonal cells. **Insert**: bending and flaccid cilia. Some cilia are fused on giant cilia (arrowheads). **F**: Well preserved tuff of cilia at the surface of the damaged epithelium. **Insert**: a normal appearing tuff of cilia. **Scale bars**: **A**= 100 µm. **B - F** = 10 µm. **Insert in A**= 10 µm. **Inserts in C**, **E**, **F**= 5 µm.

6.4. Discussion

A previous study (McCauley et al., 2000) reported the response of squids to air gun exposure and underlined the extremely important role played by cephalopods in the marine food chain as well as in fisheries in some parts of the world, and suggested a probable impact of seismic operations on these species (using thresholds at 161–166 dB re 1 μPa). These results reported similar behaviour responses as those we observed during sound exposure: several cuttlefish showed startle response by inking. Despite this similarity, the individuals in our experiments, after an initial stress reaction, remained motionless at the bottom of the tanks. After the CEE stopped, the cuttlefish presented evident symptoms of stressed behaviour and decrease of activity, loss of muscle tone, remaining motionless until they were sacrificed. However, the different experimental conditions (size of the tank and the difference of the sound sources) are a limiting factor to assess and directly compare the behavioural reaction of the exposed individuals.

We divided the controls and experimental animals into six groups: sacrified immediately after sound exposure, twelve hours, one, two, three and four days after sound exposure. While the controls showed no lesions in the statocysts (for a complete description of the control statocysts, see chapter 5), all exposed individuals presented the same lesions and the same incremental effects over time. Immediately after exposure, damage was observed on the *macula statica princeps* (*msp*) and on the *crista* sensory epithelia. The hair cells were partially ejected from the sensory epithelium. Spherical holes at the base of the hair cells were visible and a rupture of the plasma membrane was apparent, most probably due to the extrusion of the internal cellular material. A number of hair cells had lost kinocilia or presented bent and flaccid kinocilia. These lesions were gradually more pronounced in individuals after 12, 24, 48, 72 and 96 hours. The most definite lesions were visible in individuals observed 96 hours after sound exposure. In these individuals, the sensory epithelium appeared totally devastated, with practically no hair cells, most of which had been expulsed by extrusion. Only the supported cells made a holed mosaic with residual hair cells with either no kinocilia at all or very few that were bent, flaccid or fused. The hair cells' cellular bodies were extruded above the sensory epithelium into the statocyst cavity. The afferent system axons were hypertrophied, and the nerves showed endoplasmatic reticulum damage, vacuolization or a complete degeneration, although it was not possible to quantitatively determine an increase in the contact number between the hair cells and the nervous system. The almost complete extrusion of the hair cells as well as the fractures present in the epithelium are clear signs that the noise impact was acute and limited in time. On *crista* epithelium, the extrusion of internal material could be the cause of the fracture observed between two rows. The damages were present not only on the sensory epithelium (macula and crista) but also in other inner ciliated areas from the statocyst, specially on lining ephitelium, where the cilia and microvilli were bent, flaccid and disorganized in almost the totally samples, and some cilia appeared fused on giant cilia. In some individuals, the effect of the sound exposure was massive, affecting a wide range of statocyst inner areas, specially on the individuals sacrificed 96h after exposure. One explanation could be that the holes due to hair cell disappearance allowed the entrance in the epithelium of endolymph charged with high potassium ion concentration. This would increase the initial mechanical insult to hair cells together with a more progressive chemical damage as it is observed in acoustic traumatized coclea of mammals. Indeed, Amoor (1959) and colleagues determined a high concentration of potassium in *Octopus vulgaris,* thus supporting this hypothesis.

Partial or total cell extrusion and alterations on kinocillia hair cells were the most frequent lesions in the sensory epithelium of all tested groups. Although in avian and mammals, sensory hair cells carry stereocilia which are not kinocilia, similar damages to hair bundles are previously described in these species, especially in the basilar papilla of the avian inner ear and in the mammalian organ of Corti just after sound exposure. This is consistent with the process of lesion development in our case study. Two mechanisms seem to be involved in noise-induced

hearing loss in mammals: direct mechanical damage, which appears immediately after short exposures at high intensities, and metabolically induced damage, which occurs after long exposures with moderate intensities and develops over a longer period after sound exposure. In mammals, a low- or mid-intensity acoustic trauma does not lead to obvious mechanical damage to the sensory epithelia (McCauley et al., 2000, 2003; Pujol and Puel, 1999; Popper and Hastings, 2009b). Instead, lesions occur primarily at a metabolic level (Bohne and Rabbitt, 1983), leading to the fusion of the cilia, the deformation of the apex of the cells, and eventually the death of the cell over a period of several weeks. However, this process can also be observed at the periphery of a violent acoustic trauma where open holes are left following the expulsion of the cell apex. This was indeed observed in all cephalopod individuals 48, 72 and 96 hours after exposure: the sensory epithelium presented mechanical damage (partial or total loss of sensory cells) and metabolically induced damage (swollen sensory cells, vacuolization of cytoplasm, mitochondrial degeneration, damage to dendrites).

In vertebrates, it was shown that glutamate, which in normal conditions works as a neurotransmitter, has a cytotoxic effect when released in excess in stressful conditions as after exposure to loud noise (among others: Puel et al. 1998; Ruel et al. 2005). The excitotoxic effects of glutamate in response to noise could be the result of increased release or inadequate removal of glutamate, primarily due to the breakdown of recycling mechanisms (Ruel et al. 2006). This may eventually result in toxic cellular events leading to neuronal degeneration and sensorial epithelium damage. Since glutamate has also been described as neurotransmitter acting at AMPA/kainate-like receptors in statocyst afferent fibers of cephalopod species (Tu and Budelmann, 1994; Di Cosmo et al., 2006), the presence of hypertrophic afferent dendrites in our images could probably point to the great amounts of glutamate secretion as a possible cause of the sensory epithelium degeneration process. The observed impact on the statoacoustic organs of the noise-exposed cephalopods would actually suggest the occurrence of an excitotoxic process due to an excess of glutamate.

In this work, the starting of a healing process is shown for the first time on cephalopods. Because the beginning of the scarring process showed by the extension of the supporting cells can be appreciated in SEM images of individuals sacrificed 48 and 96 hours after sound exposure and in TEM images of individuals sacrificed 48h after sound exposure, these results suggest that the recovery process was operant at least two days after sound exposure. However, further experiments are needed to determine the timing of the healing process more precisely.

Reparation and regeneration of stereocilia occur in the vestibular system of bird and mammals and limited reparation is seen in the cochlea of mammals. The morphological diference of the cilia, which are microvilli in the vestibular and auditory system of birds and mammals while they are kinocilia in cephalopods statocyst sensory sytems, require further investigation to understand the kinocilia repair and regrowth process that would happen in cephalopods. In addition, the actin–myosin system could be involved in the removal of the dying hair cells (Leonova and Raphael, 1997; Raphael, 2002).

Apoptosis which is a mode of hair cell loss that does not induce inflammatory processes (Wyllie et al., 1980), contribute to phagocyte recruitment at the site of lesion (see for review: Grimsley and Ravichandran, 2003). The swift and proper removal of apoptotic cells by macrophages prevents leakage of potential cytotoxic and antigenic substances from dying cell and may induce tissue repair by releasing various cytokine and growth factors (for review: Fujiwara and Kobayashi 2005). Apoptosis is a major mode of hair cell loss in the mammalian damaged vestibular organ and cochlea (see for review: Nakagawa et al. 1997a). In addition, in the mammalian cochlea exposed to loud noise (Hirose et al. 2005; Fujioka et al. 2006) or ototoxic drugs (Ladrech et al. 2007), macrophages are believed to remove hair cell corpses. In the present study, the presence of both apoptotic cells and of macrophages in the damaged statocysts suggest that similar post-traumatic mechanisms are involved in the cephalopod damaged sensory epithelium.

In the avian cochlea and vestibular organs and in the vestibular organ of mammals, posttraumatic hair cell regeneration has been shown to occur after either acoustic trauma or drug poisoning (Corwin and Cotanche, 1988). Several mechanisms are believed to be involved, in such regeneration, including proliferation followed by differentiation of non-sensory epithelial cell, direct transdifferentiation of supporting cells into hair cells, and reparation of damaged hair cells. These processes occur generally within one or several weeks after hair cells death. In the present study, individuals were not collected after 96 hours following the acoustic trauma. This is probably a too short delay to observe the potential occurrence of regenerative processes due to cell division and differentiation. Nevetheless, in the present study, an interesting finding was the presence of almost normal kinocilia bundles in statocysts observed 96h after the acoustic trauma in areas where almost all hair cells had disappeared. This feature suggests a possible repairing process in some damaged hair cells, which survived the acoustic exposure.

These results show lesions new to cephalopod pathology and demonstrate that exposure to sounds causes massive lesions on the statocyst sensory epitheliums in cuttlefish. Their presence in all the noise-exposed individuals (vs. their absence in controls, see chapter 5) and their definite progression over time are consistent with a massive acoustic trauma induced here by exposure to relatively low intensity, low frequency sounds, while the same lesions were reported in land mammals and birds exposed to much higher sound levels. Further investigation is needed to determine threshold levels and to quantify the lesions in the statocysts; to explain the mechanism onset of these lesions, in particular to determine if the laboratory conditions can be reproduced in open environments; and to definitively understand if these animals are more sensitive to particle motion or acoustic pressure, or to a combination of both. With low frequency noise levels in the marine environment on the increase, future electrophysiological experiments coupled with post-mortem imaging techniques are needed to determine the tolerance to noise thresholds of cephalopods. However, the presence of lesions in the statocysts clearly points to the involvement of these structures in sound reception and perception. Given that low frequency noise levels in the oceans are on the increase (e.g. shipping, offshore industry, navy maneuvers), that the regular exposure to noise may compromise the statocyst sensory epithelium regeneration capacity, that the role of cephalopods in marine ecosystems is only beginning to be understood, and that reliable bioacoustic data on invertebrates is scarce, such future studies have an important contribution to make to the sustainability of the marine environment.

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7. Ultrastructural analysis of *Loligo vulgaris, Illex coindetii and Octopus vulgaris* statocysts after low frequency sound exposure

7. Ultrastructural analysis of *Loligo vulgaris, Illex coindetii and Octopus vulgaris* statocysts after low frequency sound exposure

7.1. Introduction

When exposed to relatively low intensity low frequency sounds, Controled Exposure Experiments revealed lesions in the statocysts of *Sepia officinalis* (chapter 6). Here, the aim was to validate the presence of the same lesions in other cephalopod species: *L. vulgaris, Illex coindetii* and *O.vulgaris* statocyst sensory epithelium.

L. vulgaris and *I.coindetii* are decapods, thus the structure of their statocyst is very similar to *S.officinalis* (see chapter 5). The ecology of these species is, however, very different. *S. officinalis* and *L. vulgaris* can reach a maximun depth of 200m. *I. coindetii* is a species that can live at depth between 100 and 400m, but can it can easily reach 1100m.

O. vulgaris is an octopod that lives in shallow waters (200 m of maximum depth). Its statocysts present substancial differences with decapods (see chapter 5).

It was therefore relevant to comparatively study the possible differential effects of sound exposure on species whose ecology differs from *Sepia officinalis*.

Structural Morphology

In chapter 5, the inner statocyst structures of the individuals that were used as control are detailed and documented. SEM and TEM images of the four Mediterranean Sea cephalopods species (*Sepia officinalis, Loligo vulgaris, Illex coindetii* and *Octopus vulgaris)* illustrate the structural morphological differences.

Sound perception and acoustic impact

There is a considerable lack of information concerning cephalopod sound reception and therefore on the effects of noise exposure. In sections 5.1 and 6.1 respectively, a detailed description of the state-of-the-art can be found in the provided literature review.

7.2. Material and Methods

7.2.1 Cephalopod individuals

Nine adult and subadult individuals from *Loligo vulgaris* (mantle length 15-25 cm), four *Illex coindetii* (mantle length 10-13 cm) and ten *Octopus vulgaris* (weight 200-400g), were obtained from the Catalan Coast (NW Mediterranean Sea) over a period of 2 years, between February of 2008 and August of 2010, and kept in a closed system of recirculating natural seawater (at 18-20ºC, salinity 35‰ and natural oxygen pressure) consisting of 2 mechanically filtered fiberglass reinforced plastic tanks (A and B) of 2000L capacity, that were connected to each other. In chapter 4.3 and 6.2.1, a detailed description can be found on the maintenance of individuals.

7.2.2. Sound Exposure Protocol

The same sequential CEEs were conducted as with other cephalopods spp. (see chapters 4.3 and 6.2.2). The difference here is that, since the results from the analysis with *Sepia officinalis* were known, we concentrated the study on animals sacrificed immediately and 48 hours after exposure. The same was applied to the number of the exposed and control individuals, thus limiting the suffering of these animals **(DECRET 214/1997[1], DIRECTIVE 2010/63/EU[2]**).

7.2.3 Imaging Techniques

The same imaging techniques were conducted as with other cephalopods spp. (see chapter 5.2.3 and 6.2.3).

7.3. Results

7.3.2 Light microscopy (LM)

The samples neither showed any post-mortem artefacts nor any specific lesion in any organ (see 6.3.2) except light haemorrhaging in some individuals, at mantle level, probably due to impacts against the tank walls. Apart from statocysts (see data below), the systematic comparison of the histological preparations between exposed individuals and controls did not allow determining the presence of any pathology associated with sound exposure in any of the tissues analyzed.

7.3.3 Structural and ultrastructural investigations of the statocyst sensory epithelium

7.3.3.1 Loligo vulgaris, Illex coindetii **and** *Octopus vulgaris macula*

Just after sound exposure (fig. 7.1 A, 7.2A-D) damage was observed on the *macula statica princeps* (*msp)* sensory epithelium, by SEM analysis. The hair cells were partially ejected from the sensory epithelium. There were appreciable spherical holes on the base of the hair cells and a rupture of the plasma membrane, probably due to the extrusion of the internal cellular material. Some hair cells had lost a number of kinocilia or showed bent and flaccid kinocilia.

On animals sacrificed 48h after sound exposure (fig. 7.1 B-D, 7.2 E-F), the sensory epithelium of the msp presented hair cells partially or totally ejected from the sensory epithelium. The apical ciliated apex and part of the cellular body were extruded above the sensory epithelium into the statocyst cavity. Some hair cells had totally, or in a considerable number, lost the kinocilia and rests of their roots were visible within the damaged epithelium or exhibited bent and flaccid kinocilia. Large extensions of msp epithelium presented rupture of the plasma membrane on the base of the kinocillia probably due to the swelling and extrusion of the cellular body. The spherical holes observed in animals sacrificed right after the exposure were more pronounced here, confirming the extrusion of the internal cellular material.

[1] Decret de 30 de juny de 1997 del *Departament d'agricultura, ramaderia i pesca*, D.O.Generalitat de Catalunya sobre protecció d' animals utilitzats per a l'experimentació i d'altres finalitats científiques

[2] DIRECTIVE 2010/63/EU OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 22September 2010 on the protection of animals used for scientific purposes

Illex coindetii presented the center of the macula statica princeps free of hair cells in contrast to the macula from other species of cephalopods in our study *(S. officinalis, L. vulgaris* and *O. vulgaris*).

Light microscopy and transmission electron microscopy images (fig. 7.3, 7.4) confirmed the lesions revealed by scanning electron microscopy. Some damages were recognized on the *macula statica princeps* sensory epithelium. Octopus affected by sound exposure (Fig. 7.3) exhibited a destructured epithelium, which lost its typical simple prismatic structure, showing translucient hair cell bodies, due to the extrusion of internal material. Extruded inner cellular material was visible above the sensory epithelium. The nuclei of hair cells nucleous had lost their central-basal location and were sited near the cellular apex or were simply missing. In some cases the nuclei adopted irregular shapes. The cilliated apex of hair cells was partially or totally ejected from the sensory epithelium and in some cases the supporting cells had extended to fill the space left by them (fig 7.3, 7.4,). Translucient and smaller hair cell bodies were visible as a consequence of the expulsion of its inner cellular material. Some hair cells had lost kinocilia or presented bent and flaccid kinocilia. Disorganization of endoplasmatic reticulum cisternae surface and damage on mitochondrion (lysis of the cristae and dilution of the matrix) were visible. Nerve endings showed vacuolitzaction and mitochondrial damage as a sign of metabolic exhaustion, and large neurons were hypertrophies probably due to the great amounts of glutamate secretion (fig. 7.4D, E).

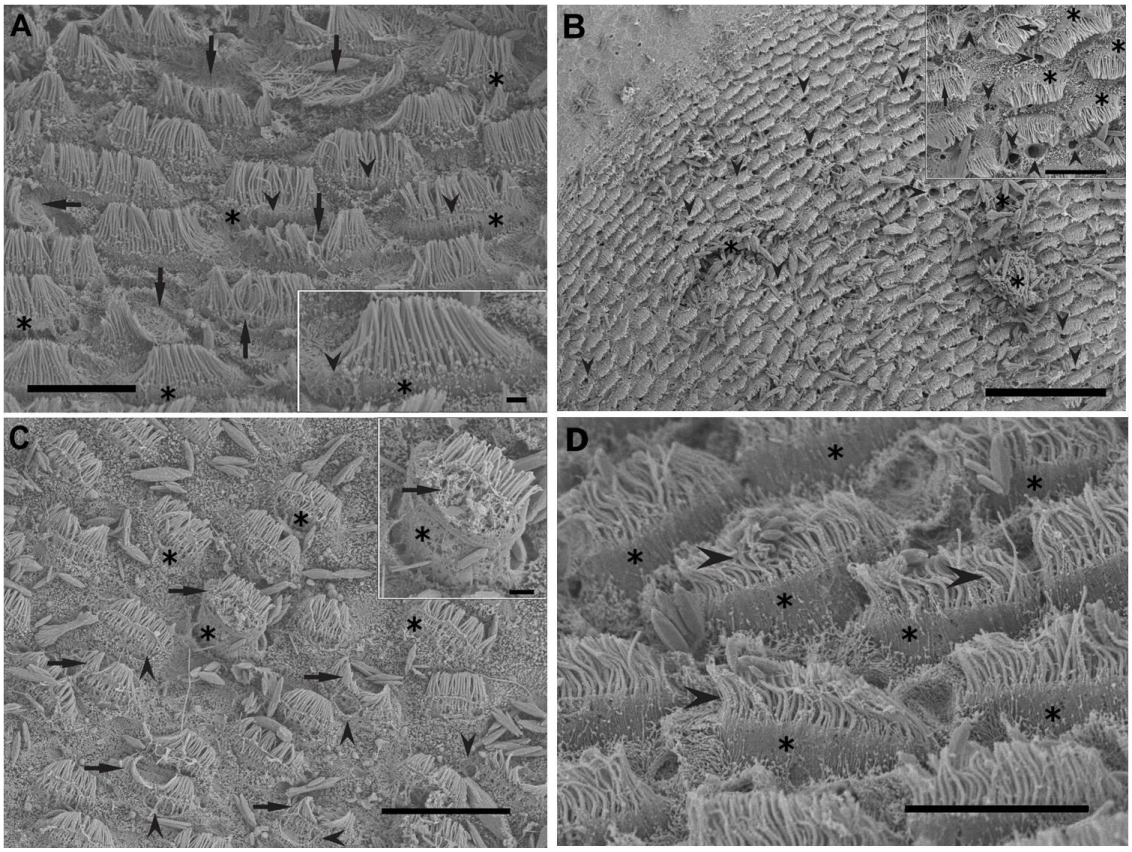

FIG. 7.1. SEM. *Octopus vulgaris* macula static princeps (msp), sacrificed immediately (A) and 48h (B-D) after sound exposure. A: The surface of the epithelium presents deformation of numerous bundles of kinocilia (arrows). Some hair cells present rupture of the plasma membrane (arrowheads). The hair cells are partially ejected from the sensory epithelium (asterisks). **Insert in A**: A hair cell shows rupture of the plasma membrane (arrowheads) and signs of cell extrusion from the sensory epithelium (asterisk). **B**: Large extension of the *msp* shows holes on the base of the hair cells (arrowheads). Some residual crystals of the statolith are visible (asterisks). **Insert in B**: Detail of B. Some hair cells present spherical holes (arrowheads) on the apical pole and rupture of the plasma membrane (arrows). The hair cells are partially extruded (asterisk). **C:** The hair cells show severe damage. Most hair cells are partially (asterisks) ejected from the sensory epithelium. A number of them have lost their kinocilia (arrows) and present rupture of the plasma membrane (arrowheads). The insert shows a partially extruded

hair cell (asterisk) with missing kinocilia (arrow). **D**: In this area of the *msp*, the cell body of most of the hair cells is protruding into the statocyst cavity (asterisk) and shows bent and flaccid kinocillia (arrowheads). **Scale bars: A, D** = 10 µm. **B** = 50 µm. **C** = 20 µm. **Insert in A** = 1 µm. **Insert in B** = 10 µm. **Insert in C** = 2 µm.

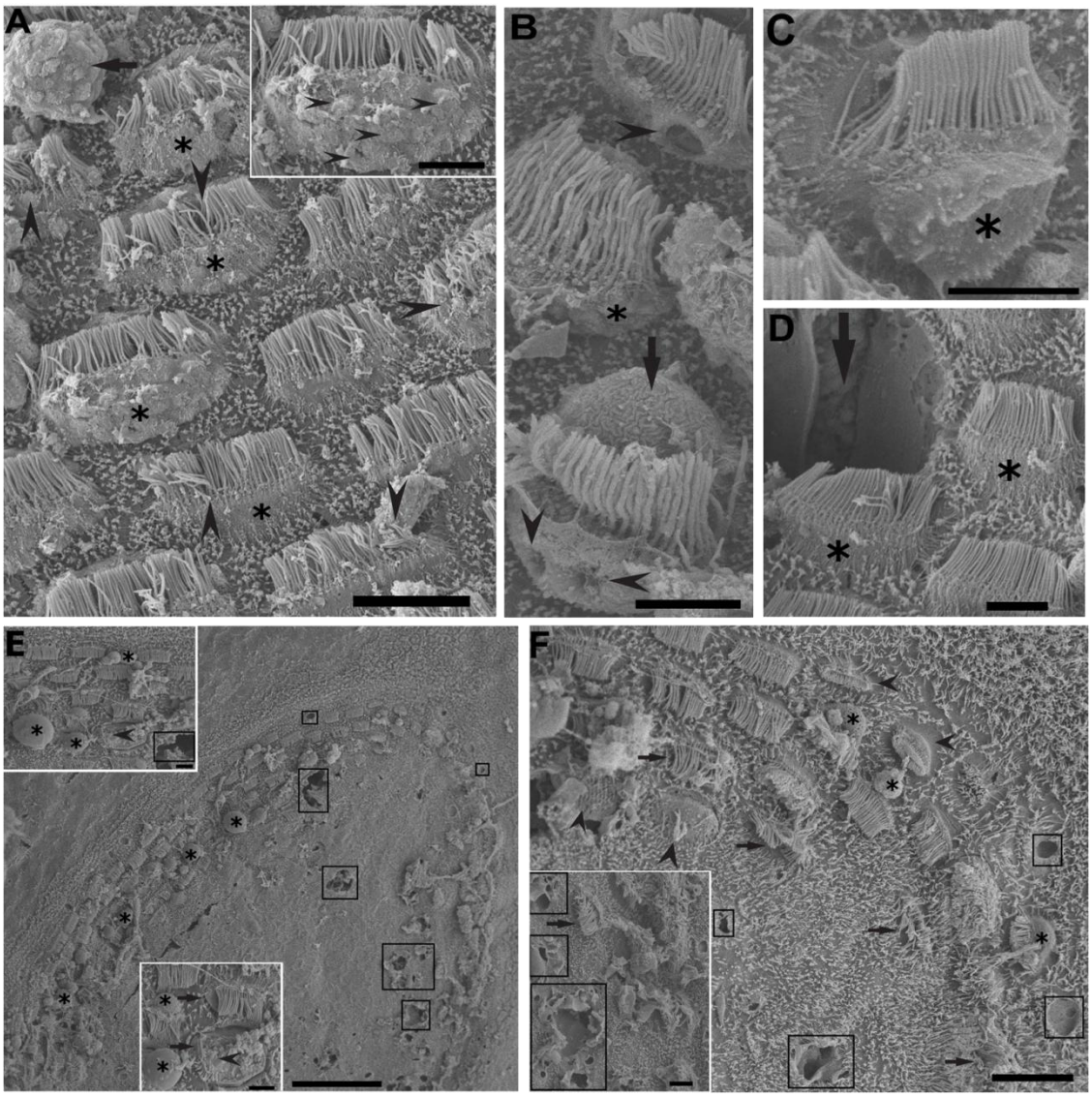

FIG. 7.2. SEM. *Loligo vulgaris* macula statica princeps (msp) sacrificed immediately (A-C) and *Illex coindetii* msp sacrificed 48h (E-F) after sound exposure. A: The surface of the epithelium presents deformation of numerous bundles of kinocilia (arrowheadss). The hair cells are partially ejected from the sensory epithelium (asterisks). Arrow shows extruded cell body of a hair cell. **Insert in A**: Little arrowheads indicate to the protusions of inner material extruding from the body of a patially protruded hair cell. **B**: Holes (arrowheads) on the base of the hair cells are shown. The hair cells are partially extruded (asterisk). Arrow shows extruded cell body of a hair cell. **C**: A hair cell is partially protruded on the statocyst cavity (asterisk). **D**: Between some partially protuded hair cells (asterisks), arrow points the place left by a totally extruded hair cell. **E**: Upper view of the *msp* of *I. coindetii* shows some hole on the epithelium superficie (squares). Some inner material is extruding (asterisk). Note the center of the macula is free of hair cells in contrast to the macula from other species of cephalopods studied. **Inserts** show some details from E. Arrowhweads indicate unestructured bundles of kinocilia. Asteriks show extruding material. Two hair cells that present rupture of the plasma membrane (arrows). **F**: In this area of the *msp*, the cell body of some hair cells is protruding into the statocyst cavity (asterisk) and show bent and flaccid kinocillia (arrows). Some hair cells have totally or partially lost their kinocilia (arrowheads). Some holes (squares) are visible on the epithelium.The insert shows a dramatical damaged area with larged holes (squares). **Scale bars: A, F** = 10 µm. **B, C, D, inserts in E, inert in F** = 5 µm. **E** = 50 µm.

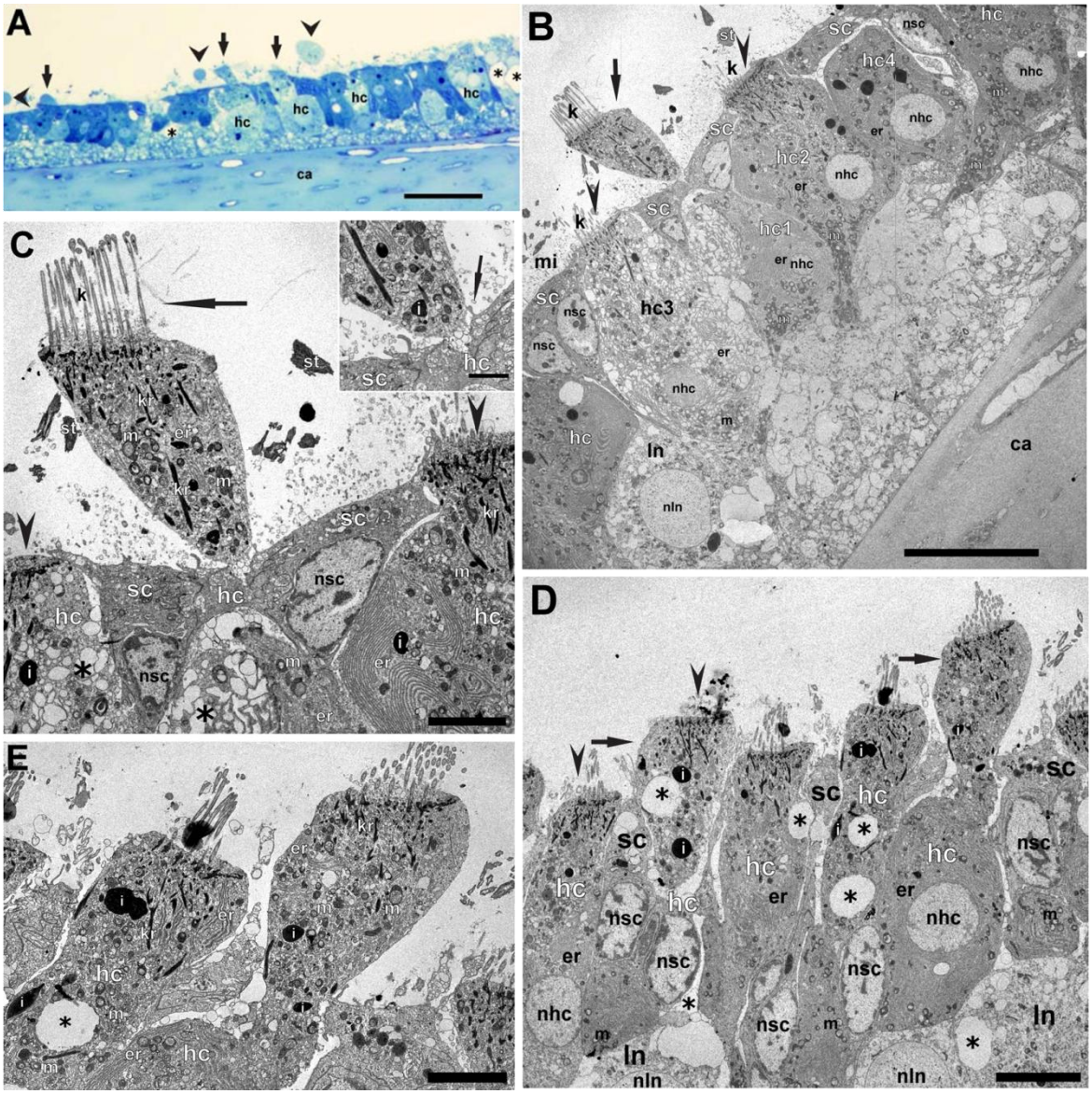

FIG. 7.3. LM (A) and TEM (B-E). *Octopus vulgaris* macula statica princeps (msp), sacrificed 48h after sound exposure. A: In the acoustically damaged epithelium, the arrangement of hair cells and supporting cells is destructurated. Some hair cells have translucient hair cell bodies (asterisks). Most of the hair cell nuclei have lost their typical mid-basal position in the cell. Note the extruded cytoplasmic material (arrowheads) and the cell body of the hair cells protruding into the statocyst cavity (arrows). **B**: Drastic ultrastructural changes are seen into the macula sensory ephitelium. The hair cell 1 (hc1) has almost lost its apical pole (arrow). Hc2 and hc3 show swelling of rough endoplasmatic reticulum cisternae (er), large vacuolization and lost of kinocillia (arrowheads). Hc4 has lost their apical pole and in its upper part a supporting cell (SC) make a continuous scar formation isolating the plate of the hair cell from its fully cell body. At the base of the sensory epithelium, the swollen nerve plexus is shown. A large neuron (ln) is visible in contact to hc3. **C:** The hair cell (hc) has almost lost their apical pole (arrow) and two supporting cell (SC) are constricting the hc to make a continuous scar formation isolating the plate of the hair cell from its fully cell body. In this extruded apical pole, destructurated kinocillia roots (kr) are visible. Note the large vacuolization (asterisks), swelling er of the rough endoplasmaticum reticulum (er), dark inclusions (i) and the lost of kinocillia (arrowheads) of the hair cells. **Insert**: Arrow shows the area where the apical pole is almost detached from its cellular body. **D**: Other large area of the *msp* shows all the apical poles of the hair cells almost extruded from the epithelium (arrows). Note the lost of kinocillia (arrowheads), the large vacuolization (asterisks) and the dark inclusions (i). **E**: Detail from D. The area where two hair cells have almost detached its apical pole is shown. Note the dark inclusions (i) and large vacuoles (asterisk). HC: hair cell, sc: supporting cell, ca: cartilaginous plate, ln: large neuron, nhc: hair cell nucleus, nsc: supporting cell nucleus, nln: large neuron nucleus, k: kinocillia, mi: microvilli, st: statolith crystal, er: rough endoplasmatic reticulum, m: mitochondria, kr: kinocillia root. **Scale bars: A** = 100 µm. **B** = 20 µm. **C, E** = 5 µm. **Insert in C** = 2 µm. D: 10 µm.

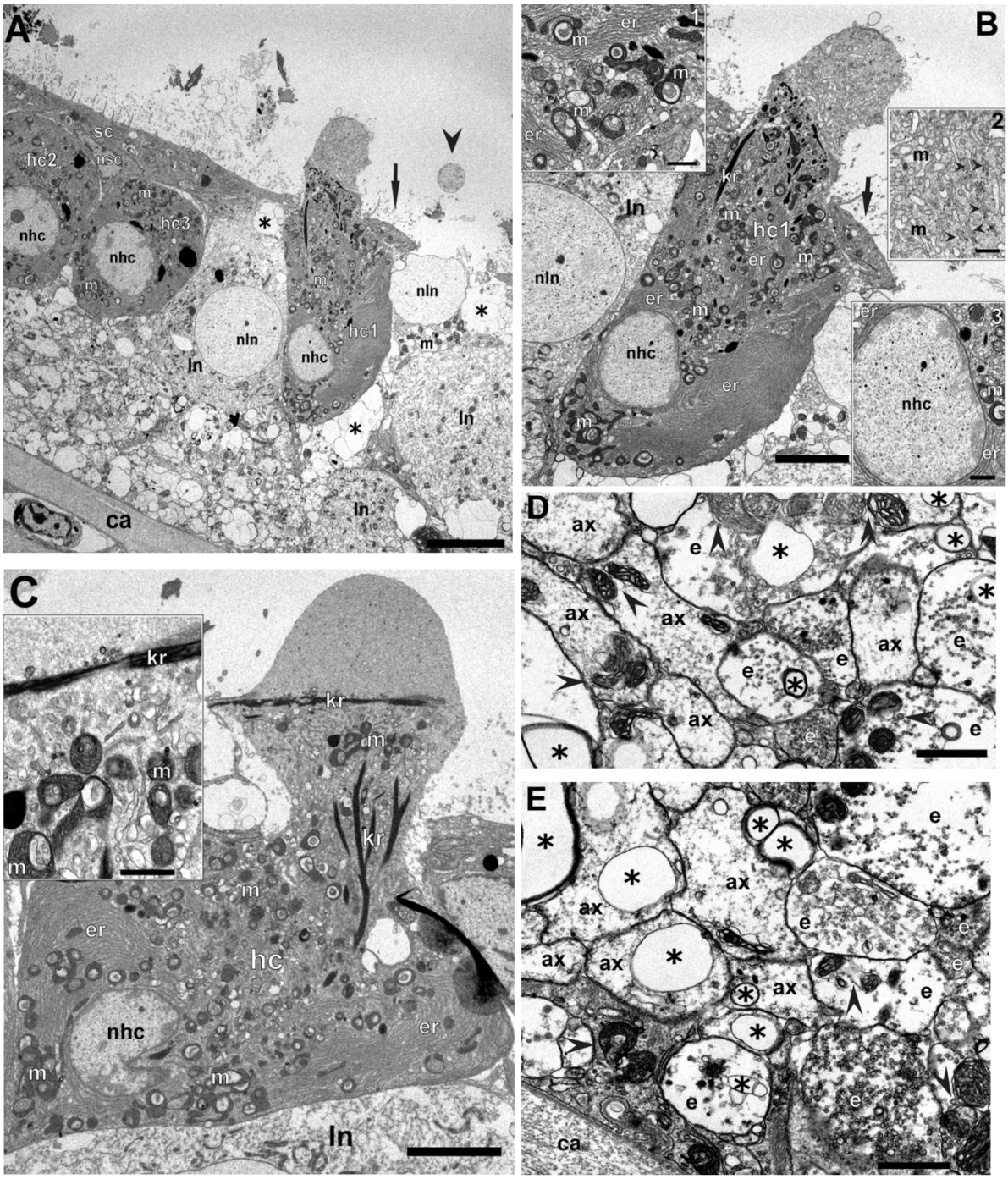

FIG. 7.4. TEM. *Octopus vulgaris* macula statica princeps (msp), sacrificed 48h after sound exposure. (HC: hair cell, **sc**: supporting cell, **ca**: cartilaginous plate, **ln**: large neuron, **nhc**: hair cell nucleus, **nsc**: supporting cell nucleus, **nln**: large neuron nucleus, **er**: rough endoplasmatic reticulum, **m**: mitochondria, **kr**: kinocillia root, **ax**: afferent axon, **e**: efferent terminal) **A:** Drastic ultrastructural changes are seen into the macula sensory ephitelium. The hair cell 1 (hc1) is partially protuding to the cavity. On its apical pole some inner material is extruding and has partially missed the kinocillia (arrow). Hc2 and hc3 have lost their apical pole and in its upper part the supporting cells (SC) make a continuous scar formation isolating the plate of the hair cell from its fully cell body. An extruded nucleus is visible (arrowhead). At the base of the sensory epithelium, the swollen and vaculized nerve plexus is shown. A large neuron (ln) is constricted between hc1 and hc2 and visible on an inusual position near the apical pole of the epithelium. On the right part of the image other swollen and vacuolitzed (asterisk) large neurons are visible. Some of them are almost extruded. A nucleous of one large neuron (nln) partially extruded is visible on the apical pole of the epithelium. **B**: Hc1 is partially extruded. On its apical pole extuding material and lost of kinocillia (arrowheads) are visible. Destructurated kinocillia roots (kr), swelling rough endoplasmaticum reticulum (er) and damage on mitochondrion (m) are shown. **Insert 1**: Detail from the swelling endoplasmatic reticulum (er) and damged mitochondrion (m). **Insert 2**: Detail from the cellular material which is extruding through the apical pole of hc1. Arrowheads show the kinocillia rests showing the 9x2 +2 structure between the extruding mass. **Insert 3**: Detail from the nucleous of hc1.

Some chromatin aggregations are visible. **C**: A hair cel is estruding from the epithelium. On its apical pole the cellular material is expelled through the microtubular structure of the kinocillia. Destructurated kinocillia roots (kr), swelling rough endoplasmaticum reticulum (er) and damage on mitochondrion (m) are shown. A kr is in horizontal position. The nucleus (nhc) has irregular shape. On its base hc contacts with a ln. **Insert:** Detail from the damaged mitochondrion. **D, E**: Detail from the nervous plexus. Nerve endings showed vacuolitzaction (asterisks) and mitochondrial damage (arrowheads).**Scale bars: A** = 10 µm. **B, C** = 5 µm. **D, E** = 1 µm. **Inserts in B (1, 2 3) and C** = 1 µm.

7.3.3.2 Loligo vulgaris, Illex coindetii **and** *Octopus vulgaris crista*

From right after (Fig. 7.6, 7.7) until 48h after sound exposure damage was recognized on the crista sensory epithelium. Spherical holes could be noticed at the base of the hair cells arranged in rows, as well as rupture of the plasma membrane, due to the extrusion of the internal cellular material. As a consequence, apical ciliated hair cells were partially ejected from the sensory epithelium. The damage on kinocilia was not extensive to all the individuals but some individuals showed bent and flaccid kinocilia in their hair cell rows.

One individual of *Illex coindetii* presented the cupula partially adhered to the inner surface of the statocyst and in an area of the inner statocyst ephithelium between two crista-cupula segments it also presented cillia fused in giant cilia.

LM and TEM images confirmed the lesions shown in SEM. Damage appeared on the crista sensory epithelium. Octopus affected by sound exposure (fig., 7.6B, 7.8) showed a destructured epithelium, losing its typical simple prismatic structure and showing translucient hair cell bodies, due to the extrusion of internal material. The cilliated apex of hair cells was partially or totally ejected from the sensory epithelium. Translucient and smaller hair cell bodies were visible as a consequence of the expulsion of its inner cellular material. Some hair cell had lost kinocilia or showed bent and flaccid kinocilia. Disorganization of the endoplasmatic reticulum cisternae surface and damage on mitochondrion (lysis of the cristae and dilution of the matrix) were visible. There was a significant increase in the number of multivesicular body. Underneath the hair cells, the nerve fibers were swollen. Nerve endings showed mitochondrial damage probably as a sign of metabolic exhaustion, and afferent dendrites had suffered hypertrophy probably due to the great amounts of glutamate secretion (fig 7.8A, F).

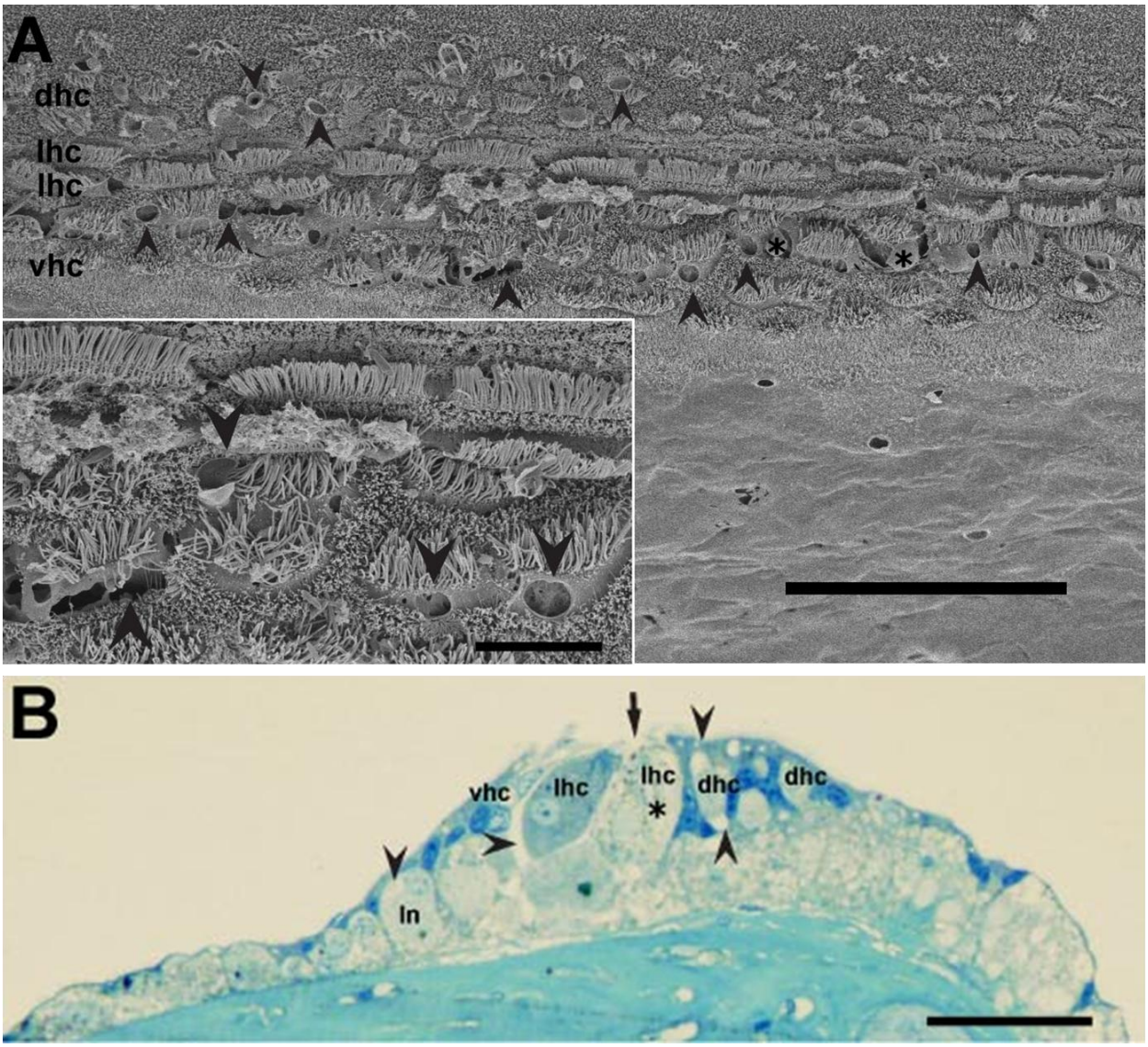

FIG 7.5. SEM (A) and LM (B). *Octopus vulgaris* crista, sacrificed immediately (A) and 48h (B) after sound exposure (**dhc**: small dorsal primary hair cell; **lhc**: regular row of large secondary hair cells; **vhc**: small ventral secondary hair cell; **ln**: large first-order afferent neuron). **A**: amongst the four rows of primary hair cells, two rows (dhc and vhc) show obvious signs of damage including bending kinocilia and cytoplasmic blebs. Some hair cells present spherical holes (arrowheads) on the apical pole. Note the cellular material extruding (asterisk). **Insert**: Detail from A show the holes (arrowheads) on the apical pole of vhc. **B**: Cross section of an odd-numbered crista segment. In the acoustically damaged epithelium, the arrangement of hair cells and supporting cells is destructurated. Some hair cells have translucient hair cell bodies (asterisks). The two central rows of lhc have started its extrusion process, its cellular body have been detached from the epithelium and their apical pole presents ruptures on the plasmatic membrane (arrow). Arrowheads sign to vacuolization of the cells. **Scale bars: A** = 50 µm. **B** = 200 µm.

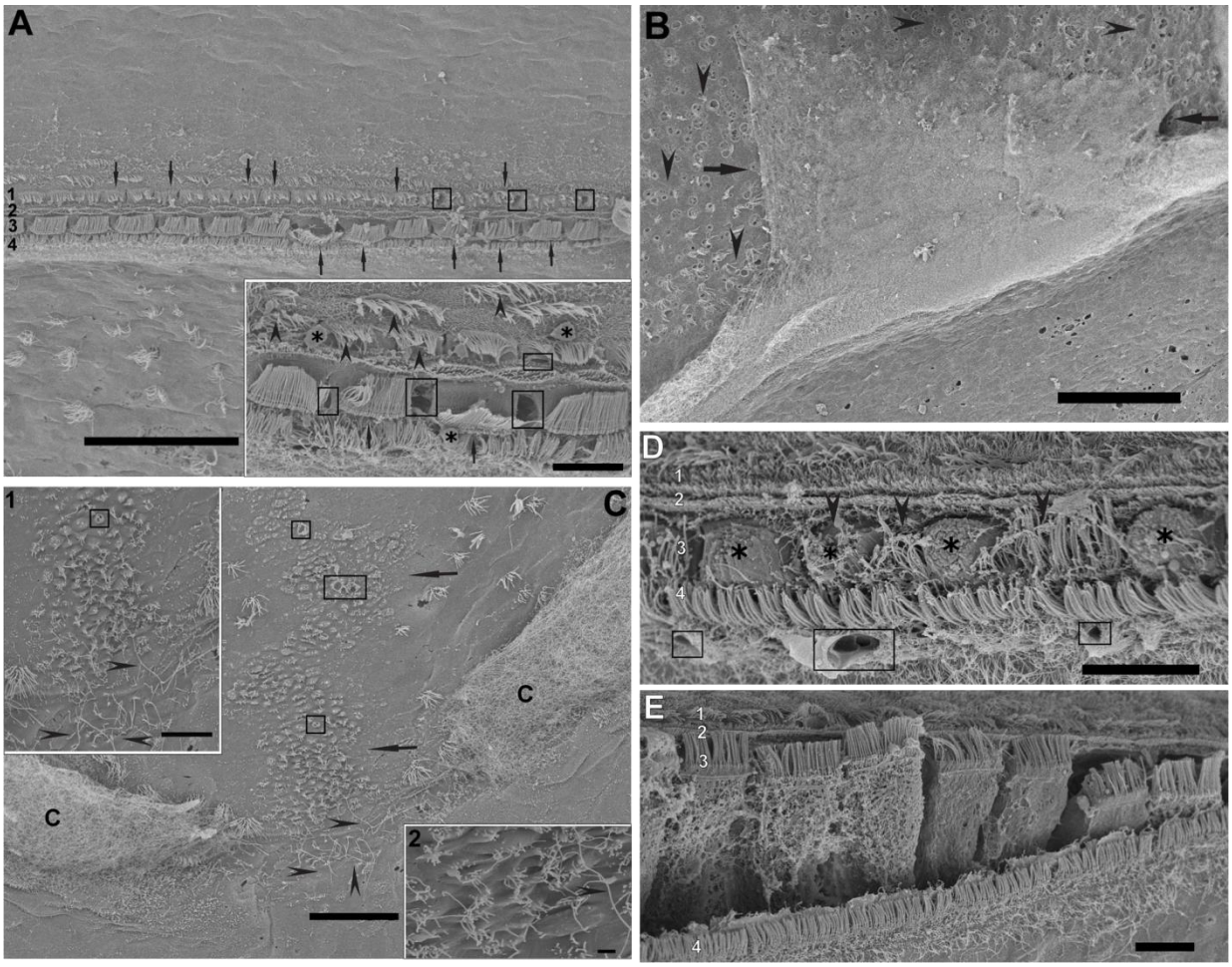

FIG. 7.6. SEM. *Loligo vulgaris* crista sacrificed immediately (A) and *Illex coindetii* crista sacrificed 48h (B-E) after sound exposure. A: amongst the four rows of primary hair cells, two rows (1and 3) show obvious signs of damage including bending kinocilia (arrows), while row 2 and 4 seem to be more preserved. Some spherical holes (squars) are visible between hair cells of the row 1. **Insert**: Detail from A. Arrowheads signs the bending kinocillia of the row 1. Squares show the holes on the row 3. Note the cellular material extruding (asterisk). **B**: The cupula appears partially adhered to the inner surface of the statocyst (between arrows). Arrowheads show numerous holes on the inner statocyst epithelium near the crista-cupula section. **C**: A large area of the inner statocyst ephithelium between two crista-cupula segments presents cillia (arrows). The cillia are fused in giant cilia (arrowheads). Squares show some spherical holes. Inserts 1, 2 show giant cillia (arrowheads). **D**: In the acoustically damaged epithelium, the arrangement of hair cells and supporting cells is destructurated. The more severe alterations are seen in hair cells of row 3 and 4. Row 3 has started its hair cells extrusion process (asterisks). Its cellular bodies were detached from the epithelium and their apical pole presents ruptures on the plasmatic membrane and missed kinocillia. Hair cells show loss of kinocilia or bent, flaccid or fused kinocillia (arrowheads). Row 4 shows some spherical holes on the base of the hair cells (squares) **E**: in other region, the epithelium is fractured between row 3 and row 4 of hair cells. Note that hair cells in row 3 are partially extruded into the statocyst cavity independently of the neigborouging cells. By contrast, kinicilia on hair cell of row 1, 2 and 4 show a healthy aspect. Scale bars: **A** = 50 µm. **B** = 100 µm. **C** = 20 µm. **D, E**, **Insert in A**, **Insert in C (1)** = 10 µm. **Insert in C (2)** = 1 µm.

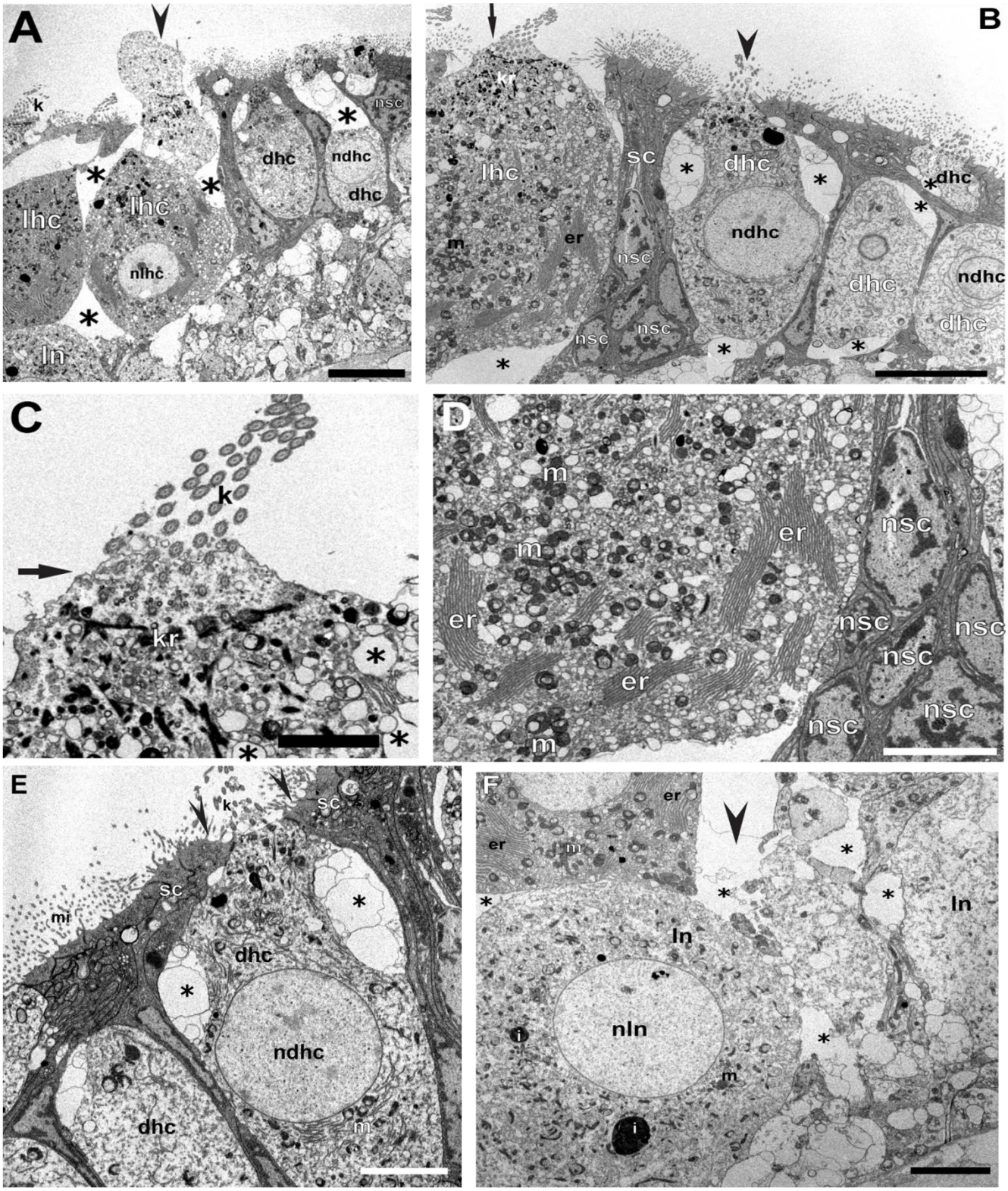

FIG. 7.7. TEM. *Octopus vulgaris* crista, sacrificed 48h after sound exposure. (**dhc**: small dorsal primary hair cell; **lhc**: regular row of large secondary hair cells; **vhc**: small ventral secondary hair cell; **sc**: supporting cell, **ln**: large first-order afferent neuron, **nhl**: nucleous of large hair cell, **ndhc**: nucleous of dorsal hair cell, **nsc**: nucleous of supporting cell, **nln**: nucleous of large neuron, **k**: kinocillia, **kr**: kinocillia root, **m**:microvilli, **er**: rough endoplamatic reticulum, **m**: mitocondrion). **A**: Drastic ultrastructural changes are seen into the macula sensory ephitelium.On the apical pole of the epithelium the cells are detached and a lhc has almost lost its damged apical pole (arrowhead). No kinocillia are visible on it and the inner material is protruding to the statocyst cavity. At the base of the sensory epithelium, the swollen nerve plexus is shown. Large vacuolization is visible in the epithelium (asterisk) **B**: In other fragment of acoustically damaged epithelium, the arrangement of hair cells and supporting cells is destructurated. The apical pole of a lhc is protruding into the statocist cavity (arrow) and a

dhc is starting its extruding proces (arrowhead). Note the large vacuaolization of the epithelium cells (asterisk). **C**: Detail from the apical pole of a lhc. The cell material is extruded through the kinocillia anchoring (arrow). Note the kr destructuration and the cell vacuolization (asterisk). **D**: Detail from the contact between a damaged lhc and a sc. The nucleous of sc are constricted and the damaged mitocondrion and destruturated rough reticulum cisternae (er) on the lhc are visible. **E**: A dhc is constricted by two sc (arrowheads) in order to make a continuous scar formation isolating the plate of the hair cell from its fully cell body. Note the large cell vacuolization (asterisk) due to the material extrusion. **F**: Contact between a lhc and a swelling ln. The rupture of the nervous plexus (arrowhead) and its large vacuolization (asterisk) are visible. Damaged mitochondria and dark inclusions (i) are shown. **Scale bars: A, B** = 10 µm. **C** = 2 µm. **D, E, F** = 5 µm.

7.3.3.3 Lining ephitelium of Illex coindetii statocyst

A part from the specific sensory areas (*msp* and *crista*), the individuals showed acoustic trauma, affecting a wide range of statocyst inner ciliated areas (fig. 7.9), even on individuals immediately sacrificed after exposure but particularly on the individuals sacrificed 48h after exposure. All tested groups showed lesions in some areas of the lining epithelium of the cavity which consists of flat hexagonal cells with oval nuclei. In some areas of the statocyst, bundles of cilia emerge between the epithelial cells. The lesions in this epithelium consisted basically in the extrusion of the cellullar material into the statocyst cavity. In individuals sacrificed at 48 hours after sound exposure there was a massive extension of holes folowing the extrusion of inner cellular material. The cilia and microvilli were bent flaccid and disorganized in almost the totally samples. In individuals sacrificed 48h some individuals presented some bent, flaccid and expoiled microvilli.

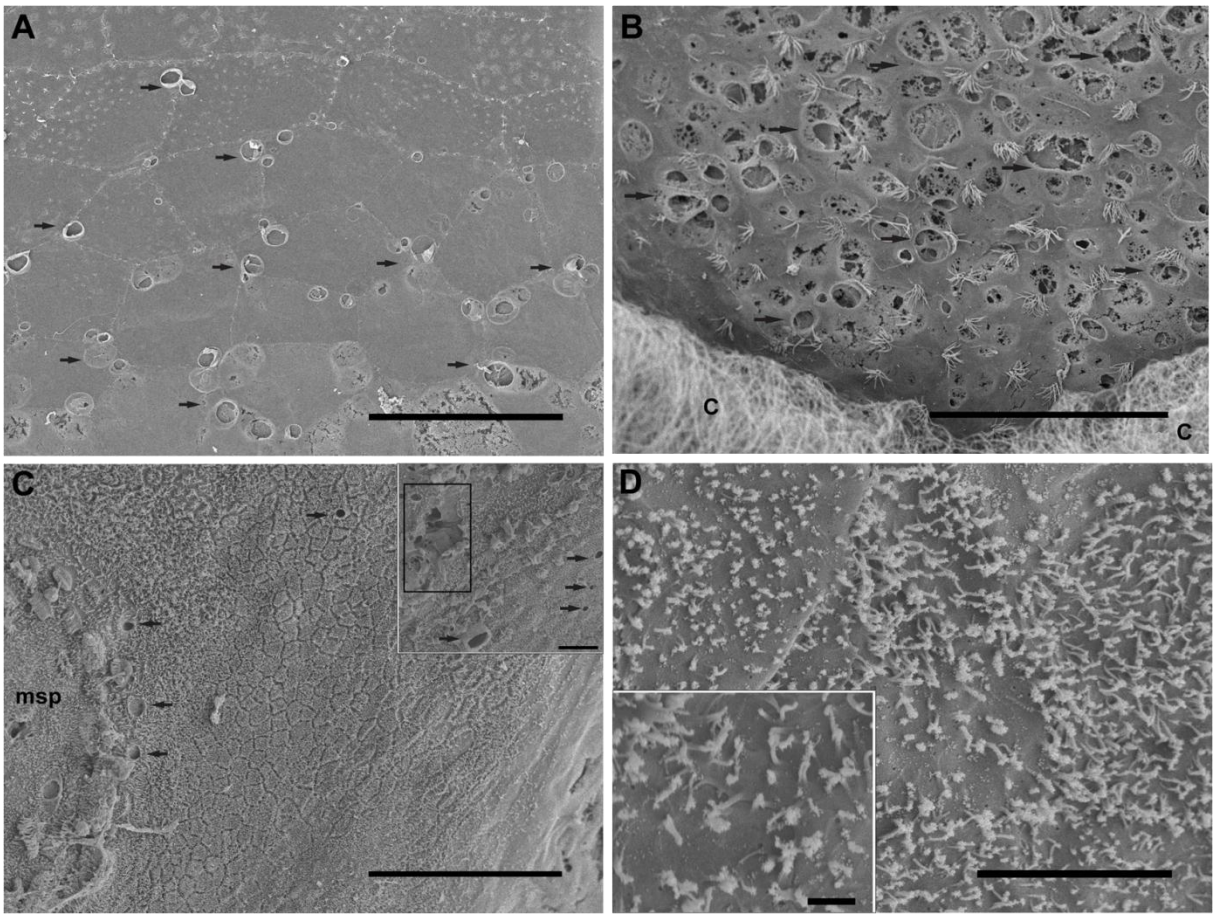

FIG 7.8. SEM. *Illex coindetii* lining epithelium of the statocyst cavity, 48h after sound exposure. A: The cilia are missing and some cells exhibit holes (arrows). **B**: Note the holes (arrowheads) in the epithelial cells and the bending and flaccid cilia (arrows) on an area near the crista-cupula system (c). **C:** Near the *macula statica princeps (*msp) an extense area shows very high density of cilia covering all the well preserved surface of the flat hexagonal cells of the lining epithelium. In some parts spherical holes (arrows) are visible. **Insert**: arrows show the spherical holes on the epithelium. Some zones are highly damaged (squares). **D:** damaged microvilli form a perimeter surrounding the

hexagonal cells. **Insert**: Detail from D. Bending, flaccid and expoiled microvilli. **Scale bars**: **A, B, C**= 50 µm. **D** = 5 µm. **Insert in C**= 10 µm. **Inserts in D** = 1 µm.

7.4. Discussion

In this chapter images of non-previously described statocyst ultrastructure of *Illex coindetii* are shown. Further analysis of different live stages of *Illex* are needed to a better description of these ultraestructures.

The observation of the statocyst ultrastructure of *Illex* showed a non-previously described feature in cephalopods: the center of the macula princeps presented no hair cells. In other species, it was demonstrated that the macula grows by accreting rings of sensory cells from the center to the periphery of the sensory epithelium (Stephen and Young, 1982). Two explanations can be discussed here: either there is never any hair cell in the center of the macula during the whole Illex live, or rings of hair cells grow iin this species from the periphery to the center of the macula.

In terrestrial vertebrates (including humans) exposure to very high sound pressure levels may result in permanent hering loss, because the sound destroys sensory hair cells of the inner ear and fractures the bones of the middle ear (Patterson and Hamermik, 1997; Henderson et al., 2008). Exposure to lower levels for longer periods can also lead to permanent hearing loss through death of sensory cells (Hamernik et al., 1994).

Data on the effects of exposure to sound on fishes are very limited as compared with data for terrestrial vertebrates. Some works reported that sound can damage sensory cells in ears of some fish species (Enger, 1981; Hastings, 1995; Hastings et al., 1996; McCauley et al., 2003). However, no study has yet determined the relationship between damage of hair cells and permanent hearing loss in fishes. The work of Enger (1981) found that some sensory cells lost their ciliary bundles in the ears of cod (*G. morhua)* after 1-5h exposure to pure tones (100-110 dB above threshold in its most sensitive hearing frequence range), examining the sensory ephitelia by SEM. In Hastings (1995, 1996) it was reported damage to auditory hair cells in hearing (*C. auratus*) happened after exposure to continous tones (120-140 dB above threshold in its most sensitive hearing frequency range) for approximately 2h, and in oscar (*A. ocellatus)* after 1h of continuous exposure to a 300 Hz pure tone. In this last case the damage was only visible in animals that were alive four days after sound exposure. This fact allows concludimg that damage caused from exposure to sound takes some time to become visually apparent. McCauley 2003 shows by electron microscopic techniques destruction of hair cells in ears of pink snapper (*P. auratus*) after exposure to sound of a seismic air gun. The damage observed in these four species was only a visual manisfestation of what may have been a much greater effect. Temporary deafness could result in a fish being unable to respond to presence of predators and to locate preys and mates.

Because of the very scarce data, it is necessary to be extremely cautious when extrapolating results between fish species or received signals, because of the differences in the hearing systems, limited data of precise stimulus (pressure and/or particle velocity) and the time course and frequency components of the signals.

The same considerations may be aplicated to the studies on the effects of sound on sensory epithelia of cephalopod statocyst. No data were avallaible until this moment. This thesis presents the first morphological and ultrastructural evidence of a massive acoustic trauma, not compatible with life, induced on individuals belonging to four cephalopod species under low frequency sound controlled exposure experiments resulting in permanent and substantial alterations of the sensory hair cells of the statocysts, the structures responsible for the animals' sense of balance and position.

The same lesions and the same increasing effect with time as in *Sepia* were observed in these three species. Interstingly, *Illex*, being a deep-sea species appeared to be affected at a same level. This would mean that the sensitivity to low frequency noise would equally alter sensory organs of any species of cephalopods, no matter their foraging ecology. However, because the experimental conditions placed the animals very close to the surface, the question remains on whether this species would also be equally affected when exposed to noise at greater depths.

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8. Effects of low frequency sound exposure to the epidermal line system of *Sepia officinalis, Loligo vulgaris and Illex coindetii* hatchlings by Scanning Electron Microscopy: Preliminary Findings

8. Effects of low frequency sound exposure to the epidermal line system of *Sepia officinalis, Loligo vulgaris and Illex coindetii* hatchlings by Scanning Electron Microscopy: Preliminary Findings

8.1 Introduction

Late embryonic stages and hatchlings of cephalopods have epidermal lines (Naef, 1928; Sundermann, 1982, 1983, 1997; Lenz et al., 1995; Lenz, 1997; Villanueva and Norman, 2008), consisting of ciliated primary sensory hair cells that carry cilia with an internal 9 x 2 + 2 tubules content (Sundermann, 1983; Hanlon and Budelmann, 1987) and non-ciliated accessory cells, running in anterior-posterior direction and located on the arms, head, anterior part of dorsal mantle and funnel. Cuttlefishes and squids show eight epidermal lines on their heads, two dorsally and two laterally (one above and one below the eye) which continue on the arms (except de line below the eyes) (fig. 8.1B), an additional paired short fifth line on the ventral side of the head (Sundermann, 1983) and a broad band of ciliated cells on the ventral funnel surface.

In *O. vulgaris* hatchlings, the unique line of the funnel is located along its midline. The other epidermal lines (dorsal, dorsoventral, ventrolateral and ventral) are paired occupying both sides of the head and right and left arms (fig. 8.1A). As opposed to cuttlefish and squids, the epidermal lines found in octopus paralarvae have not been reported in adult octopuses.

Each epidermal line consist of ciliated cells that carry between few and more than 100 kinocilia per cell, each 10-20 µm in length. Each hair cell is surrounded by several smaller supporting cells that carry only microvilli (Sundermann, 1983). Each hair cell is polarized in one direction, according to the orientation of the cilia's basal feet and the 9 x 2 + 2 tubules content (Budelmann et al., 1997). The hair cells are primary sensory hair cells and their axons run underneath the hair cells (Sundermann, 1983).

Vibration receptors and sound perception

There is a considerable lack of information concerning the cephalopod's reception of sounds (Packard et al., 1990; Bleckmann et al., 1991; Bullock, 1991; Budelmann et al., 1995; Hu et al., 2009). Cephalopods are sensitive to vibration stimuli, and the most important receptors systems in that respect are statocyst receptor sytem and the lateral line analogue system. This "lateral line system" constitutes a mechanoreceptive organ that is analogous to the amphibian and fish lateral lines. Its ciliated cells are sensitive to local water movements and are able to perceive hydrodynamic pressure. Stimulation of the lines with artificial water displacements of defined frequency and amplitude evoke receptor potentials with features very similar to the lateral microphonic potential of fish (Budelmann and Bleckmann, 1988b; Bleckmann et al., 1991; Budelmann et al., 1997).

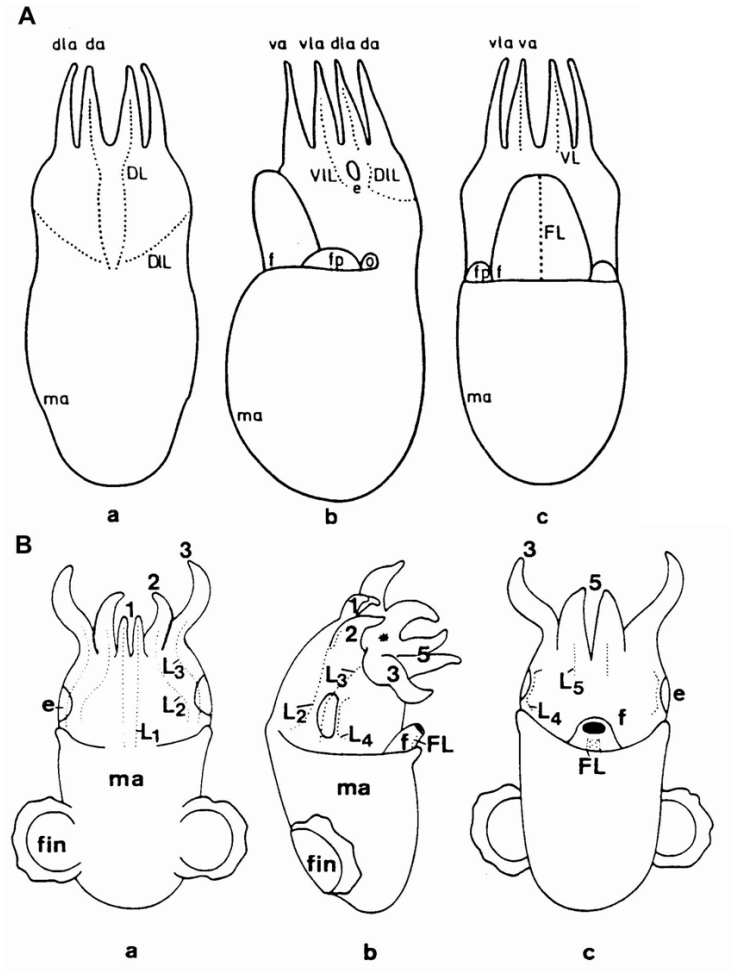

FIG. 8.1. Schematic drawings, showing the arrangements of the epidermal lines of *O. vulgaris* (A) and *S. affinis* (B). (a: dorsal, b: lateral, c: ventral sight, 1-5: number of arms; da: dorsal arm, DL: dorsal line, dla: dorsolateral arm, DlL: dorsolateral line, e: eye, f: funnel, FL: funnel line, fp: funnel pocket, L1-5: lateral lines, ma: mantle, o: olfactory organ, va: ventral arm, VL: ventral line, vla: ventrolateral arm, VlL: ventrolateral line) (Lenz et al., 1995).

Acoustic impact

The possible effects of an acoustic impact on cephalopods are reviewed in previous chapters. Here we review our knowledge on the effects of sound exposure to lateral lines of fishes. The lateral line system of fishes consist a set of receptors, found on the surface of the body, which detect water motion near of the fish. Mechanical stimulation of the lateral line of clupeids may cause damage by decopling the cupulae from the neuromasts (Denton and Gray, 1993). Loss of the attachment between the cupula and neuromast would result in dysfunction of the lateral line. Only one study (Kostyuchenko, 1973) reported damage on neuromasts (sensory structures with sensory hair cells) of the lateral line system of cod (*G. morhua*) and Atlantic hering (*C. harengus*) larva under seismic air-gun sounds exposure.

Owing to a lack of previous reports concerning acoustic trauma in cephalopods, a comprehensive study was therefore needed to assess the direct effects of acoustic impact on these species. In this study, we set out Controled Experiment Experiments to determine the lesions which would occur on the ciliated primary sensory hair cells arranged in the epidermal lines of the Mediterranean *S. officinalis, L. vulgaris* and *I. coindetii* hatchlings when exposed to sound overstimulation by low frequency sounds. Since no data was available on the effects of acoustic overstimulation in these species, the main objective of this study was to determine if the exposure to sounds would trigger lesions in the sensory cells of the epidermal lines.

8.2 Material and Methods

8.2.1 Cephalopod individuals

Hatchlings of *Sepia officinalis*, *Loligo vulgaris* and *Illex coindetii* from the Catalan Coast (NW Mediterranean Sea) were used in this study. Egg mass of *S. officinalis* and *L. vulgaris* were obtained over a period of 2 years, between February of 2008 and August of 2010, and kept in a closed system of recirculating natural seawater (at 18-20ºC, salinity 35‰ and natural oxygen pressure) until hatch. *I. coindetii* hatchlings were obtained from experiments described in Villanueva et al. (2011) using *in vitro* fertilization techniques and kindly provided by the authors of this study (Marine Sciences Institute (ICM-CSIC). Part of these hatchling animals were used as controls and were kept in the same conditions as the experimental animals until we exposed the latter to noise, in an independent tank, sacrificing them respecting the same sequential process.

8.2.2. Sound Exposure Protocol

Sequential (at different times and seasons) Controlled Exposure Experiments (CEE) were conducted on hachtlings of *S. officinalis, L. vulgaris* and *I. coindetii*. An additional set of hatchlings of the same species were used as a control and sequentially processed (same procedure as with noise-exposed cephalopods) right after being caught, before and after the CEE. The egg masses were caught by local fishermen following the same protocol and transferred to our laboratory a few minutes after capture (our facilities are located in the Vilanova i la Geltrú fishing harbour).

After keeping the egg masses until hatch in a rearing tank (ranging from a few hours to a few days) the protocol included immediate exposure of the individuals. The general analysis protocol is contained in chapter 4.3 and the specific sound exposure protocol is contained in chapter 6.2.2.

Following exposure, the non-anesthetized individuals (exposed and control hatchlings) were processed to be observed under scanning electron microscopy at different intervals, ranging from immediately afterwards to 24 hours after exposure. Except for the animals sacrificed immediately after exposure, the rest of the individuals were put in maintenance tank.

It must be emphasized again here that the experiment was not set up to find specific threshold levels, but designed to investigate if cephalopods are subject to acoustic trauma when exposed to low frequency sounds, commonly encountered associated to human activities in all oceans. The transducer did not have a constant (or linear) response over the sweep which means that the levels of individual frequencies covered a wide range. The acoustic characteristics of the tank also added to differences in levels. The level measurements presented in this study are meant to provide a global characterisation of the received levels from frequencies within the sweep (157 dB re 1 µPa was the median received SPL with 50% of the peaks falling within +- 5 dB. The maximum received SPL was 175 dB re 1 µPa), and cannot therefore be taken as references to assess thresholds levels. For the same reasons, no quantitative data are presented.

8.2.3 Scanning electron microscopy (SEM)

The specific SEM protocol is contained in chapter 5.2.3 and 6.2.2.

8.3. Results

8.3.1 Structural and ultrastructural investigations of the epidermal lines sensory epithelium

8.3.1.1 Loligo vulgaris epidermal lines

Just after sound exposure (fig. 8.4A) in comparison with the same tissues from control animals (fig. 8.3), damage was observed in the epidermal lines by SEM analysis. Some hair cells had dramatically lost almost all kinocilia and the remaining were bent and flaccid. in animals sacrificed 18h after sound exposure (fig. 8.4B-D), some hair cells on some epidermal lines had totally, or in a considerable number (8.4 B), lost the kinocilia and rests of their roots were visible within the damaged epithelium or exhibited bent and flaccid or fused kinocilia. On the other hand other epidermal lines on the same individuals show hair cells with high density of kinocilia (fig 8.4B). Additional structures (olfactory organ) with high quantity of cilia were visible on these individuals (fig 8.4C, D), but lesions in this organ were not apparent.

Most samples from animals sacrificed 24h after sound exposure (fig. 8.4 E-F) the epidermal lines showed healthy appearance with upright kinocilia on the hair cells arrangements, although some hair cells exhibited fused kinocilia.

8.3.1.2 Sepia officinalis epidermal lines

Just after sound exposure (fig. 8.5 C) in comparison with the same tissues from control animals (fig. 8.5 A, B), damage was observed on the epidermal lines by SEM analysis. Some hair cells had lost a number of kinocilia or showed bent and flaccid kinocilia.

On animals sacrificed 24h after sound exposure (fig. 8.5 D-F), some hair cells had totally, or in a considerable number (8.5 D-E), lost the kinocilia and rests of their roots were visible within the damaged epithelium or exhibited bent and flaccid or fused kinocilia (fig. 8.5F).

8.3.1.3 Illex coindetii epidermal lines

This study shows the first published images of epidermal lines of *I. coindetii paralarvae* (fig. 8.6). No previous studies have been previously conducted. The distribution of epidermal lines is very similar to other described decapods species such as *Sepiola affinis, Sepia officinalis and Loligo vulgaris* (fig.8.1B, five pairs of bilaterally symmetrical lines in head and arms, and an additional unique line on the funnel located along its middle). The epidermal lines are highly dense and present different kinocilia distribution and density on the hair cells (fig. 8.6 D, G, H, I).

Just after sound exposure (fig. 8.7 A, B) in comparison with the same tissues from control animals (fig. 8.6), damage was observed in the epidermal lines by SEM analysis. Some hair cells had totally lost their kinocilia and exhibit rest of their roots, or showed bent and flaccid kinocilia. Some holes on the sensory epithelium of the epidermal lines were visible.

In animals sacrificed 24h after sound exposure (fig. 8.7 C-F), some hair cells had totally, or in a considerable number, lost the kinocilia and rests of their roots were visible within the damaged epithelium or exhibited bent and flaccid or fused kinocilia (fig. 8.7 C-E). In other samples, the epidermal lines show healthy appearance with upright kinocilia on the hair cells arrangements (8.7F).

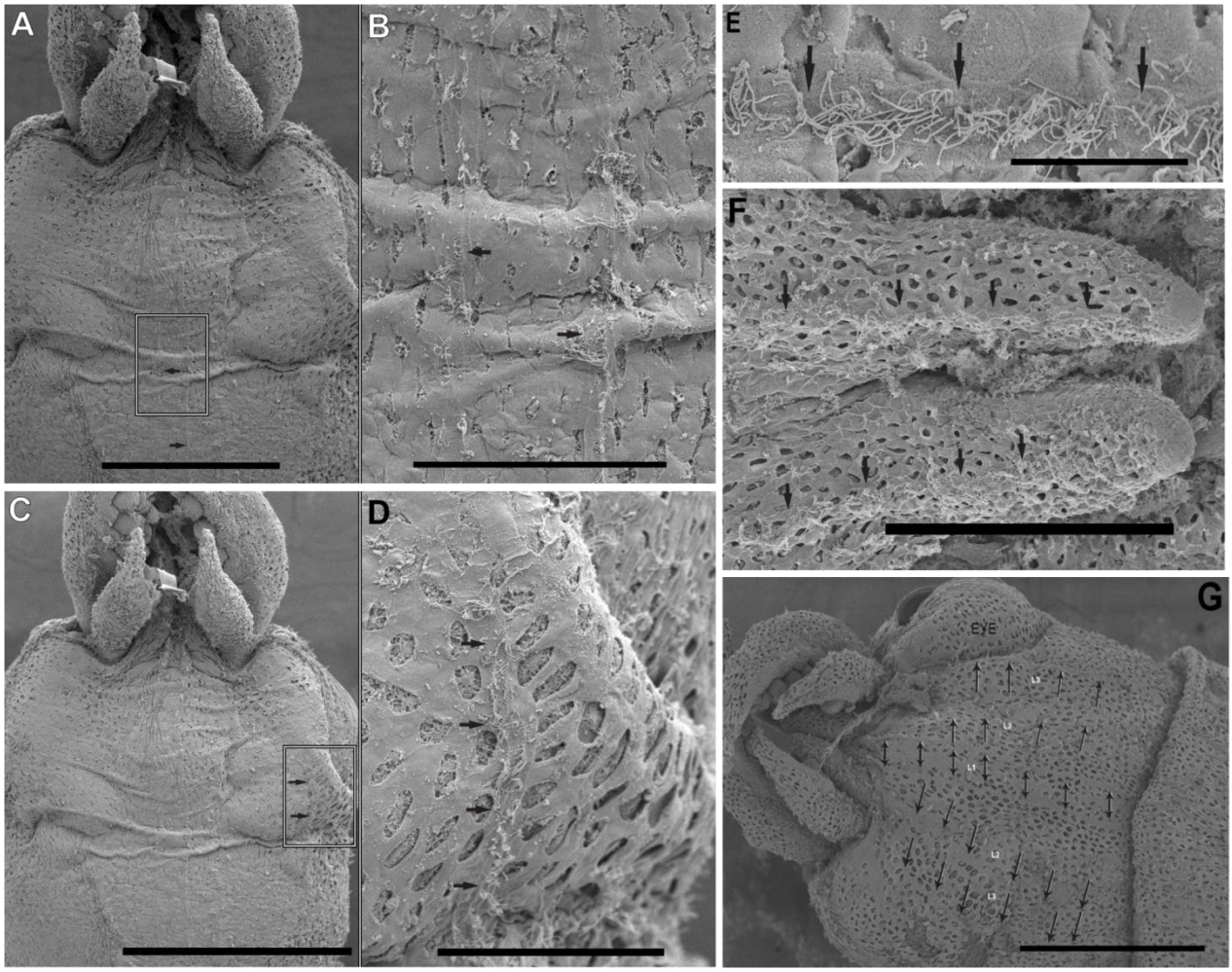

Fig. 8.2. SEM. *Loligo vulgaris* epidermal lines on head an arms. Control animals. A: Dorsal view. Arrows indicate the two lines 1. **B**: Detail from A (square). L1 (arrows). **C**: Dorsal view. Arrows indicate Line 3 running above the eye. **D**: Detail from C square. L3 (arrows). **E**: Arrows shows a detailed view from L1. Note the regular arrangements of the kinocilia hair cells. **F:** Ventral side. Arrows indicate two Lines 5. **G**: Dorsal side. Arrows show L1, L2 and L3. **Scale bars: A, C, G** = 500 µm. **E, F** = 200 µm. **B, D** = 100 µm.

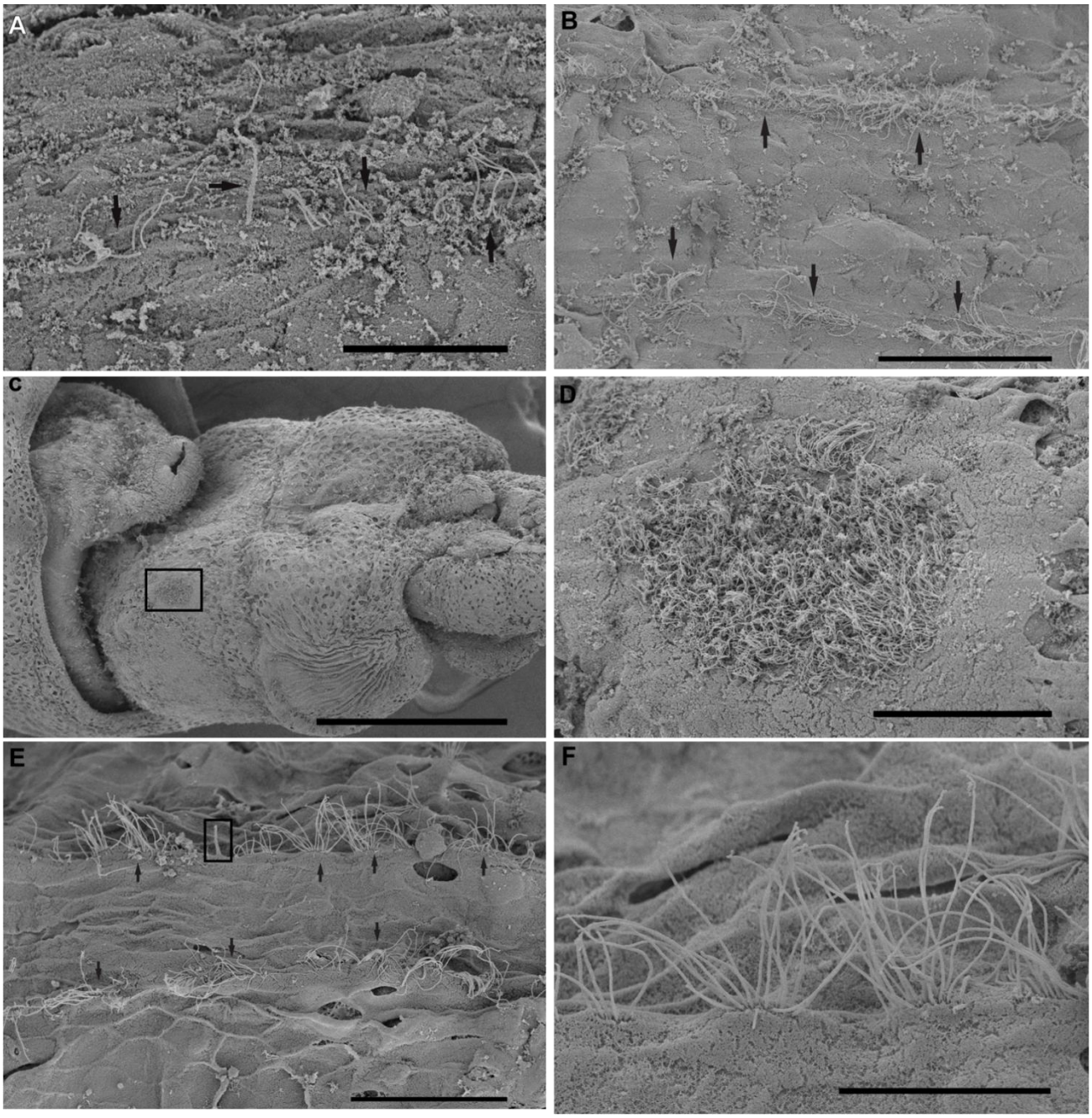

Fig. 8.3 SEM. *Loligo vulgaris* epidermal lines, sacrificed immediately (A), 18h (B-D) and 24h (E-F) after sound exposure. A: L1. Some hair cells have almost totally lost their kinocilia (arrows). The remaining kinocilia are bent and flaccid. **B**: On the top of the image arrows indicate L1, which present a higher density of kinocilia respect the other L1 (lower arrows). **C**: Larva ventral view shows the olfactory organ (square) near the funnel. D: Detail from the square of C. Note the high density of the cilia of olfactory organ. **E**: The two L1 lines (arrows) show a healthy appearance with upright kinocilia of the hair cells arrangements. On the square some kinocilia are fused. **F**: Detail from E. Hair cells' kinocilia of L1. **Scale bars: A, F** = 20 µm. **B, D, E** = 50 µm. **C** = 500 µm.

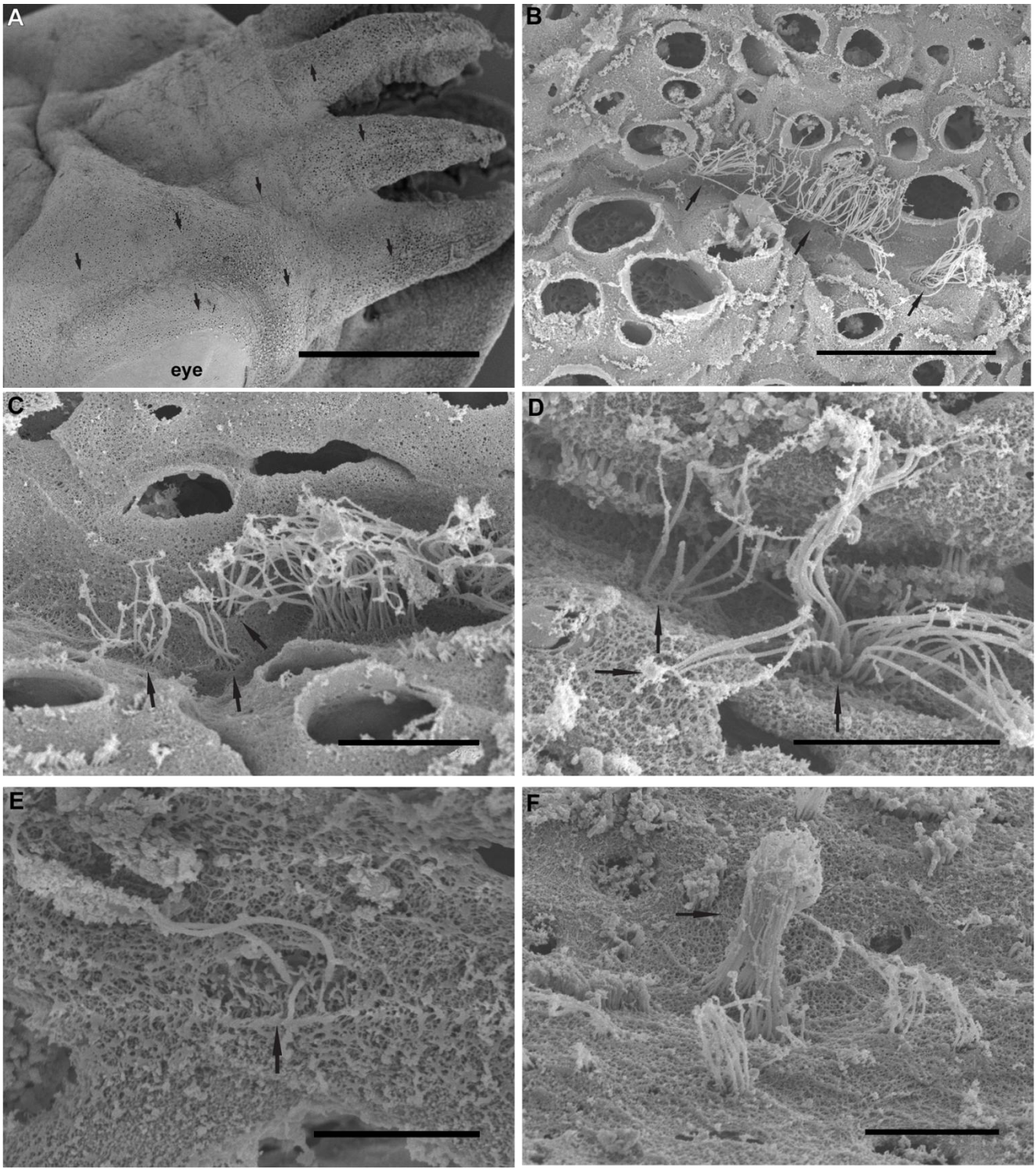

FIG. 8.4. SEM. *Sepia officinalis* epidermal lines. Control animals (A, B), sacrificed immediately (C) and 24h (D-F) after sound exposure. **A**: Arrows show lateral lines on three arms and above the eye (L1-L3). **B:** Detail from the kinolicia on epidermal lines hair cells. **C**: Arrows indicate hair cells with missing kinocilia. **D**: The hair cells have lost a number of their kinocilia (arrows) and the remaining are fused or bent and flaccid. **E**: Hair cell that has almost totally lost its kinocilia and rests of their roots are visible. **F**: An hair cell exhibits fused kinocilia. **Scale bars: A** = 1mm. **B** = 30 µm. **C, D, F** = 10 µm. **E** = 5 µm.

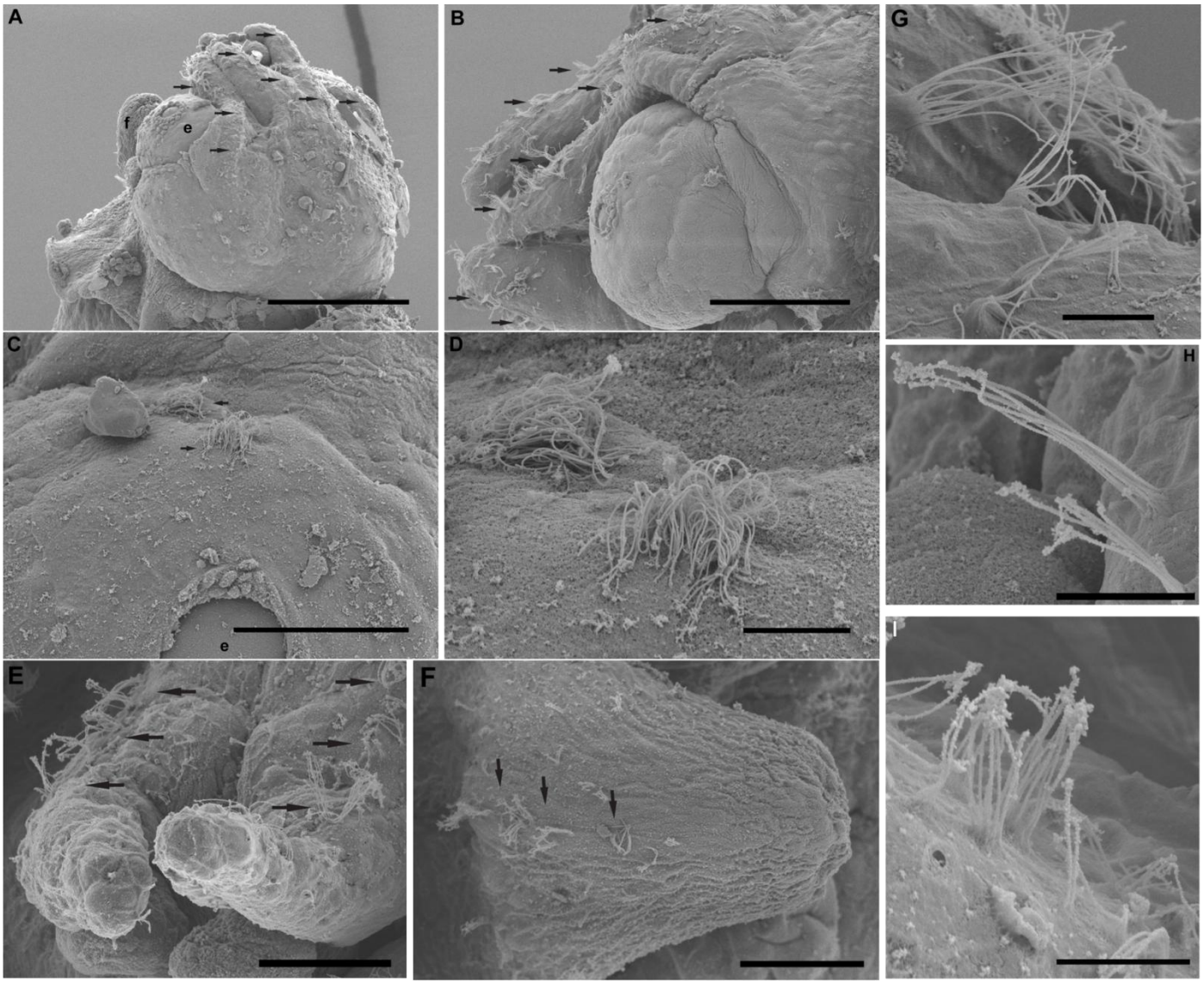

Fig. 8.5. SEM. *Illex coindetii* epidermal lines. Control animals. **A:** Arrows show the four lines on dorsal side of the head. **B**: Detail from A, arrows indicate the highly dense epidermal line. **C**: Arrows mark the bundles of kinocilia of the Line 3 running above the eye (dorsal side). **D**: Detail from C. Note the high density of the kinocilia of the hair cells. **E**: Arrows indicate the two Lines 5 on the ventral side. **F:** Unique line of the funnel located along its midline. **G, H, I**: Three different views of the kinocilia distribution on the epidermal lines hair cells. (e: eye, f: funnel). **Scale bars: A** = 200 µm. **B** = 100 µm. **C, E, F** = 50 µm. **D, G, H, I** = 10 µm.

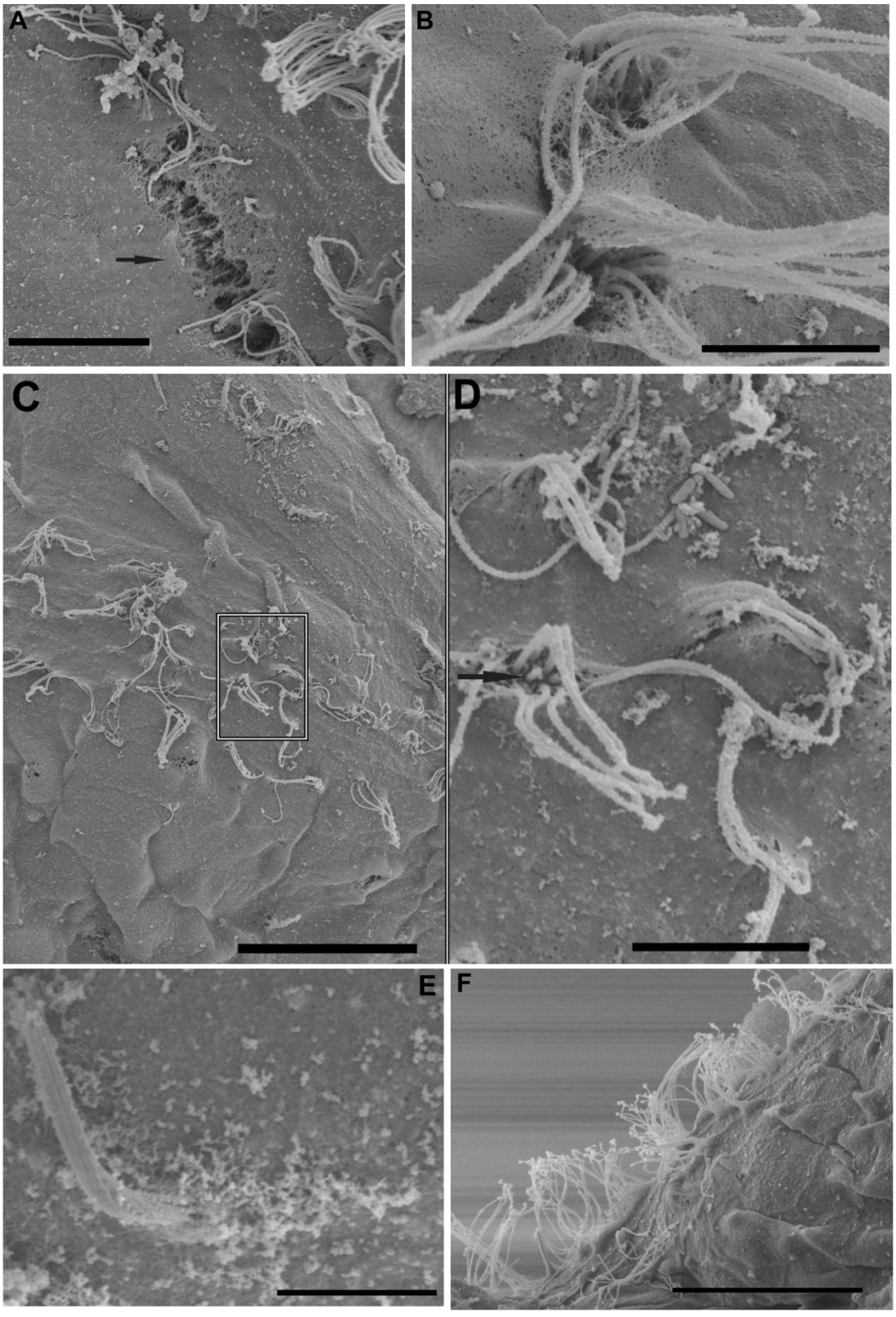

Fig. 8.6. SEM. *Illex coindetii* epidermal lines, sacrificed immediately (A, B) and 24h (C-F) after sound exposure. **A**: Some hair cells have totally lost their kinocilia and a hole on the sensory epithelium of the epidermal line L3 shows rests of their roots (arrow). The remaining kinocilia are bent and flaccid. **B**: Detail of a damaged bundle of kinocilia showing its broken basal part. **C**: Hair cell of epidermal line L3 shows bent and flaccid kinocilia. **D**: Detail from the square of C. Hair cell kinocilia are bent and flaccid. Some rest of roots (arrow) of lost kinocilia are visible. **E**: The remaining kinocilia of a damaged hair cell are fused. **F**: L1 line shows a healthy appearance with upright kinocilia of the hair cells arrangements. **Scale bars: A, D** = 5 µm. **B, E** = 5 µm. **C** = 25 µm. **F** = 30 µm.

8.4. Discussion

We divided the controls and experimental animals into two groups: sacrificed immediately after sound exposure and 24h after sound exposure. An additional set of *L. vulgaris* paralarvae was sacrificed 18h after sound exposure. While the controls showed no lesions in the epidermal lines, all the exposed individuals presented the same lesions, but the effects evolution over time was different in the different species of studied cephalopods. On *L. vulgaris* paralarvae, damage was observed on the epidermal lines immediately after exposure. A high number of hair cells had lost kinocilia or presented fused, bent and flaccid kinocilia. But, at difference of the lesions observed on statocysts structures (chapter 6, 7), the lesions on the paralarvae epidermal lines weren't gradually more pronounced in individuals after 18 and 24 hours. Some areas of the epidermal lines of the exposed animals seem to be more highly dense over time, probably due to high regeneration rate proper of the paralarvae tissues (Budelmann et al., 1997; Vickery et al., 2001). In addition, some typical ciliated structures not linked to sound perception, e.g. chemoreceptors, such as the olfactory organ with high cilia density and located near the funnel on *Loligo* paralarvae, were visible on all groups of samples. No lesions were visible on it. Only in some hair cells fused kinocilia remained even 24h after sound exposure.

In *I. coindeti* damage was observed in the epidermal lines immediately after exposure. These lesions were suspected to be more dramatic (even immediately after sound exposure, some hair cells had totally lost their kinocilia and presented holes that showed rests of their roots; the remaining kinocilia were fused or bent and flaccid) than in *L. vulgaris* epidermal lines, but as difference withthis species the recovery of the damage was not as evident. Some areas of the epidermal lines of the exposed animals seemed to be highly dense over time, probably due to a high regeneration rate of the paralarvae tissues, in a similar way as observed on the *L. vulgaris* paralarvae. However, 24h after sound exposure large areas showed damage consistent with an acoustic trauma.

In *S. officinalis* juveniles, damage was also observed in the epidermal lines immediately after exposure. A high number of hair cells had lost kinocilia or presented fused, bent and flaccid kinocilia and the lesions in the juveniles epidermal lines were gradually more pronounced in individuals after 24 hours as in the case of the adult statocyst lesions (chapters 6, 7). The different evolution in *S. officinalis* juveniles and *L. vulgaris* and *I. coindetii paralarvae* could be due to the different sizes of hatchlings from the three species which would correspond to different live stages and strategies. The larger size of cuttlefish hatchlings could be associated to a lower rate of regeneration. In addition this greater size would be consistent with the fact that cuttlefishes are a benthic species immediately after the hatching, whereas the other two species would present a planktonic phase before they sufficiently grow to become juveniles. This different lifestyle may involve differences in the typology of the lesions produced by the noise impact due to variations in the perception because of the different sound propagation in different media. The absence of comparative studies on the rate regeneration of different cephalopod larval species, in addition to the necessity to determine a possible relationship between sound and lesions, and its possible recovery due to the high regeneration rate on cephalopods hatchlings, could open new lines for future research, which would contribute to obtain results with possible medical applications.

In the avian cochlea and vestibular organs and in the vestibular organ of mammals, posttraumatic hair cell regeneration was shown to occur after either acoustic trauma or drug poisoning (Corwin and Cotanche, 1988). Several mechanisms are believed to be involved in such regeneration, including proliferation followed by differentiation of non-sensory epithelial cell, direct transdifferentiation of supporting cells into hair cells, and reparation of damaged hair cells. These processes occur generally within one or several weeks after hair cells death. In the present study, individuals were not collected after 24 hours following the acoustic trauma. This

is probably a too short delay to observe the potential occurrence of regenerative processes due to cell division and differentiation.

These preliminary results constitue a basis for future research, which would help to determine qualitatively the extent of injuries caused by exposure to sound and its subsequent recovery in the larval epidermal lines of cephalopods. It may also open lines of research to determine whether some of the epidermal lines are more sensitive than others to sound exposure. It would also be appropriate to conduct an analysis of the effects of sound exposure on the hatchlings statocysts. These results would certainly contribute to gather datato help determining whether acoustic trauma is triggeredby pressure waves or particle motion effects, since there is a still a controversial discussion going on sound perception in cephalopods (see chapter 5.1).

Further experiments are needed to determine the timing of the suspected healing process more precisely. Reparation and regeneration occur in the vestibular system of bird and mammals and in the auditory system of mammals, when apoptosis is not involved. The morphological diference of the cilia, which are microvilli in the vestibular and auditory system of birds and mammals while they are kinocilia in cephalopods epidermal lines, require also further investigation to understand the kinocilia repair and regrowth process that would happen in cephalopods.

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9. Proteomic studies of statocyst endolymph for the assessment of acoustic trauma on common cuttlefish *(Sepia officinalis)*

9. Proteomic studies of statocyst endolymph for the assessment of acoustic trauma on common cuttlefish *(Sepia officinalis)*

9.1. Introduction

Although several studies were conducted on the structural and biochemical composition of cephalopod statoliths, there are very few initiatives focused on the endolymph composition. Statoliths, calcified biomineral structures composed of calcium carbonate crystallised as aragonite with a small percentage of organic material that has been ascertained to be protein (Radtke, 1983), are found in the gravity receptor system of the cephalopods. Statoliths had becomed a useful tool to provide information on aging (Villanueva, 1992; Lipinski, 1993), food (Zumholz et al., 2006a), environmental conditions (Durholtz and Lipinski, 2000; Zumholz et al., 2007), timing of exposure to pollutants, or timing of migrations (Ikeda et al., 2003). They grow throughout the lifetime of the individuals and deposit microscopically visible daily increments. Since the two theoretical models (Morris, 1988, 1991; Lipinski, 1993) had been hypothesized to establish the physiological mechanisms by which cephalopods form statoliths, some works have contributed with new data on mineralization process (Durholtz et al., 1997; Zumholz, Hansteen, Piatkowski, 2006b). Morris proposed that the low concentration of Mg^{2+} ions in the statocyst lymph allows the $CaCO_3$ precipitation in the form of aragonite, this process being controlled by the pH of the endolymph and organic matrix. Lipinski hypothesised that strontium is responsible for the definitions of growth layers and increments in the statoliths. Only a few works were focused on the purifying and characterizing the organic matrix proteins of statolith (Durholtz, Kretsinger, Lipinski, 1999) as well as the quantification of protein concentration on statolyth and statocyst endolymph (Bettencourt and Guerra, 2000).

The most common application of proteomics involves the use of electrophoresis for comparative mapping of expression of proteins from populations of cells or tissues under conditions of treatment as opposed to those that have not been subjected to treatment. Two dimensional-difference gel electrophoresis (2D-DIGE) is a form of gel electrophoresis where up to three different protein samples can be labeled with fluorescent dyes, prior to two-dimensional electrophoresis. The important aspect of this technique is its ability to label two or more samples with different dyes and separate them on the same gel, eliminating gel-to-gel variability making the analysis more accurate. Following the separation and statistical analysis by DIGE, the identification of the differentially expressed proteins is performed usually by a combination of enzymatic digestion and peptide analysis by mass spectrometry. Identification by peptide mass fingerprinting using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF) is often the preferred instrument because it allows a high sample through put. The combination of two techniques like 2-DE followed by MALDI-MS allows the identification and sequencing of proteins before and after undergoing traumatic situations. There are several papers published in this regard like those engaged in the study of several diseases (Haas et al., 2006; Nedelkov et al., 2006)

Although the advent of contemporary proteomic technologies has ushered in definite advances to the field of auditory research (Patterson and Hamermik, 1997; Zheng et al., 2006; Coling et al., 2007; Jamesdaniel et al., 2008; Jamesdaniel, Salvi, Coling, 2009), this is the first time that proteomic techniques are used on the determination of the effects of the acoustic impact on cephalopods. These technique are particularly interesting for this project because they allow the comparison of two different situations, to analyze the effects of acoustic trauma at molecular level, comparing control individuals of cephalopods with those that suffer acoustic impact. The endolymph analysis under situations of acoustic stress allow us to establish the effects at molecular level, and identify the proteins especially sensitive to this type of trauma, and then analyze the change of the initial proteomic map of the tissue in situations of acoustic stress.

Damage on sensory ephiteliums of the cephalopod statocysts, which could be induced by metabolic exhaustion resulting in distortion of the homeostasis of statocysts, were exhibited on our previous work (André et al., 2011). An analysis of the protein fraction of the endolymph before and after of the sound exposure is needed. In this study, we set out to determine the changes on the protein composition of the endolymph which would occur on the statocysts of the Mediterranean common cuttlefish *S. officinalis* when exposed to sound overstimulation by low frequency sounds.

9.2. Material and Methods

9.2.1 Cuttlefish individuals

Fifty two adult and young individuals from *S. officinalis* (mantle length 11-18 cm) were obtained from the Catalan Coast (NW Mediterranean Sea) over a period of 2 years, between February of 2008 and August of 2010, and kept in a closed system of recirculating natural seawater (at 18-20ºC, salinity 35‰ and natural oxygen pressure) consisting of 2 mechanically filtered fiberglass reinforced plastic tanks of 2000L capacity, that were connected to each other . An additional set of live adult individuals (n=60) was used as a control and sequentially processed (same procedure as with noise-exposed cephalopods) right after being caught, before and after the CEE. In chapter 4.3 and 6.2.1, a detailed description can be found on the maintenance of individuals.

9.2.2 Sound Exposure Protocol

The same sequential CEEs were conducted as with other cephalopods spp. (see chapters 4.3 and 6.2.2).

9.2.3 Dissection of statocysts and extraction of endolymph

In all experiments isolated head preparations, obtained by decapitation without prior anaesthesia, were used. The statocysts with the surrounding cartilage were removed and 10-40 µl of endolymph were extracted from every statocyst with the help of a microsyringe. Then the endolymph was frozen at -70ºC until required for protein analysis. The same protocol was applied both on the control individuals and on the animals affected by sweep sacrificed at different regular times (0h, 24h). Three independent biological samples of 100-150 µL for each treatment were obtained following this treatment corresponding to four animals per sample. All the animals were processed at 14:00-15:00h to minimize the daily variations of the protein concentrations on endolymph.

9.2.4 2D-DIGE

All samples were adjusted to 200µL with lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 2% w/v DTT, 40mM Tris-base) before to proceed to sonication (three pulses of 10 sec). Because of the high levels of salt present in the endolymph that could interfere in the focusing of the proteins, salt content was removed from the samples before running the gels (PlusOne 2-DE clean up kit, Amersham Biosciences, Little Chalfont, Bucks, UK).

Gel	Cy2	Cy3	Cy5
1	pool	Control-A	0h-A
2	pool	24h-A	Control-B
3	pool	Control-C	0h-B
4	pool	0h-C	24h-B
5	pool	0h-D	24h-C
6	pool	24h-D	Control-D

Table 9. 1. Experimental design

The protein content of three samples from each treatment (control, treated 0h and treated 24h) was quantified (RC DC Protein Assay, Bio-Rad, Hercules, CA) before samples were labeled with cyanine dyes (Cy3 or Cy5) by the addition of 400 pmol of Cy dye in 1 μL of anhydrous N,N-dimethylformamide per 30 μg of protein. An internal standard control, consisting of a pool of the same total protein amount of every sample, was labeled with Cy2 dye, using the same method (See table 9.1 for experimental design).

After 30 min of incubation on ice in the dark, the reaction was quenched by addition of 1 μL of 10 mM lysine and additionally incubated for 10 min. Samples were finally combined according to the experimental design, at 30 μg of protein per Cy dye per gel, and diluted 2-fold with IEF sample buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 2% w/v DTT, 2% v/v pharmalytes pH 3-10). The 2-DE was performed using GE-Healthcare reagents and equipment. First-dimension IEF was performed on IPG strips (24 cm; linear gradient pH 3-10) using an Ettan IPGphor system. Samples were applied via anodic cup loading on the strips previously incubated overnight in 450 μL of rehydration buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 1% v/v pharmalytes pH 3-10, 100mMDeStreak). After focusing at 67 kVh, strips were equilibrated first for 15 min in 6 mL of reducing solution (6 M urea, 100 mM Tris-HCl, pH 8, 30% v/v glycerol, 2% w/v SDS, 5 mg/mL DTT) and then in 6 mL of alkylating solution (6 M urea, 100 mM Tris-HCl, pH 8, 30% v/v glycerol, 2% w/v SDS, 22.5 mg/mL iodoacetamide) for 15 min, on a rocking platform. Second-dimension SDS-PAGE was run by overlaying the strips on 12.5% isocratic Laemmli gels (24.6 × 20 cm), casted in low fluorescence glass plates, on an Ettan DALTsix system. Gels were run at 20 °C, at constant power 2.5 W/gel for 30 min followed by 17 W/gel until the bromophenol blue tracking front reached the end of the gel. Fluorescence images of the gels were acquired on a Typhoon 9400 scanner (GE Healthcare). Cy2, Cy3 and Cy5 images were scanned at 488 nm/520 nm, 532 nm/580, and 633 nm/670 nm excitation/emission wavelengths, respectively, at a 100 μm resolution. Image analysis and statistical quantification of relative protein abundances was performed using DeCyder V. 6.0 software (GE Healthcare). Gels were poststained using the noncovalent fluorescent stain Flamingo (BioRad, Hercules, CA). Fluorescence images were then matched to those of the DIGE analysis. Protein spots of interest were excised from the gel using an automated Spot Picker (GE Healthcare). In-gel trypsin digestion was performed using autolysis stabilized trypsin (Promega). Tryptic digests were purified using ZipTip microtiter plates (Millipore).

9.2.5 Protein Identification by MS

Tryptic digests from excised 2D gels spots were analyzed by MALDI-TOF MS on an Ultraflex TOF-TOF Instrument (Bruker, Bremen, Germany). Samples were prepared using HCCA as matrix on anchor-chip targets (Bruker). Calibration was performed in the external mode using a peptide calibration standard kit (Bruker Daltonics). The spectra were processed using Flex Analysis 3.0 software (Bruker Daltonics). Peak lists were generated using the signals in the *m*/*z* 800-4000 region, with a signal-to-noise threshold of greater than 3. The SNAP algorithm

included in the software was used to select the monoisotopic peaks from the isotopic distributions observed. After removing *m/z* values corresponding to usually observed matrix cluster ions, an internal statistical calibration was applied. Peaks corresponding to frequently seen keratin and trypsin autolysis peptides were then removed. The resulting final peak list was used for identification of the proteins by peptide mass fingerprint. Mascot 2.2 program (Matrix Science Ltd., London, U.K.). Search parameters were as follows: trypsin cleavages excluding N-terminal to P, 1 or 2 missed cleavages allowed, cysteine carbamidomethylation set as fixed modification, methionine oxidation as variable modification, mass tolerance less than 50 ppm, monoisotopic mass values.

9.3. Results and discussion

9.3.1 Protein content

Each replicate that contained 100-150 µl of endolymph was obtained from four different individuals corresponding to eight statocysts. We measured an average of 11.7 ± 6.0, 17± 6.6 and 13.1 ± 2.0 µg protein per statocyst in control (N= 24), treated 0h (N=32) and treated 24h (N=24) respectively. These results are similar to that observed by Bettencourt and Guerra (2000) who found that the protein concentration in hemolymph of *S. officinalis* changed from 1.8 µg/µl in the morning to 0.7 µg/µL in the evening. In our studies all samples were processed at the same moment of the day (14h-15h) to avoid this variable and the measured concentration of protein varied from 0.8 to 1.13 µg/µL. No important differences were found in the protein concentration between treated and untreated individuals, and these slight differences were probably due to intrinsic differences and also to the considerable oscillations along the day described previously by other authors (Bettencourt and Guerra, 2000).

9.3.2 Gel differences on DIGE gels

Representative 2D-DIGE gels from the endolymph of untreated and treated individuals are presented in Fig. 9.1 Over 900 spots per gel for each treatment were detected in gel analysis with molecular masses ranging from 15 to 150 kDa, and isoelectric point between 3 and 10. The 2D-DIGE comparison of the location and volumes associated with each spot revealed that the level of the majority of proteins remains virtually unchanged in the control and 0h-treated (Fig. 2). Variation in spot intensities was much more manifest between 24 h treated and the other two treatments. The expression levels of 18 of the matched proteins were significantly different between treatments ($P<0.05$) (Fig 9.2-3). Those differentially expressed proteins could be distributed into four patterns (Fig 9.4): pattern I, two protein differentially expressed among all three samples; pattern II, two proteins differentially expressed between control (C) and 0h treated (T0) as well as control and 24 h treated (T24), but not sample T0 and T24; pattern III, five proteins differentially expressed in C-T24 but not C-T0 and T0-T24; and pattern IV, nine proteins differentially expressed in C-T24 and T0-T24 but not C-T0. As it could be expected, the most important differences were found between control and 24h treated. A total of 16 spots (5 in pattern III and 9 in pattern IV) were expressed differentially between both treatments, furthermore nine of them were also differentially expressed between 0h and 24h treated. We only found four proteins differentially expressed just after treatment (0h treated), however we could observe important differences just after noise exposition using TEM and SEM techniques (chapter 6) at the same moment. These result showed that changes in proteome expression were displayed later than structural changes. Nevertheless those changes in protein expression that appeared at 24 hours after treatment could appear before this period of time.

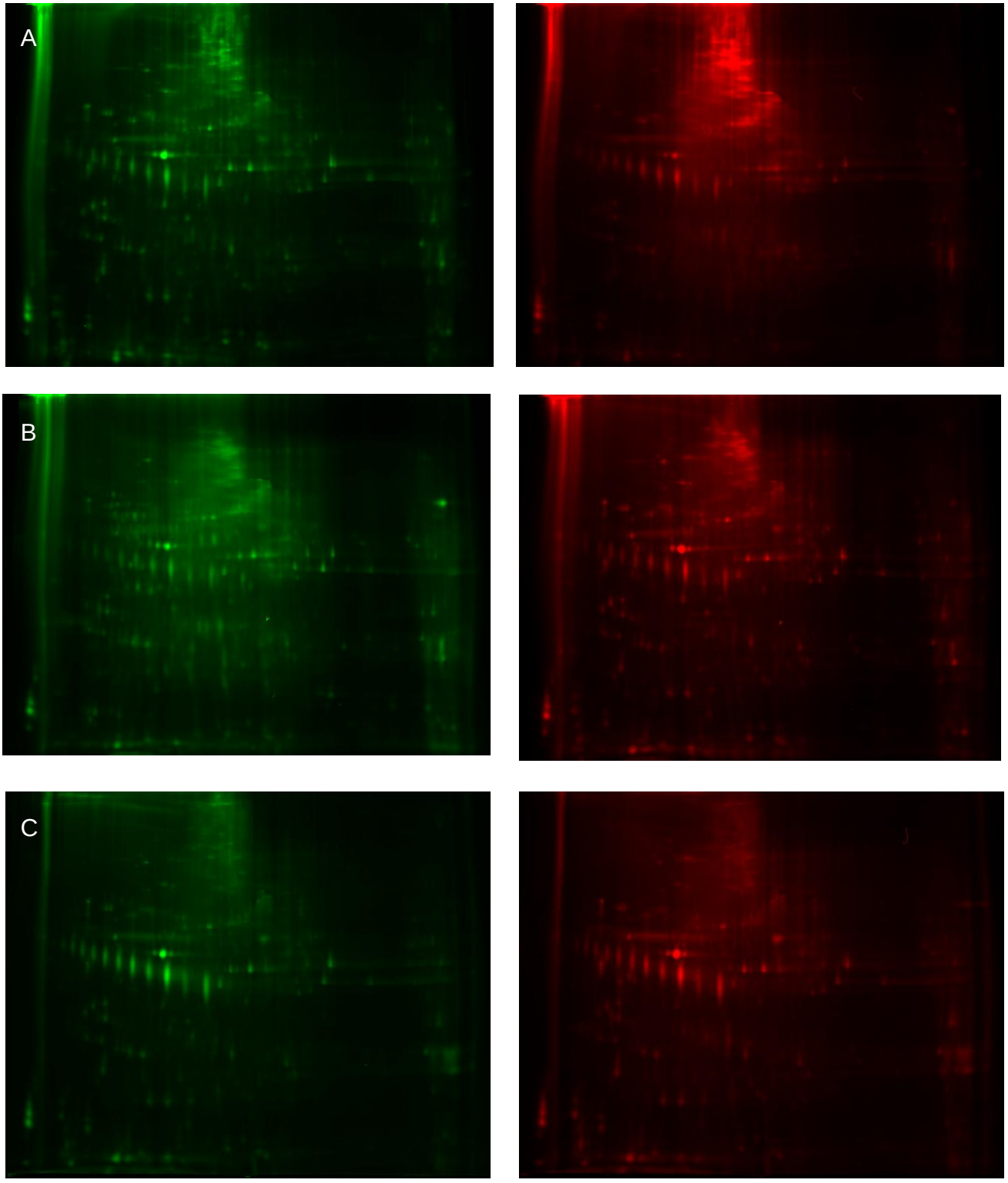

Figure 9.1. Two-dimensional difference gel electrophoresis (2D-DIGE) fluorescence images of *Sepia officinalis* endolymph (A: control Cy3-labeled and 0h treated Cy5-labeled , B: control Cy5-labeled and 0h treated Cy3-labeled and C: 0h treated Cy3-labeled and 24h treated Cy5-labeled).

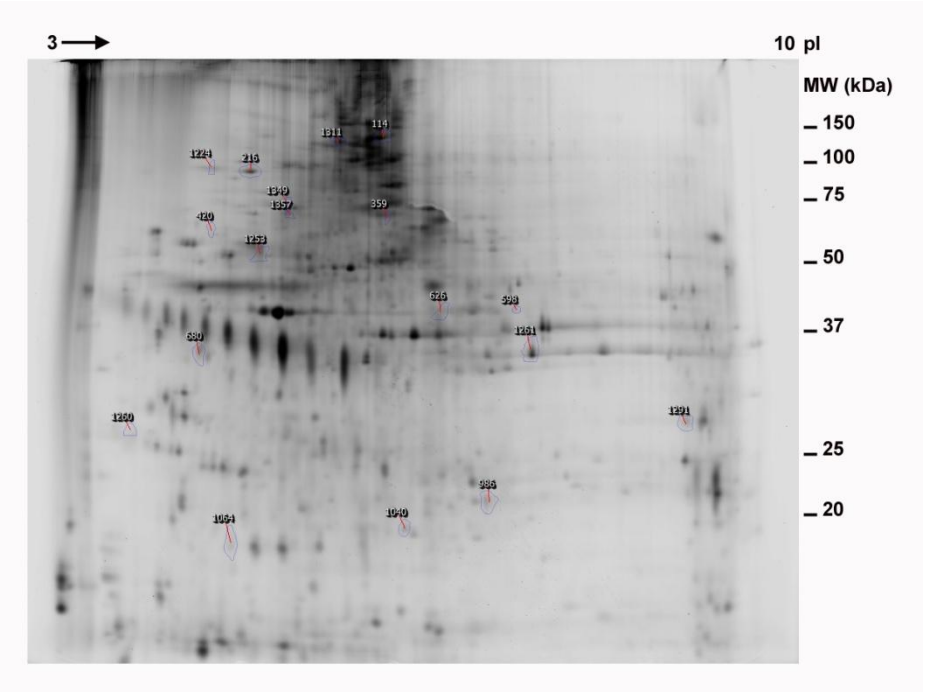

Figure 9.2 Two dimensional *Sepia officinalis* hemolymph obtained in pH range between 3 and 120 and 12.5% of SDS-PAGE and with flamingo staining. Spot number indicates differential spots.

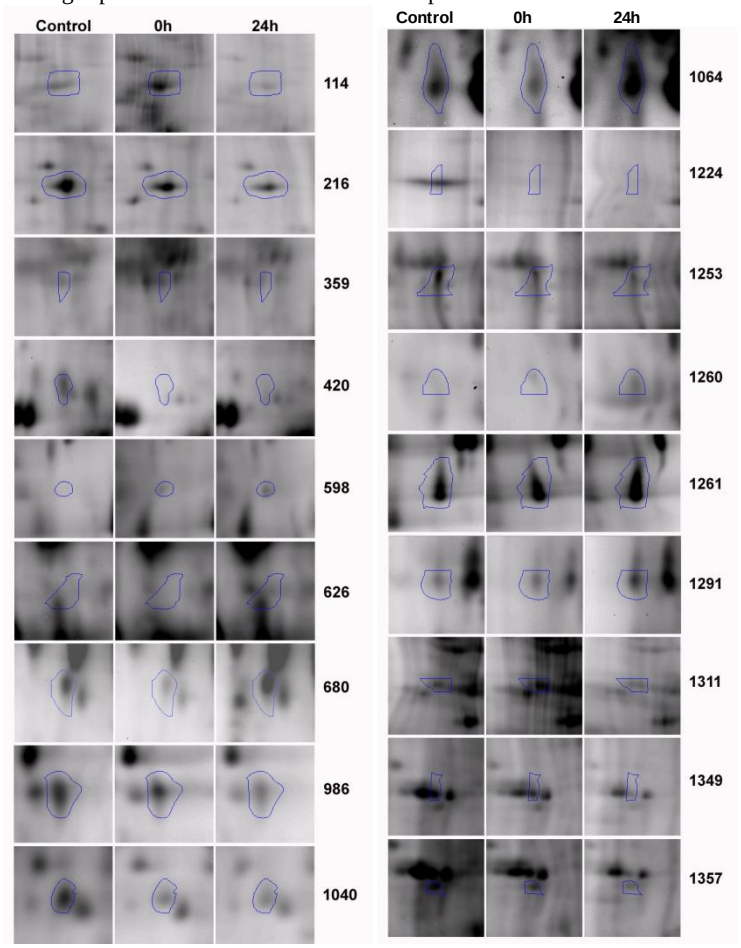

Figure 9.3 Selected 2D-PAGE gel areas related to *Sepia officinalis* hemolymph proteins differentially expressed according to treatments (C, TO and T24).

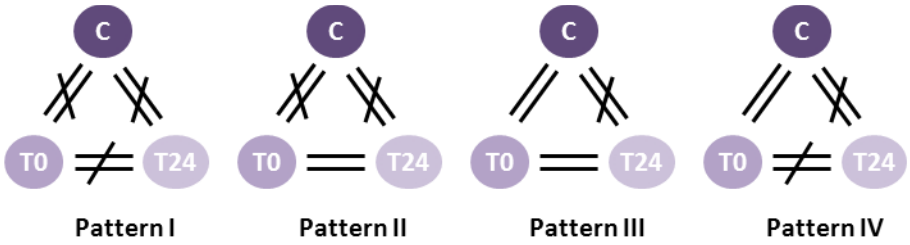

Figure 9.4 Distribution of differentially expressed protein spots. There are 15 differentially expressed proteins revealed by 2-DE gel analysis and comparisons between every two of the three samples. Those proteins were distributed into four patterns: I, two proteins differentially expressed among all three samples; II, two proteins differentially expressed between control (C) and 0h treated (T0) as well as sample control and 24 h treated (T24), but not sample T0 and T24; III, five proteins differentially expressed in C-T24 but not C-T0 and T0-T24; IV, nine proteins differentially expressed in C-T24 and T0-T24 but not C-T0.

9.3.3. Identification

Mass fingerprints were compared with known proteins from several protein databases (Swiss-Prot and nonredundant NCBI database). None of the protein peptide mass fingerprints match entries in the public databases probably because they are not described or because there are considerable variations of the trypsin hydrolytic peptide masses for homologous proteins among organisms. More studies should be directed to identify these differential proteins probably performing preparative gels and using other analytical methods (MS-MS).

9.4. Conclusions

In summary, our experiments provided evidence that protein content of endolymph changes after been exposed to low frequency sounds. These changes can be expected to affect physiology and functioning of *S. officinalis* statocyst and alter the sensory information of this species. But since we could not accomplished the identification, future work is required to understand those changes and also to see if changes are more important along the time.

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10. General Discussion

10. General discussion

The sea environment is filled with natural sounds, although increasingly many anthropogenic sources have contributed to the general noise budget of the oceans. The extent to which sound in the sea impacts and affects marine life is a topic of considerable increasing concern to the scientific and environment communities and to the general public. Sources of sound produced by human activities induce physical, physiological, and behavioral effects on marine fauna (mammals, reptiles, fish, and invertebrates), effects that can be diverse depending on the proximity to the signal source. These impacts can include a reduction in the abundance of fish species of up to 50% in zones under exploration, changes in cetacean behavior and migration routes, and a distinct range of physical injuries in both marine vertebrates and invertebrates. There may be further long-term consequences due to chronic exposure, and sound can indirectly affect animals due to changes in the accessibility of prey, which may also suffer the adverse effects of acoustic pollution (Richardson et al., 1995). These damages could significantly impair the conservation of already endangered species that use acoustically contaminated areas for migratory routes, reproduction, and feeding.

For many reasons, evaluating the acoustic impact of artificial sound sources in the marine environment is a complex and expensive proposition. First, we face the relative lack of information on the sound-processing and analysis mechanisms in marine organisms. Although we are capable of cataloging and recording the majority of these signals, we still do not know enough about the important role they play in the balance and development of populations. Second, the possible impact of sound emissions may not only concern auditory reception systems but might also interfere on other sensorial and systemic levels, possibly lethal for the affected animal. Complicating the situation even more is the fact that a prolonged or punctual exposure to a determined noise can have negative short-, medium-, and long-term consequences not immediately observed. The lack of provision and research resources contributes to the greatest difficulty in obtaining objective data that will allow the efficient control of anthropogenic noise in the ocean.

Interestingly, most of the literature arising from noise effects on marine organisms concerns endangered species that use sound for daily activities. Less attention has been paid to commercial species, in particular invertebrates like cephalopods. In a recent and extremely comprehensive review of the effects of anthropogenic sound sources on fish, A.N. Popper and M.C. Hastings (2009b) rightly conclude that without data “obtained in a systematic way with excellent controls and peer review” it is impossible to develop clear sound-exposure metrics and criteria that could help predict and manage the potential effect of sound on marine life. This study has contributed filling this gap. Indeed, reliable data in this field was extremely limited and, in light of the scope and importance of ocean systems, urgently required. Furthermore, of the three main forms of life in the seas (mammals, fish and invertebrates) cephalopods represent the group about which the very least is understood. Situated as they are in the food chain between fish and marine mammals, they are also key bio-indicators for balance in the vast and complex marine ecosystem.

This study has provided new findings and supported earlier conclusion on the effects of underwater anthropogenic sound on the marine environement with a specific focus on cephalopods. Further more, it brought discussion the effects on cephalopods under sound exposure under laboratory conditions, described the lesions on adult statocyst system and epidermal lines of hatchlings from the analyses of both SEM and TEM, and studied the effects on endolymph statocyst composition from proteomic techniques.

10.1 Characterization of the cephalopod statocyst structures in control animals

Previously to assess acoustic trauma induced by controlled exposure experiments, we needed to describe the statocyst inner structures. We revisited the ultrastructures of the statocysts of four Mediterranean cephalopod species: *Sepia officinalis*, *Loligo vulgaris, Illex coindetii* and *Octopus vulgaris*. All the four cephalopod species structures appeared to be identical as previously published (e.g. Budelmann 1973, 1977, 1979, 1987, 1990, 1995; Stephens 1982; Young 1960, 1965), thus confirming their suitability to be used as control to assess acoustic trauma during noise exposure experiments.

This work contributed new images of cephalopod inner statocyst structures to the current knowledge of these sensory organs. In addition, although *macula* and *crista* of *Sepia officinalis*, *Octopus vulgaris* and *Loligo vulgaris* have been described in previous excellent works, this thesis presents new structural and ultrastructural aspects using SEM and TEM. For instance, non-previously-described images of different ciliated structures located in the lining epithelium of the cavity which consists of flat hexagonal cells with oval nucleus are shown. In some parts of the cavity, cilia emerge between the epithelial cells. The cells of the lining membrane carry cilia on the outer side, which project into the cavity and play part in the circulation of the endolymph. In decapods, they are especially abundant ventrally from the three horizontal *crista* segments and medially from the vertical *crista*. Ciliated cells also occur on the base of anti*crista* lobes.

This study showed the first published images of *crista-cupula* system and inner statocyst cavity of *Illex coindetii*. No previous studies have been carried out on this species. As in other decapods, in some parts of its inner cavity the flat hexagonal cells carry cilia on the outer side, which project into the cavity, microvilli are present with less density than in *Loligo vulgaris* and surround the epithelium cells. Inner statocyst area near of the *macula* shows very high density of microvilli covering all the surface of the flat hexagonal cells of the lining epithelium. The *cupula* of *Illex coindetii* attached to the *crista* presents a filamentous structure similar to the other decapods. Model of the *Illex coindetii* statocyst liner epithelium is shown next to the hair cell rows that surround the main rows of *crista*. Microvilli grow in some of the liner epithelium cells.

The observation of the statocyst ultrastructure of Illex showed a non-previously described feature in cephalopods: the center of the macula princeps presented no hair cells. In other species, it was demonstrated that the macula grows by accreting rings of sensory cells from the center to the periphery of the sensory epithelium (Stephen and Young, 1982). Two explanations can be discussed here: either there is never any hair cell in the center of the macula during the whole Illex live, or rings of hair cells grow in this species from the periphery to the center of the macula. Further analysis on different live stages of Illex are obviously needed to answer this question.

A very electrondense plate, probably of a proteinical nature, was shown through the apical region of the supporting cells, between the desmosomes. We hypothesized that this electrodense plate was composed by bundles of tonofilaments, proteinaceous fibers, associated with desmosomes (*macula* adherens) to which they anchor to the cytoskeleton. Tonofilaments are filamentous structures that form part of the cytoskeleton of cells. These filaments, which are abundantly present in keratinocytes and that are found at desmosomal junctions, have a supporting function. Several epithelial cells, which are capable of continuous division in culture, continuously produce large, balanced amounts of prekeratin-like material, which is assembled in tonofilament-like structures (Werner et al., 1978). The strong assembly of tonofilaments that anchor the supporting cells to the cytoskeleton enables that the sensory epithelium has a mesh-

like structure, the basal plate, capable of strong maintaining hair cells in the precise position to capture the sensory information.

Several vesicles at the centre of the cytoplasm as well as at the contact point between two axons on the nervous plexus of *Octopus vulgaris macula* were described. No previous studies had described this type of synapses between two afferent processes**.** Their presence is probably due to exocytosis, although no definitive conclusion can be drawn here since these structures also resemble clathrins that would not be compatible with the above hypothesized exocytotic mechanism. The presence of these vesicles both in the cellular cytoplasm and open on the extracellular medium suggests a possible function related to intercellular communication, or a possible influence on extracellular medium at least.

The characterization of the cephalopod statocyst structures in control animals represents a fundamental approach to determine the presence of pathologies in individuals under external pressure from human activities. The detailed description of the four cephalopod species normal statocyst structures presented here appeared to be identical as previously published. This, therefore, confirms that these animals can be used as a control set to contrast and define lesions compatible with acoustic trauma after noise exposure experiments conducted in parallel on another set of individuals caught, handled and maintained in the exact same conditions. These results allowed to discard the possibility that the trauma observed in exposed individuals could constitute artefacts derived from the capture or the handling of the cephalopods in a captive environment.

10.2 Behaviour responses to acoustic impact

Data are lacking on the effects of anthropogenic sounds on the behaviour of fishes, on the effects on fishes close to source and further from the source; on the long-term effects exposure to sound or on cumulative exposure to loud sound; and on effects on fish encloused on experimental cage o tanks and on the natural environement. Several studies have demonstrated that anthropogenic sounds may affect the behaviour of at least a few species of fishes of the effects of anthropogenic sounds on the behaviour of fishes. A significant decline in catch rate of haddock (*Melanogrammus aeglefinus*) and Atlantic cod (*Gadus morhua*) was observed on field studies for up to 5 days after termination of use of seismic air guns for geologic exploration (Engas et al., 1996; Engas and Løkkeborg, 2002) . Parallel results were observed for blue whiting (*Micromesistius poutassou*) and Norwegian spring spawning herring (*Cuplea harengus*) (Slotte et al., 2004) and on rock fish (*Sebastes* spp.) (Skalski et al., 1992).

Some studies examined the effects of exposure to seismic airguns on six reef species of fishes held in cage (Boeger et al., 2006) and the response to different sounds on three species of fish in a pool (Turpenny et al., 1994). On both cases the fish showed small level of response.

There is thus a growing interest regarding the effects exposure to seismic air guns can have on invertebrates. In a study conducted by Wardle et al. (2001), the authors, using a video system, reported minor behavioural responses to the air-gun emissions (peak level 210 dB re 1 μPa at 16m from the source and 195 dB re 1 μPa at 109m from the source) of fish and invertebrates on a rocky reef off Scotland, (Wardle et al., 2001). A previous study (McCauley et al., 2000) reported the behaviour response of squids to air gun exposure and suggested a probable impact of seismic operations on these species (using thresholds at 161–166 dB re 1 μPa). These results reported similar behaviour responses as those we observed during sound exposure: several cuttlefish showed startle response by inking. Despite this similarity, the individuals in our experiments, after an initial stress reaction, remained motionless at the bottom of the tanks. After the CEE stopped and the animals were placed in tank B, they presented evident symptoms

of stressed behaviour and decrease of activity, loss of muscle tone, remaining motionless until they were sacrificed. However, the different experimental conditions (size of the tank and the difference of the sound sources) are a limiting factor to assess and directly compare the behavioural reaction of the exposed individuals.

10.3 Lesions on adult statocyst inner structures

In terrestrial vertebrates (including humans), exposure to very high sound pressure levels may result in permanent hering loss, because the sound destroys sensory hair cells of the inner ear and fractures the bones of the middle ear (Patterson and Hamermik, 1997; Henderson et al., 2008). Exposure to lower levels for longer periods can also lead to permanent hearing loss through death of sensory cells (Hamernik et al., 1994).

Data on the effects of exposure to sound on fishes are very limited as compared with data for terrestrial vertebrates. Some studies reported that sound can damage sensory cells on ears of some fish species (Enger, 1981; Hastings, 1995; Hastings et al., 1996; McCauleyet al., 2003). However, no study has determined the relation between damage of hair cells and permanent hearing loss in fishes. The work of Enger (1981) found that some sensory cells lost their ciliary bundles in the ears of cod (*G. morhua)* after 1-5h exposure to pure tones (100-110 dB above threshold in its most sensitive hearing frequence range), examining the sensory ephitelia by SEM. In Hastings (1995, 1996) it was reported damage to auditory hair cells in hearing (*C. auratus*) after to be exposed to continous tones (120-140 dB above threshold in its most sensitive hearing frequence range) for approximately 2h, and in oscar (*A. Ocellatus)* after 1h of continuous exposure to a 300 Hz pure tone. In this last case the damage was only visible in animals that were alive four days after sound exposure. This fact allows concluding that damage caused from exposure to sound takes some time to become visually apparent. McCauley 2003 showed by electron microscopic techniques destruction of hair cells on ears of pink snapper (*P. auratus*) after exposure to sound of a seismic air gun. The damage observed in these four species was only a visual manisfestation of what may have been a much greater effect. Temporary deafness could result in a fish being unable to respond to presence of predators and to locate prays and mates.

Aa a consequence of the very scarce available data, one must be extremely cautious in extrapolating results between fish species or received signals, because of the differences in the hearing systems, limited data of precise stimulus (pressure and or particle velocity) and the time course and frequency components of the signals.

The same considerations may be applied to the studies on the effects of sound on sensory epithelia of statocyst cephalopods. No data were avallaible until the publication of this study. This thesis presents the first morphological and ultrastructural evidence of a massive acoustic trauma, not compatible with life, induced on individuals belonging to four cephalopod species under low frequency sound controlled exposure experiments resulting in permanent and substantial alterations of the sensory hair cells of the statocysts, the structures responsible for the animals' sense of balance and position.

While the controls showed no lesions in the statocysts, all exposed individuals presented the same lesions and the same incremental effects over time. The effect of the sound exposure was massive, affecting a wide range of statocyst inner areas, specially on the individuals sacrificed 96h after exposure. Partial or total cell extrusion and alterations on kinocilia hair cells were the most frequent lesions in the sensory epithelium of all tested groups.

One explanation could be that the holes due to hair cell disappearance allowed the entrance in the epithelium of endolymph charged with high potassium ion concentration. This would increase the initial mechanical insult to hair cells together with a more progressive chemical

damage as it is observed in acoustic traumatized cochlea of mammals. Indeed, Amoor (1959) and colleagues determined a high concentration of potassium in *Octopus vulgaris*, thus supporting this hypothesis.

In mammals, a low- or mid-intensity acoustic trauma does not lead to obvious mechanical damage to the sensory epithelia (McCauley et al., 2000; Pujol and Puel, 1999; Popper and Hastings, 2009b). Instead, lesions occur primarily at a metabolic level (Bohne and Rabbitt 1983), leading to the fusion of the cilia, the deformation of the apex of the cells, and eventually the death of the cell over a period of several weeks. However, this process can also be observed at the periphery of a violent acoustic trauma where open holes are left following the expulsion of the cell apex. This was indeed observed in all cephalopod individuals 48, 72 and 96 hours after exposure: the sensory epithelium presented mechanical damage (partial or total loss of sensory cells) and metabolically induced damage (swollen sensory cells, vacuolization of cytoplasm, mitochondrial degeneration, damage to dendrites).

In vertebrates, it was shown that glutamate, which in normal conditions works as a neurotransmitter, has a cytotoxic effect when released in excess in stressful conditions as after exposure to loud noise (among others: Puel et al., 1998; Ruel et al., 2005). The excitotoxic effects of glutamate in response to noise could be the result of increased release or inadequate removal of glutamate, primarily due to the breakdown of recycling mechanisms (Ruel et al., 2006). This may eventually result in toxic cellular events leading to neuronal degeneration and sensorial epithelium damage. Since glutamate has also been described as neurotransmitter acting at AMPA/kainate-like receptors in statocyst afferent fibers of cephalopod species (Tu and Budelmann, 1994; Di Cosmo et al., 2006), the presence of hypertrophic afferent dendrites in our images could probably point to the great amounts of glutamate secretion as a possible cause of the sensory epithelium degeneration process. The observed impact on the statoacoustic organs of the noise-exposed cephalopods would actually suggest the occurrence of an excitotoxic process due to an excess of glutamate.

Apoptosis, which is a mode of hair cell loss that does not induce inflammatory processes (Wyllie et al., 1980), contribute to phagocyte recruitment at the site of lesion (see for review: Grimsley and Ravichandran, 2003). The swift and proper removal of apoptotic cells by macrophages prevents leakage of potential cytotoxic and antigenic substances from dying cell and may induce tissue repair by releasing various cytokine and growth factors (for review: Fujiwara and Kobayashi, 2005). Apoptosis is a major mode of hair cell loss in the mammalian damaged vestibular organ and cochlea (see for review: Nakagawa et al., 1997a). In addition, in the mammalian cochlea exposed to loud noise (Hirose et al., 2005; Fujioka et al., 2006) or ototoxic drugs (Ladrech et al., 2007), macrophages are believed to remove hair cell corpses. In the present study, the presence of both apoptotic cells and of macrophages in the damaged statocysts suggest that similar post-traumatic mechanisms would be involved in the cephalopod damaged sensory epithelium.

In this work, we could observe what may represent the starting point of a healing process. Because the beginning of the scarring process showed by the extension of the supporting cells can be appreciated in SEM images of individuals sacrificed 48 and 96 hours after sound exposure and in TEM images of individuals sacrificed 48h after sound exposure, these results suggest that the recovery process would be operant at least two days after sound exposure. Reparation and regeneration of stereocilia occur in the vestibular system of bird and mammals and limited reparation is seen in the cochlea of mammals. The morphological diference of the cilia, which are microvilli in the vestibular and auditory system of birds and mammals while they are kinocilia in cephalopods statocyst sensory sytems, require further investigation to understand the kinocilia repair and regrowth process that would happen in cephalopods. However, further experiments are needed to confirm and understand the kinocilia repair and regrowth process that would happen in cephalopods as well as to determine its timing more precisely.

In the avian cochlea and vestibular organs and in the vestibular organ of mammals, posttraumatic hair cell regeneration was shown to occur after either acoustic trauma or drug poisoning (Corwin and Cotanche, 1988; Raphael and Altschüler, 1991; Roberson and Rubel, 1994; Oesrtele and Rubel, 1996 and see for review: Rubel and Stone, 1996). Several mechanisms are believed to be involved, in such regeneration, including proliferation followed by differentiation of non-sensory epithelial cell, direct transdifferentiation of supporting cells into hair cells, and reparation of damaged hair cells. These processes occur generally within one or several weeks after hair cells death. In the present study, individuals were not collected after 96 hours following the acoustic trauma. This is probably a too short delay to observe the potential occurrence of regenerative processes due to cell division and differentiation. Nevertheless, here, the presence of almost normal kinocilia bundles in statocysts observed 96h after the acoustic trauma in areas where almost all hair cells had disappeared constitutes an interesting observation. It suggests a possible repairing process in some damaged hair cells, which survived the acoustic exposure.

These results showed lesions new to cephalopod pathology and demonstrated that exposure to sounds can cause massive lesions on the statocyst sensory epitheliums in cephalopods. Their presence in all the noise-exposed individuals (vs. their absence in controls) and their definite progression over time are consistent with a massive acoustic trauma induced here by exposure to relatively low intensity, low frequency sounds, while the same lesions were reported in land mammals and birds exposed to much higher sound levels. Further investigation is needed to determine threshold levels and to quantify the lesions in the statocysts; to explain the mechanism onset of these lesions, in particular to determine if the laboratory conditions can be reproduced in open environments; and to definitively understand if these animals are more sensitive to particle motion or acoustic pressure, or to a combination of both. With low frequency noise levels in the marine environment on the increase, future electrophysiological experiments coupled with post-mortem imaging techniques are needed to determine the tolerance to noise thresholds of cephalopods. However, the presence of lesions in the statocysts clearly points to the involvement of these structures in sound reception and perception. Given that low frequency noise levels in the oceans are on the increase (e.g. shipping, offshore industry, navy maneuvers), that the regular exposure to noise may compromise the statocyst sensory epithelium regeneration capacity, that the role of cephalopods in marine ecosystems is only beginning to be understood, and that reliable bioacoustic data on invertebrates is scarce, such future studies have an important contribution to make to the sustainability of the marine environment.

10.4 Lesions on epidermal lines of cephalopod hatchlings: Preliminary results

The lateral line system of fishes consist a set of receptors, found on the surface of the body, which detect water motion near of the fish. Mechanical stimulation of the lateral line of clupeids may cause damage by decopling the cupulae from the neuromasts (Denton and Gray, 1993). Denton and Gray reported working with adult cupleids that loss of the attachment between the cupula and neuromast would result in dysfunction of the lateral line. Hastings 1996 suggested no effects of exposure to sound on the hair cells of the fish lateral line system.

Whereas most studies on sound damage on fish larval stages and eggs assessed the effects on behaviour, growth (length and weight), mortality and internal organs pathology (Banner and Hyatt, 1973; Govoni et al., 2003, 2008; Jørgensen et al., 2005, only one study (Kostyuchenko, 1973) reported damage on neuromasts (sensory structures with sensory hair cells) of the lateral line system of cod (*G. morhua*) and Atlantic hering (*C. harengus*) larva under seismic air-gun sounds exposure.

This study showed the first published images of epidermal lines of *I. coindetii paralarvae*. No previous studies had been carried before. The distribution of epidermal lines is very similar to other described Decapod species such as *S. officinalis*, *S. affinis* or *L. vulgaris* (five pairs of bilaterally symmetrical lines in head and arms, and an additional unique line on the funnel located along its middle). The epidermal lines are highly dense and present different kinocilia distribution on the hair cells.

While the controls showed no lesions in the epidermal lines, all the exposed individuals presented the same lesions, but the effects evolution over time was different in the different species of studied cephalopods. The different evolution on *S. officinalis juveniles and L. vulgaris* and *I. coindetii paralarvae* could be due to different size of hatchlings of the three species which gives them different lifestyles. The higher size of cuttlefish juveniles could involve its lower rate of regeneration. In addition this higher size implies that cuttlefish is a benthic species immediately after the hatching, whereas the other two species have a planktonic phase before they have sufficient size to become juveniles. This different lifestyle may involve differences in the typology of the lesions produced by the noise impact due to variations in the perception of it because of the different sound propagation in different media. The absence of comparative studies on the rate regeneration of different cephalopod larval species, in addition to the necessity to determine a possible relation between sound and lesions, and its possible recovery due to the high regeneration rate on cephalopods larvae, could open new lines for future research which would contribute to found results with medical applications.

Partial or total lost or alterations on kinocilia hair cells were the most frequent lesions in the epidermal lines sensory epithelium of all tested groups. Although in avian and mammals, sensory hair cells carry stereocilia which are not kinocilia, similar damages to hair bundles are previously described in these species, especially in the basilar papilla of the avian inner ear and in the mammalian organ of Corti just after sound exposure. This is consistent with the process of lesion development in our case study. Two mechanisms seem to be involved in noise-induced hearing loss in mammals: direct mechanical damage, which appears immediately after short exposures at high intensities, and metabolically induced damage, which occurs after long exposures with moderate intensities and develops over a longer period after sound exposure. In mammals, a low- or mid-intensity acoustic trauma does not lead to obvious mechanical damage to the sensory epithelia (McCauley et al., 2003; Pujol and Puel, 1999; Popper and Hastings, 2009b). Instead, lesions occur primarily at a metabolic level (Bohne and Rabbitt, 1983), leading to the fusion of the cilia, the deformation of the apex of the cells, and eventually the death of the cell over a period of several weeks. This was indeed observed in all cephalopod hatchlings 18 hours after exposure.

Further experiments are needed to determine the timing of the suspected healing process more precisely. Reparation and regeneration occur in the vestibular system of bird and mammals and in the auditory system of mammals, when apoptosis is not involved. The morphological diference of the cilia, which are microvilli in the vestibular and auditory system of birds and mammals while they are kinocilia in cephalopods epidermal lines, require also further investigation to understand the kinocilia repair and regrowth process that would happen in cephalopods.

These preliminary results showed lesions new to cephalopod pathology and suggest that exposure to sounds can cause, in addition to those observed in the statocysts, massive lesions on the epidermal lines in cephalopod hatchlings. The outcomes are the basis for future research, which would help to determine qualitatively the extent of injuries caused by exposure to sound and its subsequent recovery in the larval epidermal lines of cephalopods. Lines of research must also be implemented to determine whether some of the epidermal lines are more sensitive than others to sound exposure. It would also be appropriate to conduct studies of the effects of exposure to sound in the statocysts of the hatchlings. The results of this research would

contribute to get arguments to help determining whether their origin is due to the pressure wave of sound or the effect of particle motion.

10.5 Proteomic analysis

Although the advent of contemporary proteomic technologies has ushered in definite advances to the field of auditory research (Patterson and Hamermik, 1997; Zheng et al., 2006; Coling et al., 2007; Jamesdaniel et al., 2008, 2009), this is the first time that proteomic techniques are used on the determination of the effects of the acoustic impact on cephalopods. These technique are particularly interesting for this project because allows the comparison of two different situations, to analyze the effects of acoustic trauma at molecular level, comparing control individuals of cephalopods with those that suffer acoustic impact. The endolymph analysis under situations of acoustic stress allow us to establish the effects at molecular level, and identify the proteins especially sensitive to this type of trauma, and then analyze the change of the initial proteomic map of the tissue in situations of acoustic stress.

Damage on sensory ephiteliums of the cephalopod statocysts, which could be induced by metabolic exhaustion resulting in distortion of the homeostasis of statocysts, were exhibited on our previous work (André et al., 2011). An analysis of the protein fraction of the endolymph before and after of the sound exposure were needed.

We measured an average of 11.7 ± 6.0, 17± 6.6 and 13.1 ± 2.0 µg protein per statocyst in control (N= 24), treated 0h (N=32) and treated 24h (N=24) respectively. These results are similar to that observed by Bettencourt and Guerra (2000) who found that the protein concentrationt in hemolymph of *S. officinalis* changed from 1.8 µg/µl in the morning to 0.7 µg/µL in the evening. In our studies all samples were processed at the same moment of the day (14h-15h) to avoid this variable and the measured concentration of protein varied from 0.8 to 1.13 µg/µL. Not important differences were found in the protein concentration between treated and untreated individuals, and these slight differences were probably due to intrinsic differences and also to the considerable oscillations along the day described previously by other authors (Bettencourt and Guerra, 2000).

The 2D-DIGE comparison of the location and volumes associated with each spot revealed that the level of the majority of proteins remains virtually unchanged in the control and 0h-treated .Variation in spot intensities was much more manifest between 24 h treated and the other two treatments. .. As it could be expected, the most important differences were found between control and 24h treated. We only found four proteins differentially expressed just after treatment (0h treated), however we could observe important differences just after noise exposition using TEM and SEM techniques at the same moment. These result showed that changes in proteome expression were displayed later than structural changes. Nevertheless those changes in protein expression that appeared at 24 hours after treatment could appear before this period of time.

None of the protein peptide mass fingerprints match entries in the public databases probably because they are not described or because there are considerable variations of the trypsin hydrolytic peptide masses for homologous proteins among organisms. More studies should be directed to identify these differential proteins probably performing preparative gels and using other analytical methods (MS-MS).

In summary, our experiments provided evidence that protein content of endolymph changes after been exposed to low frequency sounds. These changes can be expected to affect physiology and functioning of *S. officinalis* statocyst and alter the sensory information of this species. But since we could not accomplished the identification, future work is required to understand those changes and also to see if changes are more important along the time.

10.6 Future Research

This study presents the first morphological and ultrastructural evidence of a massive acoustic trauma, not compatible with life, induced on individuals belonging to four cephalopod species under low frequency sound controlled exposure experiments resulting in permanent and substantial alterations of the sensory hair cells of the statocysts, the structures responsible for the animals' sense of balance and position. These results indicated a need for further environmental regulation of human activities that introduce loud low frequency sounds in the oceans.

The lesions described here are new to cephalopod pathology. Their presence in all the noise-exposed individuals (vs. their absence in controls) and their definite progression over time are consistent with the massive acoustic trauma observed in other species that have been exposed to much higher intensities of sound. Why the relatively low levels of low frequency sound have caused such lesions in cephalopods demands further investigation. In particular, it will be critical to determine the mechanism onset of the acoustic trauma to definitively understand if these animals are more sensitive to particle motion or acoustic pressure, or to a combination of both. Cephalopods are sensitive to low frequency sounds and although there are some studies that presumably demonstrate that they can hear up to some kHz, recent publications point out that the interpretation of these findings could be biased by the fact that no particle motion measurements were conducted and that high frequency pressure could have generated lower frequency kinetic sound components due to the small distance of the cephalopods to the pressure release at the water surface.

Future electrophysiological experiments coupled with postmortem imaging techniques are also needed to determine the tolerance-to-noise threshold of these species, to understand the mechanism onset of the lesions, and relate them to in vivo sound perception analysis.

The absence of comparative studies on the rate regeneration of different cephalopod larval species, in addition to the necessity to determine a possible relation between sound and lesions, and its possible recovery due to the high regeneration rate on cephalopod hatchlings, could open new lines for future research which would contribute to gather results that may have medical applications. The preliminary results on hatchlings should constitute the basis for a future research, which will help to determine qualitatively and quantitatively the extent of injuries caused by exposure to sound and its subsequent recovery in the larval epidermal lines of cephalopods. It must also be determined whether some of the epidermal lines are more sensitive than others to sound exposure. It would also be appropriate to conduct studies of the effects of exposure to sound in the statocysts of the hatchlings. The results of this research would contribute to get arguments to help determine whether their origin is due to the pressure wave of sound or the effect of particle motion, since there is a controversial discussion on this matter.

On the other hand, future proteomic research could help to determine which specific proteins of the statocyst endolymph suffer permanent and substantial alterations, and consequently explain the mechanism onset of these lesions.

Further investigation is needed to determine if this study laboratory conditions can be reproduced in open environments, and to definitively understand if these animals are more sensitive to particle motion or acoustic pressure, or to a combination of both. With low frequency noise levels in the marine environment on the increase, future electrophysiological experiments coupled with post-mortem imaging techniques are needed to determine the tolerance to noise thresholds of cephalopods.

11. Conclusion

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- The ultrastructures of the statocysts of four Mediterranean cephalopod species: *Sepia officinalis, Loligo vulgaris, Illex coindetii* and *Octopus vulgaris* were revisited by imaging techniques, and their suitability to be used as control to assess acoustic trauma during noise exposure experiments was confirmed.

- New images of cephalopod inner statocyst structures added to the current knowledge of these sensory organs: amongst them are the first published images of *crista* & macula system and inner statocyst cavity of *Illex coindetii* and a non-previously described feature in cephalopods: the center of the macula *princeps of I.coindetii* presented no hair cells.

- Massive lesions, new to cephalopod pathology, were observed by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy on the statocyst sensory epitheliums of *Sepia officinalis, Octopus vulgaris (SEM &TEM), Loligo vulgaris* and *Illex coindetii (SEM)* after laboratory conditions controlled exposure to low frequency sounds.

- Preliminary results showed lesions on epidermal line systems of *Sepia officinalis, Loligo vulgaris* and *Illex coindetii* hatchlings and suggested exposure to sound can cause reversible lesions on the epidermal lines in cephalopod hatchlings.

- Evidence of protein content of statocyst2 endolymph of adult individuals of *Sepia officinalis* changes after been exposed to low frequency sounds was provided using 2-DE/MALDI-MS techniques.

12. References

12. References

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