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EXPLORING NEURAL HALLMARKS OF CONSCIOUS STATES IN OCTOPUS

An experimental approach including the development
of minimally invasive and stunning methods

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Abstract

Cephalopods molluscs are well known for the richness of their behavioral repertoire, learning and cognitive abilities, complemented by a sophisticated nervous system including a highly differentiated multi lobular brain considered to rivalling higher vertebrates for the relative brain-to-body size, to mention one characteristic. Functional analogies between cephalopod and vertebrate brains have also been suggested, further supporting evidence for sentience, complex cognition and the presence of 'elements' suggesting the existence of primary consciousness.

For the sake of this PhD project, I explored hallmarks of consciousness in an invertebrate: the cephalopod mollusc *Octopus vulgaris*.

In this PhD thesis I focused on: **a.** structural and functional analogies between the cephalopod 'brain' structures and the thalamus-cortex 'complex'; **b.** neural dynamics, both spontaneous and evoked, based on electroencephalographic (EEG) signatures in octopus. I also developed minimally invasive approaches (epidermal and subcutaneous recordings) allowing to record EEG brain signals of neuronal activities in live animals. Furthermore, I explored stunning as a way to induce temporary electronarcosis, a potential route to promote reduction and/or loss of consciousness and insensibility in the live animal, as required to develop humane slaughtering method for cephalopods.

Overall, my results are novel and provide the basis for future studies exploring the neural correlates of conscious and unconscious states in cephalopods. To the best of my knowledge, this PhD is the first designed around the experimental search of hallmarks of consciousness in cephalopod molluscs, invertebrate animals very distant from higher vertebrates.

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This PhD has been a long journey, which allowed me to gain in-depth knowledge (at least from my own perspective) of many aspects of a fascinating marine animal, which is the octopus; an invertebrate you never stop learning from and never fails to amaze. But above all, it has allowed me to meet experts in many areas of neuroscience research with whom I have been lucky enough to interact and learn from their experience. All of them provided me a generous offering of their experience and knowledge, that has been fundamental for me and greatly contributed to let this complex PhD project forward; I am sure I will benefit from this gift in the future in any path I will explore after my PhD.

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Introduction

Over several decades, studies on physiological properties of the cephalopod nervous system played an important role in neurobiological research and biology in general. One of the recurrent justifications has been the simplicity of brain structure and organization when compared to vertebrate brains. A comparative outlook allowed to identify convergent features that despite the marked phylogenetic distance, let cephalopods to be considered to rivalling higher vertebrates (Packard, 1972; Young, 1991a; Edelman and Seth, 2009).

In addition, genome sequencing (e.g., Albertin et al., 2015; Zarrella et al., 2019; da Fonseca et al., 2020; Destanović et al., 2023) provided important knowledge around the novelties that evolved in these organisms: an example of exceptional innovations acquired in taxa very distant from vertebrates during the evolutionary path, thus letting these fascinating animals to acquire further arguments for being considered an important case of study in Biology (e.g., Albertin and Simakov, 2020; Albertin et al., 2022; Schmidbaur et al., 2022; Baden et al., 2023). As member of the Phylum Mollusca, cephalopods contribute to the exploration of the evolutionary scenarios occurred during the evolution of the nervous systems (for review see for example: Arendt et al., 2008; Holland, 2016; 2020).

Within the Phylum, the Coleoid cephalopods evolved exceptionally large brains (Nixon and Young, 2003) reaching more than 30 differentiated lobes in octopus (Young, 1971). This complex lobe-organization of the cephalopod brain masses correspond to highly organized neuropils and fasciculate tract bundles (e.g., for *Octopus vulgaris* Young, 1971), very numerous neural cell counts (Young, 1963) possibly belonging to different cellular types (Young, 1932; 1972b; Styfhals et al., 2022). The relative complexity in terms of structure and organization acquired by cephalopod brains allow the identification of species- or taxon-specific cerebrotypes, appearing linked also to life-history and biological features of the species (Ponte et al., 2021).

As originally reviewed by Young (1985), cephalopods greatly contributed to neuroscience.
May be the first account can be traced to early 1900 for the discovery of the electroretinogram by Frohlich (review in Dröscher, 2016) that oriented a large series of subsequent studies aimed

at, for example, the understanding of the photoreceptors functioning, with the opening of channels to produce generator potentials occurring locally, near the site of photon absorption in a rhabdome. Indeed, the history of the use of cephalopod eyes for neuroscience merits an overview *per se*, out of the aims of this Thesis (review in Dröscher, 2016). Other biological features of relevance to neuroscience may be accounted in the characterization of the oculomotor control system (Budelmann and Young, 1985), the discovery that fast and slow movements are processed by octopus separately by an unique system of large and small cupula, with structures in the brain (i.e. the peduncle lobe) containing small cells with parallel fibres resembling the vertebrate cerebellum (review in Young, 1985; see also: Gutnick et al., 2011; Levy et al., 2015; Levy and Hochner, 2017; Gutnick et al., 2020).

In the words of Young (1985), the existence of extraocular photoreceptors in cephalopods has a long history. Taking inspiration from the work of Enrico Sereni, Young discovered a «small orange spot at the hind end of the stellate ganglion. I cut sections of it, with no hypothesis other than curiosity. It proved to be a hollow vesicle into which passed a number of projections, apparently of nerve cells. After discussion with Sereni, it was named the epistellar body […]. I was interested at the time in the vertebrate adrenals and made the hypothesis that these projections into the epistellar cavity were secretory. Ernst Scharrer eagerly seized upon this as one of the earliest examples of neurosecretion. But he and I were sadly mistaken. Forty years later Howard Bern […] thought it time to study this organ properly […, *reaching the conclusion that*] «the processes inside the epistellar body are rhabdomes […]. It is not a gland at all but a photoreceptor, though without any lens or other dioptric apparatus. Alex Mauro working at Naples and Ischia confirmed that it produces its own mini-electroretinogram […]. What can this photoreceptor be doing inside the mantle? The epistellar body is especially large in deep-sea octopods, which are transparent. One hypothesis is that it serves to detect the presence of a mass of luminous material in the mantle, which would attract a predator. The oesophagus is deeply pigmented, presumably for the same reason. However Houck […] has recently shown that in octopuses with the optic nerves cut, diurnal rhythms can still be entrained by light, perhaps detected by the epistellar bodies» (Young, 1985, p. 154).

It is worth to mention that extra ocular photoreceptors have been discovered in various cephalopod species, and in octopus shown to contribute to light activated chromatophore

response independent from the central processing associated to vision (Ramirez and Oakley, 2015).

It is without doubts that one of the greatest contributions to neuroscience provided by cephalopod molluscs is represented by the discovery of the giant fibre system of *Loligo* (Young, 1939). «It was only at this time that I discovered that the first order giant cells in the brain had been illustrated by Williams [… *in early 1900*]. His excellent monograph [… *published in the original language, thus resulting*] the giant fiber system was never mentioned throughout the succeeding years. Williams followed the large fibers into the stellate ganglion and stellar nerves, but he seems to have supposed that they ran through the ganglion without synapse. He gave no figure of them. The axons provided the material for the first direct measurements of the internal potassium concentration of protoplasm […]. The full development of the potentialities of the giant fibers occurred after the war […]. Outstanding achievements were the placing of an internal electrode and the emptying and refilling of the axon by Hodgkin and Huxley. These investigations provided the data that enabled them to deduce the equations of the ionic exchanges that are involved» (Young, 1985, p. 156). J.Z. Young in depth analysis of cephalopod 'brain' structures allowed him to discover many other characteristics including «a dozen distinct lobules, each with a different pattern of cells and neuropil. Surely these would provide a good opportunity to study higher nervous activities, such as memory. I felt that this was an opening even more important than was offered by the nerve fibers. […] But the really mysterious problems of neuroscience were hidden there in the neuropils of the higher centers. Biophysics was not ready to attack them, and still cannot do so even in 1984. However, it seemed to me that a start should be made, and Sanders and I were able to show at Plymouth that the learning power of *Sepia* is indeed dependent on the vertical lobe […]. After a long interval in the war, while studying nerve regeneration in mammals and men, I returned to the problem of memory at Naples. Octopus provides even better opportunities than *Sepia* and proved to be a splendid learner. […] With the cooperation of Boycott, Wells, Sutherland, and many others, the two memory systems of the octopus, visual and tactile, have been thoroughly explored» (Young, 1985, p. 156; for review see also Marini et al., 2017).

Thus, a long journey around cephalopod neural systems bringing important findings to Biology.

The journey initiated by J.Z. Young and coworkers in the search of neural correlates of complex behaviours (review in: Brown and Piscopo, 2013; Marini et al., 2017) facilitated the subsequent exploration of the neurophysiological features of cephalopod 'brain' structures. Examples are provided by the use of brain slices preparation (e.g., Williamson and Budelmann, 1991; Hochner et al., 2003; Shomrat et al., 2008; Shomrat et al., 2010) to *in vivo* recordings (Bullock, 1984; Bullock and Budelmann, 1991; Brown et al., 2006; Gutnick et al., 2023; Pophale et al., 2023).

To mention some, Brown and colleagues (2006) recorded spontaneous neural activity from freely behaving octopus in order to identify neural correlates of resting/activity cycles of the animal. They used a semi-invasive technique to record spontaneous activity from the vertical lobe (an area of the octopus brain, dorsal to the supraoesophageal mass); they found single spikes and spike trains suggesting 'body off/brain on' type of activation. The vertical lobe, once again, attained its primary character in this endeavour of neuroscience (e.g., Muntz, 1961a; Maldonado, 1963; Gray and Young, 1964; Sanders, 1975; Fiorito and Chichery, 1995; Young, 1995; Hochner et al., 2006; Shigeno and Ragsdale, 2015; Turchetti-Maia et al., 2017): a little structure in the supraoesophageal mass accounting for about 12% in volume in respect to the total mass, but counting more than 60% of the number of neural cells in the same mass.

The features of neural activities recorded by Brown and colleagues let them to confirm a consolidated view of the vertical lobe as part of the neural circuitry involved in learning and memory processing (e.g., Maldonado, 1963; Maldonado, 1965; Sanders, 1975; Hochner et al., 2006; Turchetti-Maia et al., 2017), also displaying a vertebrate-like Long Term Potentiation (e.g., Hochner et al., 2003; Shomrat et al., 2008; Shomrat et al., 2011).

Detecting EEG signatures in nervous systems including cephalopods

Local field potentials are generated by neurons located in the nervous system, and electroencephalography (EEG) is one of the oldest and most widely utilized method to collect these field potentials from brains. EEG provides a graphical representation of the differences in voltage between two cerebral locations plotted over time (Olejniczak, 2006) and allow recording of the current flow through the tissues between the electric generator (bunch of neurons) and recording electrode. The most significant source of EEG potentials is synaptic activity, both

excitatory and inhibitory postsynaptic potentials (Coenen, 1995; Olejniczak, 2006). EEG measures the difference in potential between an active electrode, placed above the area where the neuronal activity takes place, and an indifferent electrode, located at a certain distance from the first one. Neurons with similar spatial orientation in the brain with synchronized activation would produce an electrical activity detectable from the surface of the head, and electric field would be different than one generated by un-synchronized or orthogonally oriented neurons (Coenen, 1995). As a consequence, the amplitude of the EEG signal depends on the synchronization of the activated cells. If synchronous activation of this group of cells repeats many times, the resulting EEG will be characterized by broad and rhythmic waves (Brienza and Mecarelli, 2019). There are different reasons why EEG is one of the most used methods to collect neural electrical activity. Time resolution in electroencephalography is really high (up to a millisecond) and can be used to interpret many aspects of neuronal communication and computation (Buzsáki et al., 2012), also it appears useful to monitor cognitive abnormalities and diseases. Moreover, EEG is considered a non-invasive approach, as electrodes are generally placed on the head of the subject via electrode caps (and electroconductive gel to optimize bioelectrical signals) and do not require surgical intervention for implantation. In addition, and as an alternative method to collect brain signals, electrodes can be inserted subcutaneously in correspondence of specific regions of the cortex (Scriba et al., 2013; Duun-Henriksen et al., 2015; Liu et al., 2022) responsible for neural evoked activity after sensory stimulations; this approach is seen as requiring minimally invasiveness.

During EEG recording (review in e.g., Bronzino, 1984; Shibata et al., 2024), different kind of stimulations are employed to correlate changes in electrical neural activities, with the intention to understand how the nervous system is influenced by sensory inputs (e.g., visual, olfactory, auditory stimuli). The use of light flashes or images of different shapes and colours during electrophysiological recordings are some examples of visual stimulation commonly utilized to assess normal and/or abnormal functioning of the visual system. Responses to visual stimuli result in the Visually Evoked Potential (VEP) and is considered as a gross electrical signal generated by given brain areas in response to visual stimulation. A VEP requires an intact and functional visual pathway from the retina to the primary visual cortex (Sokol, 1976; Liu et al., 2022). Its waveform, the timing, duration of each peak are all related to a number of conditions (Sokol, 1976).

Several studies in various animal species adopted VEP as approach to detect and evaluate sensory evoked potentials in brains of mammals (Liu et al., 2022), birds (Yano, 1976; Engelage and Bischof, 1990) and fish (Bullock et al., 1990a; Bullock et al., 1990b; Hofmann and Meyer, 1993; Karamürsel and Bullock, 1994), to mention some. In cephalopods, Bullock (1984) observed voltage fluctuations as the result of summed spike potentials. In addition to spontaneous activity VEPs from an unanesthetized and unrestrained cuttlefish have been detected through electrodes implanted in different brain regions (Bullock and Budelmann, 1991). What the Authors found has been interpreted as vertebrate-like ongoing background activity, appearing different from signals identified in other invertebrates (at least at the time of the study), with VEPs recorded in cuttlefish appearing to «have relatively long latencies, multiple peaks extending for several hundred milliseconds, some relatively slow and long-lasting waves and slow recovery as well as low following and fusion frequencies» (Bullock and Budelmann, 1991, p. 148).

Recently, Pungor and colleagues (2023) used two-photon calcium imaging for detecting and measuring visually evoked responses in *O. bimaculoides*, at the level of the optic lobe. Authors found similarities in neural visual processing between octopus and other species, including a retinotopic organization of responses, ON/OFF pathways and size selectivity, thus reporting visual processing dynamics across the layers of the octopus optic lobe for the first time (Pungor et al., 2023). Gutnick and colleagues (2023) recorded electrical activity from the brain of freely behaving *O. cyanea* implanting four 1-mm-spaced electrodes on the dorsal side of octopus brain (at the level of the superior frontal and vertical lobe) connected to a Neurologger data loggers inserted in the mantle of the animal. Behavioral activity patterns have been correlated to oscillations frequency (around 1.5-3 Hz); slow, large scale electrical transients with durations between 15-45 s have been noted with individual transients with rise times around 10–50 ms and decay time constants of 100–200 ms. In the words of the Authors «In the brain activity, we identified several distinct patterns that appeared consistently in all animals [N = 3]. While some resemble activity patterns in mammalian neural tissue, others, such as episodes of 2 Hz, large amplitude oscillations, have not been reported» (Gutnick et al., 2023, p. 1171). In addition, «transients with rise times around 10–50 ms and decay time constants of 100–200 ms [… *have been noted, with*] peak amplitudes vary over more than an order of magnitude and occasionally exceed the 2 mV range of our recording equipment. At different times during the recording, we observed at single recording sites similar transient shapes with opposite signs» (Gutnick et al.,

2023, p. 1176). Despite methodological improvements that allow to detect neural signals from untethered animal for a relatively long time (in this case about 12 hours), this study represents an interesting piece of work attempting to finding correlations between neural traces and octopus behaviour.

In an elegant study Pophale et al. (2023) examined neural activity and body patterning dynamics during sleeping and waking in *Octopus laqueus*. Authors developed methodologies to sophisticated behavioural recording and quantification and utilized Neuropixels probes for acquiring local field potentials (LFP) in live octopus. Neuropixels recordings were performed in nine different regions of the supraoesophageal mass, with sampling rates at 2.5kHz (for LFP signals) and 30kHZ (for extracellular spike signals) and synchronized to videorecordings of animal behavioural states; the study focused on quiet- (QS) and active sleep (AS). Authors detected LFP signals from discrete areas in the octopus brain (e.g., superior frontal and vertical lobes) and correlated with AS/QS states, also proving spectrograms of the corresponding LFP signals (see for example figure 3 in Pophale et al., 2023). Frequency oscillations of LFPs ranging between 0.1–10 Hz and 20–150 Hz recorded during the wake phase were noted. According to Authors AS resembles vertebrate REM sleep, when considering wake-like neural activity accompanying eye and body twitches. LFPs activity differed across brain regions (with strongest activity registered at the level of the vertical lobe system) with large increases in LFP activity in AS when compared to QS. In this last case, octopus brain appeared relatively silent (LFP activity lower than that of AS across frequencies and recorded brain areas) with brief flashes of skin coloration accompanied by LFP activity resembling that of waking across brain regions. For this finding it is not possible to rule out if this is the case of brief periods of waking, or micro-arousal states of the octopus, as possibly expected considering being an active predator. Occasional 12-18 Hz oscillatory events (LFP oscillations resembling mammalian sleep spindles), not associated with behavioral changes, have been also recorded (Pophale et al., 2023).

Hallmarks of Consciousness, the cephalopod case

The entire set of above-mentioned studies, despite the original aims and the possibilities to finally correlate behavioral with neural signatures at higher level of signal integration, bring us to

something that is also relevant to this Thesis: the subjective awareness of ourselves and our environment, i.e. consciousness. Thinking of cephalopod molluscs, one may ask if there is the possibility that subjective awareness can be addressed in these marine invertebrates.

Following Edelman & Seth - and many others - there are abundant and increasing behavioral and neurophysiological evidence suggestive of the existence of conscious states in some animals (Edelman and Seth, 2009; Birch et al., 2020; Ehret and Romand, 2022). A requisite is the capacity of exhibit complex cognitive abilities and behaviors, and the possession of strikingly elaborate brains.

Consciousness as a process builds from the interaction between brain, body, and environment, highlighting the integrated nature of conscious experience into a unified picture of the world from a subjective perspective, encompassing various sensory modalities and motor actions. Developing this, Dr Gerald Edelman identified two distinct classes of consciousness: the first-order or primary consciousness[1], and the higher-order consciousness[2] (Edelman, 1989; 2003). In the philosophical discourse on consciousness, Chalmers (1995) proposed a distinction between the "easy" and "hard" problems of consciousness. The first refers to describing how the brain functions during both conscious and unconscious states - a task that, while complex, is possible within the bounds of scientific investigation. The hard problem, on the other hand, concerns the subjective nature of conscious experience - why and how physical processes in the brain give rise to subjective, qualitative experiences. This aspect of consciousness remains one of the most challenging and controversial topics in both philosophy and neuroscience.

Considering a behavioural perspective, consciousness has been associated with behavioural flexibility (e.g., plasticity), among other attributes. Marked learning capabilities and interindividual differences in temperament have been observed across several species, including cephalopods, with the existence of ‘personalities' in octopus (e.g., Borrelli et al., 2020; O'Brien et al., 2021; Dissegna et al., 2023) and other cephalopods being considered an «interesting manifestation of individual differences», but questioned as a demonstration of «consciousness

[1] This refers to the basic ability to perceive and describe one's immediate environment and interactions with it. It involves direct experience of sensory inputs and motor outputs, allowing an organism to be aware of its surroundings and its own actions.
[2] Building upon primary consciousness, this form involves the interpretation of primary conscious contents. It includes a sense of self and, in its most developed state, the ability to reflect on past experiences and imagine future scenarios. It enables abstract thinking, self-awareness, and the construction of complex mental models.

or self-monitoring» (Mather, 2008, p. 43). The large spectrum of temperaments (i.e. 'shy-bold continuum') observed in some cephalopods support the hypothesis that even in these molluscs each individual develops its own unique response to a given stimulus, forming a specific workspace and an appropriate representation of the environment (Baars, 1994; review in Ponte et al., 2022). Although the large body of evidence for high-level behavioural flexibility and capacity might suggest the representation of a multimodal set of perceptual and motor events in the cephalopod nervous system, it seems that this sole 'element' would not be sufficient to conclude for the existence of primary consciousness in these animals. Advanced cognition does not necessarily coincide with conscious experience, even in human beings (Vallortigara, 2017).

To achieve a given degree of 'consciousness', animals must possess highly elaborated neural structures capable of generating a 'global workspace' in which sub-networks of signals, from otherwise disparate inputs, are bound together within a single dynamic network (Baars, 1994). Global Workspace Theory was clearly formulated with the mammalian brain in mind: conscious gestalts arise specifically from signalling both within the cerebral cortex and between cortex and thalamus. After further refinements through physiological and imaging data – derived primarily from human subjects – the extension of the theory to investigations of consciousness in animals quite distant from the mammalian (or even vertebrate) line should be possibly explored following the matching against the fulfilment of different criteria, namely three well-established brain correlates and a number of 14 distinctive properties (for review see Ponte et al., 2022). Key features being EEG signatures, existence of cortex and thalamus analogous structures, and widespread brain activity. For an overview of features, dimensions, and hallmarks of consciousness see Ponte et al. (2022), as far as other recent reviews summarizing evidence (Mather, 2008; Godfrey-Smith, 2013; 2016; Birch et al., 2021; Mather, 2021a; b; 2022) to whose I am referring; a short account around these matters is not given in this Thesis, despite interest and relevance.

Extending this framework to cephalopods, a logical starting point would be the assessment of behavioural and cognitive abilities, and the identification of reliable neural correlates analogous to those observed in conscious, awake vertebrates.

EEG recordings reveal distinct patterns associated with different states of consciousness. Different types of brain rhythms indicate that simultaneous activity of the brain cortex neurons depend on the subject 'mental state'. The frequency of brain waves is reported to

indicate/correlate sleep, consciousness, cognition, and some mental disorders. Slow brain waves are seen in some conditions such as sleep, coma, brain death, depression, obsessive–compulsive disorder, while rapid waves are generally reported in conditions such as epilepsy, anxiety, posttraumatic stress disorder, to mention some. Brain waves have been categorized into several main groups based on their frequency (see later in this Thesis) in relation to different states of the brain activity. Conscious experiences correlate with widespread brain activation (Tononi et al., 1998; Srinivasan et al., 1999); when a stimulus is presented during a conscious state, activity is observed across multiple brain regions. However, similar stimuli presented during unconscious states elicit only localized activation in sensory areas (Dehaene et al., 2001). Finally, research suggests that in mammals the neural basis of consciousness is primarily associated with the thalamus and cortex. These structures and their interactions appear to be crucial for generating and maintaining conscious experiences.

The sophisticated behavioural repertoire and cognitive abilities of cephalopods (and octopus) strongly support the hypothesis of the presence of conscious states in these animals (Mather, 2008; Ponte et al., 2022). The diverse and complex behaviours exhibited by cephalopods often match or exceed those observed in some vertebrate species, leading researchers to re-evaluate conventional ideas about the cognitive limits of invertebrates (Ponte et al., 2022). To make a compelling case for consciousness in cephalopods, a thorough understanding of their neuroanatomy is essential. In addition, cephalopods exhibit several neurophysiological features that bear striking similarities to those observed in vertebrates. As mentioned above EEG-like signatures and evoked compound-field potentials in cephalopods resembling recordings from vertebrate brains have been reported, together with evidence of widespread brain activity in these animals, further supporting the notion of complex neural processing. These neurophysiological observations provide a preliminary foundation for exploring the possibility of consciousness in this taxon. However, to draw more definitive conclusions, particular attention must be paid to advance our knowledge around neural signature, and the search of anatomical structures analogous to the thalamocortical system in mammals.

Establishing these analogies could offer valuable insights into the evolution and nature of consciousness across diverse animal taxa; a little contribution towards this end is represented by this PhD thesis.

Aims of this PhD Project

The idea of the PhD project emerged from the increasing need of additional experimental evidence in support of the possibility of examining properties and conditions at the basis of consciousness in cephalopod molluscs, thus in at least some invertebrates.

Despite the original aims were broader - also including the exploration of behavioural components and a more attentive morphological investigation around the presence of neural substrates analogous to mammalian cortex and thalamus - I focused my attention to the identification of neurophysiological dynamics considered to resemble the functional signatures of conscious states in mammals.

I am referring here to EEG signature (see for example, Seth et al., 2005; review in: Edelman and Seth, 2009; Ponte et al., 2022).

Cephalopods are iconic species for the humanity (Nakajima, 2018; Nakajima et al., 2018; O'Brien et al., 2019; Zarrella et al., 2019) that contributed to important achievements in the scientific endeavour including e.g., **a**. the preparation of the squid giant synapses as a gold standard for the electrophysiological investigation of properties of excitable membranes, and the analysis of ionic currents involved in action potential and its propagation in neural systems (e.g., Stanley, 1990); **b**. the basis of search for neural correlates of behavioural plasticity, including learning (review in Marini et al., 2017), to mention some.

Cephalopods, and octopus in particular, intrigued scientists for marked analogies – mostly functional – between the taxon and mammals; these are based on biological adaptations, neural organization, cognitive abilities and genome innovations (Packard, 1972; Roth, 2013; Roth and Dicke, 2013; Roth, 2015; Albertin and Simakov, 2020). For example, analogies between the brains of cephalopods and avian and mammalian brains, and the putatively analogous neural attributes provide further argument for a systematic exploration of the boundaries of higher cognitive functions, including consciousness (Edelman and Seth, 2009; Ponte et al., 2022).

For the sake of this PhD project, I explored:

i. Possible morphological and functional analogies between the mammalian (and vertebrate) thalamus and parts of the octopus' brain in the search of evidence of one of the required "hallmarks".
ii. The development of minimally invasive electrophysiological techniques to acquire EEG signatures for both spontaneous and evoked neural signals from live octopus. Through electrical recordings I have been able to detect general characteristics of the neural traces in amplitude, frequency domain and oscillations. This approach is unprecedented, to the best of my knowledge.
iii. The effects of electrical stunning as a way to potentially interfere with neural brain functioning to induce a status where a reduction/impairment of consciousness is observed, in terms of its neural correlates.
iv. I also planned and finalized preliminary experiments examining possible biological correlates of the effects of stunning on brain functions, at molecular level (the outcomes of such experiments are not included in this Thesis, for lack of time).
v. The identification of mammalian like EEG signatures and widespread brain activity considering frequencies of neural signatures and the corresponding frequency-bands of EEG signal normally detected in mammals.

In my view, the above represent a little but significant advancement in our current path to the understanding of neural plasticity and explore hallmarks of consciousness in octopus. Despite the limitations and exploratory character of my results, I hope that this work will provide the basis for future studies around this very important research questions.

The thalamocortical “hallmark”: exploring functional analogies in cephalopods as a working hypothesis

The quest to understand consciousness across the animal kingdom has long been a central challenge in neuroscience and philosophy (e.g, Edelman and Seth, 2009; Allen and Trestman, 2017; Birch et al., 2020).

As mentioned in the Introduction of this Thesis and in line with a behavioural perspective, criteria/hallmarks for consciousness have been associated with several attributes of cephalopod behaviour (review in e.g., Edelman et al., 2005; Edelman and Seth, 2009; Hanlon and Messenger, 2018; Ponte et al., 2022) including behavioural flexibility/plasticity, learning capabilities, interindividual differences in temperament. The large body of evidence for the sophisticated cognitive abilities of these animals suggest the representation of a multimodal set of perceptual and motor events in cephalopods, referred to octopus in the greatest number of cases (review in: Mather, 2008; Birch et al., 2021; Mather, 2021a; b; 2022; Ponte et al., 2022). However, the behavioral perspective *per se* is not considered sufficient to make the case for primary consciousness in these organisms sufficiently robust (Edelman and Seth, 2009; Vallortigara, 2017; Gutfreund, 2018; 2019). Another ‘feature’ to evince any degree of consciousness corresponds to the possession of highly elaborated neural structures capable of generating a ‘global workspace' in which sub-networks of signals from various inputs are bound together within a single dynamic (vast/broad) network.

The thalamocortical system in mammals has been considered a hallmark of consciousness, crucial for integrating sensory information and generating conscious experiences. It represents one element of the multimodal framework for assessing primary consciousness in animals: exploring functional analogies in cephalopods represents a working hypothesis. The remarkable cognitive abilities recognized for cephalopod molluscs, challenge this vertebrate-centric view, and necessitate a broader, more inclusive approach to studying consciousness (review in e.g., Edelman and Seth, 2009). The approach of exploring consciousness beyond mammalian brain structures may be considered as grounded on a concept: a dynamic process involving brain, body, and environment, allowing to explore how consciousness-like phenomena might arise from diverse neural architectures.

Cephalopods, including cuttlefish, squid and octopus, have emerged as compelling subjects for investigating invertebrate consciousness. Features as sophisticated problem-solving abilities, capacity for learning and memory, and complex nervous systems have prompted researchers to reconsider a marked homology principle in the search of the neural prerequisites for consciousness. Edelman and Seth (2009) – among other works from the Authors – identified potential candidates among the structures in cephalopod brains, suggesting that these maybe considered to serve as analogous to mammalian thalamus and cortex. Based on other arguments, Shigeno et al. (2018) provided elements to a discussion around the existence of functional analogies between neural structures in the cephalopod brain (e.g., dorsal basal- and sub-vertical lobes, inferior frontal lobe, and frontal and vertical lobes) and vertebrate/mammalian structures (see table 1 in Shigeno et al., 2018). In particular, the Authors recognize **a.** the vertical lobe system (i.e. superior frontal- vertical-lobes) to possibly correspond to cerebral cortex (pallium), hippocampus, amygdaloid complex, **b.** the dorsal basal lobe to the thalamus, **c.** the anterior basal lobe to basal ganglia, to mention some. Neurons and neural circuits in cephalopod brains have been thus suggested to perform functions like those of the thalamocortical system in mammals, despite significant anatomical differences (e.g., Edelman and Seth, 2009).

This chapter briefly outlines lines of research that should be investigated to test this hypothesis, encompassing cellular and molecular analyses, morphological studies, functional investigations, connectivity mapping, and comparative studies. By exploring functional analogies to the thalamocortical system in cephalopods, my aim is to contribute to a more nuanced and inclusive framework for exploring the thalamocortical 'hallmark' in these animals; here we[3] explore functional brain analogies in cephalopods as a working hypothesis.

The so-called brains of cephalopods originate from shortening and rearrangements of tracts and connectives, linking ganglia of molluscan origin (Kandel and Kupfermann, 1970; Kandel, 1979), that acquired unusual features that distinguish them from the nervous systems of other molluscs (review in Ponte et al., 2021). In brief, to mention some, we recognize ***a.*** highest degree of

[3] This chapter has been elaborated after discussion with Dr G. Fiorito and with the help of Ms Catherine Golinvaux, an IMBRSea Intern of OctoLab (Stazione Zoologica, Napoli, Italy; Tutors: Drs G. Ponte and G. Fiorito) who enthusiastically contributed to and early draft of this chapter.

centralization among invertebrates (insects excluded), ***b.*** neurons of small size (e.g., nuclear diameters of 2–5 µm), allowing a greater cell density, ***c.*** the existence of a blood-brain barrier (unique among molluscs), ***d.*** compound field potentials, similar to those recorded in vertebrate brains (for review see e.g., Brown and Piscopo, 2013), **e.** a large variety of putative neurotransmitters and neuromodulators (review in Ponte and Fiorito, 2015).

During its evolution, a radical shift in the gross neural organization of the cephalopods resulted from a change in position and relative volume of the different areas of the nervous system that occurred with the addition or loss of ganglia, thus bringing to the emergence of a central brain subdivided into a variable number of lobes (depending on species). The central nervous system varies across different cephalopod genera, with levels of neural complexity considered to parallel the complexity of sensory inputs and of behaviours modulated and performed. The greatest degree of nervous system centralization is recognized to occur in octopuses (Nixon and Young, 2003).

Overall, the octopod brain is more ‘centralized’ than the one of decapods (cuttlefish and squid (cuttlefish and squid; e.g., Young, 1976; Young, 1977; Young, 1979) where brachial and pedal lobes are fused and the superior buccal lobe encapsulated the inferior frontal lobe, much simpler in architecture when compared to octopuses. This view brings to the identification of taxon-specific ‘cerebrotypes' in cephalopods, providing another analogy with brain architectures of vertebrates (Ponte et al., 2021). In the case of cephalopods, quantitative differences between the brains of different species reflect variations in habitat and lifestyles. This supports the idea that cephalopod taxa evolved different sensory and cognitive strategies to cope with the differential demands of life in the ocean; complexity and diversity evoking comparisons to similar adaptations found among vertebrates (e.g., Packard, 1972; Amodio et al., 2019; Albertin and Simakov, 2020; Schnell et al., 2021).

The thalamocortical system

Koch et al. (2016) emphasize that the maintenance of cortical information processing during conscious states depends on preserved communication between the cortex and key subcortical structures, with the thalamus being the most significant (Shine et al., 2023).

The thalamus is a highly conserved subcortical structure highly interconnected with other brain areas at micro-, meso- and macro-scale. In mammals, excitatory cell types project to distinct layers of the cerebral cortex making 'contact' with specific cellular subpopulations, thus generating different impacts on cortical computations. At mesoscale, the thalamus is considered to control the core and the matrix excitability of distributed regions of the cerebral cortex via projections that 'augment' local or more wide distributed resonance in corticothalamic loops. At macroscale, the thalamus is deeply interconnected with the entire cortex, providing an intricated distributed network considered to be at the neural basis of the conscious experience.

Both convergent and divergent architectures within the same neural system are noted to exist: i.e. multiple areas in the cortex projecting to a given thalamic area and single thalamic areas projecting to various/multiple cortical areas, respectively (Whyte et al., 2024). In terms of neurochemical fingerprint, it appears remarkable that the excitatory projections of thalamic neurons are embedded in a dense inhibitory neural network (i.e., GABA). Furthermore, the thalamus represents a hub «connecting the ascending sensory pathways and arousal system from the brainstem with the massive projections from the cerebellum, basal ganglia […], as well as the vast reciprocal connections with the entire cerebral cortex» (Whyte et al., 2024, p. 1613).

In addition, and as mentioned above, convergence is another feature facilitating efficient information processing in the brain.

Furthermore, the arrangement in nuclei serving as the primary pathways for sensory information from the body to the cortex, and those facilitating information transfer between different cortical areas, allows for complex inter-regional communication within the brain (Sherman, 2007; 2016; Shine, 2021). Thus, the thalamus acts as a sensory relay centre, channelling almost all sensory inputs (except for olfactory information) to the cerebral cortex or pallium (Riss et al., 1972; Swanson, 2007).

The thalamocortical system plays a pivotal role in various leading theories of consciousness, including Dendritic Integration Theory, Global Neuronal Workspace Theory, Integrated Information Theory, Recurrent Processing Theory, and Predictive Processing and Neurorepresentationalism. While these theories differ in their specifics, they share a common thread: the recognition of interconnectedness within the thalamocortical system as crucial for consciousness; a feature that is here reiterated. The shared emphasis on neural interactions, facilitate by both local and long-range connections in thalamocortical networks, and is key to

understanding the emergence of conscious experiences awareness (Storm et al., 2024). Furthermore, thalamic cells also play crucial computational roles in shaping experiential properties, with characteristic neuroanatomy of the core and of matrix 'role' of the thalamic nuclei, and their embedding in the rest of the brain support key properties of the contents of consciousness (integration/unified, segregation/differentiated, continuity). The relative importance of these properties differs across theories of consciousness, but such concepts «have a long history in the philosophy and science of consciousness and have been discussed by a wide range of theorists with divergent starting assumptions» (Whyte et al., 2024, p. 1617).

In a fascinating account Edward G. Jones (1985) provides a detailed comparative overview of the anatomy of the thalamus among vertebrates. In his words, the diencephalon of fish, amphibians, reptiles, and birds led the identification of a basic pattern of neural organization with a distinct preoptic area characterized by a thick periventricular cell layer and dispersed cells laterally and anteriorly penetrated by medial and lateral forebrain bundles passing towards the telencephalon. Throughout the length of the diencephalon in all species (with exception of birds and a few reptiles) dorsal-, ventral-thalamus and hypothalamus are characterized by «a thick periventricular layer of cells, interrupted at the sulci of Herrick and laminated dorsoventrally to varying degrees […]. In cyclostomes and many urodele amphibians, virtually all neurons are in the periventricular layer […]. In other groups of nonmammals there are varying degrees of migration of cells into the surrounding neuropil. In most cartilaginous fish, there is relatively little nuclear differentiation in the cells outside the periventricular layer. In teleosts and anuran amphibians, a few distinct nuclei are seen among the looser packed cells; more nuclei become visible in most reptiles […], while in some reptiles and all birds the periventricular layer has virtually disappeared […], the surrounding neuropil has become tightly packed with neurons, and many nuclei, some with further cytoarchitectonic divisions, are distinguishable among them» (Jones, 1985, p. 769). Jones provides a through description of the anatomy of the thalamus and its inter-relationship with other brain structures of the major taxa of vertebrates. In his survey of the nonmammalian thalamus the diversity of this structure emerge revealing that in many instances thalami appear different from one another "as they are from those of mammals". However, basic patterns emerge, and in particular; **a.** a periventricular thalamus with afferent fibres terminating in a lateral neuropil suggesting the evolution of a structure (i.e. the thalamus) that is then characterized by nuclei migrated into the neuropil (e.g., as in anuran amphibians in

comparison with urodeles). Remarkable appear to resolve a common origin of the reptilian and avian thalamus, although differences between reptiles (large and differentiated thalamus) and birds (small, poorly differentiated) appear in line with the observed diversification known for some mammalian species (Jones, 1985). Apart from the morphology, patterns of connectivity appear to be the key and are recognized in "all vertebrate thalami". Jones refers to afferent connections to the thalamus (from all sensory systems), thalamic projections to defined areas of the pallium or towards non mammalian-like structures of cartilagineous fish (central telencephalic nucleus), reptiles (dorsal ventricular ridge) and birds (hyperstriatum), with thalamo-striate connectivity recognized in almost all species. In his words «[w]hether these are fundamentally homologous aspects or examples of parallel evolution can only be guessed at, but they show far greater parallelism between the mammalian and the nonmammalian thalamus than had been anticipated from the studies of Herrick and his contemporaries» (Jones, 1985, p. 800).

A detailed description of thalami across vertebrates is out of the scope of this chapter. I would like only to recall a couple of other interesting pieces by Cassel and Pereira de Vasconcelos (2021), who reviewed the historical identification and/or characterization of the thalamus from antique to our times, and a route into the evolution of thalamus among vertebrates with a glance at the neuromodulatory fingerprint and functional perspective with a comparative outlook (Butler, 2008).

The centrality of the thalamocortical system in theories of consciousness raises an intriguing question: How can we conceive of consciousness in non-mammalian species that lack these specific neural structures? This challenge invites us to reconsider our understanding of consciousness and its potential manifestations across diverse forms of life starting with one of the most fascinating classes in the Mollusca phylum: Cephalopoda.

Exploring analogies of the thalamus in cephalopods and possible future directions

As mentioned above, Shigeno and coworkers (2018) overviewed various areas/parts of the nervous system of cephalopods in the search of possible evolutionary convergence of the neural organization in cephalopods when compared to vertebrates' neural systems. Following the Authors, key-features of the vertebrate thalamus (i.e. sensory relay centre, gatekeeper to the cortex, existence of various nuclei with distinct sensory fields) can be traced in the sub-vertical and dorsal basal lobes in the supraoesophageal mass of cephalopods (mainly *O. vulgaris*). According to Young (1963; 1971) the subvertical lobe (originating from the ancestral cerebral ganglion), is a structure strictly connected to the vertical lobe (lying dorsally) and consisting of a wall that in several regions is folded to form islands of cells, with transverse and longitudinal fibre bundles and patches of neuropil between each other. The lobe counts 810,000 neural cells, many of them being less than 5 µm in diameter and very few larger than 10 µm. Following the original description by Young, at both sides of the sub-vertical lobe there are cells with diameter of 5 – 10 µm, but also neurons that are the largest of the whole supraesophageal mass of *O. vulgaris*, reaching about 25 µm (see also: Gray and Young, 1964; Ponte, 2012). Ventrally to this structure in the supraoesophageal mass (more in a ventral position) are located a series of lobes belonging to the basal lobe system. This is considered the most 'higher' centre for motor control (see also: Young, 1991a; Zullo et al., 2009) and constituted by six lobes or parts with cells of different dimensions and either distributed in layers or dispersed in the neuropile. The dorsal basal lobe counts about 2 million neural cells mostly of about 5-10 µm and many less than 5 µm in diameter. In the original description for *O. vulgaris* Young refers to be organized in islands of neurons separated by neuropil and by islands of neuropil surrounded by neural cells. The lobe is organized into a "complicated" pattern of cell islands and "promontories" (as Young describes), that looks like associated to the arrangement of interweaving bundles of transverse fibres (from optic lobes) and longitudinal ones running from the subvertical lobe and between the parts of same dorsal basal lobe. In general, the characteristic arrangement of the lobe in octopus is with two subdivisions (anterior and posterior parts) separated by a vertical wall of cells. Dorsally in the mid-line this wall carries extensions anteriorly and posteriorly, serving partially to divide both parts into right and left halves. Although accurate description of the anatomy of the brain of decapods (squids and cuttlefish) is available, we have no similar refined description to refer to in

the case of the dorsal basal lobe in *Loligo* (Young, 1977). When compared with the description of *Loligo*, a more complex architecture of the subvertical lobe results for the case of *O. vulgaris* (see Young, 1971 vs. Young, 1979).

Based on the overall description, connectivity and putative function Shigeno and coworkers suggested that the two lobes (i.e. subvertical and dorsal basal lobes) «could be considered as candidates for analogs to the vertebrate thalamus» (Shigeno et al., 2018, p. 8). These lobes receive numerous input fibres via both direct and indirect pathways from the various brain territories thus suggested to function as relay centres for the outermost frontal and vertical lobes, much like the thalamus does for the cortex in vertebrates. While the exact number of neural fibres in these structures is unknown, Young identified several tracts (about 10) originating from or terminating at these lobes in *O. vulgaris* (Young, 1971).

In terms of modulatory fingerprint these two structures also recall somehow what is known to characterize the vertebrate/mammalian thalamus. Despite the limited details available about the distribution of cell-types and localization of neuromodulators (their transporters and receptors) in these two areas, the information available to us support the view of important contribution of GABA, and dopamine, noradrenaline (to mention some others) - as recognized in mammalian thalamus (review in Jones, 1985; e.g., Arcelli et al., 1997; Kim et al., 1997; García-Cabezas et al., 2007) - is well represented in the octopus brain, where several cells and fibres are positive to GABA (Figure 1), dopamine, noradrenaline (Ponte, 2012; Ponte and Fiorito, 2015; Shigeno and Ragsdale, 2015). Muscarinic receptors have been putatively considered to occur in the octopus brain (Fiorito et al., 1998); a recent mapping of receptors and transporters linked to the neuromodulator fingerprint in octopus has been recently initiated by Pizzulli (2023).

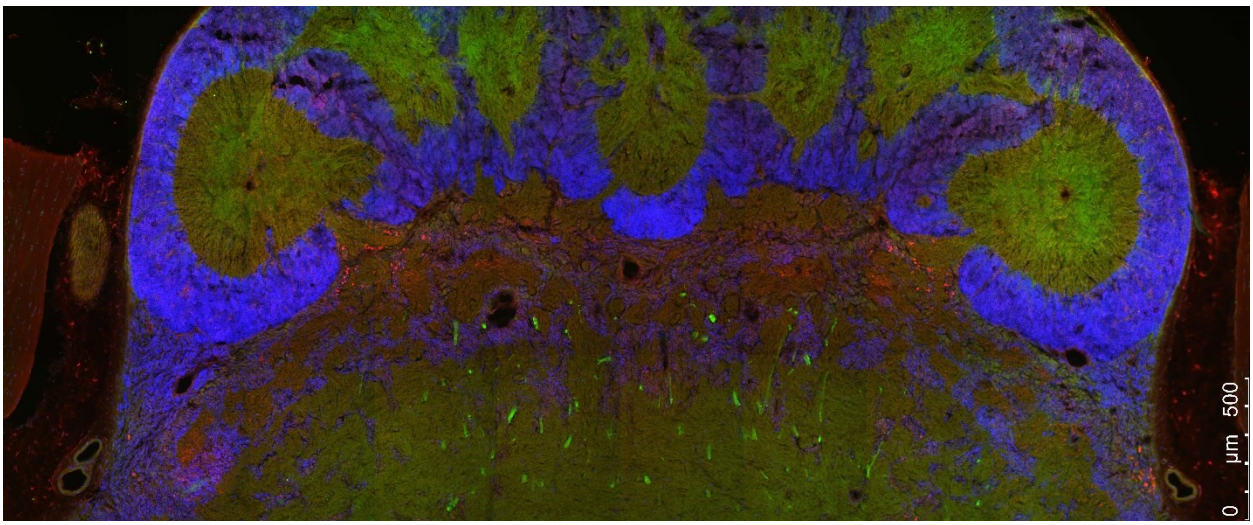


Figure 1. Double-immunolabelling IR of GABA and 5-HT in a coronal section of the supraoesophageal mass (SEM) at the level of the vertical lobe (5 gyri on the dorsal side, top of image) including the area belonging to the sub-vertical (SVL) lobe. The two neuromodulators are not uniformly distributed in the brain of the octopus, and in some cases tracts (or nerves) are mainly GABAergic with positive serotoninergic labelling surrounding. In the SVL are clearly evident longitudinal fibres depicting a putative inhibitory control along the anterior/posterior axis of the SEM mimicking GABAergic inhibition operating in thalamocortical networks (see for a comparative overview Shigeno et al., 2018). DAPI (blue) nuclear staining, GABA-immunoreactivity (green), and serotonin-immunoreactivity (red). Figure courtesy of Dr G. Ponte.

A mention should be given to the vertical lobe systems; the lobe contains large populations of uniquely distributed small interneurons (amacrine cells), parallel-running fibres, and reverberating circuitry across different lobes (Young, 1971; Young, 1979; Young, 1991a; 1995), a neural organization allowing for the integration, refinement, and maintenance of sensory information. Notably, these areas exhibit synaptic plasticity through NMDA-independent long-term potentiation (LTP), a mechanism considered as the cellular analogous of long term memory (Hochner et al., 2003; Shomrat et al., 2008; Shomrat et al., 2011; Turchetti-Maia et al., 2017). The heterogeneity of neurochemical identity in these lobes (Ponte and Fiorito, 2015; Shigeno and Ragsdale, 2015) further suggests a high level of adaptability and complexity.

While cephalopods lack direct homologues of the vertebrate thalamocortical system, the dorsal basal and sub-vertical lobes are considered as sensory "gatekeepers" to the frontal and vertical lobes, suggesting that they may serve as adequate analogues.

It is worth to report here that Pizzulli (2023) recently attempted also to explore neural connectivity of octopus brain finding that, for example, in *O. vulgaris* neural network the most connected regions are the medial basal lobe (32 links) and the subvertical lobe (31 links; followed

by the optic lobes with 30 links). The subvertical lobe also has the greatest indegree (n = 17) of the entire octopus brain network. Again, the structure possesses hub characteristics and rich club organization (high-degree strongly interconnected nodes).

Thus, the analogies with the mammalian thalamus maybe seen as supported by other more recent evidence.

What next?

First, analysis of morphological analogies and of the cytoarchitecture of the octopus brain (and a comparative overview of other cephalopod species) are required in our view. There are a number of features within the layered arrangement of thalamus that despite apparent missing of layers in the brain of cephalopods (possibly at the level of the subvertical and vertical lobes) can be explored to confirm the hypothesis of existence of input and output neurons. In brief in mammals, input from the dorsal thalamus terminates on neurons in layer 4 of the neocortex. These neurons project to layers 2/3 neurons, which in turn connect with layer 5/6 pyramidal neurons. These neurons send axons to subcortical regions. An equivalent circuit is present in the avian pallium where input from the dorsal thalamus terminates in primary sensory nuclei. A similar architecture of neural connectivity is possible to explore in octopus brain; interestingly it looks different when compared between species (cuttlefish vs octopus; Shomrat et al., 2011). A possible hypothesis to explore is for looking at the analogy within the vertical and subvertical lobes of putative pyramidal cells and inhibitory interneurons, i.e. large cells, their morphology and connectivity, vs amacrine small in the lobes with fine connectivity (Gray and Young, 1964).

In addition, reentrant connectivity in brain areas devoted to neural control/processing of perception and those involved in memory feedback loop (e.g., in mammals Ledergerber and Larkum, 2010).

Another aspect to consider is neural connectivity. We are just at the infancy in understanding how at macro- and mesoscale neural networks of octopus are organized. However, Pizzulli (2023) and colleagues opened the route for the identification of hub and/or small world properties in the octopus brain, with structures suggested functionally analogues of the mammalian thalamocortical, being highly candidates for possessing similar neural networks features (see also Whyte et al., 2024).

Exploration of functional analogies maybe achieved also through mapping of neuromodulatory fingerprint, via gene expression analysis and spatial transcriptomics.
Recent investigations of brain cell-type evolution in all Tetrapoda converged to suggest that the telencephalon is not composed of phylogenetically old and new regions, but of a mosaic of conserved and novel cell types; with inhibitory interneurons largely conserved in the telencephalon of different species in contrast to excitatory neurons appearing less conserved (for review see Nieder, 2023). It is noteworthy to remind that several genomic novelties has been recognized in cephalopods (and again octopus in particular), with specific ones interesting the neural/brain territories (e.g., Styfhals et al., 2019; Fiorito et al., 2020; Albertin et al., 2022; Petrosino et al., 2022; Schmidbaur et al., 2022). The analogy explored between birds and mammals speculated that transcriptional similarities should be identified at the functional level thus suggesting convergent evolution between taxa. A feature that attracted scientists in exploring cephalopods innovations at different levels.

Finally, optogenetic and chemogenetic techniques to manipulate specific cell types and neural circuits hypothesized to be analogous to thalamocortical components, and analysis of the effects of these manipulations on behaviours associated with consciousness-like states in cephalopods, properties of the nervous system (EEG-like) or response to stimulation suggesting arousal (e.g., Redinbaugh et al., 2020) may represent further future (sci-fi) hypothetical scenarios to explore, at least for some of these properties.

By integrating these approaches, future studies can help identify and characterize potential functional analogues to the thalamocortical system in cephalopods.

The exploration of functional analogies to the thalamocortical system in cephalopods offers a promising avenue for expanding our understanding of consciousness across diverse animal phyla. Our working hypothesis is that cephalopod brains may contain neural structures and circuits functionally analogous to the mammalian thalamocortical system. The proposed research directions provide a possible framework for testing this hypothesis and advancing our understanding of consciousness in invertebrates.

Recording peripheral and central bioelectrical signals in octopus. Approaches based on published evidence

In the last decades, studies on cephalopod nervous system played an important role in neurobiological research, particularly due to the simplicity of brain structure and organization (in comparison with higher vertebrate) still maintaining typical invertebrate features. Also, the centralized multilobular structure of the brain is helpful to experimentally highlight the brain's functional organization (Hochner, 2008).

Recordings of bioelectrical signals from cephalopod nervous system started in the first decades of 20th century. Signal collected from the giant axon in squid (Hodgkin and Huxley, 1939), provide first insight on the biophysical bases of action potential generation and the mechanisms of chemical synaptic transmission (Hochner, 2004).

Several studies have been conducted with the aim to evidence how the peripheral nervous system in cephalopods works and if its functions are analogous to the most known vertebrate system (Budelmann, 1995; Williamson and Chrachri, 2004; Shigeno et al., 2018; Wang and Ragsdale, 2019).

More recently, Butler-Struben et al. (2018) demonstrated the use of a non-invasive monitoring of neural signal in pallial nerves, to show the effectiveness of anaesthetic agents ($MgCl_2$ and EtOH) at achieving immobility and loss of consciousness in *Sepia bandensis, Abdopus aculeatus* and *Octopus bocki*. Authors show loss in afferent and efferent neural activity of octopus immersed in magnesium chloride (starting from a solution of $MgCl_2$ 330mM in seawater) reached within 5-10 minutes from the beginning of the induction; a long recovery (in respect to EtOH) was required for the neural signal to reappear when changing the solution where the animal was immersed back to seawater.

Peripheral system also includes the arm nerve cords and suckers complex, described as a 'complex nervous apparatus' (Bullock, 1965); the arrangement of the nervous tissue in the arm has been suggested to present marked analogies with the vertebrate spinal cord (Graziadei, 1971; Shigeno et al., 2018).

As anticipated in other sections of this Thesis, many Authors explored neural correlates of complex behaviours in some cephalopod species, including brain slices preparation (Williamson and Budelmann, 1991; Budelmann, 1995; Shomrat et al., 2008; Shomrat et al., 2010) to *in vivo* recordings (Bullock, 1984; Bullock and Budelmann, 1991; Brown et al., 2006).

Between the others, Brown and colleagues (Brown et al., 2006) recorded spontaneous neural activity from freely behaving octopus, trying to identify different 'rest-activity' cycles of the animal during the experiments. They used an invasive technique to record spontaneous activity in the vertical lobe; Authors introduced an electrode through the skull and the jelly covering the brain, to reach the supraoesophageal mass in a position close to the mid vertical lobe. The signal recorded is characterized by single spikes and spike trains. Neural activity appeared more frequent during resting period, indicating a 'body off/brain on' type of activation. Spike trains were noted to last for tens of seconds, with frequencies ranging from about 10 to 40 Hz (Brown et al., 2006). Since the vertical lobe is involved in learning and memory processing (Young, 1991b; Turchetti-Maia et al., 2017), also displaying a vertebrate-like LTP plasticity (Shomrat et al., 2008; Shomrat et al., 2011), signals recorded from this area in resting octopus was suggested to be related to memory consolidation and their presence associated with higher cognitive functions, in analogy to what is known from vertebrates (Brown et al., 2006).

For the aims of this PhD project and as a first experimental step, I attempted to replicate some of the electrophysiological approaches utilized in cephalopods (mostly *O. vulgaris*) to explore neural correlates in the live animals. I selected preparations of both peripheral and central nervous systems in order to assess the viability of *O. vulgaris* preparations - i.e. recording neural signals from live, sedated animal - in my experiments.

In particular, I adapted protocols by Butler-Struben et al. (2018), Zullo et al. (2011) and Brown et al. (2006) to record spontaneous and/or evoked neural activity from pallial and brachial nerves (peripheral nervous system) and supraoesophageal mass (SEM; central nervous system).

Methodological information is available in the Supplementary Info (STAR methods - Recording from Peripheral Nervous System).

Neural recording from octopus pallial nerves

Extracellular recordings using hook electrodes at the level of the pallial nerves (N = 9) allowed to record responses to mechanical stimulation in 90% of experiments.

Pinching at the level of the ipsilateral margin of the octopus' mantle, distal to the recording site, I recorded bursts of spikes clearly correlated to the stimulation (Figure 2).

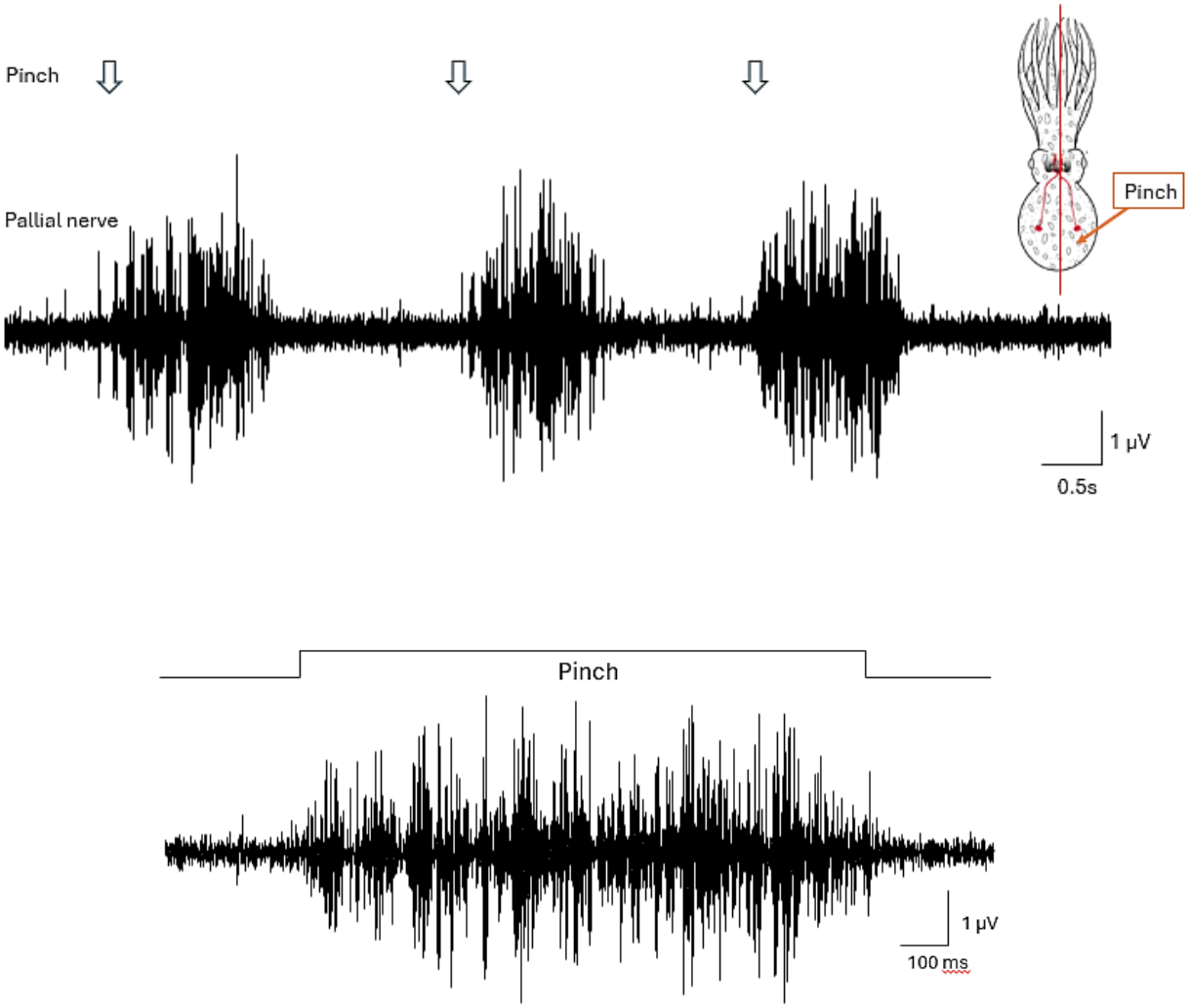


Figure 2. Extracellular recording from a pallial nerve (right side of the mantle) during mechanical stimulation (pinch of the mantle, ipsilateral). Arrows correspond to a single pinch.
Lower panel: Extended time scale of a single response evoked by a pinch. Signal is smoothed (time constant=0,8 ms).

As pinching is a manual procedure, duration and amplitude of the evoked response strictly depend on the operator accuracy, and variation maybe high between the trials

Neural recordings in my experiments appears with amplitude of the evoked potentials reaching a maximum of about 6 µV; reduction of the overall amplitude is detected after a period of time which is largely variable (from 30 minutes to > 1 hour after induction in mix solution – data not shown).

Moreover, electrical activity appeared also correlated with the contraction of the mantle during strong breathing activity (Fig. 3).

Figure 3. Extracellular recording from pallial nerves on both body sides show synchronous spike bursts. Rhythmic bursts related to mantle contractions during ventilation are shown. Contractions and expansions of the mantle linked to breathing event and burst activity on the monitor were observed and found to be in synchrony.

Patterns of neural signals after stimulation were coherent between the animals.

In a series of further experiments, I utilized von Frey filaments as mechanical stimulation (N = 6). I utilized a filament capable of applying a pressure of a standardized force (26g) to given natural landmarks located on octopus body (Figure 4-A). In particular, when midline mantle spots 1, 3, 5, 6 (see Fig. 4) were stimulated, evoked bursts were collected from both left and right pallial nerves, suggesting that mechanosensory neurons in the dorsal midline region of the mantle projects axons either on the left and/or right pallial nerve or branch (Figure 4).

Stimulation of spot 2 or 4 on octopus mantle (Fig. 4) resulted in bursts recorded only from the ipsilateral pallial nerve to the stimulation site; no neural signal was noted in correspondence of the contralateral recording electrode (Fig. 4). Pressure applied on the left side of the mantle (spot 2) did not evoke a neural response at the level of the right pallial nerve and, in the same way,

there were no neural responses from contralateral side when von Frey filament was used on spot 4 (right side).

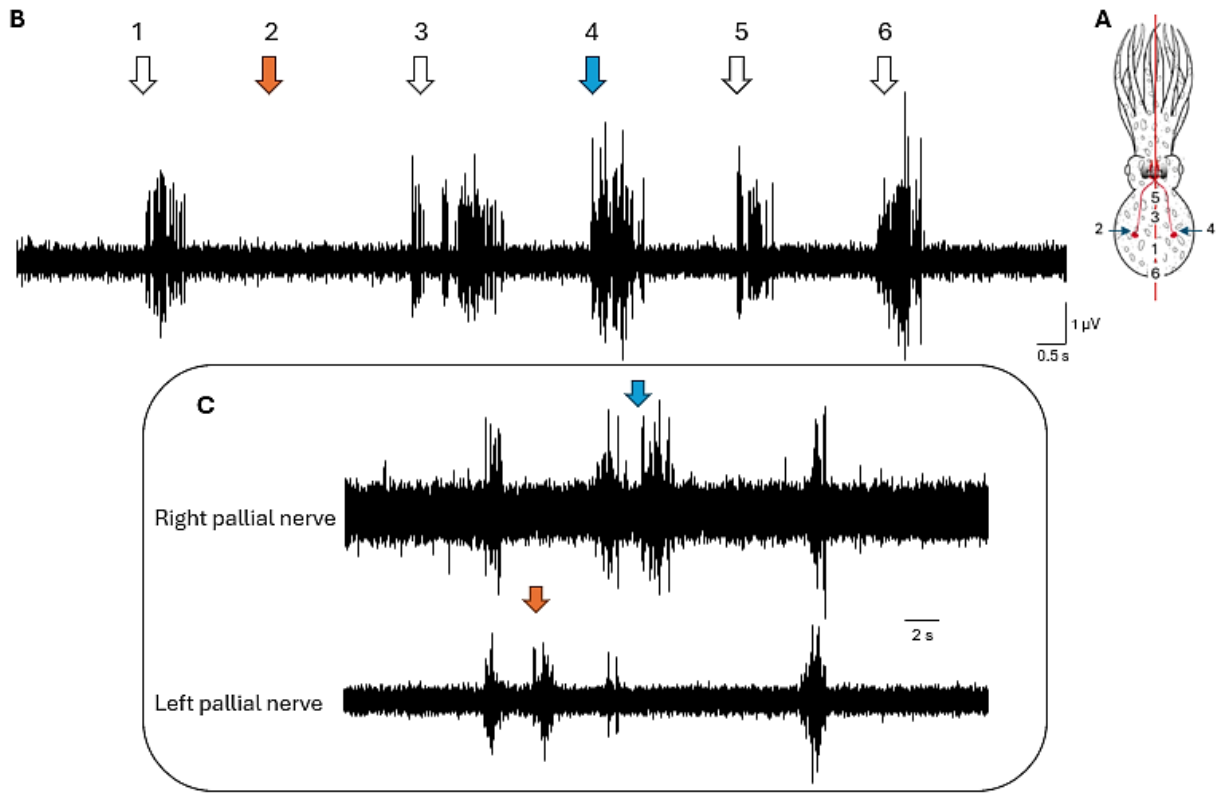


Figure 4. **A**: standardized landmarks on the mantle of the octopus, corresponding to "long mantle papillae" (Borrelli et al., 2006) and mantle tip.
B: Extracellular recording from the right pallial nerve in response to mechanical stimulations (see Fig. 4A) applying 26g von Frey filament (signal is smoothed - time constant= 0.2 ms).
C: Recordings from right and left pallial nerves of *O. vulgaris* after stimulation applied by von Frey filament (26g) in correspondence of the six landmarks of the animal mantle.

Neural recording from arm nerve cord

Bipolar silver wire electrodes anchored to the arm nerve cord were used to record neural signals at the level of the nerve between two ganglia (N = 10); electrodes were positioned at about 10 cm distance from the arm crown region. For the sake of these experiments I followed the approach utilized by Zullo et al. (2011). Pinching was applied to the skin and a sucker distal to the recording site on arm R1 (Fig. 5); the stimulation evoked spike bursts on nerve cord (R1). Vice versa, pinching skin of R2 arm evoked spike bursts in the R2 nerve cord (n = 4 preparations).

For these experiments I also applied a variant to the original strategy adopted by Zullo and coworkers (2011). I utilized two identical bipolar recording electrodes positioned simultaneously on arm R1 and R2, exposing the arm nerve cord (no more than 1 cm) at the level of the interganglionic region where the recording electrodes were positioned. In my experimental conditions a stimulation applied to arm R1 induced – as reported – an evident neural response recorded at the level of the arm nerve cord of the same arm, but not in the adjacent arm (i.e. R2; Fig. 6).

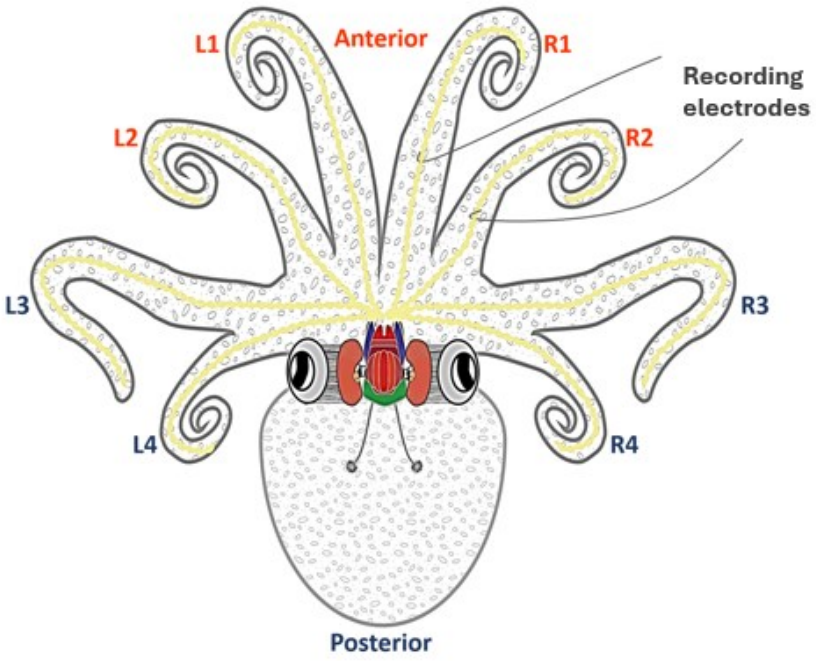


Figure 5. Schematic drawing of octopus showing the classification of the eight arms, divided by anterior (red)-posterior (blue) disposition. Right arms R1 and R2 were tested during the described procedure.

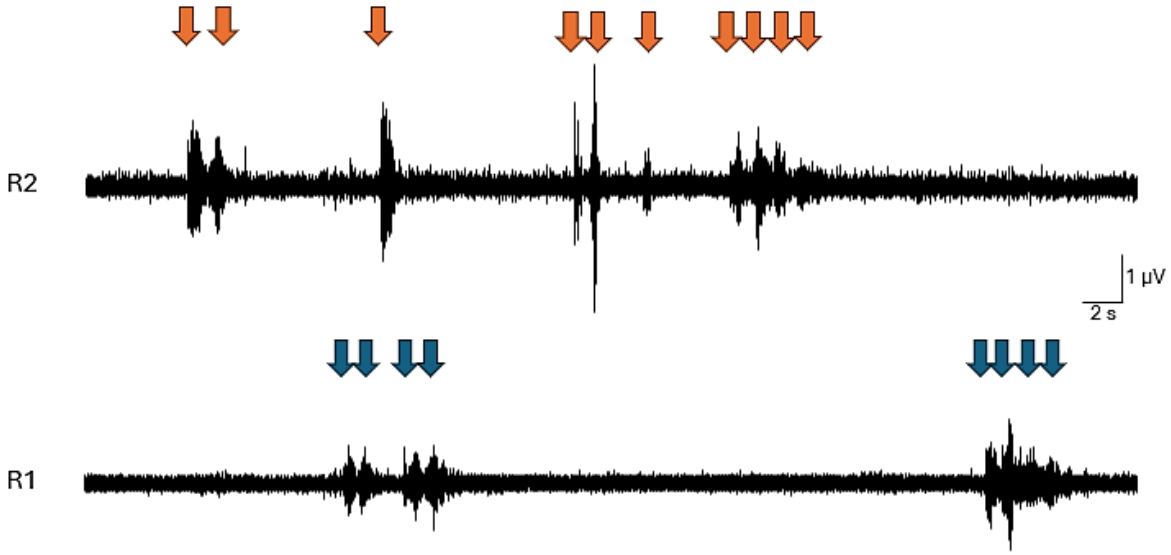


Figure 6. Arm nerve cords R1 and R2 recording. Pinching on R1 (blue arrows) provokes responses only on the same arm nerve cord, the same happens when R2 is stimulated (orange arrows). Signal is smoothed (time constant=0,6 ms).

In two preparations, a couple of bipolar silver electrodes were implanted in different regions of the nerve cord of the same arm (proximal and distal). Electrophysiological traces obtained from the two recording sites located at about 2 cm from each other, showed neural responses to mechanical stimulation from both locations when the stimulation was applied distally (Fig. 7). Under my experimental conditions, a delay of about 13 ms was noted in the neural responses. This contrasts with what occurred when stimulation was applied in correspondence of the region between the two recording sites.

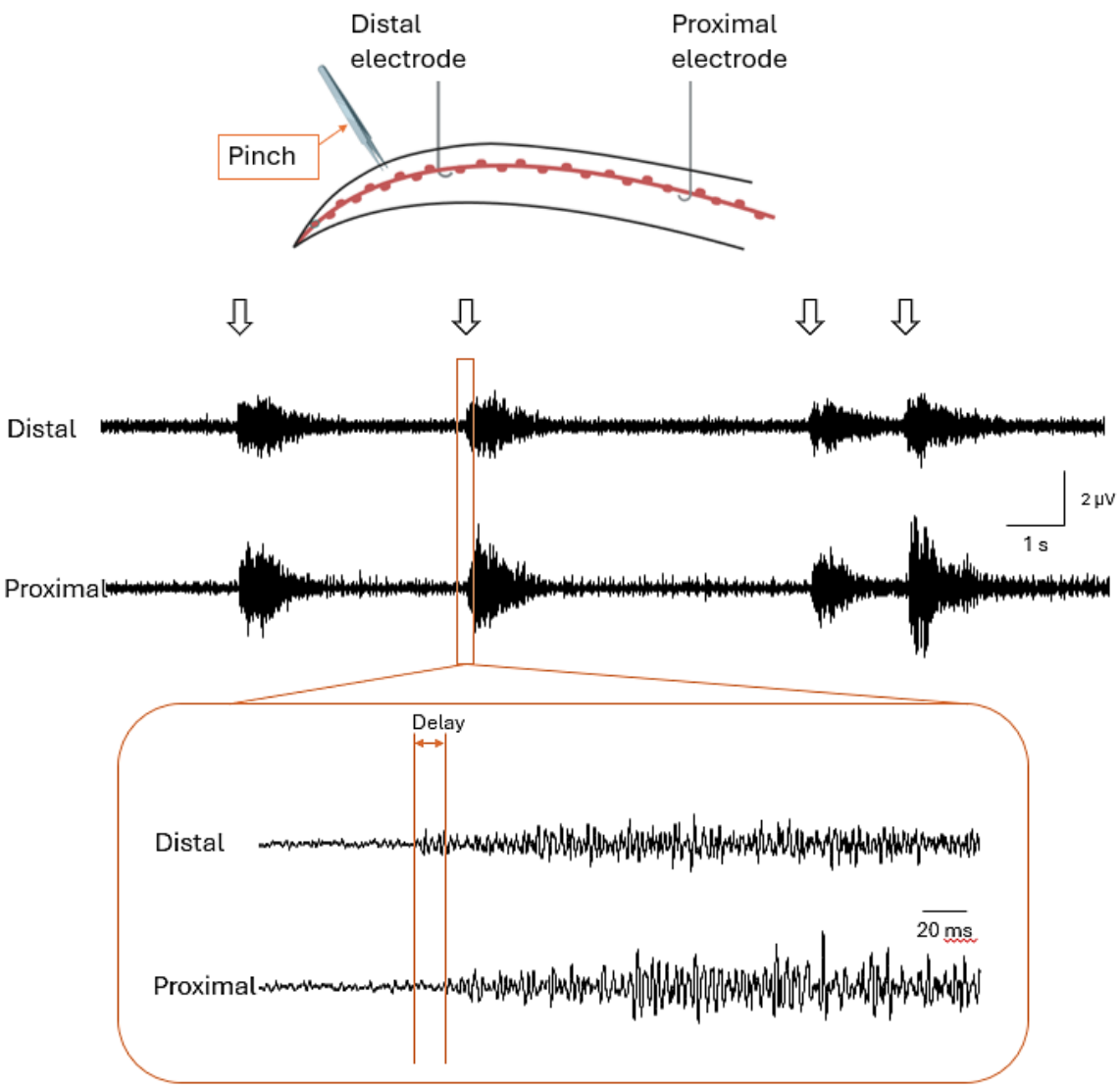


Figure 7. Arm nerve cord (R2) traces from two silver electrodes located at about 2 cm distance during pinching in correspondence of the distal region of the arm. Both electrodes record evoked response to stimulation with a delay of about 13 ms between the two locations (zoomed traces). DC removed (time constant= 0.4s) and signals were smoothed (time constant=0,6 ms).

In the latter case, electrical signals in response to the pinch are clearly visible in correspondence of the proximal electrode (Fig. 8). It is noteworthy to report that a signal characterized by smaller amplitude was recorded in correspondence of distal electrode (Fig. 8).

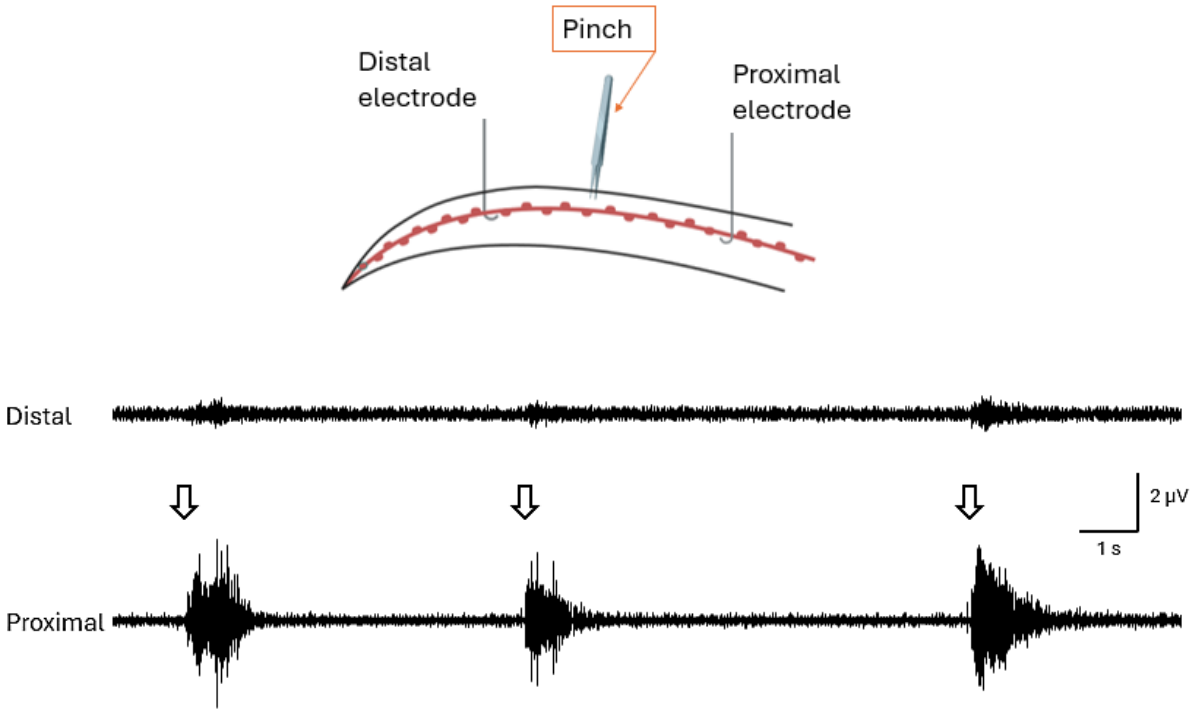


Figure 8. Arm nerve cord (R2) traces recorded from two silver electrodes located at about 2 cm distance each other during pinching in correspondence of the tract between the two recording electrodes. Traces show big signal on the proximal recording site, and a significative reduced response from the distal electrode. Signals were smoothed (time constant=0.6 ms) and DC removed (time constant = 0.4s).

In addition to mechanical stimulation, electrical stimulation of the arm nerve cord was conducted on the same preparation, using two hook electrodes recording on the same arm nerve cord. This time, the proximal electrode was used as stimulating electrode by applying 10 ms electric shocks (1 mA); interval between the stimuli was set at 1.8 s (Fig. 9).

Electrical pulses evoked neural responses on the nerve in correspondence of the distal electrode. Total duration of the elicited neural activity differed when stimulations are repeated in time. As shown in the example given in Figure 9, when delivering a train of three stimuli the first one evokes spikes activity which lasts about 500 ms, while for successive stimuli resulted shorter (< 150 ms).

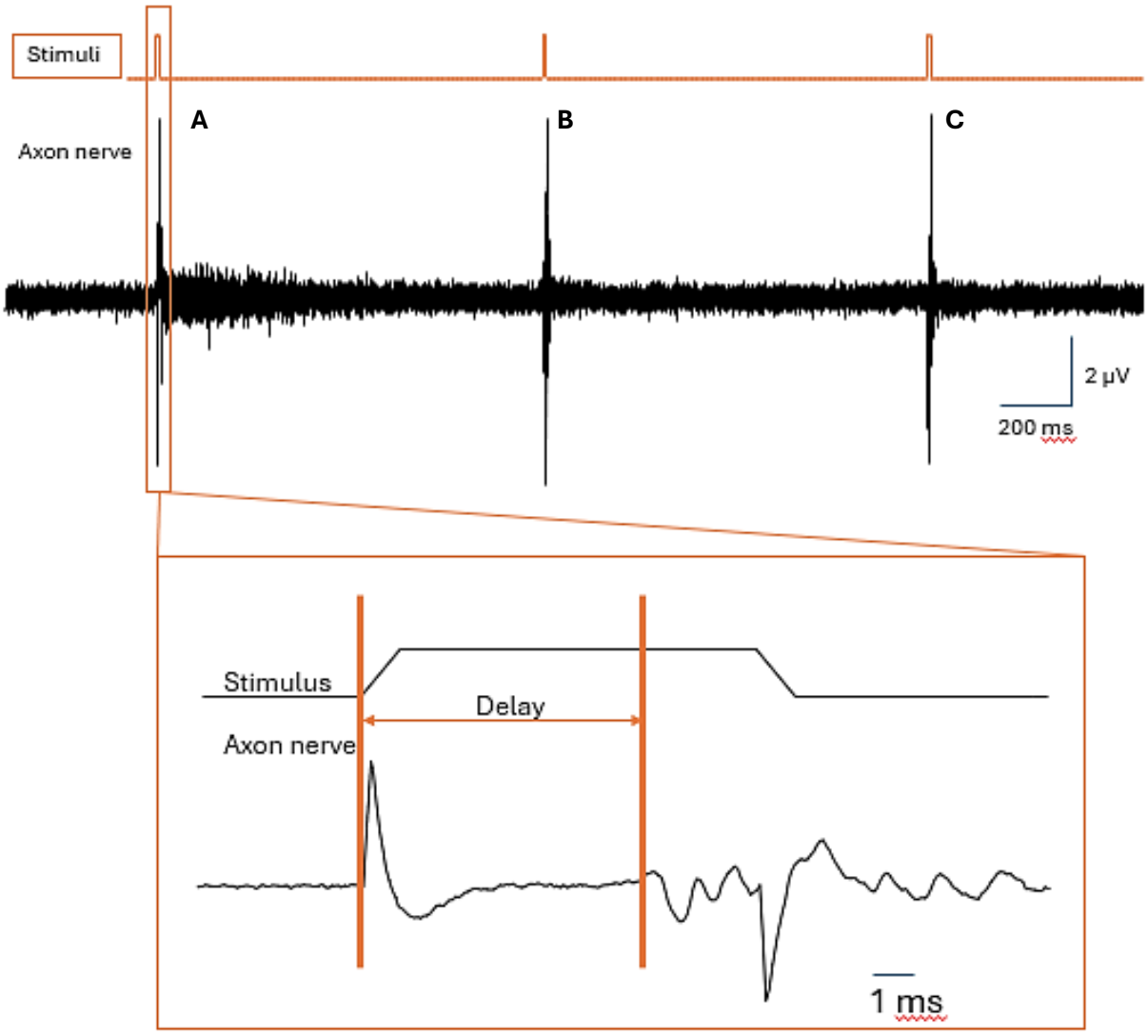


Figure 9. Neural recording of axon nerve cord of R2 using two silver wire hook electrodes anchored to the nerve at about 2 cm from each other. One electrode (proximal) was used to deliver three short electrical pulses (10 ms/each, interstimulus time=1,7s, amplitude of the pulse=1mA, 5V). The first stimulus (A) evoked neural activity with a delay (lower trace) >6 ms and a total response duration of about 500 ms (50-times longer than the stimulus); second and third stimulus (B-C) evoked an activity of about 150 ms.

Maximum amplitude of the signal is stable between the trials (around 8 µV). Increased activity evoked from the electrical stimulation appeared again after a pause of about 15 s from the previous train of stimuli (Fig. 10).

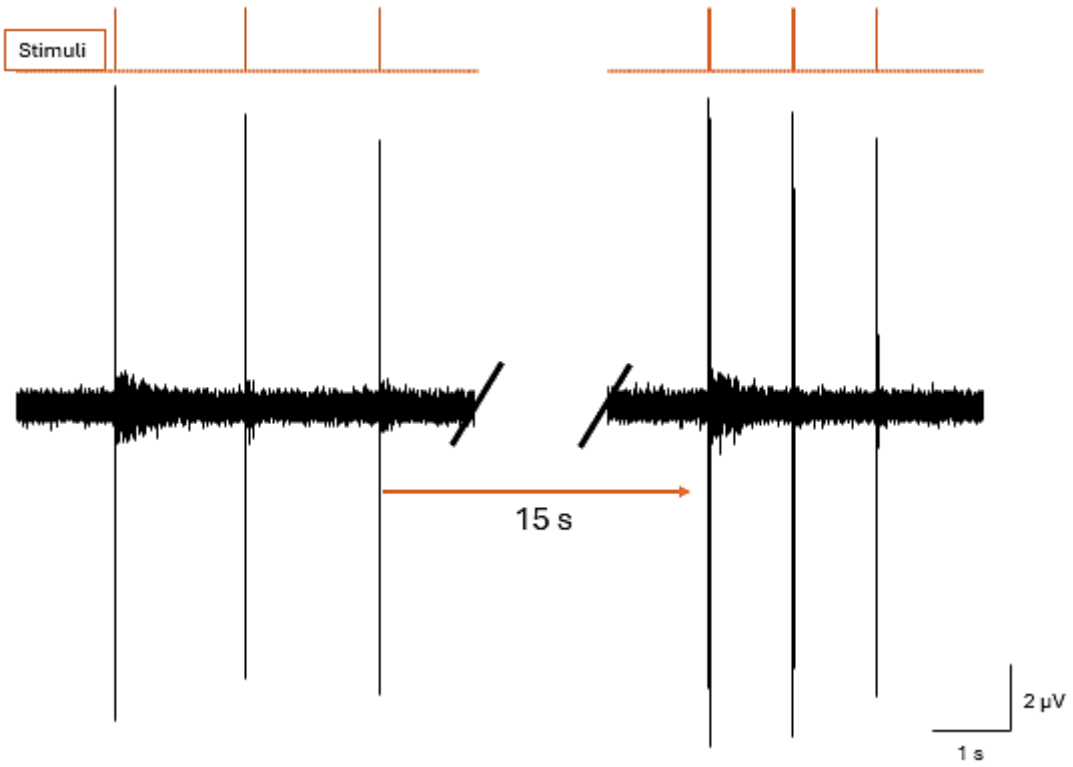


Figure 10. Neural recording of axon nerve cord of R2 using two silver wire hook electrodes anchored to the nerve at a distance of about 2 cm (same preparation as fig.9 - first train of stimulation showed in detail in fig 9). Starting of a second train of electrical stimulation in correspondence of the proximal electrode evokes again an increase of the response after about 15 s.

Neural recording from *O. vulgaris* supraoesophageal mass (SEM)

Spontaneous activity was collected using a pair of tungsten electrodes positioned on the SEM in regions corresponding to the superior frontal and vertical lobes (for materials and methods see Supplementary info, “Recording from the octopus brain”). In all instances, electrical traces recorded resulted to be spontaneous neural signals. In few cases (n=2), one of the electrodes (inverted) was moved away from the brain and positioned in the water bath, at a distance of about 4 cm from the non-inverted electrode and about 2 cm from the body of the animal.

In three of the animals tested, spontaneous ‘spike’ trains were observed (an example in Figure 11). These Local Field Potentials (LFPs) started without apparent inputs and did not result to be correlated to any behaviour from the animals.

Durations and frequencies of the observed trains were highly variable, as variable was the total duration of a single LFP (mean duration of the trains=299 s, mean frequency < 2 Hz; Figure 12).

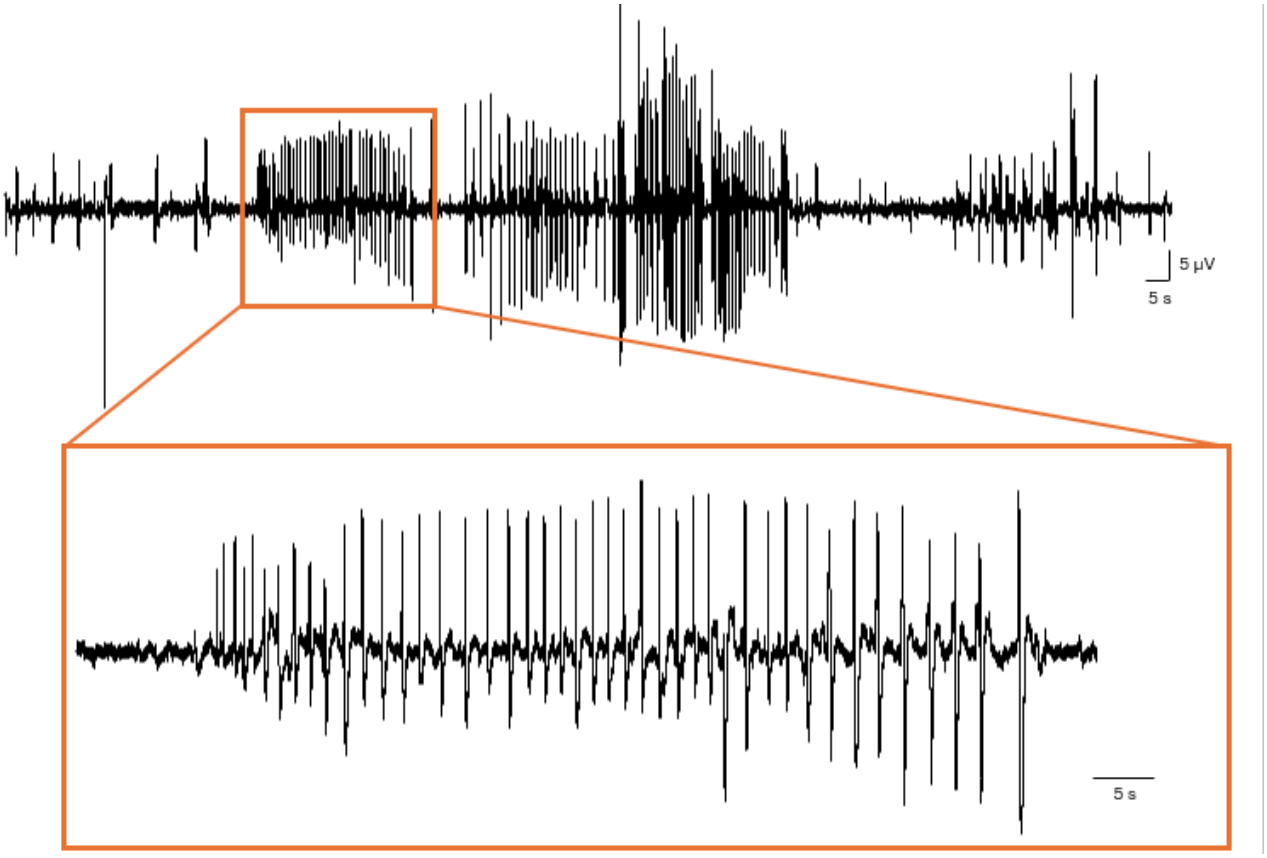


Figure 11. Spontaneous activity collected with tungsten electrodes placed on the surface of VL and SFL complex. Upper panel shows spike trains with different frequency during all the duration of the recording from one animal. Lower panel shows an enlargement of a spike train.

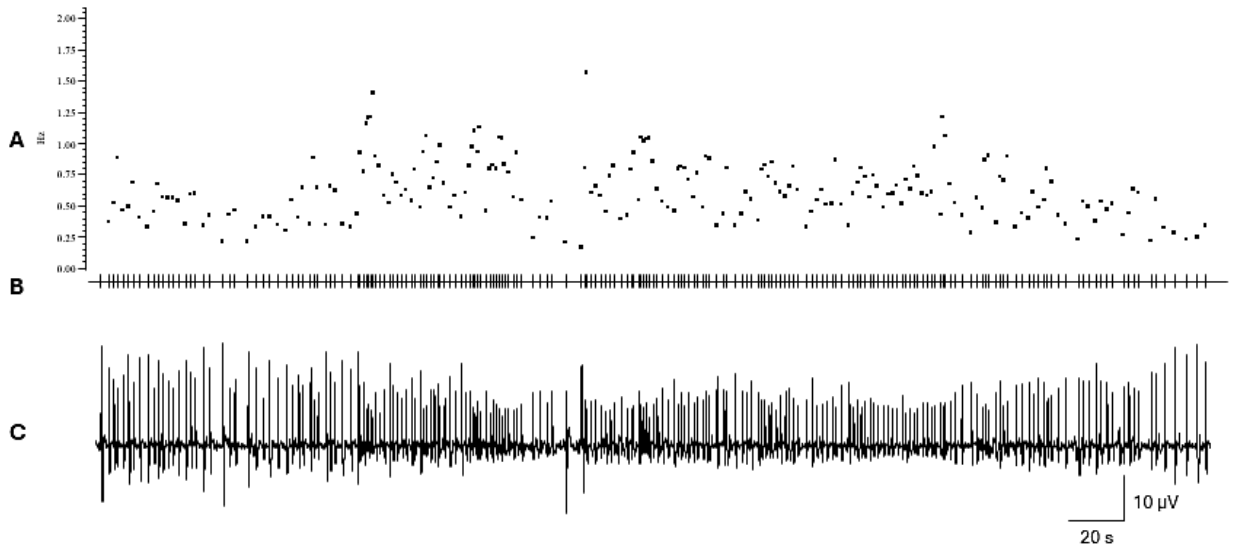


Figure 12. Spontaneous electrical activity from SEM represented by a spike train with a total duration of 400 s (**C** - lower trace). Upper panel shows instant frequency of the events (spikes - **A**), each of them marked in **B**.

During my experiments, LFPs recorded in the SEM appear analogous to the spontaneous activity recorded by Brown and colleagues (2006; Fig. 13); I did not identify clear pattern of behavioural/locomotor activity in the animal, and frequency and amplitude changes between the different trains corresponded to findings from Brown et al. (2006).

When giving a closer inspection to the neural traces recorded in my experiments, it is evident that in the great majority of cases the total duration of a single LFP ranged between 600 and 800 ms, with signal amplitude highly variable reaching values >40 µV. These values appear different from previous findings: a duration of single spike reported to be about 0.5-1 ms (Brown et al., 2006).

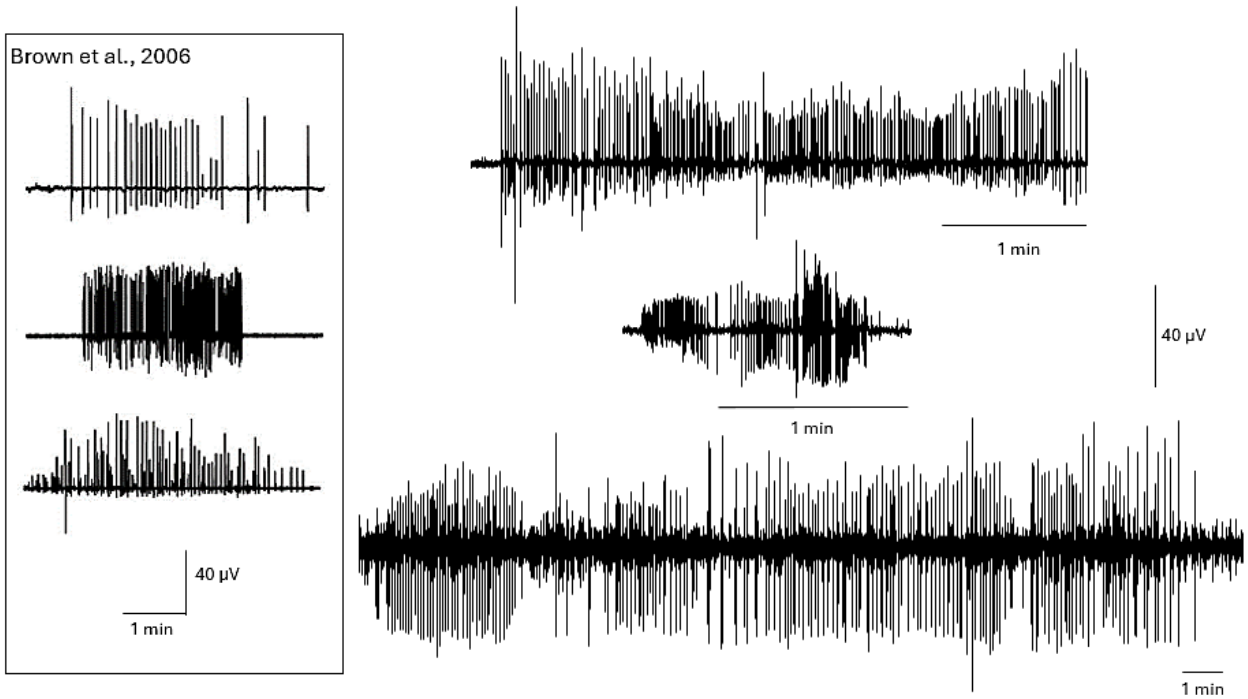


Figure 13. Comparison between spontaneous electrical activity from SEM in three octopus (this PhD) and recorded activity reported in Brown et al. (2006; left panel). Note different time scales and the variation in amplitude and frequency of the spikes collected in the two studies.

In order to confirm that the neural activity I recorded is spontaneous and not elicited (see Figures 14, 15), I also stimulated the arm or the pallial nerve. The stimulation of the pallial and arm nerve cord did not elicit any observable change in the recorded neural signal profile at the recording site (i.e. anterior part of the vertical lobe, in proximity to the superior frontal lobe: supraoesophageal mass), as expected for the known functioning of the octopus' brain. Of course,

I cannot rule out that a stronger stimulus may have evoked some neural traces at this level of the cephalopod central nervous system, but this has not been explored in my experiments.

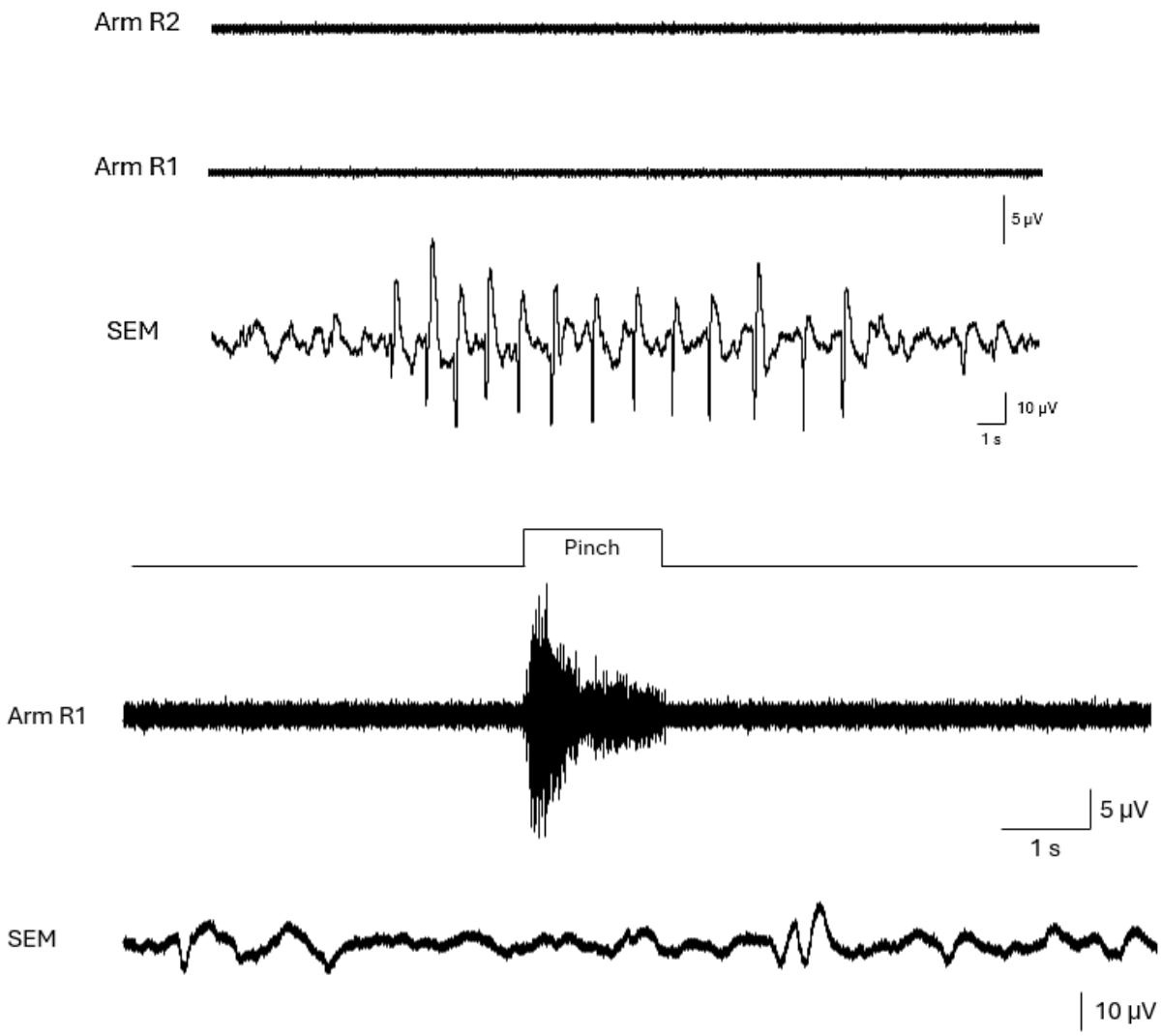


Figure 14. **Top**: Example of recording from two arm nerve cords (R1, R2) and SEM. Traces show that LFPs start without any stimulation to the arm nerve.
Bottom: Example of recording from arm R1 nerve and SEM. Mechanical stimulation of arm axon nerve elicit spikes in the nerve, but no evoked activity is observable in the brain.

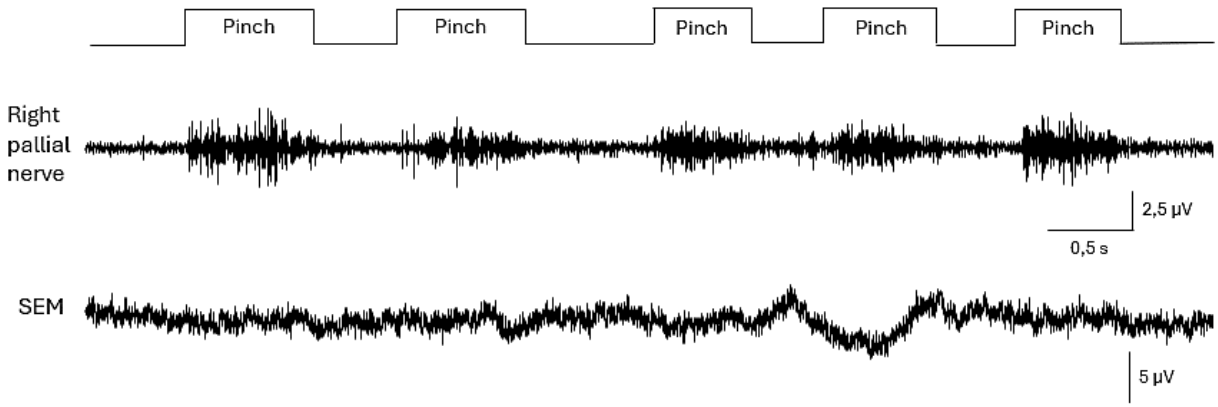


Figure 15. Example of recording from right pallial nerve and SEM. Mechanical stimulation of pallial nerve elicit spikes in the nerve, but no activity is evoked at the level of the brain. Signals are smoothed (time constant=0.8 ms).

Discussion

In my experiments electrophysiological recordings carried out at the level of the pallial nerves and arm nerve cords provided evidence of evoked neural activity in response to mechanical and electrical stimulations. This confirms previous studies (Zullo et al., 2011; Butler-Struben et al., 2018).

Based on the approach followed by Butler-Struben et al. (2018) I collected neural trace from sedated animals using low concentrations of a mixture of $MgCl_2$ and EtOH in seawater.

My findings resulted comparable to the ones reported by Butler-Struben et al. (2018) in the case of evoked neural activity recorded from a freely moving animal and in conditions reported by Authors during the induction phase of anesthesia, i.e. at 1 min induction phase as shown in original figure 6 (Fig. 16). Under my experimental conditions I have been able to record clean and strong neural traces from the pallial nerve characterized by a maximum amplitude of 6 µV.

In the original study, responses from freely behaving animal are found to reach a maximum of 1 µV; the neural signals appeared almost completely abolished after 2 minutes from induction (Butler-Struben et al., 2018).

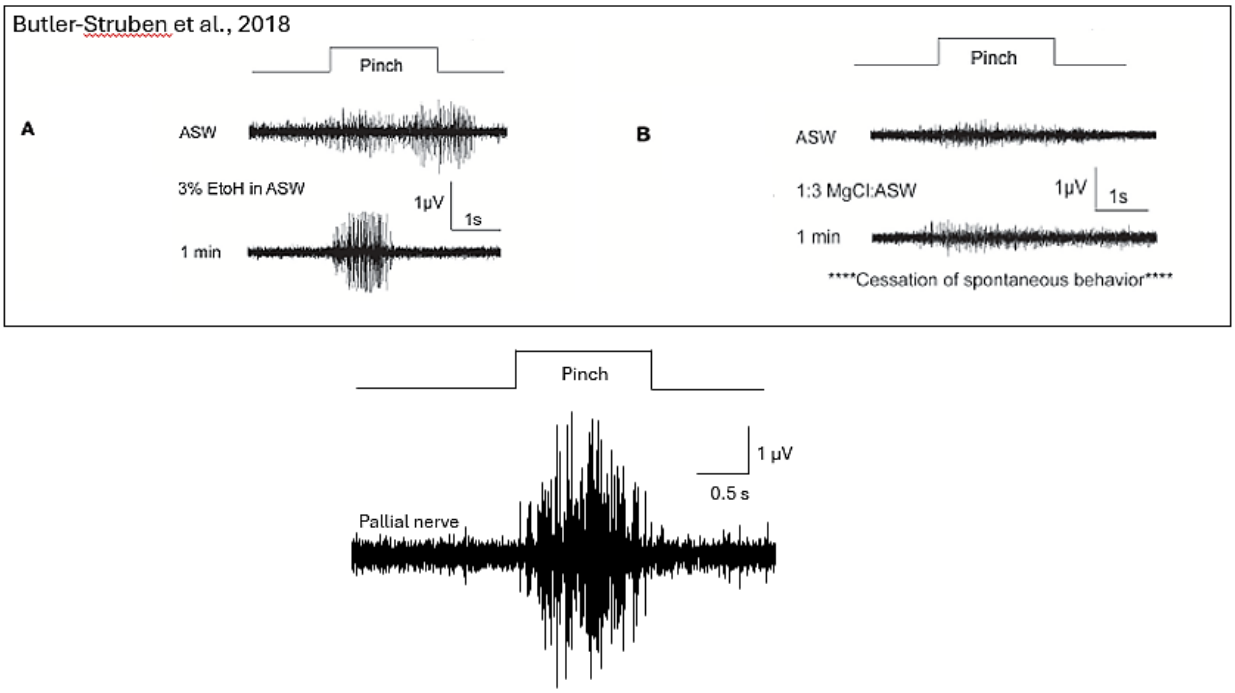


Figure 16. **Upper panel**: traces from Butler-Struben et al. (2018) showing evoked responses to pinch in octopus on ipsilateral mantle region before and during anaesthetic induction (1 min) in EtOH (**A, *n*=6**) and MgCl (**B, *n*=5**). Modified after figure 6 Butler-Struben et al. (2018) and reproduced under the terms of CCAL permission to reuse and reproduce materials in published articles.
Lower panel: trace from my experiments recorded on sedated *O. vulgaris* using mix solution after 15 minutes from induction.

In all instances evoked neural signatures have been detected even after more than 30 minutes from induction. In the experimental settings adopted by Butler-Struben and colleagues (2018) immersion of the animals in $MgCl_2$ and/or EtOH induced a marked reduction of basal and evoked neural signals after 4-5 minutes in the anaesthetic solution.

My data confirm previous findings from Shomrat et al. (2008) that a mixture of $MgCl_2$+EtOH does not impair neurophysiological properties of the tissues. Mixture of ethanol and magnesium chloride has proven to not interfere with the neural responses in octopus, as clearly shown by Hochner, Fiorito and colleagues (e.g., Shomrat et al., 2008). Using the maximal concentration of $MgCl_2$/EtOH in seawater (55 mM $MgCl_2$ and 1% ethanol) and in order to assess the viability of the slice to neural recordings, Shomrat and colleagues found that LTP is induced in the brain slices even in the presence of anaesthetic solution, with most of it gained while the slice was kept also in the presence of the mixture; only marginal additional LTP was obtained by a second set of high frequency stimulations after washout (Shomrat et al., 2008).

In addition, in my experimental conditions the concentration of the two chemicals was reduced after about 15 minutes (after 'mounting' of the specimen, e.g., setting up of the nerves or brain, placement of the electrodes in target areas), bringing the final concentration in the holding container to about 50% (by diluting with fresh seawater) of the original one. This further facilitated a status of sedation of the octopus and did not interfere with neural signals, as my results clearly demonstrated. This is further confirmed when comparing neural recordings by Butler-Struben et al. (2018) with the ones here presented; I found evident, robust, stable neural activity in *O. vulgaris* for long time in a sedated animal.

Therefore, neural signals detected in my experiments appear linked to both the use of a combination of anaesthetic agents that does not affect physiological and cellular properties, and the sensible reduction of the concentration of them in the container where the animal is placed (for details see also Supplementary Information: Sedation).

The method used for recording pallial nerve activity did not allow me to define if the evoked responses, visible after mechanical stimulation in correspondence of the ipsilateral side of the mantle, are likely to be representative of afferent and/or efferent activities, then the neural reaction provoked by the stimulus could have run from the nerve to the brain and/or vice versa. This is not surprising considering that Budelmann and Young (1985) thoroughly described the neural connectivity (fibres etc.) of the pallial nerve with the higher structures, i.e. at the level of the palliovisceral lobe in the suboesophageal mass. According to the Authors, only a small number of fibres passes through the palliovisceral commissure to the contralateral side and therefore may pass information from one side of the octopus' mantle to the other. This confirms that the 'connection' between the two nerves at the central level, probably allows only a selective transmission of the neural signal from one side to the other. Possible neural tracing experiments combined with electrophysiological recordings may provide in the future evidence, for instance, for threshold of stimulation and/or selectivity of neural signals, and exchange of information between the two sides. It is to note that the "hierarchical" organization of the nervous system of cephalopods may not necessarily provide a pass-through signalling between a paired nerves system as the case of other organisms.

I also observed that stimulation on a given area of *O. vulgaris* mantle induces neural responses, that I measured with electrodes on the two (left and right) nerves, either on both pallial nerves or in one of the two, with ipsilateral being consistent with location of the stimulation and neural

correlate. In my experiments, and once stimulation was applied in proximities of the Long mantle papillae[4] (Packard and Sanders, 1971; Borrelli et al., 2006), I was able to detect specific neural responses in the two nerves, at the same time. It is worth to remind that each pallial nerve contains around 16000 fibres with a differential composition/classification based on their size and putative role (Young, 1965; Budelmann and Young, 1985). The two nerves enter on the ventral part of the stellate ganglion (facing mantle cavity) on the same side. Twenty-six to 40 stellar nerves (Young, 1972a) arise from its dorsal region. The ganglia count around 120,000 monopolar neurons, distributed on an external cortex which surrounds the central neuropil (Young, 1972a; Monsell, 1977). Tracings of the pallial nerve revealed the presence of centripetal cells inside the ganglion, with the axons pointing toward the pallial nerve, considered sensory neurons (Monsell, 1977; Monsell, 1980; Saidel and Monsell, 1986). The stellar nerves provide the main innervation to the various mantle fields, but not exclusively. According to various studies motor neurons of each stellate ganglion innervate only one-half of the mantle musculature, although during – for example - breathing, swimming the mantle acts as a single, coordinated structure without any indication of dual innervation (Saidel and Monsell, 1986; see also Imperadore et al., 2019). Cells bilaterally localized in the various motor areas of the subesophageal lobes have been considered the source of simultaneous stimulation to motor neurons in both stellate ganglia for coordinated muscle behaviour of the mantle. Considering the sensory response, it is worth noting that each stellar nerve has two roots, each departing from a part of the ganglion and joining at the ganglion surface. The ventral root contains only large fibres, while the dorsal contains both large and small fibres (review in Imperadore, 2017). The skin of octopus (and cephalopods in general) is a highly innervated structure, a feature that is also linked to the refined innervation of the chromatophore muscles (Packard, 1988). Specific arrangements are found, with extensive overlapping of chromatophore fields due to adjacent dermal nerves, occurring mostly in the dorsal skin. Morphological and physiological data obtained from preparations of the skin concur in suggesting that single chromatophores (and probably single chromatophore muscles) are innervated by more than one axon.

[4] According to the definition for this textural component (for review see Borrelli et al., 2006), these papillae appear as quadrangle on the dorsal surface of the mantle of octopus, in a well-defined position and easily recognizable so that these are widely recognized as “landmarks” in the identification of and for the relative position of other components of octopus body patterning. Two of them correspond to the mid-line of the mantle (one anterior towards the head, one posterior towards the mantle tip) and two are raised on the sides of the mantle (one on the left, the other on the right side; review in Borrelli, 2006). These textural components are visible on the skin surface even in cases they are not extended, being identifiable because of a slight localized ‘corrugation’ of the skin.

I do not have evidence of the colour/texture counterpart of the stimulation I applied, since for a matter of timing I have been unable to examine video recordings of these preliminary experiments. It will be interesting in the future to extend further this approach for contributing to this end.

Overlapping of the motor field of the several stellar nerves has been identified in various cephalopod species at different extent, but it is worth to underline that Boyle and Froesch (1979) shown extensive overlapping of the sensory fields of the stellar nerves in *O. vulgaris*.

Data collected from recording electrophysiological responses from the arm nerve cords further support that under my experimental settings stable, robust basal and evoked responses are collected also in this case.

In these experiments I found no evident neural response from arm R2 when stimulating the adjacent arm (R1). These findings are in line with inter brachial physiological connectivity between arms in octopus as shown by Kuuspalu et al. (2022). This recent study allowed the identification of a well-defined connectivity between the eight arms in octopus, allowing for a potential path for interarm communication outside the central brain. In particular, Authors identified that every intramuscular nerve cord (INC) of an arm connects through oral INC pathways to arms that are two-arms away from the adjacent – either on right or left side.

Thus, my finding of the lack of neural responses from adjacent arms after mechanical stimulation, could be explained from the absence of direct fibres connecting arm R1 and R2.

Simultaneous multiple recordings from the same arm nerve cord suggested the presence of both afferent and efferent neural signals, even if this assumption needs further investigations. Results on mechanical stimulation of distal regions of the arm, show that neural signal evoked could be seen by both recording electrodes and that signals could be a merge of activation of afferent and efferent motoneurons which bring the information of the stimulation to/from the brain, possibly from neurons of the brachial lobe in the suboesophageal mass. This could be in part confirmed by my results in the case of stimulation conducted in correspondence of a part of the arm between the two recording electrodes, where the proximal electrode clearly shows activity elicited from a group of neurons, while the distal recording site collects a small amplitude signal compared to that previously picked up during the distal stimulation. Many reasons could explain

this situation, probably the transmission pathway from proximal to distal ganglion is different from the inverse pathway, and this results in a weaker transmission of information within the arm.

In addition, proximal electrode collected a big, evoked signal (in amplitude) when distal stimulation is applied, while a more proximal stimulation evoked a weaker reaction. This may be in accordance with evidence on the difference of the general neural and muscular structures between distal and proximal parts of a single arm. Distal regions of the arm are known to possess a larger quota of neural components (Margheri et al., 2011) and possibly a larger amount of sensory receptors compared to the more proximal areas of the arm, as deduced by various evidences.

Zullo et al. (2011) explored information flow proximal to distal and distal to proximal in the arm nerve cord of *O. vulgaris*. By using suction electrode, they stimulated electrically and recorded from ganglia, observing nervous information flow in both directions. The transmission velocity in proximal recordings (distal to proximal, DTP) was significantly higher than in distal recordings (proximal to distal, PTD). This suggests that sensory information from suckers follows two paths; in the DTP direction signals may pass directly from ganglion to ganglion and through the axonal tract. The slower velocity in the PTD direction suggests that this faster axonal transmission is either completely lacking or is only a minor component of the signal. That is, in this direction, signals appear to be mainly transmitted directly from ganglion to ganglion (Zullo et al., 2011). In my experiments, we found no significant correlation between stimulus strength and response amplitude. This finding raises the possibility that discrimination between effective and ineffective stimuli are achieved at the suckers as suggested by Rowell (1966). Responses to stimuli of the same intensity were statistically larger in DTP than in PTD recordings (Zullo et al., 2011). This fits to my data, although we did not stimulate suckers.

A larger number of fibres (from both ganglia and axial nerve cord) may thus project to the next proximal ganglion (towards the brain), while only the fibres involved in the sucker arc-reflex generate a response in the distal ganglia (away from the brain). In sum, it is possible to suggest that once the sucker response is induced, the evoked information follows two different pathways along the arm. One is via slower, direct ganglion-to-ganglion connections (see also Sakaue et al., 2014; Bellier et al., 2017) and may transfer information to the neighbouring sucker, as in the sucker reflex arc. The other, faster pathway may pass through the axial nerve cord to the brain

(see also: Budelmann and Young, 1985; Sakaue et al., 2014; Bellier et al., 2017). This dual projection may ensure that information from effective stimuli is both conserved at the peripheral level for local processing and transported to a higher central level for more global computation.

I observed a decrease of responses duration after repetitive electrical stimuli are in line with the idea of adaptation of the neurons to the stimulation (Benda, 2021), a phenomenon that is recovered after a certain period of time. A similar phenomenon (i.e. adaptation) has been reported in various instances and in different organisms. For example, Fischer et al. (2011) reported that in *Aplysia californica* low-threshold sensory neuron (Unidentified Low-Threshold - ULT) inter-stimulus-interval dependent reduction in neural activity is observed, with neurons exhibiting decreased excitability at shorter stimulus interval; the expected excitability of the circuit is fully recovered with longer inter-stimulus-interval (Fischer et al., 2011).

We also observed a sort of distribution of sensory information. In insects this occur also without control by the brain. In locusts and crickets exteroceptors (tactile hairs) and proprioceptors (chordotonal organs, i.e. stretch receptors associated with leg joints) on the legs or body segments may project into several other ganglia (Hustert, 1978). In the locust, sensory information from a middle leg is transmitted by interneurons to neurons controlling movements of the hind leg. Intersegmental interneurons in a mesothoracic neural population receive inputs from extero- and proprioceptors on a middle leg making direct synaptic connections with non-spiking local interneurons and motor neurons controlling the movements of the ipsilateral hindleg (Laurent and Burrows, 1989).

We have no evidence whether intersegmental distribution of sensory information in insects can be compared with the situation in an octopus arm. The arm (octopus) is not a segmented structure in the sense of segmented body (insect) that has a segmented nervous system. Our speculation here it is that it would be more comparable to an arm or leg in vertebrates. The coordination of arm and leg segments is not done in the appendices but in the respective ganglia of the central nervous system, meaning that all sensory information reaches the brain and there it is distributed to local and intersegmental neurons. A rapid look at the lamprey and leech literature brings the view that segmental sense organs - e.g., edge cells in lamprey and stretch

receptors in leech – and tactile receptors in leech affect local networks. We could not find studies on effects on networks in other segments, but that does not mean that these studies are not existing.

While attempting to record at the level of the brain areas, and similar to Brown et al. (2006), I positioned tungsten electrodes on the surface of the dorsal part of the supraoesophageal mass. I recorded LFPs from a small region of the neural tissue and found spontaneous signals which could be result of the sum of potentials from a bunch of neurons that synchronously activate.

To the best of my knowledge, the superior frontal lobe counts about 1,780 thousand of neural cells (equals to about 1.8 million), while the vertical lobe reaches over 25 million. Considering the position of the recording electrodes I can assume that at the recording site neural signals are contributed by several hundred of cells, most of them being of about 5 μm.

The duration of a single spontaneous activity resulted to be >600 ms, that appears remarkable different from the 0.5-1 ms found by Brown and colleagues (2006). On the other hand, findings from the spontaneous activation of neural activity in my experiments result quite closer to potentials showed by Bullock (1984) and (Pophale et al., 2023).

In sum my findings allowed to: ***i.*** identify neural activities from the peripheral nervous system in octopus; ***ii.*** correlate neural signals to mechanical and electrical stimulations, analogous to those observed in other published papers; ***iii.*** neural electrical activities, both evoked and spontaneous, obtained from animals in a mild sedation status confirmed the reliability of the live neurophysiological 'preparation' using a mix solution of $MgCl_2$ and EtOH at low concentration, a condition that allowed to record from peripheral and central nervous system, without interfering with general neural functions - confirming previous findings from Shomrat et al. (2008) in that the mixture ($MgCl_2$+EtOH) does not impair neurophysiological properties of the tissues and do not interfere with neural activity responses in octopus; ***iv.*** use of the mix solution allows to record neural traces for a long period of time (also in comparison with the previous studies).

Development of minimally invasive techniques to record neural activity from octopus' brain

Electrophysiological recordings from octopus' brains can be dated back to 1980s with study from Bullock (1984), who noted "ongoing compound field potentials" from octopuses, attributing the observed voltage fluctuations to summed spike potentials; this suggests a complex brain activity pattern similar to those found in mammals (Bullock, 1984). Subsequent studies by Bullock and Budelmann recorded spontaneous activity and VEPs in unrestrained cuttlefish, finding (again) that background neural activity was similar to the one found in vertebrates than other invertebrates, with VEPs displaying long latencies, multiple peaks, and slow recovery (Bullock and Budelmann, 1991).

As previously mentioned, EEG is the most common technique for recording brain activity in vertebrates, including humans. Here I explored spontaneous neural activity and VEPs in octopus to attempt to address gaps in current methodologies for studying cephalopods hallmarks of cognitive states.

Most of the studies on neural activity in live cephalopods rely on implanted electrodes requiring surgical procedures. Here, I aimed at developing a non- minimally invasive EEG like approach using epidermal and/or subcutaneous electrodes positioned on various areas on the head of the animal. I hope my findings may advance cephalopod research offering insights into the neural activities of octopus. Methodological information is included in Supplementary info (see Minimally invasive electrophysiological methods).

Using Ag/AgCl Plate electrodes in octopus

I combined visual stimulation using a blue-light LED with Ag/AgCl plate electrodes to record VEPs (Visually evoked potentials) from structures of *O. vulgaris* central nervous system (N = 16); in my experiments the maximum amplitude recorded originate mainly from the ipsilateral optic lobe.

Difference in amplitude and shape of the responses was clearly visible when non-inverted (+) electrode was moved between three main regions of the head, in correspondence of ipsilateral OL, SEM and contralateral OL (Fig. 17).

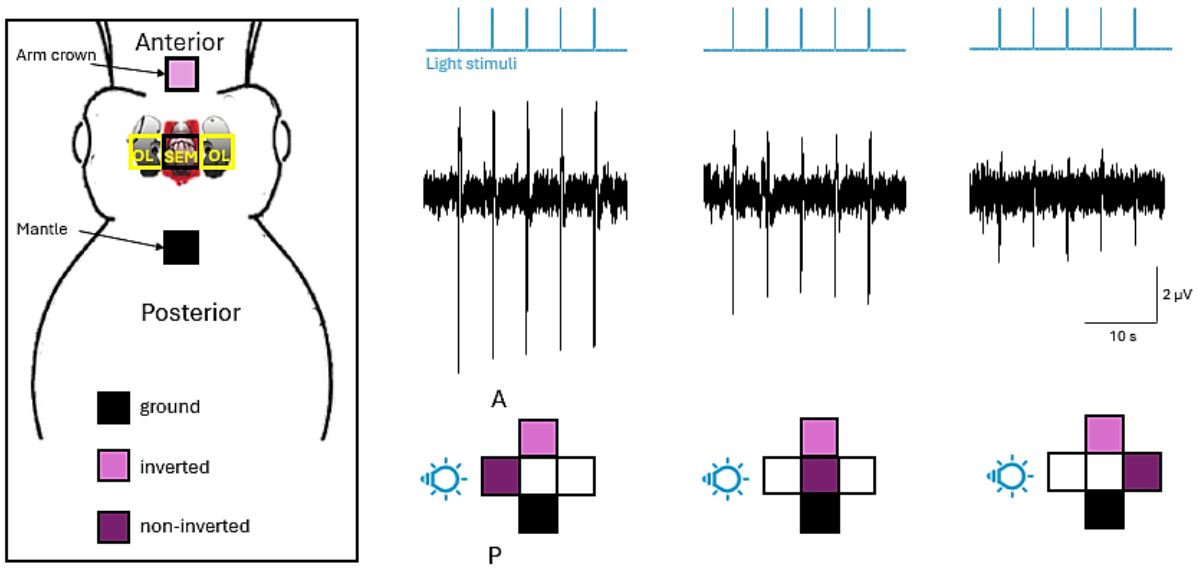


Figure 17. Visually evoked potentials (VEPs) recorded in correspondence of three main central nervous system structures' (starting from the left: Left Optic lobe, SEM, right Optic lobe) after blue Led stimulation directed to the left eye. Single stimulus of 100 ms, light intensity was set at 127,0 µW/cm^2

Visual stimulation using train of single pulses of 100 ms elicited VEP with mean durations of 1.41 seconds in ipsilateral OL and 1.27 seconds in SEM. Potentials were characterized by latency (delay of the neural response after the onset of the stimulus) and a first hyperpolarization followed by a depolarization linked to the response of several neurons potentially responsible for the sum of potentials recorded (Figure 18-A). In some cases, inversion of the shape (first depolarization followed by hyperpolarization) of the evoked signal was observed. Differences in latency between the three regions of the central brain are not statistically significant. However, latencies measured from the optic lobe directly stimulated by the light resulted to be shorter than that collected in correspondence of the others (Table 1).

Table 1. Average and standard deviation (SD) values of Latency of evoked responses recorded from different locations corresponding to brain masses in *O. vulgaris*.

Recording location	**Average (ms)**	**SD (ms)**
Ipsilateral OL	14.4	2.61
SEM	20.2	6.14
Contralateral OL	22.7	12.50

A single stimulus of 2 seconds elicited a VEP which could be collected from all the recording sites. Resulting VEP was characterized by a difference in the shape of the evoked signal when light stimulus was onset or offset. Onset of the light evokes first a hyperpolarization followed by a depolarization, while offset elicit an inverse shape (Fig. 18-B).

I observed heterogeneity of the ON/OFF responses in the case of a shorter stimulation was applied; this may suggest a merge of the two different potentials evoked in responses to onset and offset of the light, which are instead evident when the stimulus has a longer duration (Fig. 18).

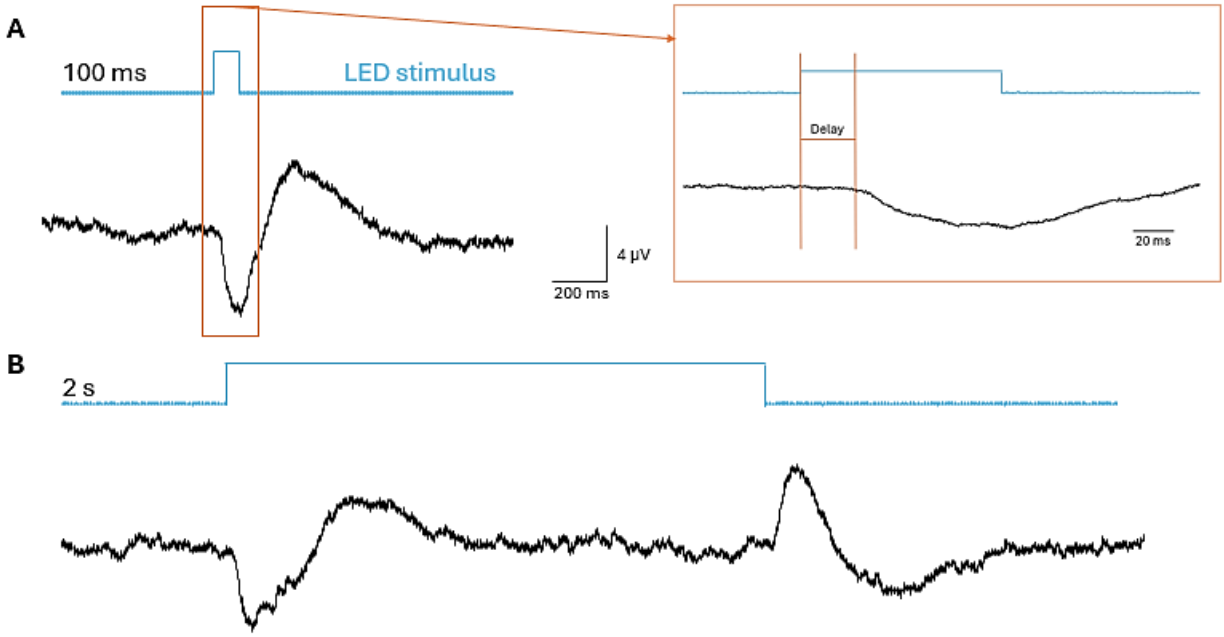


Figure 18. Traces of VEPs evoked from *O. vulgaris* optic lobe using a short (100 ms - **A**) and a long duration (2 s - **B**) stimulus. Short light pulse evoked a negative deflection of the trace showing no differences in the response when light is onset or offset (**A**); zoom on the right side shows an example of delayed response of the neurons when the eye is stimulated with blue light pulses. Long light pulse instead shows a negative deflection when light is ON and a positive one when light turn OFF (**B**).

A difference can be observed in the amplitude of the evoked neural signals between the recording sites (Table 2) when stimulation with same light intensity was applied. Mann-Whitney

test between the amplitudes of the two optic lobes resulted with a $p = 0.046$, while same test conducted between SEM and both optic lobes was not statistically significant.

Table 2. Average and standard deviation (SD) values of peak-peak amplitude of evoked responses recorded from different locations corresponding to brain masses in *O. vulgaris*.

Recording location	**Average (µV)**	**SD (µV)**
Ipsilateral OL	5.9	4.1
SEM	3.4	1.3
Contralateral OL	2.1	0.9

A closer inspection on the traces of evoked activities, gives better insight in the differences in latency and amplitude between brain structures.

In few cases it was possible to observe a difference in the shape of the neural signal evoked between the recording sites. Figure 19 offers an example of the traces during light stimulation; this resulted in a first depolarization followed by a polarization of the VEP collected at the level of contralateral OL, showing an inverse shape compared to the ipsilateral OL and SEM evoked activity. A closer look at the first milliseconds from the onset of the light stimulus, clearly show the inverse response of the potential evoked (Fig. 20-C).

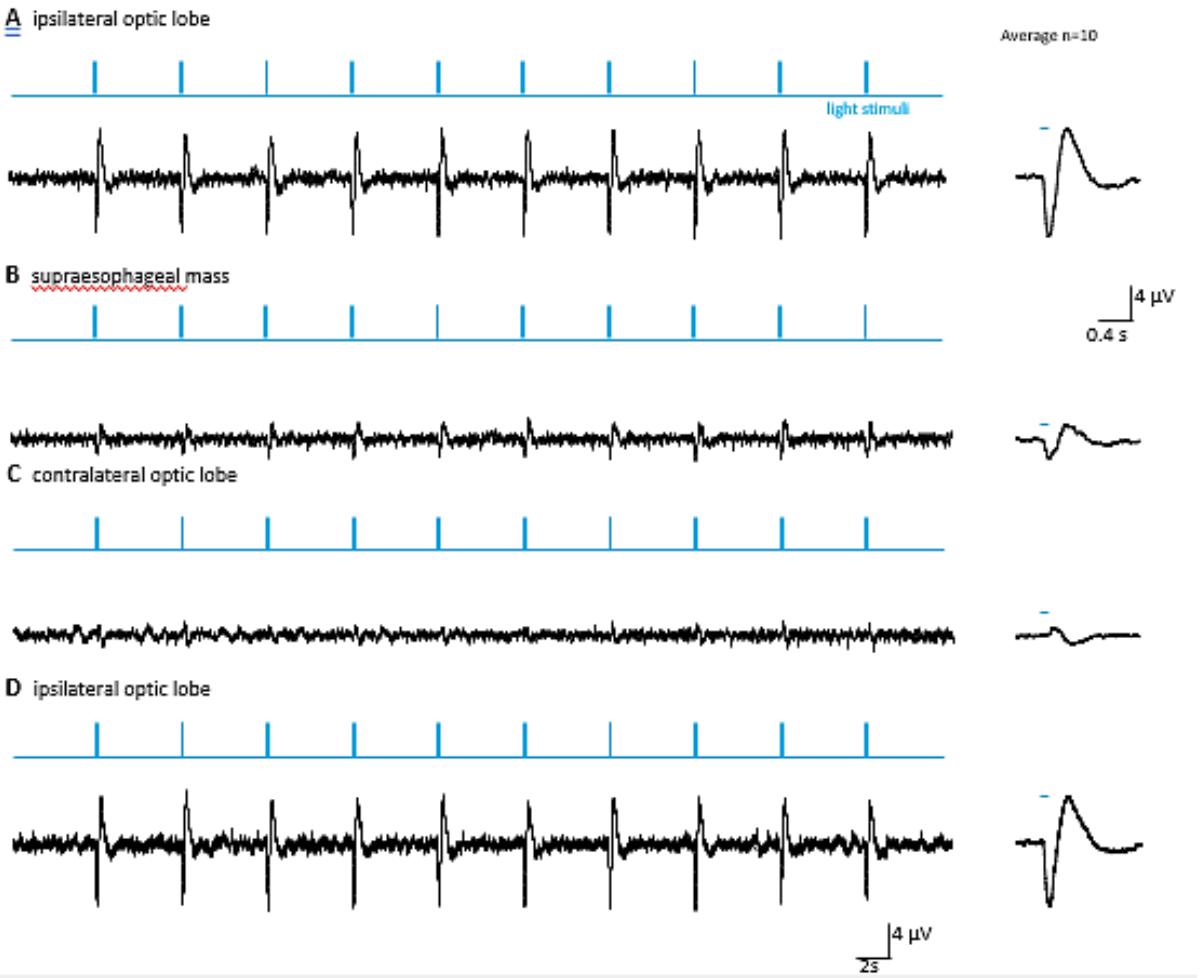


Figure 19. Electrophysiological traces from ipsilateral OL (**A**), SEM (**B**) and contralateral OL (**C**) during a train of 10 light pulses (100 ms) directed to the left eye (light intensity=127,0 µW/cm^2); last trace (**D**) is again from ipsilateral OL. On the right, corresponding waveform average of 10 stimuli for all the traces, clearly show differences in the amplitude and duration of the responses between the recorded locations.

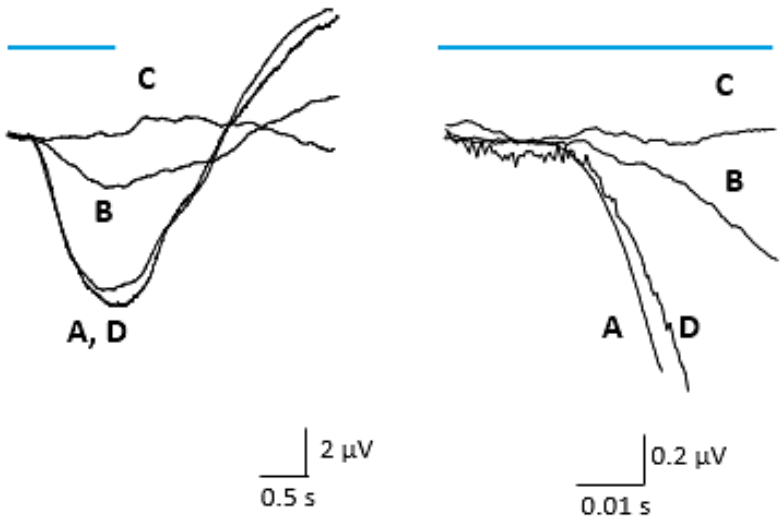


Figure 20 Latency of the averaged evoked responses (see figure 19), from the onset of the light stimulus (blue line) from the recording areas (**A**: ipsilateral OL; **B**: SEM; **C**: contralateral OL; **D**: ipsilateral OL). Right image shows in detail latencies of evoked neural signal with a smaller time scale.

Wire electrodes

In the following set of experiments epidermal-VEP recording technique has been substituted with the use of subdermal method adopting silver chloride electrodes positioned about 1.5 mm under the skin, at the midpoint on the head of sedated *O. vulgaris* (N = 8). In particular, I positioned the wire-electrodes at the midline of the head in axis between the two eyes in areas corresponding to the supraoesophageal mass (SEM), and on the center of the eye bulb (i.e. corresponding to the mid optic lobe). To standardize the locations where wire electrodes have been positioned, I used a *point de repère* approach developed by Langella (2000) for *O. vulgaris*.

Subdermal electrodes (Ag/AgCl) allowed me to collect both spontaneous and evoked potentials. I was able to record simultaneously from the three brain masses (SEM and both OLs) using three recording electrodes with shared inverted and ground electrodes.

I recorded VEPs analogous in latencies and shapes to the one acquired with silver plate electrodes previously described. Differences in latencies are not statistically significant (data not shown),

but comparison of the changes in amplitude between SEM-OL and ipsilateral OL-contralateral OL resulted significantly different (all comparisons $p < 0.05$ after Mann-Whitney U test).

In these experiments, stimulation of the left or right eye evoked the bigger (in amplitude) neural signals in correspondence of the ipsilateral OL. Moreover, in all the experiments, a train of light stimuli, with the same intensity directed to the eye, caused a gradual decreasing in total amplitude of the evoked responses, both in the case of short and longer stimuli (Figure 22).

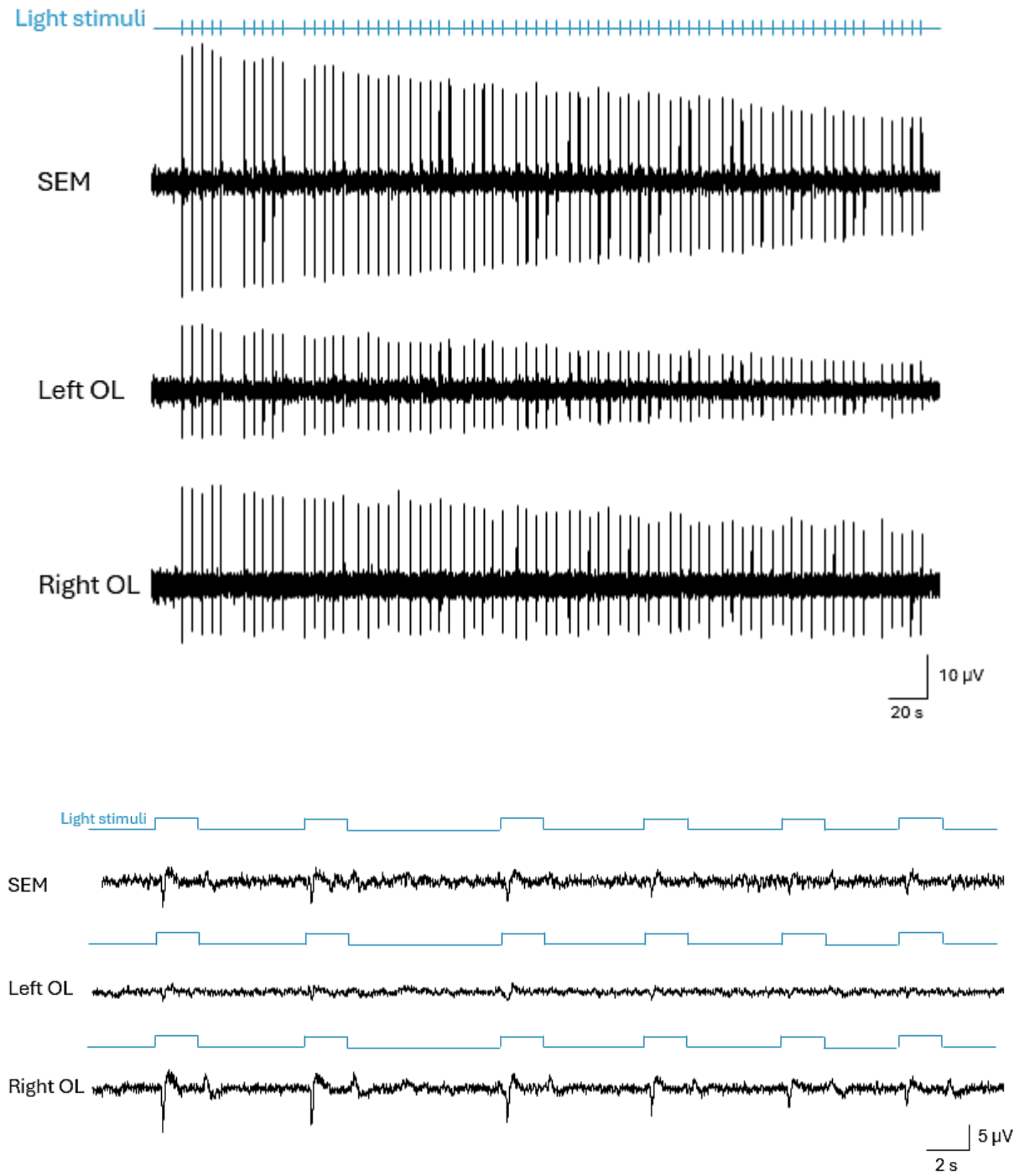


Figure 22. **Top panel**: Example of traces recorded from the three channels during repetitive LED light stimulation (100 ms/stimulus) directed to the right eye of *O. vulgaris*. Gradual decreasing of the neural responses in all the channels is observed. **Bottom panel**: Traces from the three channels during repetitive LED light stimulation (2 s/stimulus) directed to the right eye. Gradual decreasing of the neural responses in all the channels is observed also in this case.

In addition, I was able to record spontaneous neural activity from the three masses of octopus brain (Figure 23). Changes in neural activity during spontaneous recordings were not commonly

observed. In some cases, it was possible to record electrical signals correlated to movements of muscles around the head and the eyes which were detected and noted afterwards through accurate analysis of video recordings.

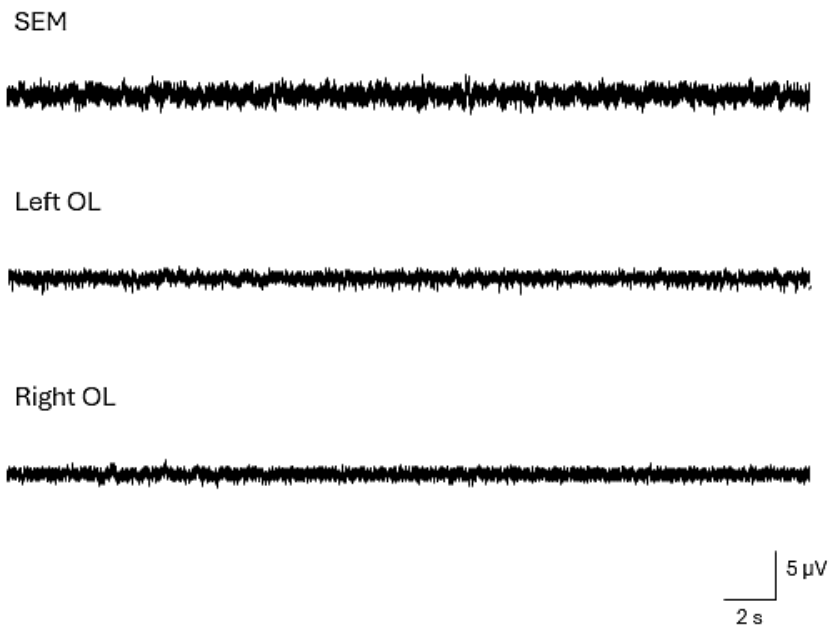


Figure 23. Example of recording of spontaneous electrical activity from the three channels corresponding to the three main areas.

In order to provide a preliminary analysis of the characteristics of the spontaneous neural activities recorded, I used a power spectrum analysis of the traces (Fig. 24). In all the cases, frequency range with the maximum density resulted to be within the range 2,5-13 Hz.

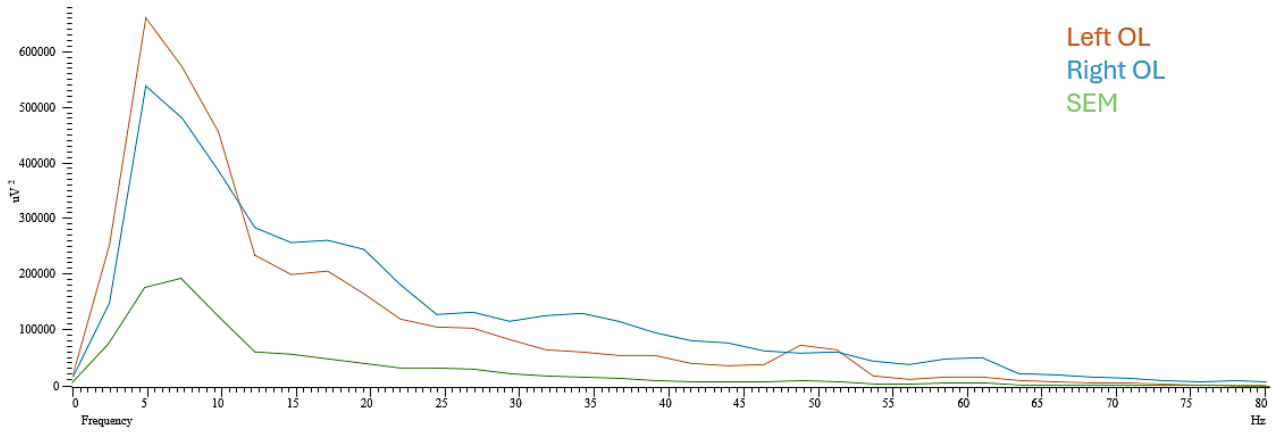


Figure 24. Example of Power spectrum of spontaneous neural signal from left and right OL and SEM from one animal.

Discussion

In this part of my PhD project, I implemented a new electrophysiological approach to record electrical activities from the central nervous system of octopus *in vivo*. This method is novel to the best of my knowledge.

I was able to record neural activities from the octopus brain detecting spontaneous and evoked signals by plate electrodes (diameter 8 mm) positioned on the dorsal surface of the animal head in locations corresponding to the areas where optic lobes and/or supraoesophageal mass are located.

In all instances head width of specimens was measured post-mortem. Based on my data, each plate electrode positioned on areas proximal to the optic lobes (and eyes) of the animal may have collected signals originating from these structures. In the case of the electrode positioned on the midline of the head this resulted to be close to the most dorsal part of the supraoesophageal mass. Based on the data available to me, the supraoesophageal mass extends for about 4 mm (latero-lateral) and 6 mm (antero-posterior) in an octopus in the average of body size utilized for this study (data not shown, Dr G. Ponte, personal communication; OctoLab repository, SZN). Furthermore, at the most dorsal side of the SEM more than 25 million neural cells are found (vertical lobe), representing more than about 45% of the overall number of neural cells constituting the supraoesophageal mass(Young, 1963; Grimaldi et al., 2011).

It is noteworthy to mention that the distance between the dorsal part of the SEM (and/or OL) from the skin surface where electrodes are positioned is not higher than about 8 mm as it is possible to visualize through ultrasound inspections of the live animal as shown in Figure 21 (see also Grimaldi et al., 2007).

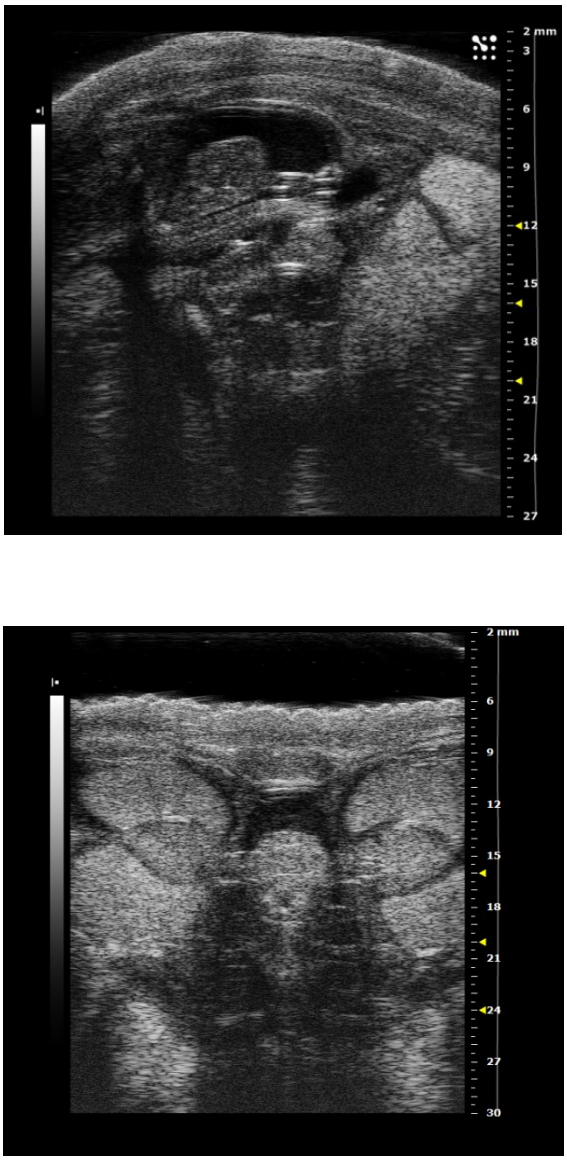


Figure 21. Ultrasound images acquired during sonographic examination of a live *O. vulgaris* at the level of the octopus head in longitudinal (**Top**) and coronal (**Bottom**) planes to reveal the brain in its natural relationship with other structures including the skin (border of the skin, on top of the images), muscle, skull, jelly and – in the coronal plane towards the sides – below the skin layers, white bodies and optic lobe (on both sides of the supraoesophageal mass). Courtesy of CephRes-ETS, VEVO High Frequency ultrasound.

Under my experimental conditions blue LED stimulation reliably elicited VEPs which seemed to originate from all the structures of the brain subjected to electrical recording. As mentioned above - also considering the relative size of plate electrodes and brain masses proximal to the location of the electrode on the head of the octopus - it is possible that each electrode may also picked-up neural signals from the surrounding masses. Accordingly, it was almost impossible to clearly identify which of the neural structures was responsible for the evoked responses

observed. Moreover, neural signals collected in correspondence of SEM and contralateral optic lobe, could be generated from their own neurons but we should also take in consideration the possible propagation of the signal generated by the ipsilateral OL.

Studies conducted on VEPs in cephalopod species (Bullock, 1984; Bullock and Budelmann, 1991; Pungor et al., 2023) have not investigated the evoked response from the contralateral side, to the best of my knowledge. Therefore, my data represent the first attempt along this line of research; further investigation is therefore needed.

It is worth mentioning that interocular transfer between the two optic lobes has been explored in classical studies on learning in *O. vulgaris* (Muntz, 1961b; c; a; Wells, 1967). According to the available evidence, transmission of neural information could be favored by the fibers of the optic commissure which connect the two optic lobes through the supraoesophageal mass (Wells, 1967; Young, 1971). Therefore, I cannot exclude that neural signals evoked by light stimuli should be somehow reverberated in the other areas of the brain (apart from the ipsilateral OL) including the contralateral optic lobe and transit and/or sending neural information at the level of the supraoesophageal mass.

This hypothesis is supported by my findings. In particular, I observed a delay in the progression of the evoked responses from the ipsilateral optic lobe (about 14 ms) to the supraoesophageal mass after about 6 ms to about 8 ms for the latency registered after blue LED stimulation at the level of the contralateral OL (see also Table 1).

The use of subcutaneous electrodes to record bioelectrical signals from the central nervous system is a method widely used in humans and mammals (e.g., Liu et al., 2022); EEG-like neural signals are acquired with no need of impacting surgical interventions.

As for the electrodes placed subcutaneously, it is worth to mention that the layered skin, at the level of the octopus head, is known to be characterized by an epidermis that may reach about 30-40 µm thickness, followed by a deep fibrous layer/dermis consisting of connective tissue matrix; ventrally, bundles of mostly obliquely striated muscles fibers occur (review in Packard, 1988). The total thickness may reach several millimeters.

Ultrasound examination of *O. vulgaris* at the level of the head in correspondence of the supraoesophageal mass (SEM) and optic lobes (OL) reveals an average thickness of the skin layers of about 3 mm (see Figure 21).

Few considerations must be made.

First, in our conditions we believe we do not see electrical muscle activity while using the sticky and wire electrodes, because of the high frequency content of muscle spikes. To the best of my knowledge muscle spikes are short, about 1.7 ms and the frequency of the muscle spikes is probably too low to produce summed action potentials (SAP). With the wire electrodes we measure single spikes and SAPs. With the plate electrodes it is likely that we only measure SAPs because of the distance to the muscle fibre and because the skin muscle is not very thick in regions we record (and in general in the animal; see also above). Typical SAPs are the on- and off-components of the visually evoked responses.

EEG provides a measure of the electrical activity of large, synchronously firing populations of neurons in the brain; i.e. EEG recordings results from SAPs.

The amplitude of summed electrical activity of a muscle depends on the number of motor units (each motor unit consists of the muscle fibres innervated by a single motor neuron). The plate electrode I utilized when placed – for example- on a human arm or leg will pick up the electrical activity of many motor units. On the other hand the activity of a wire electrode placed subcutaneously or into a muscle will be generated by the muscle fibres that are closest to the non-insulated part of the wire thus representing the activity of a single motor unit.

In order to control for any possible “interference” of muscle activity in my experiments, we ran a couple of experiments and recorded electrical activity from a patch of isolated head skin preparation from octopus placed in a petri dish; I utilized copper wires for recording and steel pins for stimulation (Figure 25). The muscle generated spontaneous bursts of spikes (“muscle action potentials”) about every 10 seconds (Fig. 25) with average duration of about 1.7 ms. An electrical stimulus (see Fig. 25) generated single muscle spikes during the stimulus and seven bursts of spikes after offset of the stimulus (Figure 26).

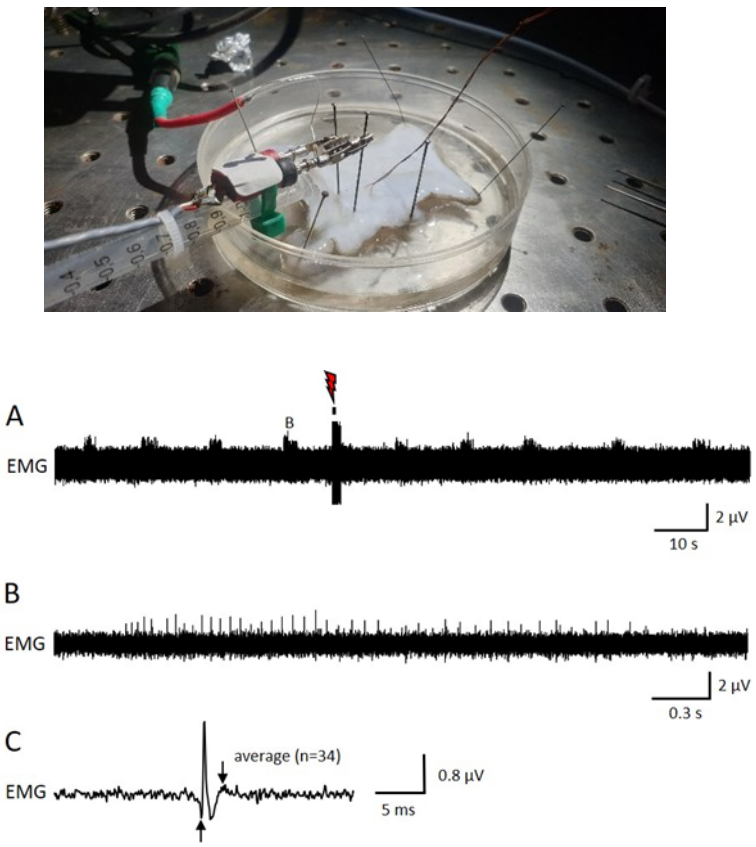


Figure 25. Electromyogram recording (EMG) of head skin muscle. **Top**: The skin was pinned inside out in a Sylgard lined Petri dish. Black arrow points to EMG electrodes. Blue arrow points to stimulation electrodes.
Bottom, A: Muscle fibres generated spontaneous spike bursts. Red symbol indicates a 0.5 s electrical stimulus (see Fig. 26 for details). **B**: spontaneous spike burst as indicated in **A**. C: Average of 34 spikes from burst shown in **B**. Arrows indicate an average spike duration of 1.7 ms.

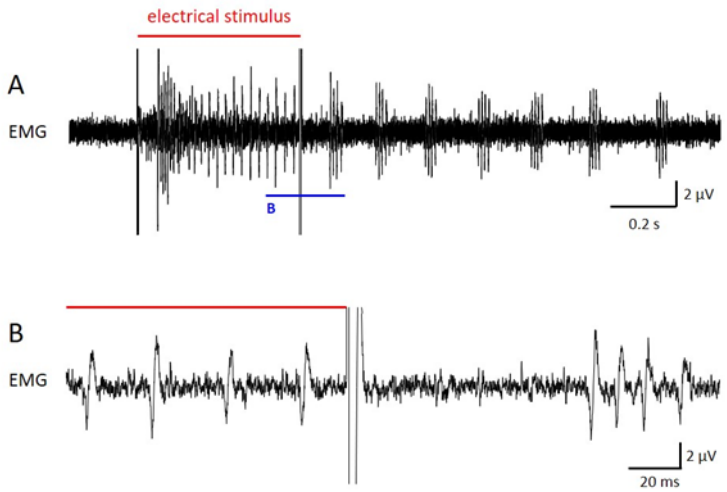


Figure 26. Electrical stimulation of head skin muscles evoked spike activity and bursting in muscle fibres. **A:** Muscle fibres generated spikes during a 0.5 s electrical stimulus (red line) and 7 bursts after stimulus offset. Large vertical lines at onset and offset of stimulus are clipped stimulus artefacts. **B:** Clipping from **A** as indicated by blue line.

The spikes have larger amplitudes compared to the spontaneous spikes in Fig. 26. Such an activity originated from skin contractions (recorded via videos; data not shown). In Figure 26-B end of the stimulus and first burst are provided from the original traces; the spikes lasted longer compared to the spontaneous ones with duration of about 5 ms. We assume that the electrical stimulus triggered fast muscle fibers that exhibit larger spikes or more muscle fibers producing summed potentials. Still, the spikes are shorter when compared to visually evoked SAPs in the brain.

While doing frequency analysis in an EEG-like setting (see also below in this Thesis), we low-pass filter the signal and down sample the recording. This restricts the frequency content of the analysis. This produce a lost of high frequencies, i.e. we destroy spikes.

Figure 27 shows the recordings after down sampling (three burst of muscle spikes from previous figure). The signal was recorded with a sampling frequency of 12,500 Hz, a frequency that already has a small impact on spike amplitude, considered negligible. In Fig. 27-B the recording has been down sampled to 2,500 Hz, the sampling rate utilized for EEG-like recordings with plate and wire electrodes.

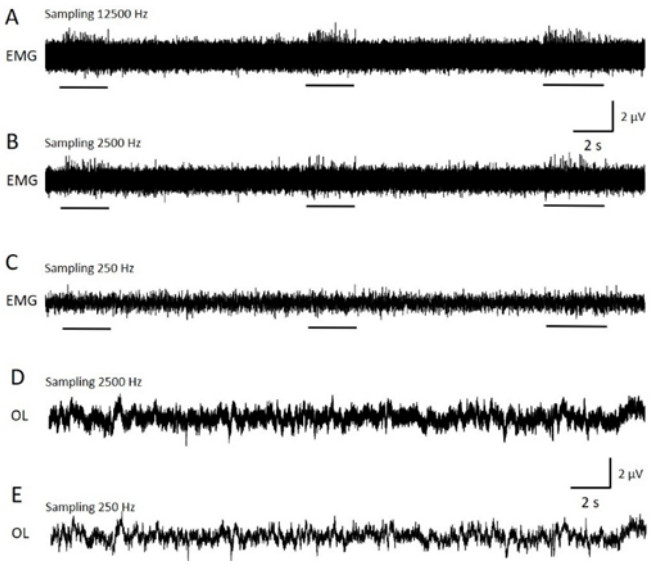


Figure 27. Impact of down sampling on the display of fast muscle potentials (spikes). **A:** Three spike bursts indicated by black lines from the same EMG recording shown in previous figure. Spikes clearly surpass the noise level. **B:** Reducing the sampling rate from 12500 Hz to 2500 Hz impacts a proper display of spikes but burst activity is still obvious. **C:** Reducing the sampling rate to 250 Hz does not allow the display of spikes. **D:** Typical recording of *O. vulgaris* optic lobe activity with a wire electrode placed under the skin (sampling rate is 2500 Hz). Spike activity is not obvious. **E:** Same recording as in **D** but down sampled to 250 Hz. It would be not possible to display spikes as shown in **C**.

Following this down sampling a loss of information occurred: the spikes are shorter, and some do not pass the noise level. In Figure 27-C the recording was again down sampled to 250 Hz. In this case, we did no observe spikes anymore. A sample frequency of 250 Hz restricts the EEG-like frequency analysis to frequencies between 0-125 Hz.

Figure 27-D shows a typical wire recording from above the optic lobe. Neither at a sampling frequency of 2500 Hz, nor at a frequency of 250 Hz spikes are observed, which of course would not be expected in the latter case.

In sum, in my data we did not observe typical muscle spikes using in the plate or the silver wire electrodes. In addition, the settings of the EEG-like frequency analysis were such that they would filter out spikes. Furthermore, I have no notes of movements of the electrodes, as ascertained via inspection of video recordings from experiments. Such movements would indicate skin muscle activity and could cause artefacts. This is something we attentively inspected and removed from my analysis in the few instances it occurred.

The approach I implemented turned out to be fruitful for a series of reasons.

First and as shown above, I observed a significant reduction/absence of any potential interference from movements of the octopus' skin. This is probably due to the high frequency content of muscles spikes; muscles spikes are about 1 ms or less and the frequency of the muscle spike is probably too low to produce summed action potentials (SAPs). With both wire and plate electrodes it was possible to measure only SAPs. The strongest activity which can be detected by electrodes in muscle will be produced by the muscle fibers that are closest to the non-insulated part of the wire, resulting in an activity of a single motor unit, and this is why spikes are short in comparison to an SAP. Moreover, the setting of the frequency analysis was such that they would filter out fast spikes.

Second, the subcutaneous location allows a closer positioning to the target neural structures. Even though the potential area from where the signal is reduced, the averaging is truly beneficial limiting potential interference from the other brain areas, as in the case of the plate electrodes.

It is noteworthy to note that subcutaneous electrodes are one of the most widely utilized methods in other vertebrate species, because generally located in correspondence of delimited

brain regions and it allows to record a more specific neural signal mainly evocated in the sensory area under investigation.

Under my experimental conditions, VEPs recorded with both methods resulted analogous, as general characteristics of shape and latency observed can be considered comparable.

I used two minimally invasive methods in both cases avoiding intensive surgical interventions to implant electrodes: I did not adopt any invasiveness to acquire neural recordings. However, subdermal method resulted the most reliable – in my experimental conditions - when considering the measurement of the coefficient of variation (CoV) of the amplitude of VEPs. In particular, CoVs of the N1 amplitudes, measured from the baseline to the most negative peak, detected in correspondence of the optic lobe for the plate resulted equivalent to 180%, while this was reduced to 62% in the case of wire electrodes. As discussed above, larger amplitude and variations noted while using plate electrodes are probably a consequence of the dimension of the recording site (8 mm), a feature that may somehow reduce precision of recording of the target areas and be affected by LFPs generated from different regions of the head of the animal.

A more in-depth analysis of data collected with these minimally invasive approaches follow in the next sections of this Thesis.

Investigation into the use of Electrical Stunning as part of slaughtering method for cephalopods

Cuttlefish, squid and octopus are well-established components of our diet and exploited for millennia as food by humans across the world and in many different culinary cultures. As food, cephalopods provide good nutritional sources of protein, minerals, omega-3 fatty acids, as well as micronutrients, with the notable benefit of an extremely low-fat content. In particular, the cephalopod mantle, arms, and ink have long been consumed, both fresh and raw (review in Mouritsen and Styrbæk, 2018). Reports from FAO, estimated that a total of 3.9 million tons of cephalopods have been caught in 2022, representing the 11.3% of the total marine capture fisheries. Cuttlefish, squid, and octopus species count the seven percent of the world's total exports of aquatic animal products (FAO, 2024). Cephalopods are predominantly exported as frozen products, representing 72 percent of the total value in 2022, followed by canned (22%) and fresh (4%). In the last three years, octopus supply has been impacted by prices increment possibly linked to the COVID-19 pandemic and increasing demand (FAO, 2024).

Cephalopods intended for human consumption are generally captured by a variety of fishing methods, depending mostly on species (i.e., trawl, purse-seine, and "other gears" carried by traditional multipurpose fishing boats such as traps, setnets, and jigs) as recently reviewed for the purpose on establishing recommendation for capture and transport of live cephalopods for research purposes (Pieroni et al., 2022; Sykes et al., 2024). During industrial and artisanal fishery practices, animals are generally processed in a fast way – a characteristic of the fishing pipeline – with little attention to their welfare, thus does not necessarily prevent potential negative outcomes on the quality of flesh (e.g., Morzel and Van De Vis, 2003; Poli, 2009; Terlouw et al., 2021; Garcia and McGlone, 2022). Once on the boat they are rarely killed by human intervention.

Anoxia, a condition requiring more than ten minutes of oxygen deprivation, is the most frequent method of killing of cephalopods in the fishery context, followed by ice chilling, and less frequently by mantle reversal, destruction of the brain using blunt force or a knife. Even if the animal seems to be dead, all these methods of killing are not supported by physiological and behavioural evidence confirming instantaneous death, for the best of my knowledge. The impact

of these activities can be considered detrimental for animal welfare and quality of flesh, as mentioned.

Council Regulation (EC) 1099/2009 on the protection of animals at the time of killing (European Council, 2009) states that «Killing animals may induce pain, distress, fear or other forms of suffering to the animals even under the best available technical conditions. […] Business operators or any person involved in the killing of animals should take the necessary measures to avoid pain and minimise the distress and suffering of animals during the slaughtering or killing process». No one of the above 'considerations' is currently applied to cephalopods.

It is also worth to recall that cephalopods have been included in European Directive 63/2010 for the protection of live animals used for scientific research (European Council, 2010; Smith et al., 2013; Fiorito et al., 2015), and to date they are the sole invertebrate class regulated at the same level as vertebrate species. As reported in the Directive 2010/63/EU, «animals […] must be killed with minimum pain, suffering and distress […], and with an appropriate method of killing as set out in that Annex IV» (European Council, 2010). Last March 2024, the European Commission published an update of the Annexes III and IV (European Commission, 2024) to adapt and integrate information of the annexes on the care and accommodation requirements for the species (this time including recommendations for cephalopods) and on the humane killing methods, also in this case including information relevant to cephalopod species. In this update a short paragraph has been included in Annex III providing general requirements to maintain cephalopods species in laboratory conditions - e.g., Water supply and quality, Lighting, Diet, Enrichment and handling; see also Ponte et al. (2023). In addition, and as relevant for this PhD project, Annex IV now includes recommended methods for humane killing of cephalopods as laboratory animals. At the moment, the sole provided guidance for humane killing of cephalopods is to confirm death after induction of anesthetic overdose. Recommendations to develop further methods of killing have been provided by European Commission to members of the research team where my PhD project has been carried out, with high interest for electronarcosis and other methods of stunning (Dr G. Ponte, personal communication).

There is no available evidence for specific or systematic slaughter practices for cephalopod species, to date.

Throughout this project, I attempted to explore possible ways of applying electrical stunning on *Octopus vulgaris* (and a few attempts with *Sepia officinalis*). For methodological information related to the stunning procedures refer to Supplementary info (Electrical Stunning).

The following approach has been utilized to explore neural correlates and how these may be impacted by electrical stunning, thus to explore if a status of temporary unconsciousness and insensibility may be induced through stunning in cephalopods.

Electrical stunning – effects on animal responses

After a series of preliminary experiments (see Supplementary Information) a custom-made, more flexible, stunning apparatus was utilized providing a range of frequencies (range: 10Hz-5kHz, sine waves) and voltage up to 620V peak-peak. As described in Supplementary Information I recorded *O. vulgaris* neural activity and then applied electrical stunning event(s).

In brief, electrical parameters were selected on the basis of the experience of my co-tutor in other species (salmon and fish), but not designed around an informed guidance via bioimpedance (see Supplementary Information: Considerations around condition factor/index and bioimpedance). As there are no previous studies on the use of electrical stunning in octopus and cephalopods in general, we selected various frequencies and voltage resulting in various currents passing through the animal (Table 4).

Table 4. Number of animals (N = 32) and list of electrical parameters utilized during electrical stunning experiments of *O. vulgaris*

Frequency (Hz)	**12**	**100**	**200**
28	-	5	5
50	4	8	3
60	4	-	-
100	-	3	-
total	8	16	8

The effects of stunning on the neural activity of *O. vulgaris* was recorded via minimally invasive electrophysiological recordings (as described above) before, during and after stun. In addition, a series of behavioural and appearance indicators were noted[5] during the phases (before, during and immediately after stunning event) of these experiments to identify correlation between octopus' reactions and the stunning, if any. Baseline neural activity and VEPs were recorded just before and after electrical stunning (around 20-40 seconds after the stunning event). Finally, VEPs were elicited by visual stimulation (blue led).

Experiments were carried out on *O. vulgaris*. Stunning parameters utilized, animals' responses, patterns exhibited are presented in a tabularized overview in the following pages (Table 3). Details about the coding of various parameters collected are included in the legend to Table 3.

Once positioned in the tube and for the entire duration of the experiments, sedated *O. vulgaris* behaved as in other occasions in this project: octopuses continued to show breathing rates comparatively uniform and stable (BR: Mean ± SEM = 7.21 ± 0.81 mantle contractions ^{x} min^{-1}) before stunning, in line with what reported in a longitudinal behavioural study with a larger sample of live octopuses (about 8-10 mantle contractions/min for days, see Borrelli, 2007).

Because of strong muscular contractions of the animal during the stunning, it was not possible to detect any breathing response (i.e. BR) that is assumed equals to zero in this phase.

[5] Two observers (myself and one independent MSc student, see also below) annotated videorecordings of the experiments (see also Table 3) in order to collect data on patterns and responses exhibited by *O. vulgaris* before, during and after the stunning event.

Table 3. *O. vulgaris* data, parameters and appearance indicators (raw data) collected throughout the course of the stunning experiments (N=33, 1 animal excluded from the PCA analysis, see main text). For simplicity, the most relevant parameters measured for each animal are reported here (per condition). The variables (columns) are listed by phases [Before Stun, During Stunning (variables marked with 'S_'), After Stunning (variables marked with 'P_')] including octopus' morphometric data, gender (sex), body weight, mantle length (ML) and width (MW). Breathing rate (BR), as the number of expansions and contractions of the mantle for 60 s providing a measure of the octopus' physiological condition (see Borrelli, 2007; Borrelli et al., 2020 for definitions), has been also noted. Inking responses are reported whenever occurred. Status of the eyes, pupil and skin patterning and texture (see following pages for description) are also included. Stunning parameters (Frequency, Voltage, Current and duration) are reported for every animal.
For readability, the table is organized in two sections: the first (Table 3A) refers to animals morphometric measures and other indicators of their status before stunning (as noted in the experimental tube) applied with a given set of parameters; this is followed by a second section (Table 3B) where states for each indicator are included (per animal) during and immediately after the stunning event.

Table 3A

							Before Stun				Stunning Parameters			
#Animal	Sex	Weight(g)	ML (cm)	MW (cm)	BR (60 s)	Ink	Eyes	Pupil	SkinDisplay	SkinTexture	Freq (Hz)	Volt (V)	Curr (A)	Duration (s)
#40/24	F	420	11.5	8.4	8	no	closed	nd	pale	smooth	28	100	1.56	2
#42/24	M	580	13.8	8.8	4	no	open	broad rectangle	pale	smooth	28	100	2.11	2
#47/24	F	672	13.6	8.8	5	no	open	broad rectangle	pale	smooth	28	200	4.62	2
#55/24	F	484	12.6	8.5	10	no	open	broad rectangle	pale	smooth	28	100	1.90	2
#57/24	F	224	9.9	6.4	12	no	closed	nd	pale	smooth	28	100	1.59	2
#87/23	M	638	13.8	8.7	9	no	closed	nd	pale	smooth	50	100	0.53	1
#25/24	F	570	13.4	9.6	4	no	open	nd	pale	smooth	50	12	0.19	2
#26/24	M	342	11.7	7.6	18	no	open	broad rectangle	pale	smooth	50	12	0.24	2
#45/24	M	674	13.4	9.2	3	no	open	broad rectangle	pale	smooth	50	100	2.58	2
#46/24	F	562	13.2	9.1	3	no	closed	nd	pale	smooth	50	100	2.69	2
#50/24	M	574	12.5	8.8	4	no	closed	nd	pale	smooth	50	200	4.44	2
#17/24	M	1116	15.7	11.3	2	no	semi closed	nd	pale	smooth	60	12	0.15	2
#19/24	F	528	12.6	8.4	12	no	open	broad rectangle	pale	smooth	60	12	0.04	2
#20/24	F	514	12.2	9.0	6	no	closed	nd	light brown	smooth	60	12	0.10	2
#41/24	M	602	13.1	9.2	6	no	open	broad rectangle	pale	smooth	28	100	2.15	2
#48/24	F	242	10.0	6.5	6	yes	open	broad rectangle	pale	smooth	28	200	4.37	2
#45/23	M	456	11.2	8.2	3	no	open	slit	pale	smooth	50	200	1.39	1
#46/23	M	704	11.2	8.4	3	no	open	broad rectangle	pale	smooth	50	200	2.30	1
#51/23	F	310	11.1	7.5	20	no	open	broad rectangle	pale	smooth	50	100	1.14	1
#86/23	F	484	10.1	8.0	4	no	open	broad rectangle	light brown	smooth	50	12	0.10	1
#88/23	F	430	8.1	7.0	14	no	open	broad rectangle	pale	smooth	50	100	0.46	1
#43/24	M	604	12.8	8.7	5	no	open	broad rectangle	pale	smooth	50	100	2.19	2
#82/23	M	382	9.5	7.8	3	no	open	broad rectangle	pale	smooth	100	100	0.70	1
#83/23	F	608	11.2	8.4	6	no	open	broad rectangle	pale	smooth	100	100	1.03	1
#84/23	F	478	11.4	8.5	4	no	open	broad rectangle	pale	smooth	100	100	0.40	1
#49/24	F	802	13.6	10.0	4	no	semi closed	nd	mottle	smooth	28	200	4.13	2
#56/24	M	208	8.4	5.9	10	no	closed	nd	mottle	smooth	28	200	2.64	2
#58/24	M	460	12.0	8.7	4	no	closed	nd	light brown	smooth	28	200	3.37	2
#03/24	M	368	11.5	7.7	8	no	open	broad rectangle	pale	smooth	50	100	0.84	1
#21/24	F	424	12.0	8.5	4	no	semi closed	broad rectangle	light brown	smooth	60	12	0.06	2
#02/24	M	395	11.3	7.4	8	no	open	broad rectangle	pale	smooth	50	100	0.87	1
#27/24	F	612	13.8	9.8	16	no	open	broad rectangle	pale	smooth	50	12	0.22	2
#18/24	M	614	12.3	8.6	10	no	semi closed	nd	pale	smooth	60	50	0.42	2

Table 3B

	During Stunning											After Stunning								
#Animal	S_Ink	S_Contr	S_Contr (duration)	S_Contr (strength)	S_ML	S_MW	S_Eyes	S_Pupil	S_Skin Display-MH	S_Skin Display-A	S_Skin Texture	Shock Lat (s)	P_ML	P_MW	P_BR	P_Eyes	P_Pupil	P_Skin Display_MH	P_Skin Display_A	P_Skin Texture	Cluster grouping
#40/24	no	yes	2.1	very strong	9.3	8.0	closed	nd	reddish-brown	reddish-brown	grained	6.0	10.0	8.4	0	closed	nd	light brown - yellowish	pale	smooth	1
#42/24	yes	yes	2.2	very strong	9.8	6.1	closed	nd	reddish-brown	reddish-brown	grained	5.0	13.3	8.5	0	closed	nd	mottle	pale	smooth	1
#47/24	no	yes	2.3	very strong	9.7	7.3	closed	nd	reddish-brown	reddish-brown	grained	12.0	11.6	7.3	0	semi closed	broad rectangle	light brown	pale	smooth	1
#55/24	yes	yes	2.1	very strong	6.7	6.2	closed	nd	reddish-brown	reddish-brown	grained	5.1	11.2	8.0	6	closed	nd	mottle	mottle	smooth	1
#57/24	yes	yes	2.1	very strong	8.7	4.3	closed	nd	uniform dark brown	uniform dark brown	grained	8.1	9.5	6.4	6	closed	nd	light brown	pale	smooth	1
#87/23	no	yes	1.1	strong	10.7	5.2	closed	nd	uniform dark brown	uniform dark brown	grained	9.0	12.3	7.8	10	semi closed	nd	mottle	mottle	smooth	1
#25/24	no	yes	2	weak	10.3	9.6	closed	nd	pale	pale	smooth	7.0	13.4	9.6	4	semi closed	broad rectangle	pale	pale	smooth	1
#26/24	no	no	2	weak	11.7	7.6	closed	nd	pale	pale	smooth	1.0	11.7	7.6	20	open	broad rectangle	pale	pale	smooth	1
#45/24	yes	yes	2.4	very strong	12.2	7.6	closed	nd	reddish-brown	reddish-brown	grained	6.0	12.2	7.6	0	closed	nd	light brown - yellowish	pale	smooth	1
#46/24	yes	yes	2.2	very strong	10.0	6.1	closed	nd	reddish-brown	reddish-brown	grained	6.5	10.9	7.3	0	closed	nd	light brown - yellowish	pale	smooth	1
#50/24	no	yes	2	very strong	10.0	6.4	closed	nd	reddish-brown	reddish-brown	grained	15.0	10.0	8.2	8	closed	nd	mottle	mottle	smooth	1
#17/24	no	no	2	weak	14.1	11.3	semi closed	nd	pale	pale	smooth	3.0	14.9	11.3	0	semi closed	nd	pale	pale	smooth	1
#19/24	no	yes	2.2	strong	9.9	6.6	closed	nd	pale	pale	smooth	9.0	12.6	8.4	8	open	broad rectangle	pale	pale	smooth	1
#20/24	no	no	2	weak	10.7	9.0	closed	nd	light brown	pale	smooth	3.0	11.5	9.0	2	closed	nd	light brown	pale	smooth	1
#41/24	no	yes	2.2	very strong	10.3	6.9	closed	nd	reddish-brown	reddish-brown	grained	6.0	10.7	8.6	0	semi closed	dilated	mottle	pale	smooth	2
#48/24	no	yes	2.2	very strong	8.7	4.4	closed	nd	reddish-brown	reddish-brown	grained	8.4	8.7	5.9	0	open	dilated	light brown	pale	smooth	2
#45/23	yes	yes	1.2	very strong	11.9	5.9	closed	nd	reddish-brown	reddish-brown	grained	6.0	10.9	7.2	0	open	broad rectangle	light brown	light brown	smooth	2
#46/23	yes	yes	1.3	very strong	10.9	5.8	closed	nd	reddish-brown	reddish-brown	grained	3.5	10.8	6.5	6	open	broad rectangle	light brown	pale	smooth	2
#51/23	yes	yes	1.3	very strong	11.1	6.0	closed	nd	uniform dark brown	uniform dark brown	grained	3.7	11.1	7.5	18	open	slit	pale	pale	smooth	2
#86/23	no	yes	1.1	strong	8.4	5.6	closed	nd	light brown	pale	grained	7.0	9.1	7.4	8	open	broad rectangle	pale	pale	smooth	2
#88/23	yes	yes	1.3	very strong	7.3	3.7	closed	nd	reddish-brown	reddish-brown	grained	8.0	7.1	6.0	6	open	broad rectangle	light brown	pale	smooth	2
#43/24	yes	yes	2.2	very strong	11.9	5.4	closed	nd	reddish-brown	reddish-brown	grained	4.5	10.6	8.4	0	semi closed	dilated	light brown - yellowish	pale	smooth	2
#82/23	yes	yes	1.2	strong	10.0	5.1	closed	nd	reddish-brown	reddish-brown	grained	4.0	8.5	5.9	0	semiclosed	nd	mottle	mottle	smooth	2
#83/23	yes	yes	1.3	strong	12.3	4.8	closed	nd	uniform dark brown	pale	grained	3.0	9.7	6.5	2	open	broad rectangle	pale	pale	smooth	2
#84/23	yes	yes	1.3	strong	9.7	5.1	closed	nd	uniform dark brown	pale	grained	7.0	10.2	7.3	8	open	broad rectangle	pale	pale	smooth	2
#49/24	no	yes	2.2	very strong	11.3	6.7	closed	nd	uniform dark brown	uniform dark brown	grained	10.0	9.1	7.7	8	closed	nd	mottle	mottle	smooth	3
#56/24	no	yes	2.4	very strong	6.2	4.3	closed	nd	reddish-brown	reddish-brown	grained	6.0	6.6	5.9	4	semi closed	broad rectangle	mottle	mottle	smooth	3
#58/24	yes	yes	2.2	very strong	11.1	6.3	closed	nd	reddish-brown	reddish-brown	grained	6.0	12.0	9.6	6	closed	nd	mottle	mottle	smooth	3

Table 3B

	During Stunning											After Stunning									
#Animal	S_Ink	S_Contr	S_Contr (duration)	S_Contr (strength)	S_ML	S_MW	S_Eyes	S_Pupil	S_Skin Display-MH	S_Skin Display-A	S_Skin Texture	Shock Lat (s)	P_ML	P_MW	P_BR	P_Eyes	P_Pupil	P_Skin Display_MH	P_Skin Display_A	P_Skin Texture	Cluster grouping
#03/24	yes	yes	1.3	very strong	10.4	5.1	closed	nd	reddish-brown	reddish-brown	grained	3.1	9.1	7.7	10	open	broad rectangle	mottle	mottle	smooth	3
#21/24	no	no	2	weak	9.6	9.3	closed	nd	light brown	light brown	smooth	4.0	10.8	8.5	0	open	broad rectangle	light brown	light brown	smooth	3
#02/24	yes	yes	1.2	very strong	10.1	3.8	open	dilated	reddish-brown	reddish-brown	grained	8.0	10.3	6.6	10	open	broad rectangle	mottle	pale	smooth	4
#27/24	no	no	2	weak	13.5	9.8	open	broad rectangle	pale	pale	smooth	2.5	13.8	9.8	16	open	broad rectangle	pale	pale	smooth	4
#18/24	yes	yes	2.2	very strong	8.4	6.1	closed	nd	uniform dark brown	pale	grained	9.0	10.5	8.1	2	closed	nd	light brown	pale	smooth	excluded

Supporting description to Table 3: To standardize the coding of each indicator I adopted the following definitions/status for each of the parameters collected to describe individual *O. vulgaris* reactions/patterns during the stunning experiments. For each animal the conditions before stunning are considered as reference.

Breathing rate (BR) - Number of expansion/opening of the mantle cavity during spontaneous breathing (see legend to Table 3 for references). The measure was taken from videorecordings.

Inking - release of ink (Borrelli et al., 2006; see also Bennett and Toll, 2011; for review see also Hanlon and Messenger, 2018)

Mantle Length (cm) - length of the octopus' mantle (in cephalopods mantle length is preferred to body weight as a reference variable of body size); a standardized measure has been adopted, following protocols available in OctoLab. Mantle length is measured as the maximum antero-posterior extension from the V shaped location on the head of the animal, to the foremost posterior tip of the mantle, measured immediately at the end of the anaesthesia overdose thus to achieve the maximum relaxation of the animal body.

Mantle Width (cm) - the maximum latero-lateral expansion of the mantle of octopus taken measured immediately at the end of the anaesthesia overdose thus to achieve the maximum relaxation of the animal body.

Measures of **Mantle Length** and **Mantle Width** were also taken from video recordings and estimated through snapshots from frames where the animal was well visible in the three phases of the experiment (i.e. before, during and after stunning event).
Eyes – I refer to the "status" of the skin covering the eye bulb. Three conditions were noted in my experiments, as defined below:

Eyes Open: the skin surrounding the eye bulbs does not cover the eyes, pupil and other parts of the eye bulb are clearly visible.
Eyes semi closed: skin around the eye bulb is partially covering the eyes, only a small part of the pupil and other parts of the eye are visible.
Eyes Closed: skin around the eye bulbs completely covering both eyes, pupil and other part of the eye is not visible.

Pupil: as defined by Packard and Sanders (1971; cited in Borrelli et al., 2006) in normal conditions, the pupil is horizontally aligned and may appear as a very narrow slit or, more commonly, as a broad dark rectangle. This provides a variable degree of black appearance to the eyes; following the original description, at its extreme degree of expansion, it may appear as a rounded black circle (i.e. pupil dilated). In my experiments I noted the following states:

Slit: pupil horizontally aligned as narrow slit
Broad rectangle: pupil horizontally aligned appearing as a broad rectangle, taking almost half of the total eye space
Dilated: extreme degree of expansion, pupil appears as a rounded black circle

Skin displays: I am referring to the appearance of the animal as body patterning (for review and definitions see Borrelli et al., 2006) known to be under direct neural control (central nervous system). A body pattern is produced by a potentially continuous change and combination of chromatic, textural, postural and locomotor components. For the purposes of this study, I adopted the classification of cephalopod body patterns and referred to chromatic components and/or body patterning as originally defined (review in Borrelli et al., 2006). In my experiments the following states (i.e. chromatic components/patterns) were identified:

Pale: defined as 'Uniform Light Gray' is a condition when the animal 'wear' a light shade of grey tinged with brown or even very lightly with green, providing an appearance of a light grey background usually exhibited when the animals are at rest
Light brown-yellow: an uniform coloration of the entire body of the animal due to most of the chromatophores retracted and the resulting overall appearance darker than on pale, but with yellow and brown chromatophores expanded to provide a yellow-brownish appearance.
Light brown Mottle: mottling of all visible areas of the octopus skin on an uniform coloration of with patches and spots present, but at low intensity.
Reddish brown: a darker appearance of the skin of the entire animal mostly exhibited on a mottled phase of the body pattern.
Uniform dark brown: maximal expansion of the chromatophores over the body, resulting in a uniform darkening of the skin.

Skin texture: the skin of octopus (and cephalopods) can actively change the texture (review and definitions in Borrelli et al., 2006) with the network of grooves and patches changing over the entire body of the animal. This happens as a consequence of the contraction of the underlying musculature. In my experiments I noted the following states.

Smooth: the muscles of the skin are relaxed, the patches and grooves of the trellis lie on the same plane and the grooves appear shallow and broad, thus giving a "smooth" effect to the whole octopus. In these conditions, no papillae are raised over the body of the animal
Grained: The network of grooves and patches changes its texture over the entire body of the animal as a consequence of the contraction of the underlying musculature, thus the grooves become deep and narrow, creating a "rugose" effect.

During my experiments, body patterns displayed by animals were not uniformly distributed, after stunning, on the entire body of the animal, but exhibited with different appearance on arms vs the remaining parts of the body (i.e. mantle and head). To account for such differences the various states have been coded into the variables: S_Skin Display-MH (mantle and head, during stunning), S_Skin Display-A (arms, during stunning) and P_Skin Display-MH (mantle and head, after stunning), P_Skin Display-A (arms, after stunning).

To describe the effects of the stunning on the animals I also evaluated **muscle contraction** by measuring ***i.*** the duration of the contraction of the body (S_Contr, duration) in seconds, and ***ii.*** the strength of the contraction (S_Contr, strength). In this last case, I attributed the different extent of contraction of the entire animal on an arbitrary scale as: ***Weak***, ***Strong***, ***Very Strong***. 'Levels' have been estimated through video recordings and assessed by an evaluation of the degree of body contraction (i.e. shortening, mantle contraction/expansion) after consensus between the observers and one of my Tutors. I cannot rule out if the coding was somehow biased by other factors (e.g., duration of the contraction, darkening of the body of the animal). However, concordance between two independent observers[6] resulted to be higher than 90%.

Finally, I estimated **Shock latency** as the time interval between maximum muscles contraction reached during electric stunning event and the recovery to a total/maximal relaxation of the body of animal, as seen in the video recordings.

[6] Observation and coding of the parameters from video recording were carried out independently by me and by a MSc student (D. Lombardi, Univ. Bologna, Italy) to whom I am grateful.

On average, few minutes (about 1.5 min) after the stunning event, it was possible to detect spontaneous mantle contractions of *O. vulgaris,* identified as resuming of breathing rate (after stunning, BR: Mean ± SEM = 5.09 ± 0.96 mantle contractions x min^{-1}). However, a more attentive look at the individual data revealed that in about 36% of animals (12 out 33), spontaneous breathing did not occur (i.e. BRs equal to zero). These octopus did not resume their respiration, despite I was able to continue to detect neural signals also in most of these animals[7]. Overall, the average decrease of BR after stunning was significant when compared with their status before stunning ($Z = -2.94$, $N = 33$, $p = 0.003$, after Wilcoxon signed-rank test), but not significant when only octopuses that resumed breathing rate were considered ($Z = -1.02$, $N = 21$, $p = 0.309$, after Wilcoxon signed-rank test).

In all cases, except one octopus, no inking behaviour was observed at the beginning of the experiment, confirming that all the manipulations with live animals have been carried out limiting as much as possible any aversive reaction in the animals. Inking occurred in more than half of *O. vulgaris* (51.5%) immediately after (and during) the electrical stunning and appeared as a consequence of the marked whole-body contraction induced by the stunning event. The occurrence of inking did not appear correlated to the electrical parameters utilized to set the stunning conditions.

Maximal contraction of the entire body of the animal (e.g., mantle ballooning, shortening and 'widening' of the arms) lasted few seconds (Mean ± SEM = 1.84 ± 0.08, range = 1.1 ÷ 2.4 s) and largely correspond to the duration of the stun (1 or 2 s; average = 1.66 s). After the stunning event, animals required few seconds to recover a "normal" posture (in most of the cases almost equivalent to the previous) as measured here by 'Shock latency' (Mean ± SEM = 6.22 ± 0.51 s), indicating that despite the intensity of the stunning single event *O. vulgaris* recovered their posture and in more than two-thirds of cases also spontaneous respiration. Spearman rank correlations comparing electrical parameters (Voltage, Current) with the duration of the contraction (in seconds) revealed significant correlation coefficients in cases of Duration of the contraction vs. voltage ($\rho = 0.83$, $p < 0.001$) and current ($\rho = 0.45$, $p = 0.009$).

[7] For a matter of timing, I have been not able to evaluate differences in amplitude (for example) of the neural potentials detected after stunning comparing animals that resumed respiration with those where I was not able to detect any BR.

A Principal Component Analysis (PCA) followed by hierarchical clustering (Ward's method) was applied to identify patterns of changes and correlations between expression levels of descriptors of *O. vulgaris* states before, during and after stunning (Table 3). The computational strategy adopted followed Zar (1999), Abdi and Williams (2010) and the approaches utilized to reveal relationships between qualitative behaviour assessment and outcomes in the context of welfare measures in other species (e.g., Andreasen et al., 2013; Minero et al., 2016; Battini et al., 2018; Friedrich et al., 2021). This allowed me to identify a general overview of the changes occurring as a consequence of the electrical stunning. I ran a single PCA considering descriptors collected during the three phases of the experiment (before, during, and after stunning). A Varimax rotation with Kaiser normalization was applied to obtain a Rotated Component Matrix and the relative regressed values for each of the resulting components (Table 5).

In order to evaluate sample adequacy, I assessed Kaiser-Meyer-Olkin Measure resulting above the cut-off value (usually considered equal to 0,5). Our data resulted acceptable with KMO = 0.696 (scored as middling/mediocre). This is largely justified by the multitude of variables considered and the relative variance of descriptors in some of them; the restricted variability observed in some cases can be considered linked to the overall conditions of the animals in the three phases of the stunning experiment.

Table 5. Rotated Component matrix calculated after PCA analysis (Rotation Method: Varimax with Kaiser Normalization; rotation converged in 7 iterations) for variables considered to evaluate *O. vulgaris* response to electrical stunning (see Table 3 for coding). Marked in boldface are statistically significant correlation values and their attribution to PCA components.

Rotated Component Matrix

	Component					
	1	2	3	4	5	6
Cod_eyes	.021	**.832**	-.321	.051	-.083	.110
COD_pupil	.114	**.837**	-.210	.100	-.010	.128
COD_skin	-.230	-.184	**.760**	.034	-.083	-.272
S_COD_muscles_contr	**.803**	.081	-.130	-.247	-.163	-.302
S_COD_contr_strength	**.911**	-.013	.024	-.187	-.029	-.071
S_COD_eyes	-.245	.101	-.096	.083	-.002	**.874**
S_COD_display_MantleHead	**.866**	-.125	.132	.074	-.275	-.130
S_COD_display_Arm	**.855**	-.198	.224	-.123	.059	.096
S_COD_texture	**.921**	-.010	.042	-.009	-.249	-.118
Shock_latency (s)	.307	-.142	.037	**-.679**	-.251	-.080
P_COD_eyes	-.182	**.819**	-.051	.337	-.007	.048
P_COD_pupil	-.104	**.881**	.003	.014	.027	-.113
P_COD_display_MantleHead	.573	-.162	**.617**	-.238	.078	.239
P_COD_display_Arm	.261	-.207	**.777**	-.051	.027	.035
D_MLstart_stun	.007	.194	-.063	**.886**	-.098	.026
D_MLstart_post	-.260	.091	-.438	.364	**.622**	.045
D_MWstart_stun	**-.738**	-.137	.002	.010	.452	.011
D_MWstart_post	-.293	-.076	.098	-.034	**.867**	-.012

The six components accounted for a total of 81.45% of variance explained by the PCA analysis (eigenvalue: 0.99). The following allocation of variables/descriptors within components resulted after PCA:

Component 1 (34.27% of the total variance) included: descriptors of contraction during stunning (S_COD_muscles_contr; S_COD_contr_strength), changes in the size of the head (D_MWstart_stun) and in body patterning (S_COD_display_MantleHead; S_COD_display_Arm; S_COD_texture)

Component 2 (20.19% of the total variance) included: the status of octopus eyes before (Cod_eyes; COD_pupil) and after the stunning event (P_COD_eyes, P_COD_pupil)

Component 3 (7.73% of the total variance) included descriptors of body patterning of the animal before (COD_display) and after the stunning (P_COD_display_ManteHead; P_COD_display_Arm)

Component 4 (6.89% of the total variance) included variables measured for accounting the time that octopuses required to return to a "normal" posture post-stunning

(Shock_latency) and changes in the mantle length of the animal before and during stunning (D_MLStart_stun)

Component 5 (6.20% of the total variance) included measures of the changes in the body size of the animal before vs post-stunning (D_MLStart_post, D_MWstart_post)

Component 6 (5.51% of the total variance) included the status of the octopus eyes during stunning (S_COD_eyes).

This variegated attribution of descriptors to the component supports the suggestion that – at least with the resolution of descriptors I adopted - the animals "behave" effectively different before, during and after the electrical stunning, independently from the electrical parameters utilized for inducing the stunning (see also Figure 28).

I observed a significant change in the octopus body shape as outcome of the stunning, with overall shortening and mantle contraction (mantle ballooning/shortening/expansion and increase of the head width) that appear to not return to normal even after several seconds after the stunning event (all changes significant after Wilcoxon signed-rank test).

However, one of the interesting findings of this part of my PhD is related to the body patterning exhibited by *O. vulgaris* as a consequence of the electrical stunning. During stunning almost all animals became dark, assuming an overall contraction of chromatophore muscles in the skin, with marked expansion of the chromatophores. The resolution of my videorecordings does not allow to verify if all chromatophores expanded, i.e. the yellow, orange, brown and dark brown (Froesch and Packard, 1979; Packard, 1985; 1988; 1992; 1995; Messenger, 2001).

Wilcoxon signed-rank test revealed that the changes in body pattering did not occur uniformly, as expected for an overall contraction (driven at local and/or central neural level) in response to the stunning. The patterning exhibited by *O. vulgaris* resulted significant different when mantle and head are compared to the body patterns of the arms, suggesting that the electric shock given with the stun possibly induce a different response in brain centres at the level of the suboesophageal mass where posterior- and anterior chromatophore lobes are located, controlling mantle and head and arms, respectively. Comparison of patterns noted on the skin before, during and after stunning revealed a return to a previous/benchmark state after the stunning at the level of the arms (all statistically significant apart from COD_Skin vs P-COD_display-Arm after Wilcoxon signed-rank test, that is marginally significant, $p = 0.046$; Sign

test exact p = .180). Of course, this result (and suggested explanation of some sort of 'dissociation' in the central circuit regulating body patterning) is mentioned here as a way of suggestive hypothesis.

As mentioned above, I carried out a hierarchical cluster analysis (Figure 28), following Ward (1963) method on the regression scores obtained for each principal component resulted (n = 6) after PCA. We assumed that values of the PC scores reflected octopuses' performance throughout the phases, and the values of the PC scores reflecting (some of) the characteristics of the animals as described with the states/indicators considered (see Table 3 and its legend for details). The hierarchical cluster analysis method allowed to graph patterns of similarity and dissimilarity among subjects. The ultimate goal was, thus, to find a potential explanation for measured differences and to encourage a hypothesis for the observed variability.

In Figure 28 the resulting dendrogram is coded – at the level of each branch origin (i.e. animal) – according to the corresponding set of main electrical parameters (frequency, voltage) utilized for the stunning experiment. No evident relationship was found between the distribution of animals in clusters and the electrical parameters set for stunning, thus confirming that the response of the animals was related to stunning parameters, but not directly linked to a given parameter.

The four clusters correspond to different responses of the animals to the electrical current flowing across the body, tissues and the nervous system.

In the following pages, I will attempt to summarize what I observed from these experiments in the search of how neural correlates change in response to electrical stunning.

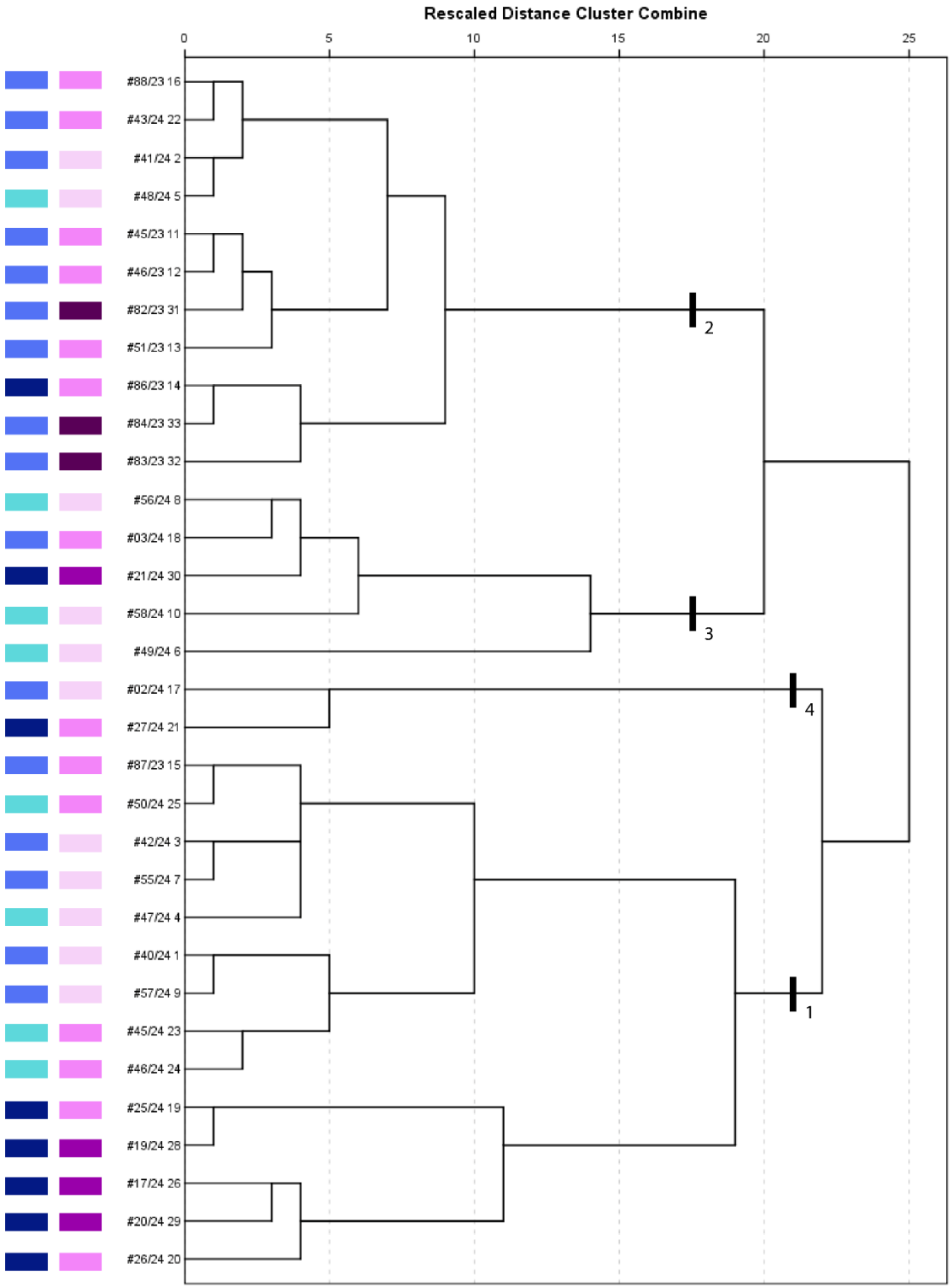


Figure 28. Dendrogram after Ward's hierarchical clustering showing the relationship among octopuses (N = 32; one removed from the analysis) based on the animals' responses during stunning experiments included in this thesis. The clusters (n = 4) recognized are numbered from 1 to 4 (see text for details). Colour coding refers to electrical parameters utilized for the stunning (left column, Voltage: 12 V ; 100 V ; 200 V ; second column, Frequency: 28 Hz ; 50 Hz ; 60 Hz ; 100 Hz ;).

Effect of Electrical stunning on Visually Evoked Potentials (VEPs)

During my experiments on electrical stunning and in more than 60% of animals, I was able to observe a general increase in the amplitude of the visually evoked potentials recorded from brain masses (see for example Figure 29).

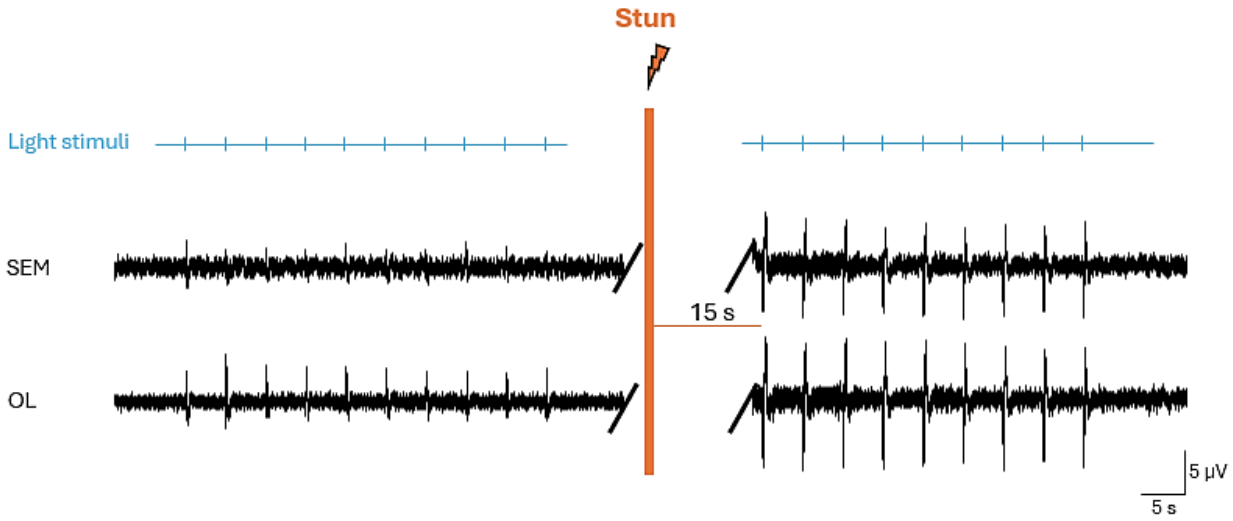


Figure 29. VEPs evoked by trains of LED stimulation (single stimulus=100ms) collected in correspondence of right OL and SEM before and after 15 seconds from the electrical stunning (50 Hz, 12 V, 1 s).

On average, the neural signal recorded after stunning resulted to be more than doubling in amplitude when compared to the pre-stunning condition.

Latencies and Amplitude of evoked neural responses measured pre-stunning (i.e. baseline) at the level of the ipsilateral optic lobe, for example, resulted to be significant different ($p < 0.05$, after Mann-U Whitney test) when applying single electric shock (see Figure 30).

The increase of VEPs amplitude resulted not correlated to given values of the electrical parameters set for the stunning (i.e., Frequency, Voltage and Duration; data not shown). I observed an increased in neural responses both with low (0.04-0.4 Amp) and high (1.9-2.6 Amp) currents passing through the body of the octopus.

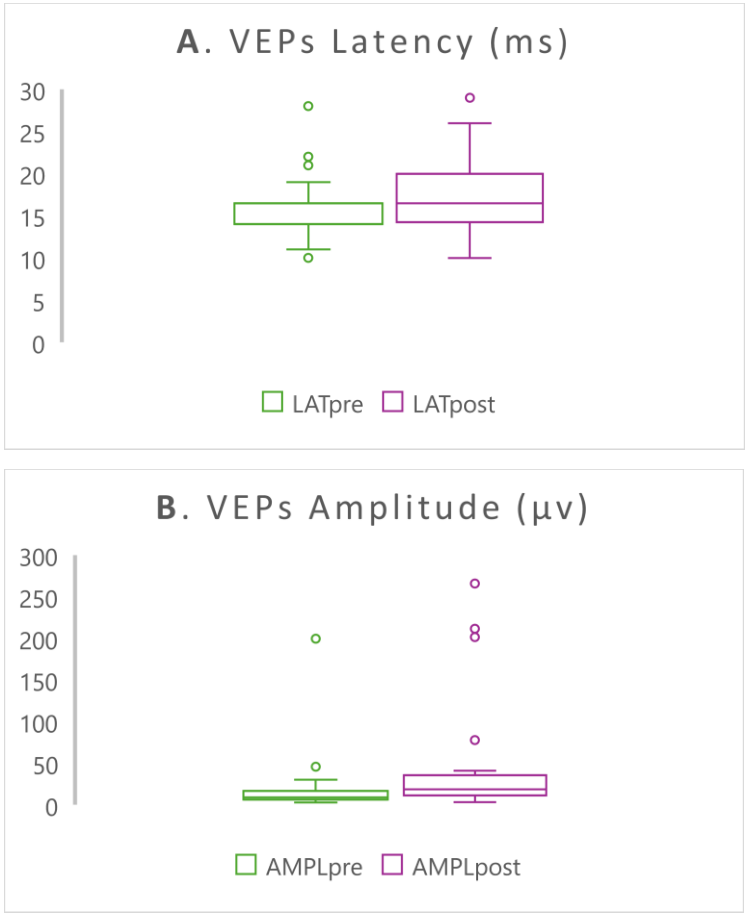


Figure 30. Boxplot of the distribution of Latency (**A**) and Amplitude (**B**) values recorded before (pre) and after (post) electrical stunning at the level of ipsilateral optic lobe of *O. vulgaris*. VEPs were recorded after LED stimulation using stimuli of 100 ms duration.

The increase of the amplitude of the recorded neural signal was observed after about 15-20 seconds from the offset of the electrical stunning; this was the time necessary to reconnect the recording electrodes to the electrophysiological setup.

In order to search for differences in the VEP detected in these experiments we developed custom made scripts using MatLab (R2023a).

First at all, grand average of the evoked responses detected at the level of the three areas on the body of the animal (i.e. ipsilateral optic lobe, supraoesophageal mass and contralateral optic lobe) for the two conditions (pre- vs post-stun) were plotted, as shown in Figure 31. It was possible to define, for every condition, different groups of event related potential (ERP) based on the profiles of the response (shape, amplitude and duration), except for the pre-stun condition in both optic lobes, which showed only one profile of evoked responses (Fig. 31).

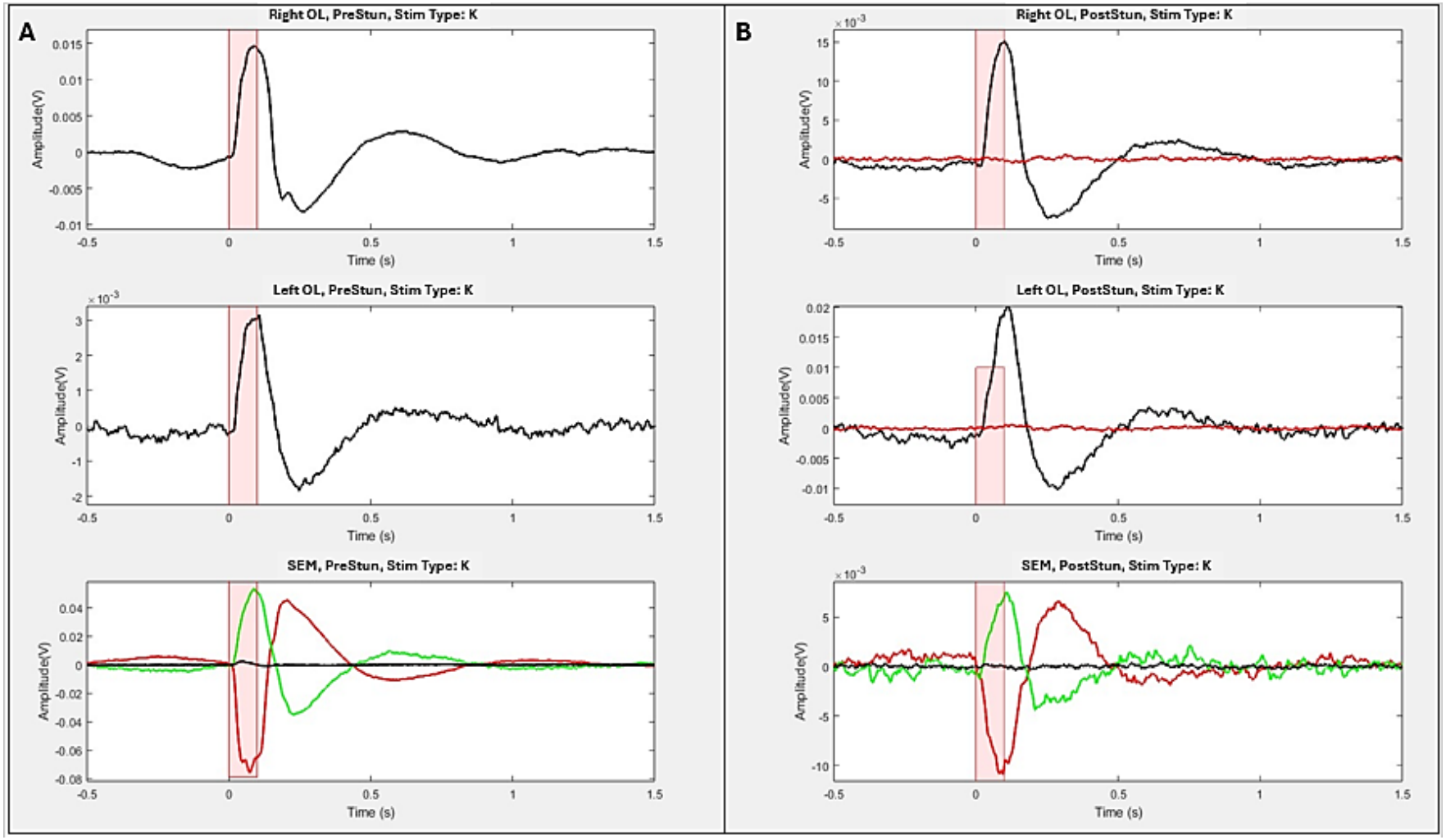


Figure 31. Plot of grand average of ERP in the two conditions Pre (**A**) and Post (**B**) stun recorded from at the level of right, left optic lobes (OL) and supraoesophageal mass (SEM). Different colours highlight groups of ERP based on the profiles of the potentials recorded.

Clusters of ERP were defined on the basis of the number of light stimuli the animal was subjected to. Based on my data we observed that increasing number of light pulses directed to one of the eyes resulted in a difference on 'behaviour' of the profile of the evoked responses. Figures 32-37 show grand averages and clustering of the ERPs recorded from the right OL, left OL and SEM for both pre- and post-stun conditions. Neural responses were elicited by trains of 100 ms light pulses. Figures allow inspection on mean amplitude of all ERPs (32-37, **A**), clustering of ERP on the basis of the peaks' values of amplitude and time (32-37, **B**) and mean of ERP clusters amplitude (32-37, **C**).

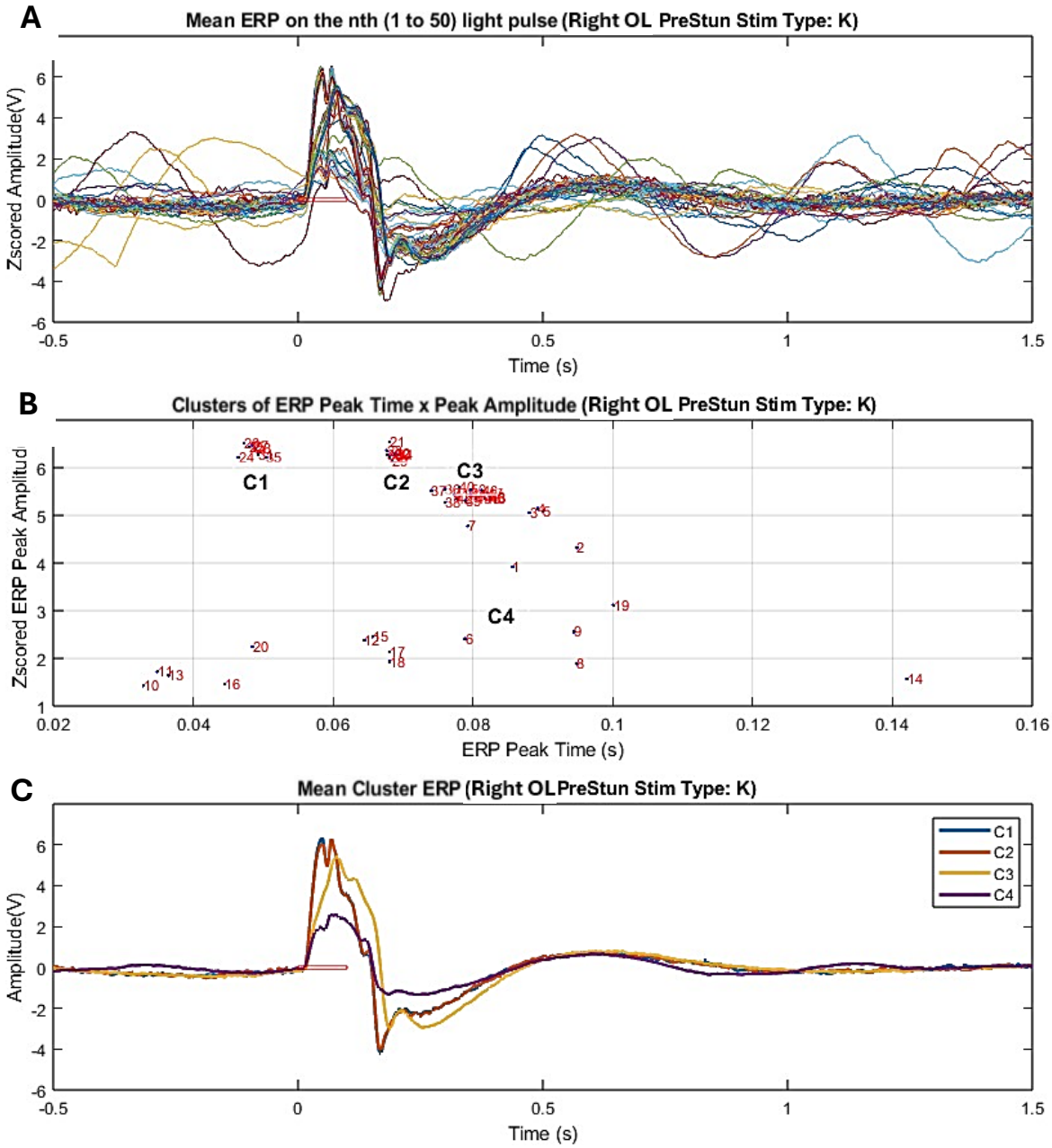


Figure 32. Grand average and clustering of ERPs detected at the level of the right *O. vulgaris* optic lobe (OL); Pre Stun (see text for details).

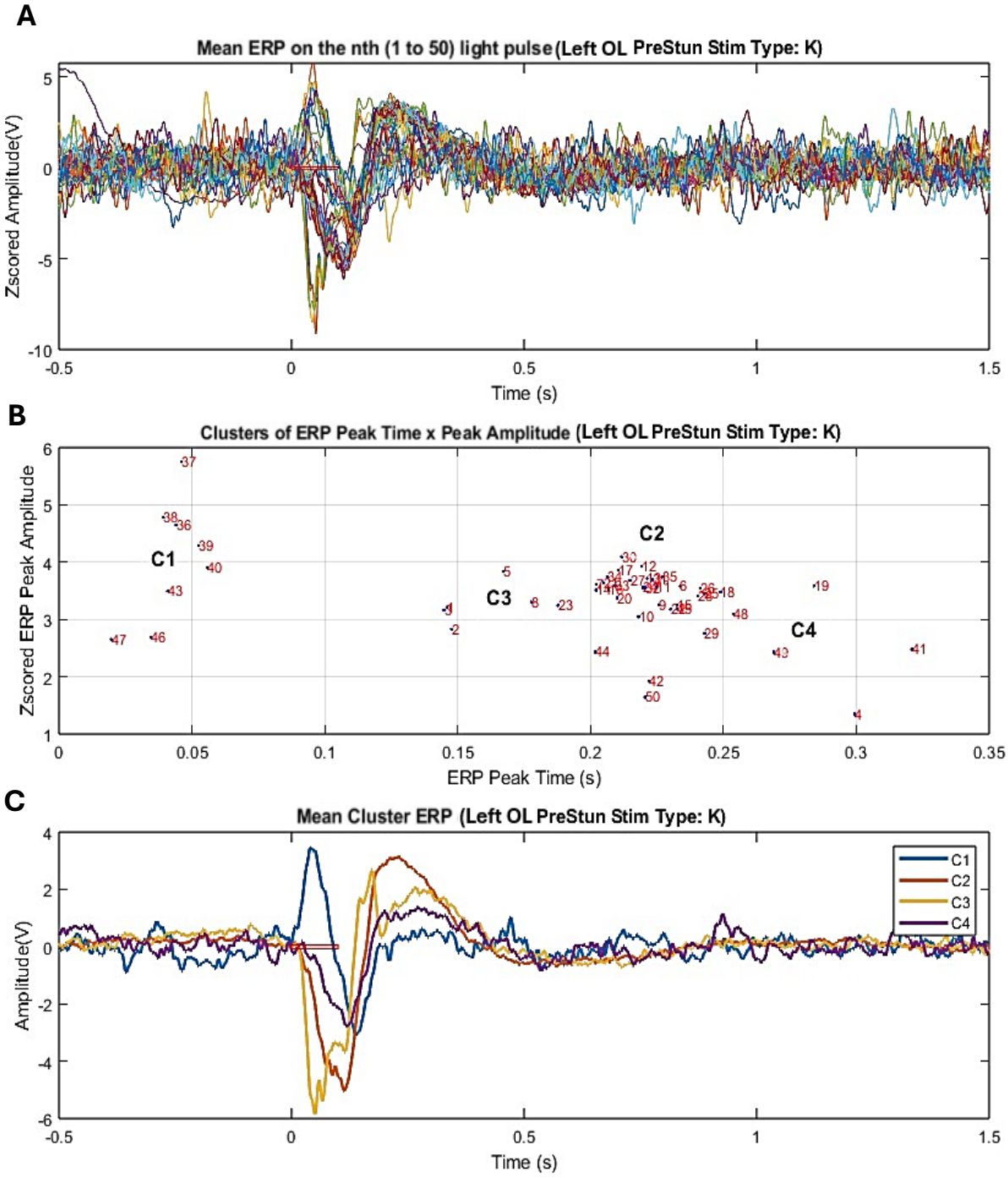


Figure 33. Grand average and clustering of ERPs detected at the level of the left *O. vulgaris* optic lobe (OL); Pre Stun (see text for details).

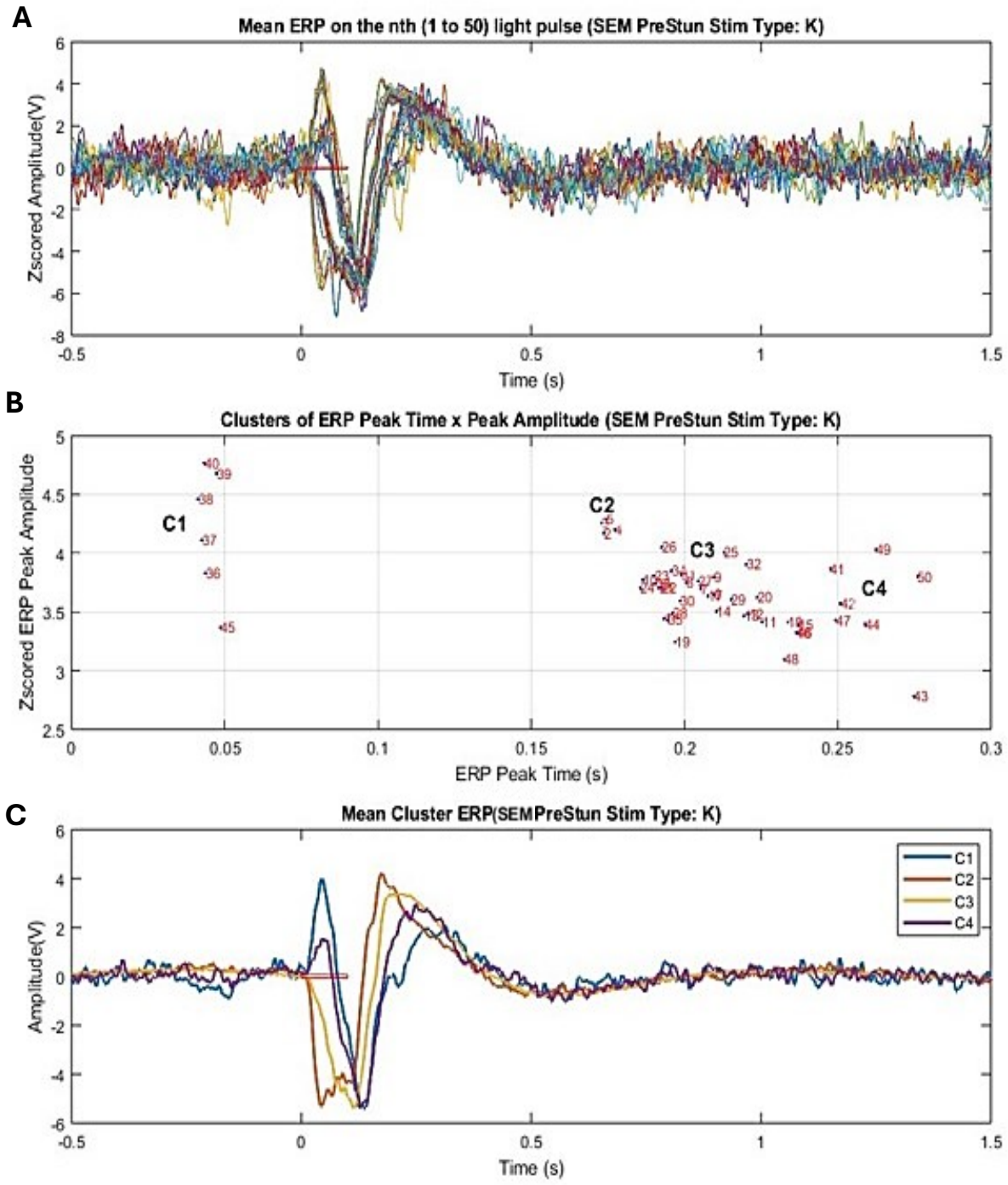


Figure 34. Grand average and clustering of ERPs detected at the level of the supraoesophageal mass, SEM, of *O. vulgaris;* Pre Stun (see text for details).

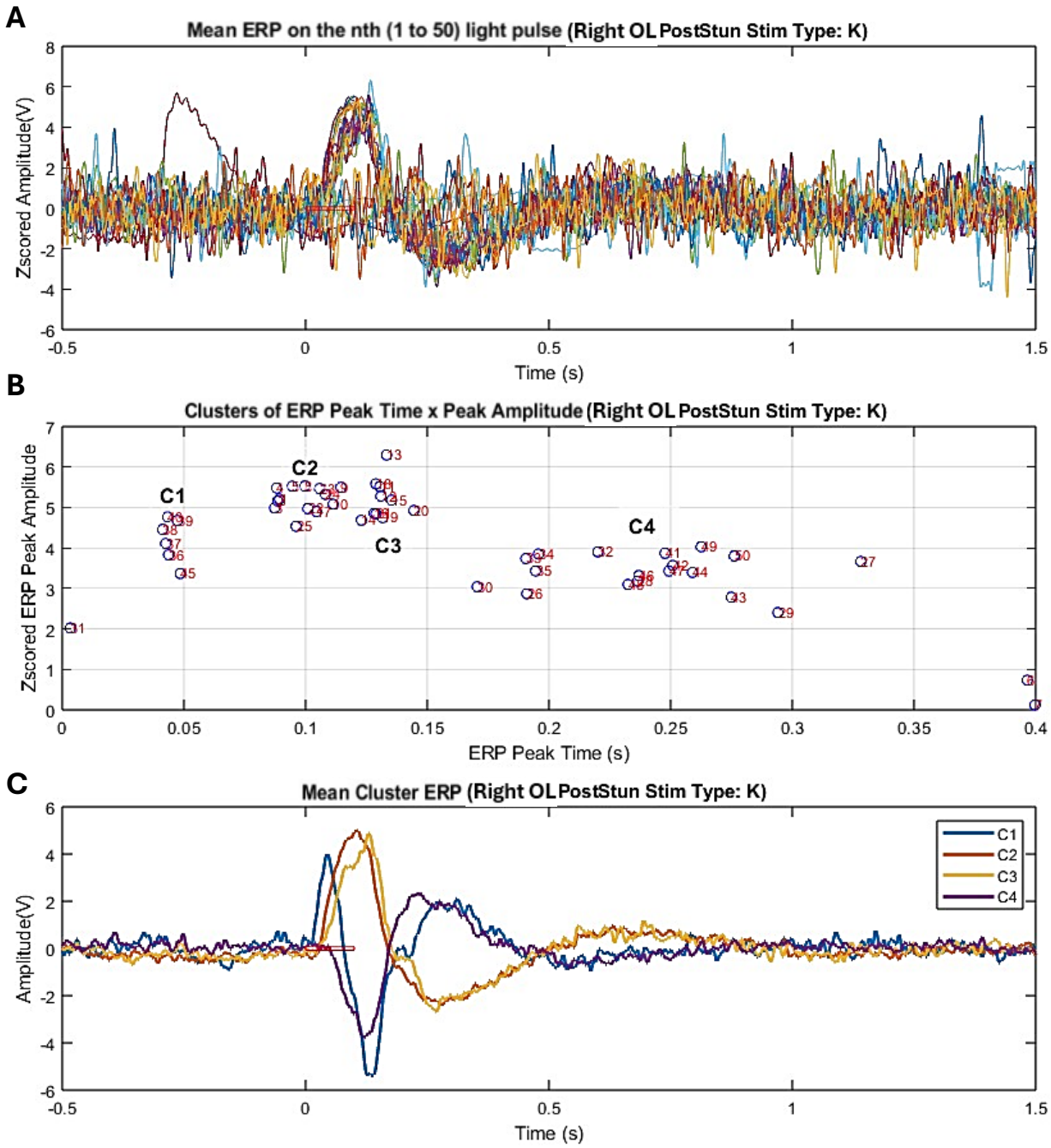


Figure 35. Right OL – Post Stun; Grand average and clustering of ERP (see text for details).

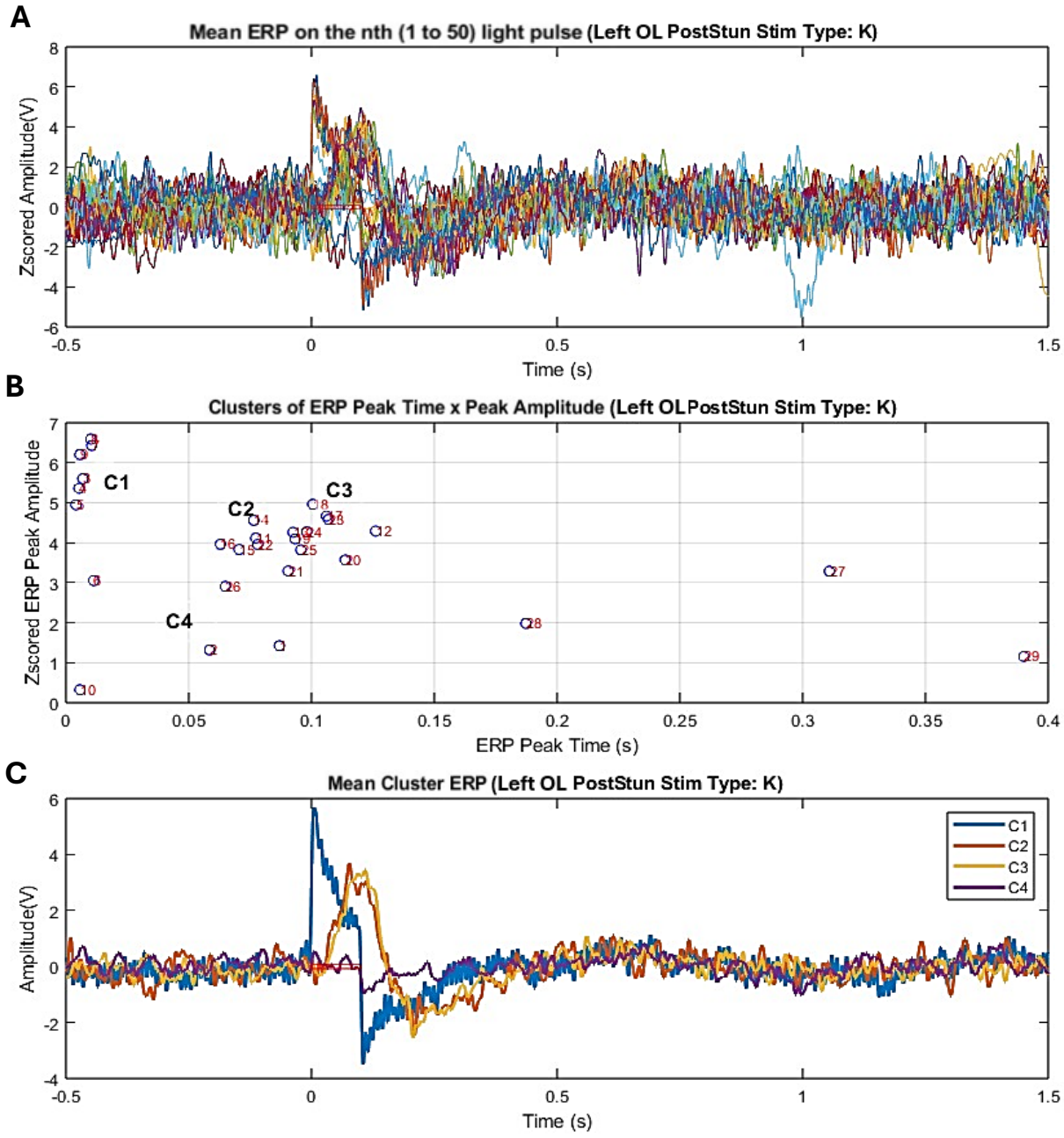


Figure 36. Left OL – Post Stun. Grand average and clustering of ERP (see text for details).

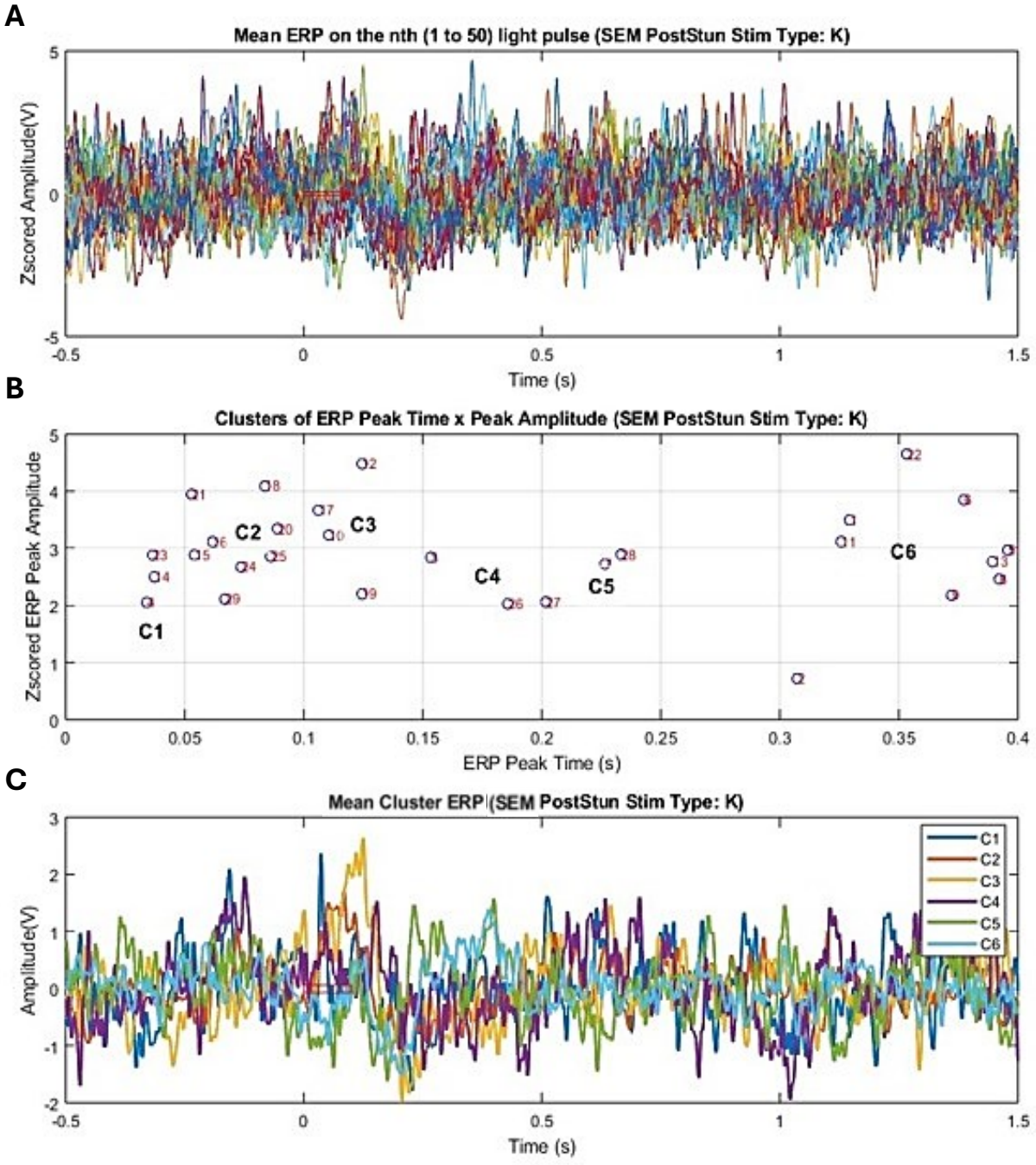


Figure 37*. O. vulgaris* SEM - Post Stun. Grand average and clustering of ERP (see text for details).

At the level of the supraoesophageal mass (SEM) it can be noted that the ERPs are similar (in general shape) but clearly different from the ones detected on the left optic lobe (left OL). In addition, the recordings from the right and left side (right and left OL, respectively) look very different, as ERP waveforms are reversed between the two and latencies observed in correspondence of the ERP peak in left OL are longer than those on the right side. Even latency of the negative ERP peak in right OL is longer than the latency of the positive peak in the same OL.

It is clearly visible the effect of the electrical stunning on the VEPs. For the SEM it is almost impossible to define ERPs clusters anymore, but also for the two optic lobes the neural signals appear very noisy and less responsive to strong light stimulation. Apart from the difference in the waveform shape of VEPs, also latencies from the onset of the light stimuli seem probably affected by the electric shock causing a delay between the pre and post conditions.

Effect of the Electrical stunning on spontaneous activity (resting state)

To test the effect of different light conditions on the electrical activity frequency domain of the central nervous system in octopus, I introduced an alternating light on/light off condition during electrophysiological recordings.

Both period of light and dark had a duration of 30 seconds, during which the animal was not stimulated. Electrical neural signals from optic lobes and supraoesophageal mass were recorded 10 minutes before, and again at about 2 minutes after the electric shock delivered as stunning (Figure 38).

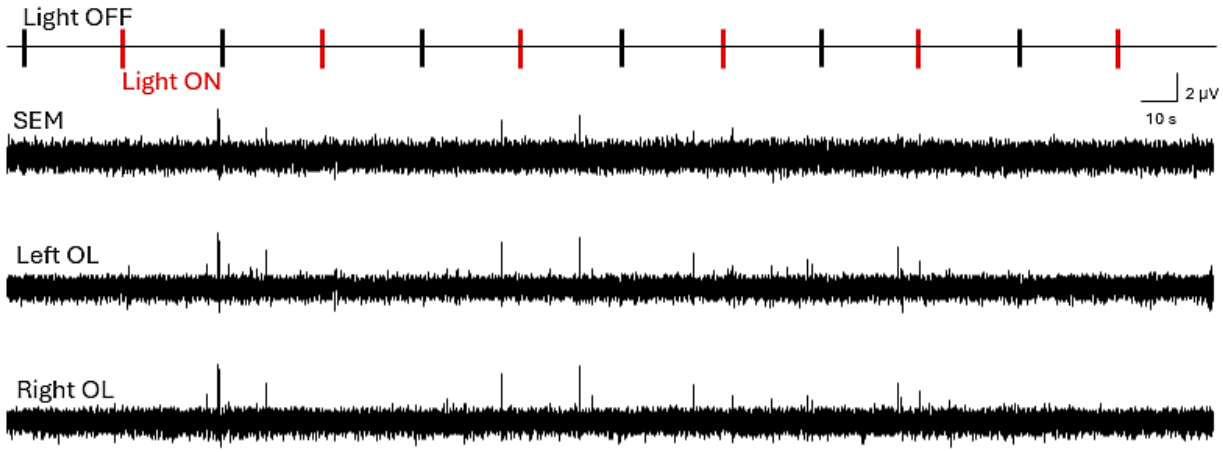


Figure 38. Example of spontaneous recording from the three channels during alternating period of light off (black) and light on (red) before electrical stunning.

All the resting state data were organised and analysed by a custom-made MATLAB script.

Analysis on power spectra and spectrogram were carried out for all the recording channels (related to the right and left OL and SEM) and computed for light conditions (ON/OFF) and frequency bands: delta (1-4Hz), theta (4-8 Hz), alpha (8-12 Hz), beta (12-30 Hz) and gamma (>30 Hz).

Spectrograms from the three brain structures highlight the differences in the power density of the frequencies between conditions of light ON/light OFF (Figure 39).

As shown in the figure all the frequencies included in the selected range (1-40 Hz) resulted with a general increase of the power during light ON condition. This appears particularly evident for signal detected from the supraoesophageal mass and from the right OL (Fig. 39).

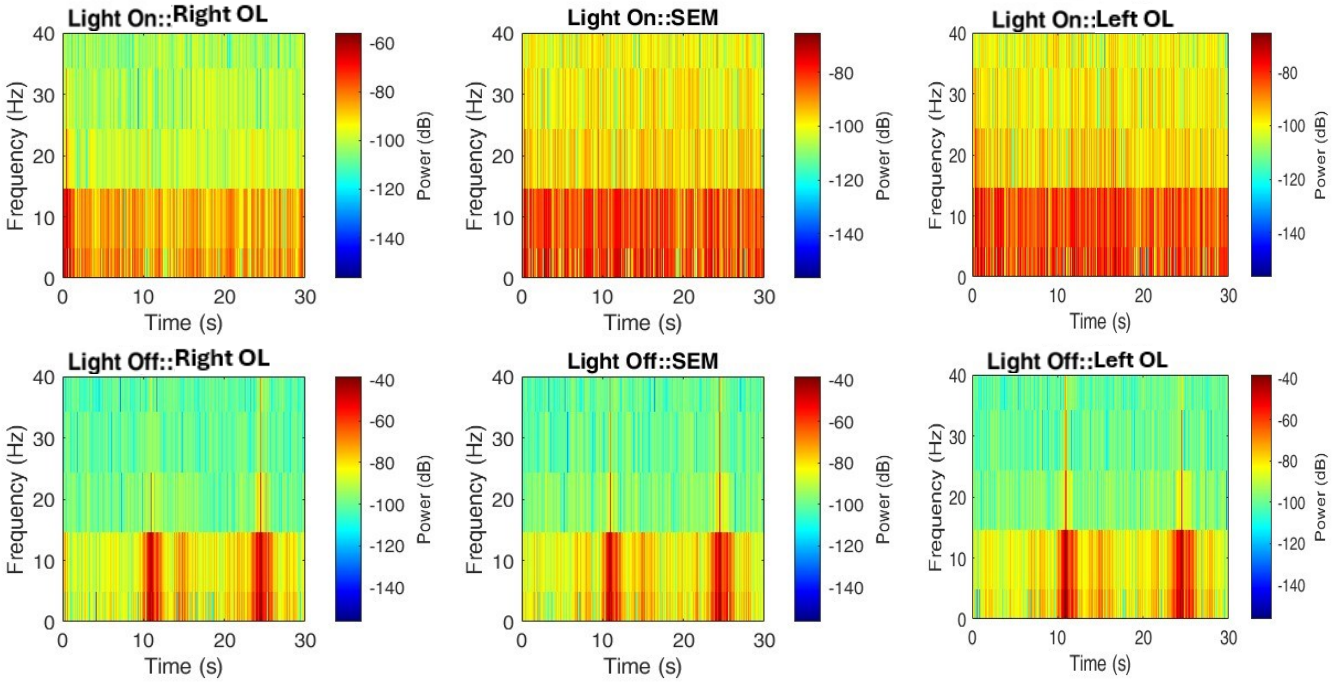


Figure 39. Grand average spectrograms of all resting state recordings (time=30 seconds) before the electrical stunning. The three channels in condition of light ON (top row) and light OFF (bottom) are presented. Frequency range 0-40 Hz.

These results are confirmed by my results after the electrical stunning. Light ON condition resulted in a higher power density for all the frequencies. However, also neural recordings acquired during OFF condition showed a visible difference in frequencies compared to the pre stunning phase (Fig. 40).

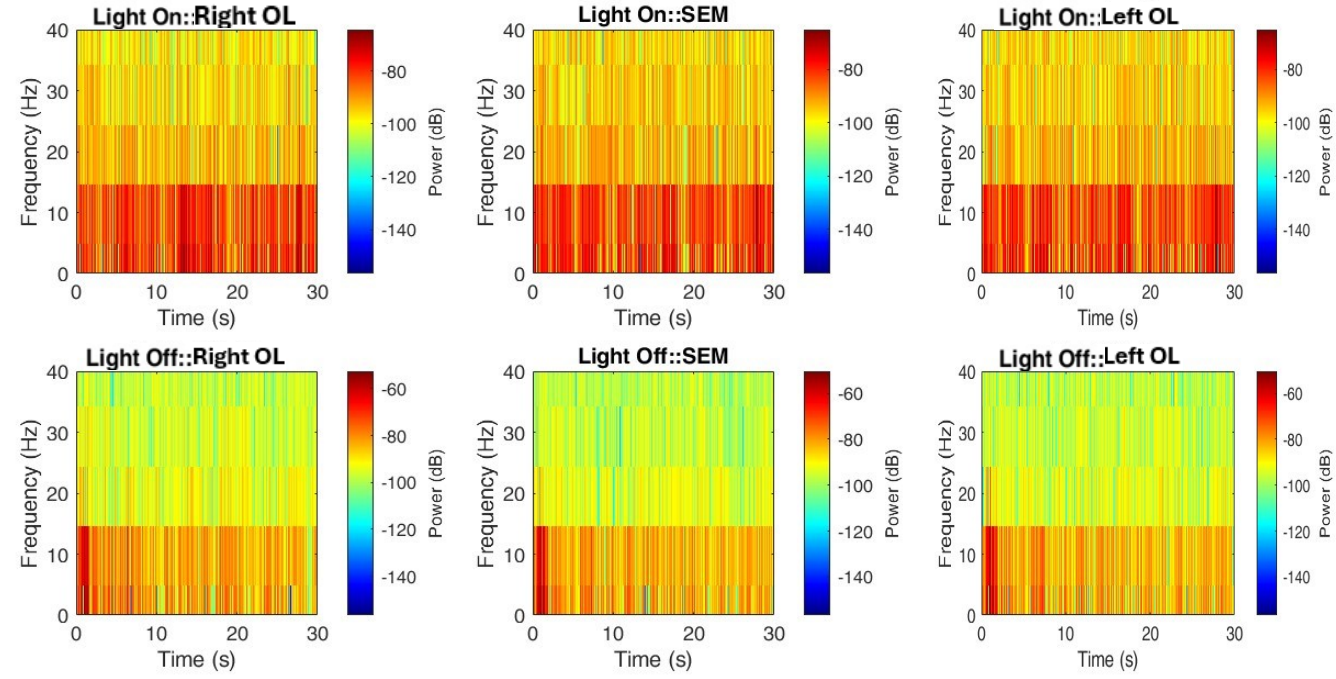


Figure 40. Grand average spectrograms of all resting state recordings (time=30 seconds) after the electrical stunning. The three channels in condition of light ON (top row) and light OFF (bottom) are presented. Frequency range 0-40 Hz.

Power increase was confirmed through plots and analysis of the power spectral density. Neural signal detected pre- and post-stunning highlight a difference in the frequency-content correlated to the presence/absence of environmental light (Figure. 41 A-D). Neural signals detected from the right optic lobe present an increased power for frequencies from 4 to 8 Hz (Fig. 41-B), while SEM showed a general increase of power densities, particularly higher in the ranges of 3-9 Hz and 15-23 Hz (Fig. 41-D).

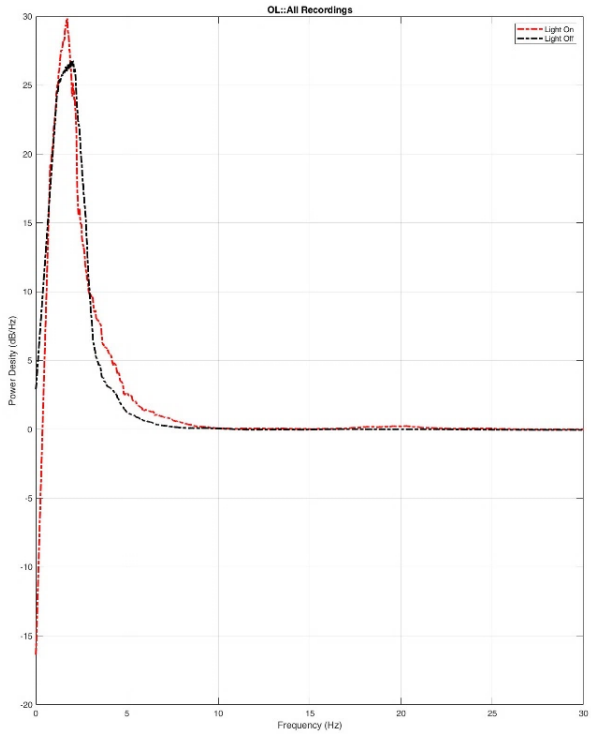


Figure 41 A. Power spectral density (PSD) of the right OL in condition of light ON (red line) and OFF (black line) before electrical stunning.

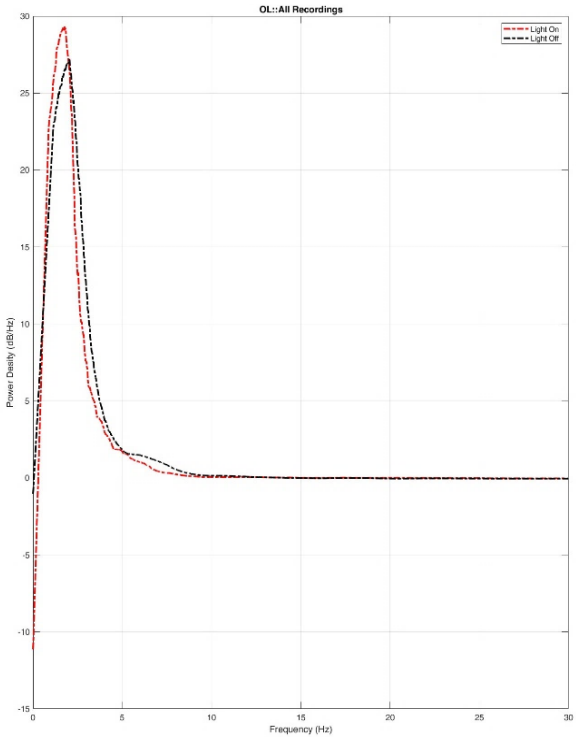


Figure 41-B. Power spectral density (PSD) of the right OL in condition of light ON (red line) and OFF (black line) after electrical stunning.

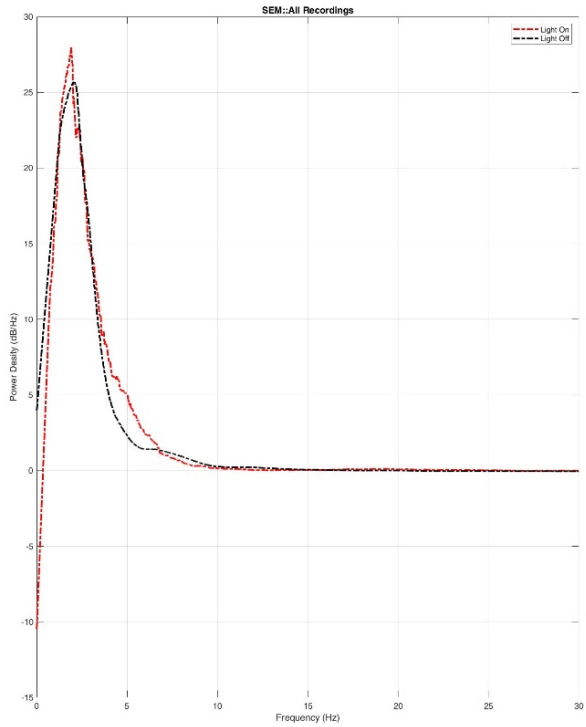


Figure 41 C. Power spectral density (PSD) of the SEM in condition of light ON (red line) and OFF (black line) before electrical stunning

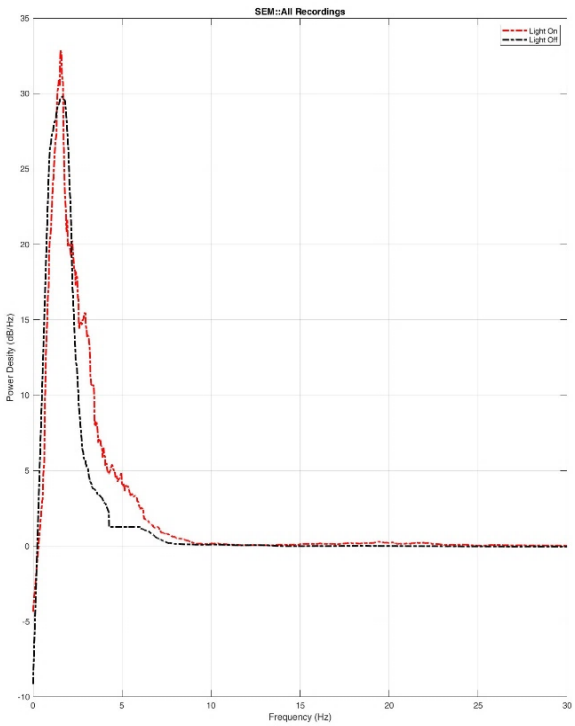


Figure 41 D. Power spectral density (PSD) of the SEM in condition of light ON (red line) and OFF (black line) after electrical stunning.

We also split the neural signal into the five frequency band waves (delta, theta, alpha, beta, gamma). Plots of all band power correspondent to OL, left and right side, and SEM in all conditions (i.e. pre-post stun; ON/OFF) were used to analyse the trend of the power for every band wave on time. We calculated band power on every second and during a time range (30 seconds) after Light ON and OFF for every single band before and after stunning.

Figure 42 shows plots of the power density for the selected band waves (Delta=1-4 Hz; Theta=4-8 Hz; Alpha=8-12 Hz; Beta=12-30 Hz; Gamma>30 Hz) in all the conditions considered and from recordings acquired in correspondence of the three brain areas of *O. vulgaris*.

Overall, it is observed a general increase in the power of all the bands after stunning (OL_L, OL_R, SEM – **2**), both in condition of light and dark.

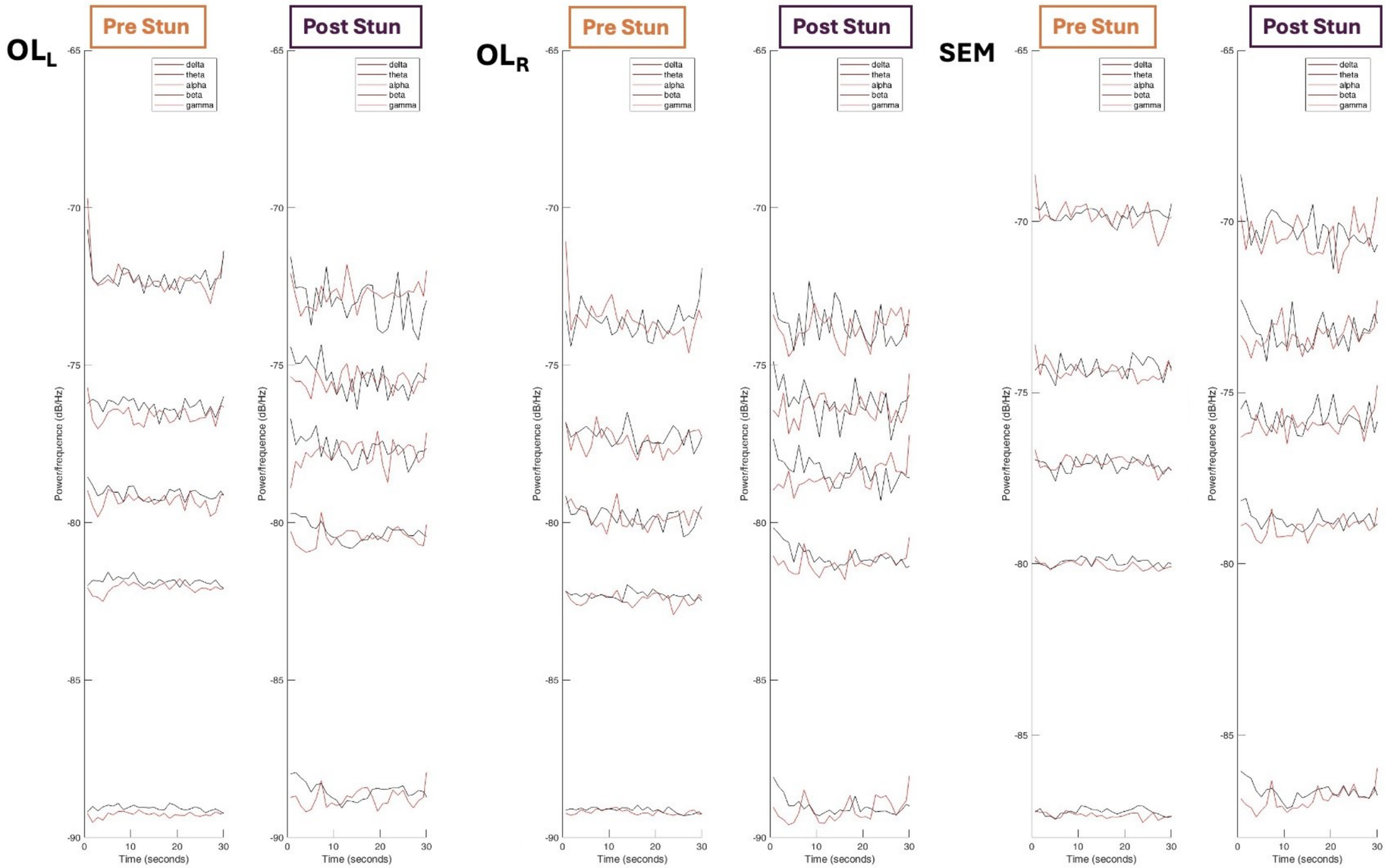


Figure 42. Band Power/Frequency (Delta=1-4 Hz; Theta=4-8 Hz; Alpha=8-12 Hz; Beta=12-30 Hz; Gamma > 30 Hz) in *O. vulgaris* detected from neural signals recorded in areas close to the left (**OL_L**), right Optic Lobes (**OL_R**) and supraoesophageal mass (**SEM**), pre- and post stunning. Power density refers to light ON (red line) and light OFF (black line) conditions. For frequency bands observed in the power plots refers to the in-figure legends.

Based on the above results, we decided to explore if there were significant differences in the frequency domain and power between the animals that have been grouped in clusters after the analysis of indicators of animal status before and after stunning via PCA and Ward's hierarchical clustering approaches. This was done again by replicating the above reported analyses (spectrograms, power spectral density PSDs, frequency bands PSDs) for the three main clusters detected, here identified as Cluster 1 (C1), Cluster 2 (C2) and Cluster 3 (C3) and results compared between the animal groupings.

Spectrograms (Figure 43) and PSDs from *O. vulgaris* grouped in the three main clusters confirmed my results. Neural signals detected pre- and post-stunning revealed a difference in power density of the frequencies characterizing the traces collected from all the brain structures, from the right optic lobe for Cluster 1 (C1), from the supraoesophageal mass for C2 and from all the three recording areas for C3.

In order to further explore differences in the frequency bands PSDs, a series of paired samples t-test (Student's) were carried out to compare band powers from neural traces detected before and after the stunning within the clusters.

Cluster 1: For the right OL, all comparisons resulted significant ($p < 0.05$) except for Delta power in condition of light and dark (ON/OFF). All comparisons for the left Optic lobe resulted with a $p < 0.05$ except for Theta and Beta power in condition of light ON. For the SEM, only Alpha power (in condition of darkness, OFF) resulted with a $p > 0.05$.

Cluster 2: All paired comparison for the right and left OLs and SEM resulted to be significant ($p < 0.05$) in both conditions (light ON and OFF).

Cluster 3: For the right OL, all comparisons resulted significant ($p < 0.05$) except for Theta power in condition of light ON. All comparisons for the left Optic lobe resulted with a $p < 0.05$ while for the SEM, only Delta power in both ON/OFF condition resulted not significant.

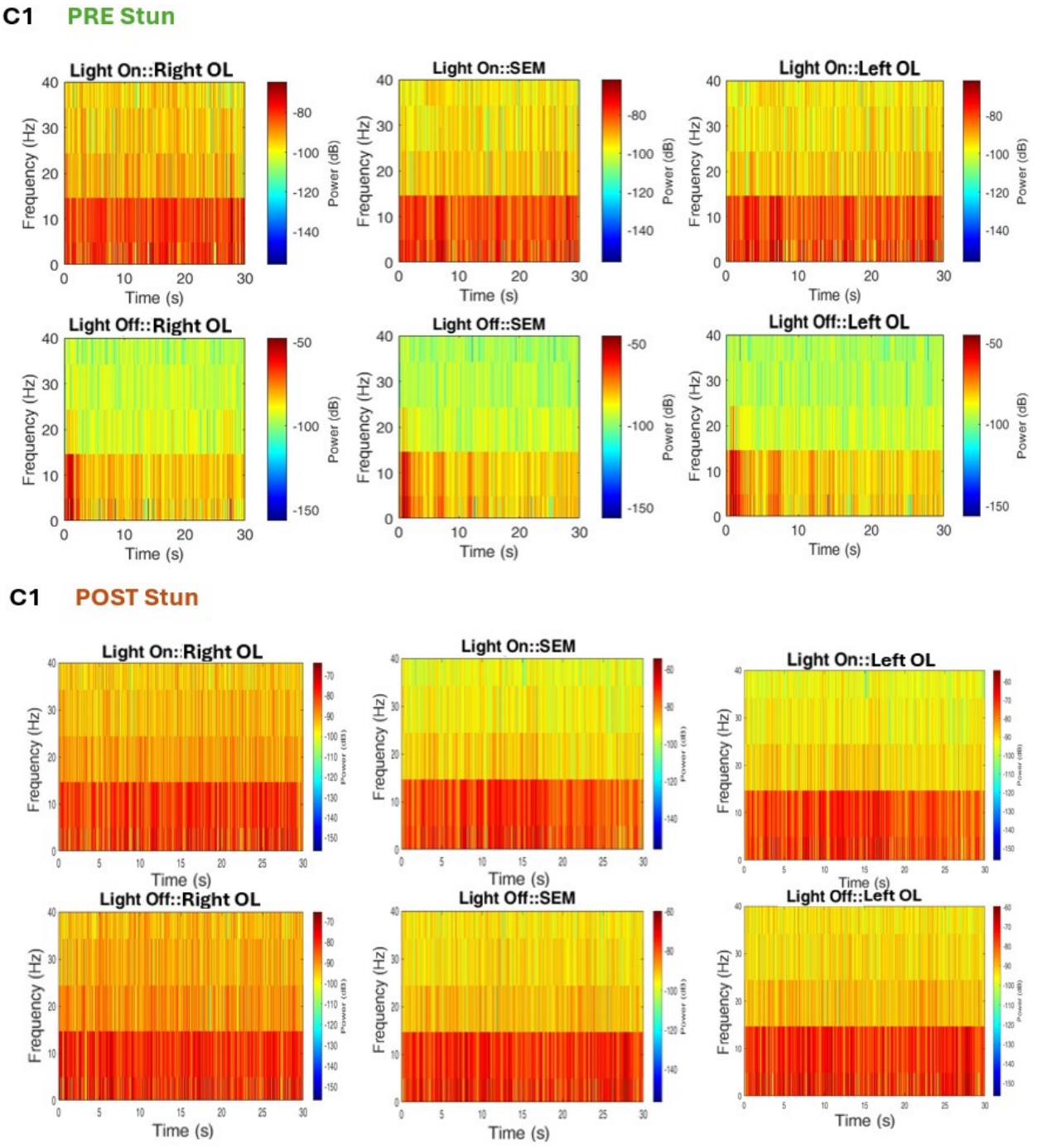
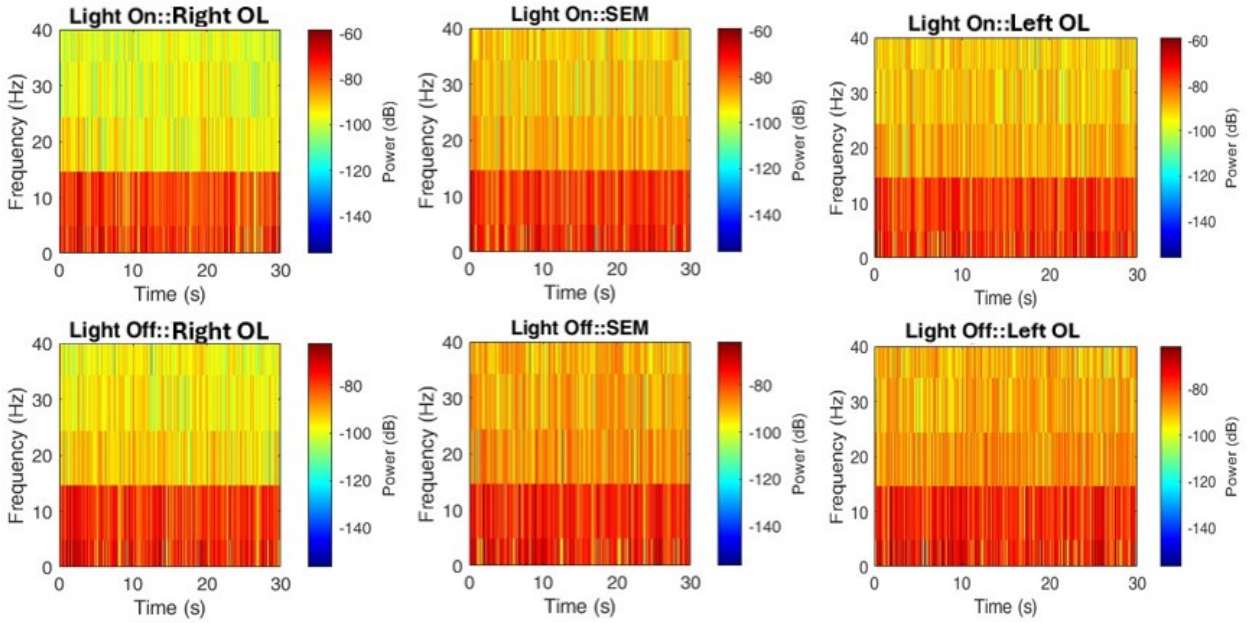


Figure 43. Spectrograms (and following pages) corresponding to the average of frequency domain detected from the three neural structures (left OL, right OL, SEM) in *O. vulgaris* before (PRE stun) and after (POST stun) electrical stunning, for cluster 1 (C1; this page), cluster 2 (C2) and cluster 3 (C3); see also Figure 28.

C2 **PRE Stun**



C2 **POST Stun**

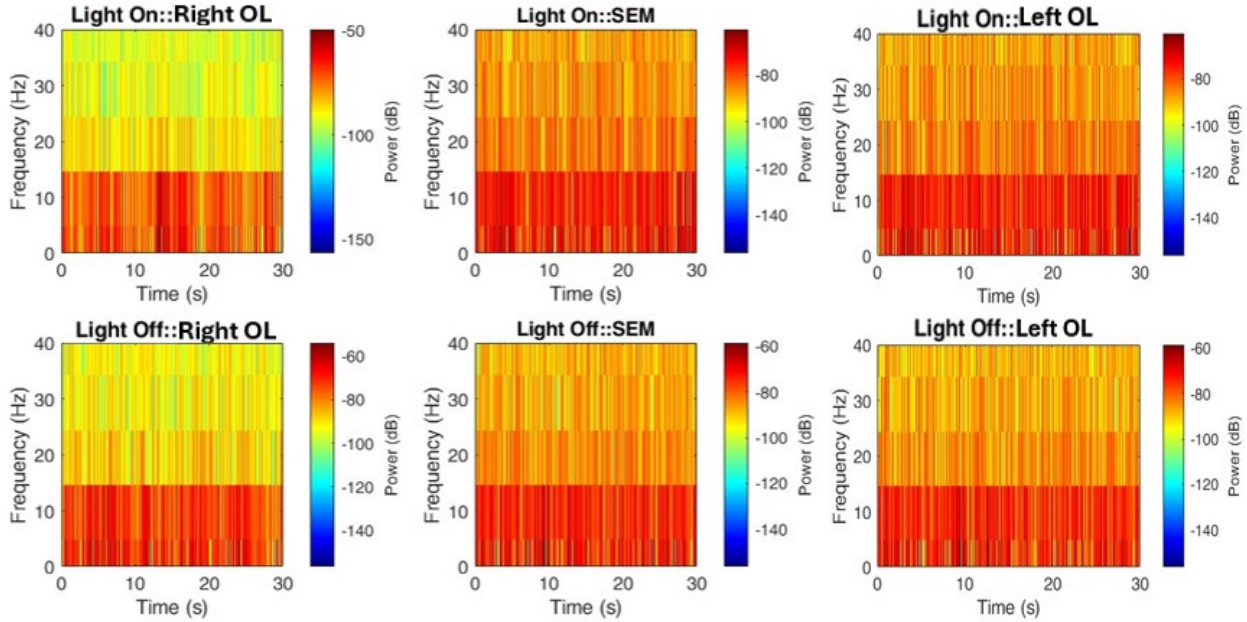
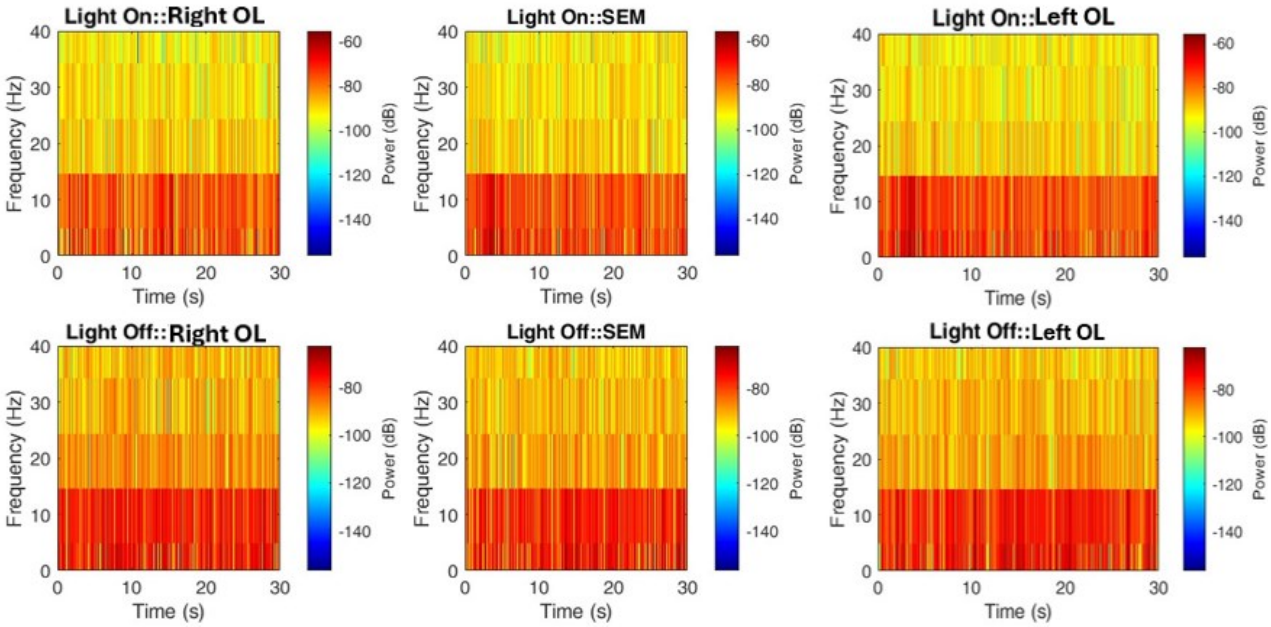


Figure 43 - *continued*. Spectrograms (previous and following page) corresponding to the average of frequency domain detected from the three neural structures (left OL, right OL, SEM) in *O. vulgaris* before (PRE stun) and after (POST stun) electrical stunning, for cluster 1 (C1), cluster 2 (C2; this page) and cluster 3 (C3); see also Figure 28.

C3 PRE Stun



C3 POST Stun

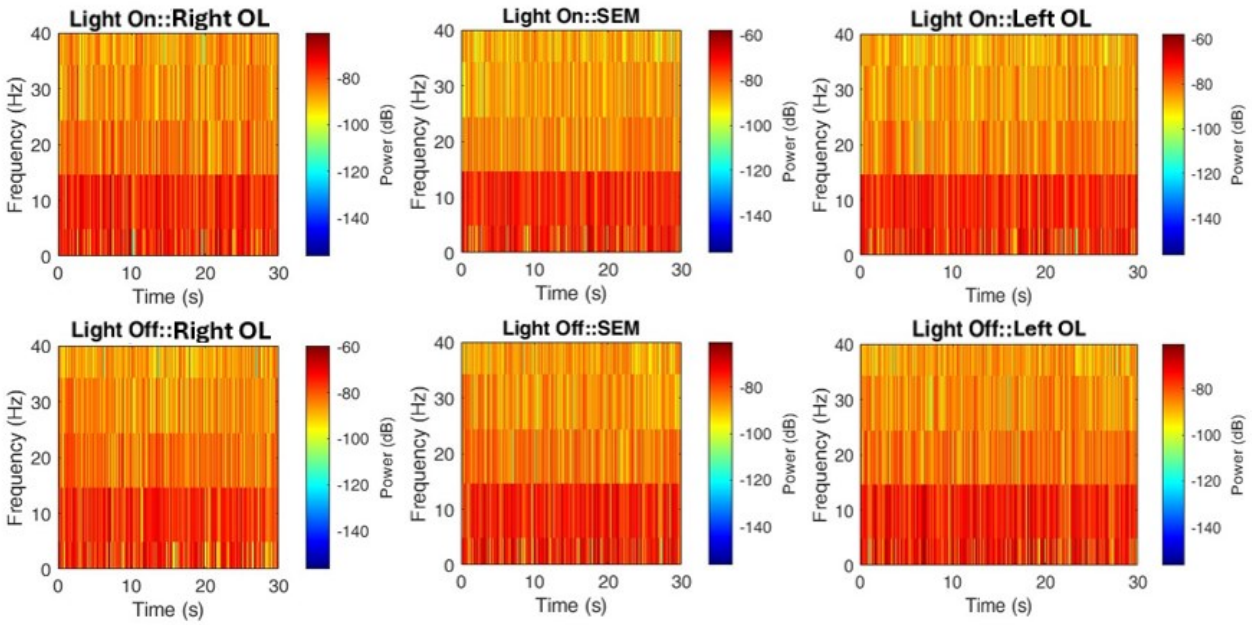


Figure 43 - continued. Spectrograms (*this and previous two pages*) corresponding to the average of frequency domain detected from the three neural structures (left OL, right OL, SEM) in *O. vulgaris* before (PRE stun) and after (POST stun) electrical stunning, for cluster 1 (C1), cluster 2 (C2) and cluster 3 (C3; this page); see also Figure 28.

In addition, a series of Independent Sample t-Test have been carried out to explore for possible differences in band powers before and after stunning comparing performance recorded from animals belonging to different clusters.

Cluster 1 vs Cluster 2: all comparisons resulted to be significant ($p < 0.05$) for left and right OL, and SEM, except for the Delta band recorded during light ON condition in correspondence of left OL.

Cluster 1 vs Cluster 3: all comparisons with $p < 0.05$.

Cluster 2 vs Cluster 3: comparisons for left and right OL resulted to be statistically significant, but for SEM alpha and gamma bands (light ON condition) resulted not significant in pre stun, while theta and beta bands in condition light OFF show a $p > 0.05$ after stunning experiment.

In sum, my data show that the approach I followed for analysing descriptors of the 'behaviour' of animals during the stunning experiments, possibly allow to identify groupings of *O. vulgaris* that appear to be sorted into clusters identifiable by different neural responses to the conditions tested from area of the body of the animal proximal to the optic lobes and the supraoesophageal mass (see also Figs 44-46).

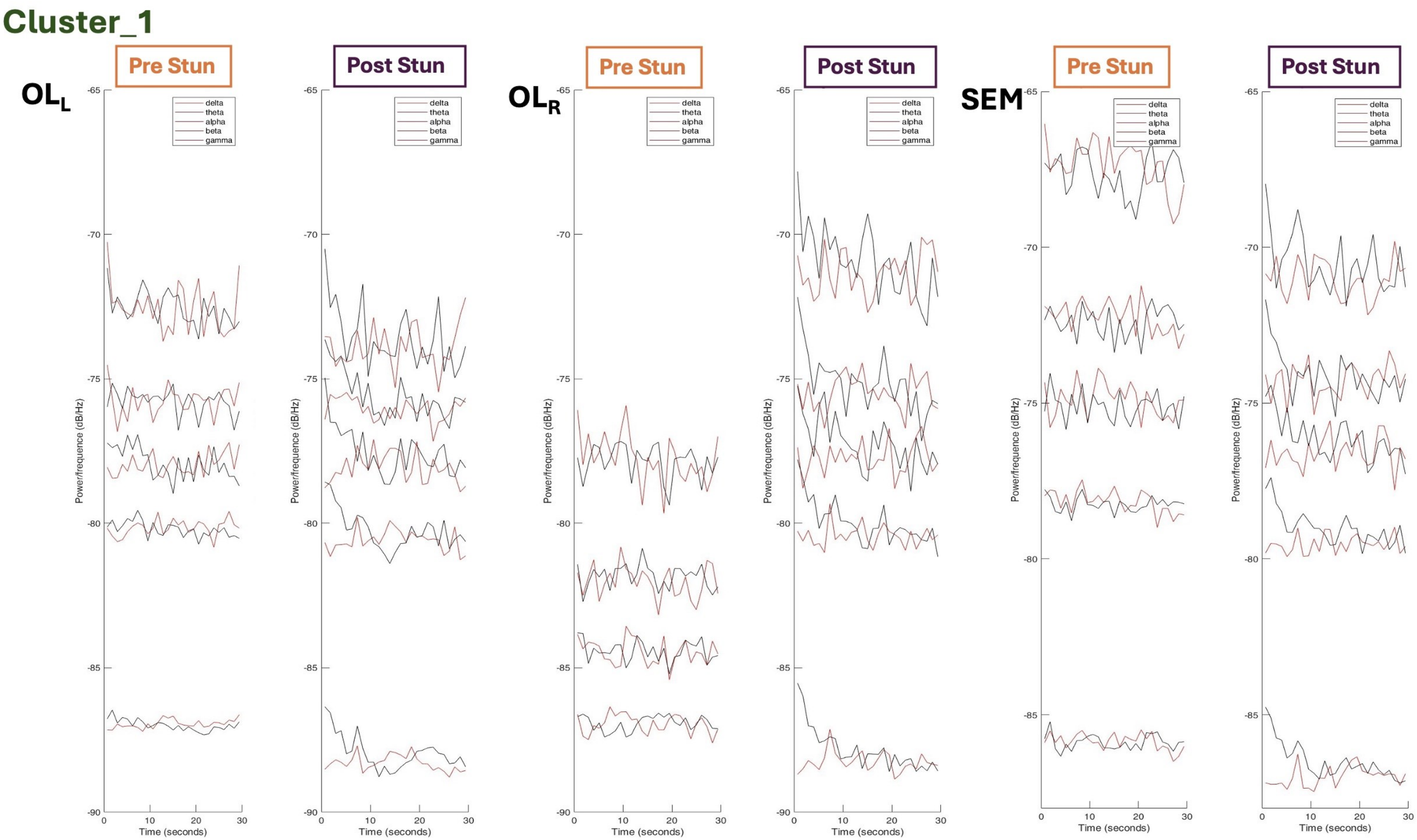


Figure 44. Band Power/Frequency (Delta=1-4 Hz; Theta=4-8 Hz; Alpha=8-12 Hz; Beta=12-30 Hz; Gamma>30 Hz) in Cluster 1 from neural signals recorded in areas close to the left (**OL_L**), right Optic Lobes (**OL_R**) and supraoesophageal mass (**SEM**), pre- and post stunning. Power density refers to light ON (red line) and light OFF (black line) conditions. For frequency bands observed in the power plots refers to the in-figure legends.

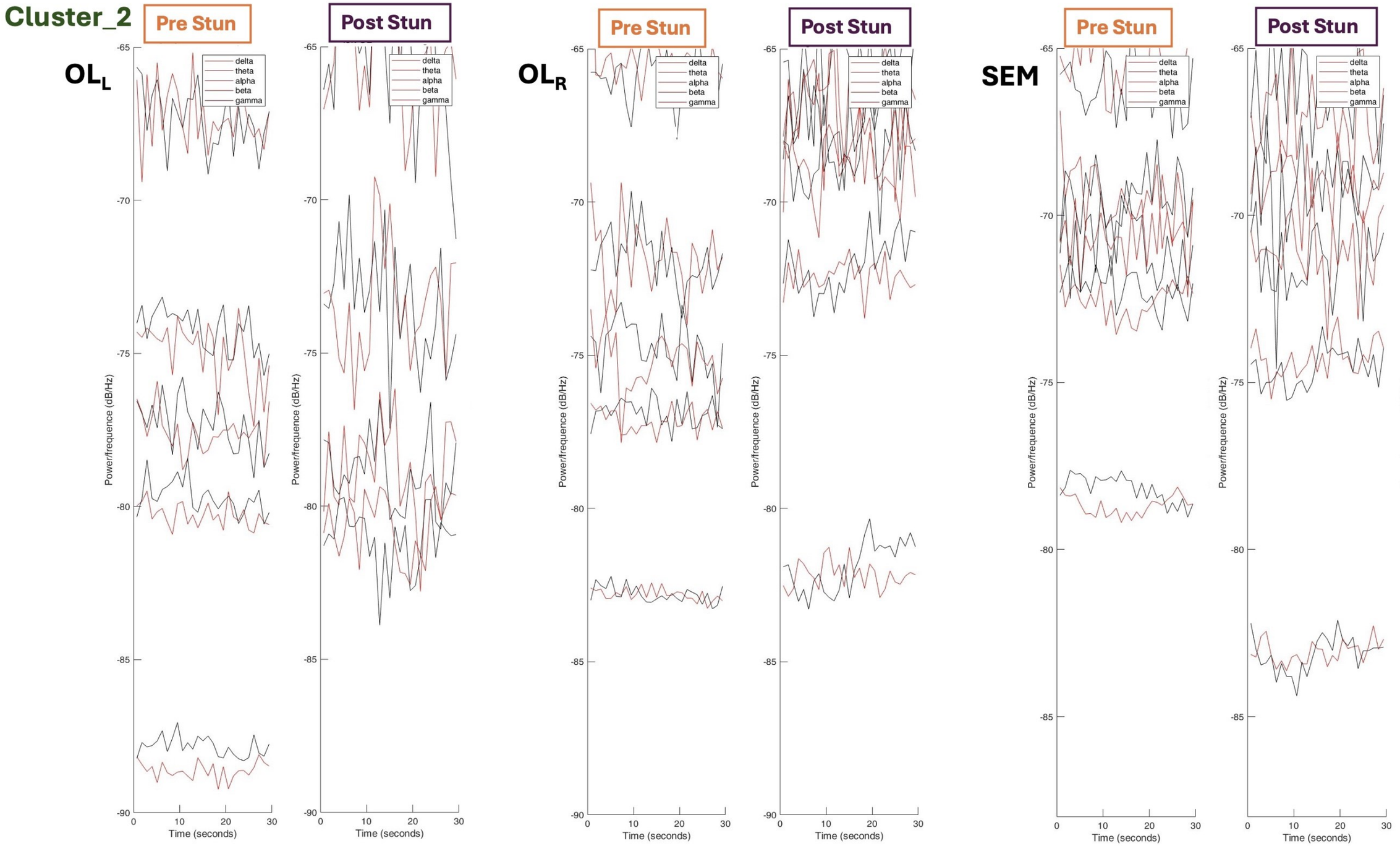


Figure 45. Band Power/Frequency (Delta=1-4 Hz; Theta=4-8 Hz; Alpha=8-12 Hz; Beta=12-30 Hz; Gamma>30 Hz) in Cluster 2 from neural signals recorded in areas close to the left (**OL_L**), right Optic Lobes (**OL_R**) and supraoesophageal mass (**SEM**), pre- and post stunning. Power density refers to light ON (red line) and light OFF (black line) conditions. For frequency bands observed in the power plots refers to the in-figure legends.

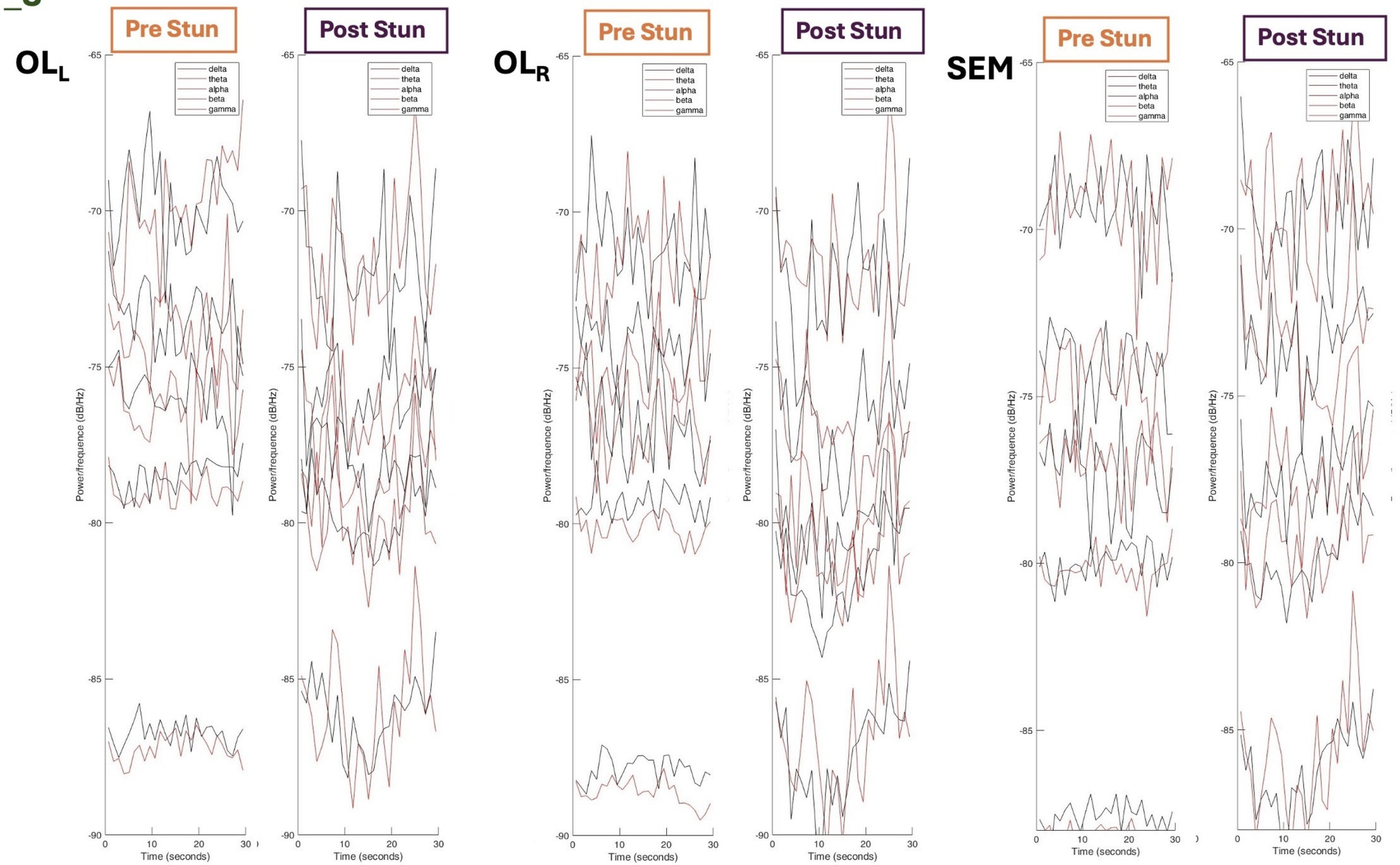


Figure 46. Band Power/Frequency (Delta=1-4 Hz; Theta=4-8 Hz; Alpha=8-12 Hz; Beta=12-30 Hz; Gamma>30 Hz) in Cluster 3 from neural signals recorded in areas close to the left (**OL_L**), right Optic Lobes (**OL_R**) and supraoesophageal mass (**SEM**), pre- and post stunning. Power density refers to light ON (red line) and light OFF (black line) conditions. For frequency bands observed in the power plots refers to the in-figure legends.

Based on the above band power/frequency analyses of octopuses grouped in the three different clusters, I also evaluated if power densities recorded at the level of the brain areas (OL left, OL right, SEM), and during ON/OFF conditions resulted statistically different when time ranges (i.e. 0-5, 5-10, 10-20, 20-30 s) are considered.

Table 6 provides a tabularized overview of these data. For example, animals grouped in Cluster 1 revealed a significant difference in power density between the two light conditions mostly observed during first 5 seconds and between 20/30 sec for the higher frequencies (OL left). Alpha, beta and gamma ranges show significative differences also in the right OL, particularly during for post stunning condition. In correspondence of supraoesophageal mass power densities (ON/OFF conditions) resulted mostly different at the 10-20 seconds interval. For Cluster 2, Theta, Beta and Gamma ranges resulted significantly different for the two light conditions at 5-10 and 20-30 sec intervals, while at the level of the right optic lobe, significant differences for alpha, beta and gamma frequencies are observed. Finally, for octopuses included in Cluster 3 higher frequencies (>8Hz) resulted significantly different for the entire time interval (30 seconds) in correspondence of both left and right OLs (Table 6).

Table 6. Mean values (and SEM) of frequency bands power densities recorded at the level of the brain areas (OL left, OL right, SEM) from *O. vulgaris* - grouped in the three main clusters (C1, C2, C3) – while exposed to the two light conditions (ON/OFF) at different time ranges (0-5, 5-10, 10-20, 20-30 s). Significance (boldface) differences, F and *p* values, are listed after one-way ANOVA statistical (see also Figures 44, 45, 46; previous pages). The dataset has been split into the frequency band power (Delta 0-4Hz, Theta 4-8Hz, Alpha 8-12Hz, Beta 12-30Hz, Gamma >30Hz) and considering the stun phase (Pre/Post).

Cluster1_OL_left		**0-5**			**5-10**			**10-20**			**20-30**		
		ON/OFF Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*
Delta	Pre	-72,68±0,73 -72,71±0,35	.004	.955	-73,06±0,67 -73,26±0,35	.381	.554	-73,44±0,47 -73,15±0,39	2.04	.172	-73,36±0,61 -73,36±0,50	.001	.981
	Post	-74,32±0,31 -73,56±0,36	10.4	**.018**	-74,35±0,37 -74,31±0,67	.019	.893	-73,97±0,30 -74,21±0,36	2.37	.143	-74,21±0,56 -74,24±0,32	.018	.894
Theta	Pre	-76,42±0,41 -76,29±0,36	.210	.663	-76,39±0,36 -76,25±0,19	.590	.465	-76,30±0,24 -76,26±0,28	.117	.737	-76,13±0,30 -76,38±0,38	2.59	.127
	Post	-76,31±0,36 -75,19±0,18	30.1	**.002**	-76,18±0,07 -75,75±0,70	1.87	.209	-76,07±0,43 -75,92±0,45	.537	.474	-76,10±0,37 -76,03±0,37	.168	.688
Alpha	Pre	-78,90±0,51 -78,22±0,24	5.93	***.051***	-78,58±0,30 -78,42±0,23	.836	.387	-78,69±0,20 -78,89±0,28	3.12	.097	-78,43±0,36 -78,70±0,36	2.62	.125
	Post	-78,62±0,31 -77,31±0,41	25.9	**.002**	-77,81±0,22 -77,71±0,43	.230	.644	-77,65±0,40 -77,75±0,39	.280	.604	-78,40±0,22 -78,00±0,22	14.8	**.001**
Beta	Pre	-80,94±0,14 -80,75±0,21	2.07	.200	-80,67±0,07 -80,56±0,29	.663	.439	-80,65±0,18 -80,67±0,16	.071	.793	-80,62±0,24 -80,80±0,14	3.87	.067
	Post	-81,03±0,19 -79,86±0,26	53.7	**<.001**	-80,64±0,32 -80,26±0,14	5.91	**.041**	-80,29±0,16 -80,39±0,19	1.36	.260	-80,76±0,30 -80,38±0,21	10.0	**.006**
Gamma	Pre	-87,40±0,05 -87,26±0,03	21.7	**.003**	-87,34±0,06 -87,30±0,10	.697	.428	-87,25±0,19 -87,37±0,13	2.41	.140	-87,26±0,04 -87,38±0,11	9.18	**.008**
	Post	-88,47±0,12 -87,59±0,13	94.6	**<.001**	-88,26±0,14 -87,99±0,17	7.08	**.029**	-88,03±0,19 -87,99±0,15	.245	.627	-88,49±0,19 -87,97±0,19	33.5	**<.001**

Cluster1_OL_right		0-5			5-10			10-20			20-30		
		ON/OFF Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*
Delta	Pre	-75,33±0,55 -75,87±0,38	2.60	.158	-75,35±0,61 -76,26±0,43	7.44	**.026**	-76,24±0,84 -76,18±0,61	.027	.871	-76,15±0,39 -75,84±0,38	2.90	.108
	Post	-75,93±0,52 -75,39±0,53	2.11	.196	-76,15±0,57 -75,87±0,64	.538	.484	-76,33±0,40 -75,96±0,64	2.16	.161	-75,86±0,77 -76,38±0,55	2.72	.118
Theta	Pre	-80,05±0,57 -80,01±0,44	.014	.910	-79,95±0,56 -80,12±0,30	.342	.575	-80,02±0,28 -79,96±0,27	.219	.646	-80,08±0,57 -79,94±0,40	.364	.555
	Post	-79,56±0,35 -78,07±0,65	16.2	**.007**	-79,68±0,65 -79,06±0,49	2.86	.129	-79,02±0,35 -78,91±0,54	.239	.631	-79,16±0,27 -78,85±0,36	4.17	.058
Alpha	Pre	-82,47±0,20 -82,60±0,57	.197	.673	-82,93±0,39 -82,87±0,24	.091	.771	-82,54±0,32 -82,60±0,40	.131	.723	-82,50±0,33 -82,77±0,39	2.53	.131
	Post	-81,89±0,32 -80,57±0,49	20.7	**.004**	-81,50±0,32 -80,72±0,43	10.3	**.013**	-81,27±0,33 -80,85±0,48	4.65	***.047***	-81,23±0,50 -80,82±0,36	4.16	.058
Beta	Pre	-85,49±0,33 -85,18±0,16	2.927	.138	-85,09±0,21 -85,46±0,18	8.84	**.018**	-85,03±0,14 -85,17±0,26	1.95	.182	-85,22±0,21 -85,21±0,14	.012	.914
	Post	-83,94±0,12 -82,66±0,29	64.8	**<.001**	-83,99±0,27 -83,33±0,28	14.7	**.005**	-83,77±0,27 -83,38±0,24	10.4	**.005**	-83,72±0,23 -83,25±0,21	20.8	**<.001**
Gamma	Pre	-92,21±0,24 -92,08±0,04	1.13	.328	-91,91±0,07 -92,15±0,12	14.8	**.005**	-91,85±0,14 -91,92±0,10	1.60	.224	-91,93±0,09 -92,05±0,15	4.55	**.049**
	Post	-91,27±0,15 -89,97±0,24	86.6	**<.001**	-91,10±0,19 -90,69±0,21	10.3	**.012**	-90,95±0,19 -90,69±0,18	8.96	**.009**	-91,01±0,15 -90,59±0,13	40.1	**<.001**

Cluster1_SEM		**0-5**			**5-10**			**10-20**			**20-30**		
		ON/OFF Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*
Delta	Pre	-68,18±0,31 -68,86±0,34	8.66	**.026**	-68,52±0,30 -69,11±0,63	3.57	.096	-68,56±0,33 -69,31±0,43	17.1	**.001**	-69,37±0,57 -69,07±0,52	1.36	.261
	Post	-70,32±0,49 -70,48±0,76	.113	.748	-70,76±0,72 -70,91±0,22	.195	.671	-71,23±0,38 -71,22±0,36	.005	.945	-71,57±0,44 -71,34±0,33	1.58	.227
Theta	Pre	-73,54±0,48 -74,08±0,20	4.25	.085	-73,56±0,18 -73,99±0,30	7.32	**.027**	-73,83±0,30 -74,12±0,30	4.39	***.052***	-74,04±0,46 -73,76±0,44	1.72	.208
	Post	-74,82±0,27 -73,84±0,23	30.2	**.002**	-74,52±0,37 -74,50±0,38	.013	.913	-74,62±0,36 -74,47±0,39	.646	.433	-74,39±0,17 -74,47±0,38	.306	.588
Alpha	Pre	-76,58±0,47 -76,52±0,40	.038	.851	-76,82±0,61 -76,93±0,36	.111	.748	-76,42±0,26 -76,81±0,29	9.10	**.008**	-76,74±0,20 -76,88±0,42	.812	.381
	Post	-77,13±0,04 -76,71±0,53	2.56	.161	-77,12±0,35 -76,74±0,32	3.26	.109	-77,10±0,38 -76,60±0,33	9.01	**.008**	-76,96±0,26 -76,96±0,49	.001	.978
Beta	Pre	-79,59±0,17 -79,67±0,21	.336	.584	-79,55±0,24 -79,63±0,23	.257	.626	-79,67±0,19 -79,74±0,17	.717	.410	-79,72±0,27 -79,66±0,13	.335	.571
	Post	-80,00±0,18 -79,24±0,29	20.2	**.004**	-80,04±0,26 -79,46±0,20	15.7	**.004**	-79,87±0,16 -79,48±0,19	21.5	**<.001**	-79,79±0,18 -79,69±0,26	.972	.339
Gamma	Pre	-86,72±0,12 -86,72±0,12	.011	.921	-86,76±0,14 -86,86±0,14	1.31	.285	-86,74±0,15 -86,85±0,15	2.63	.124	-86,84±0,19 -86,78±0,13	.785	.389
	Post	-87,54±0,11 -86,72±0,14	85.6	**<.001**	-87,42±0,28 -87,03±0,16	7.12	**.028**	-87,35±0,21 -87,10±0,16	8.07	**.012**	-87,28±0,13 -87,20±0,13	2.00	.176

Cluster2_OL_left		0-5			5-10			10-20			20-30		
		ON/OFF Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*
Delta	Pre	-73,57±1,12 -73,13±0,38	.548	.487	-73,05±0,47 -73,55±0,47	2.78	.134	-73,64±0,64 -73,74±0,61	.111	.743	-73,72±0,57 -73,44±0,60	1.00	.332
	Post	-70,25±1,12 -70,02±0,65	.120	.741	-70,31±0,65 -70,09±0,47	.373	.558	-70,07±1,11 -69,93±0,87	.098	.758	-69,85±0,76 -70,68±0,75	5.41	**.033**
Theta	Pre	-79,01±0,38 -78,58±0,54	1.73	.236	-79,09±0,26 -78,61±0,31	6.92	**.030**	-79,28±0,25 -78,96±0,37	4.67	**.046**	-79,81±0,51 -78,83±0,55	15.3	**.001**
	Post	-74,11±0,35 -73,58±0,53	2.82	.144	-74,75±0,75 -73,70±0,33	8.29	**.021**	-73,47±1,11 -73,84±0,93	.580	.457	-74,12±0,49 -74,10±0,82	.006	.939
Alpha	Pre	-81,45±0,37 -81,49±0,29	.028	.872	-81,97±0,36 -81,48±0,41	4.04	.079	-81,89±0,60 -81,59±0,43	1.46	.245	-82,28±0,55 -81,70±0,47	5.80	**.028**
	Post	-77,44±0,39 -77,18±0,66	.434	.535	-77,14±0,74 -76,93±0,21	.355	.568	-76,68±0,66 -76,91±0,74	.455	.510	-76,99±0,55 -76,78±0,49	.723	.408
Beta	Pre	-84,59±0,34 -84,17±0,45	2.23	.186	-84,60±0,13 -83,98±0,21	29.9	**.001**	-84,38±0,17 -84,26±0,23	1.75	.204	-84,64±0,23 -84,24±0,22	14.1	**.002**
	Post	-79,78±0,26 -79,68±0,37	.199	.671	-79,70±0,40 -79,56±0,37	.332	.580	-79,57±0,18 -80,06±0,51	7.18	**.016**	-79,42±0,46 -79,77±0,32	3.50	.080
Gamma	Pre	-91,30±0,04 -90,92±0,11	44.9	**.001**	-91,31±0,05 -90,80±0,18	38.6	**<.001**	-91,39±0,15 -91,01±0,12	34.2	**<.001**	-91,47±0,22 -91,06±0,17	20.1	**<.001**
	Post	-90,13±0,19 -89,67±0,13	16.6	**.007**	-89,98±0,26 -89,93±0,30	.079	.786	-89,76±0,27 -89,86±0,21	.878	.363	-89,88±0,29 -90,01±0,23	1.21	.286

Cluster2_OL_right		**0-5**			**5-10**			**10-20**			**20-30**		
		ON/OFF Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*
Delta	Pre	-70,56±3,16	1.02	.351	-71,40±0,38	.492	.503	-71,78±0,49	3.81	.069	-72,12±0,34	.598	.451
		-72,23±0,97			-71,61±0,57			-72,27±0,57			-71,94±0,59		
	Post	-70,34±0,59	.070	.800	-69,28±0,67	.056	.819	-70,02±1,03	4.87	**.042**	-69,69±0,76	3.52	.079
		-70,22±0,70			-69,43±1,23			-69,10±0,70			-69,01±0,78		
Theta	Pre	-75,43±1,01	.043	.843	-75,82±0,60	1.57	.245	-75,68±0,32	.766	.394	-75,77±0,50	.255	.620
		-75,54±0,27			-75,43±0,36			-75,85±0,48			-75,87±0,22		
	Post	-69,98±0,72	.293	.608	-70,18±0,97	.185	.678	-70,16±0,71	.022	.884	-70,93±0,63	15.7	**.001**
		-69,72±0,65			-69,97±0,57			-70,12±0,37			-69,81±0,57		
Alpha	Pre	-77,66±0,29	.360	.571	-78,68±0,50	22.2	**.002**	-78,34±0,52	.169	.687	-78,22±0,56	.406	.533
		-77,89±0,70			-77,49±0,25			-78,25±0,40			-78,35±0,27		
	Post	-72,06±0,81	3.64	.105	-72,61±0,65	15.9	**.004**	-71,40±0,41	.692	.418	-71,61±0,58	.036	.852
		-71,03±0,73			-71,29±0,37			-71,60±0,58			-71,67±0,78		
Beta	Pre	-80,71±0,23	.281	.615	-80,86±0,25	15.9	**.004**	-80,77±0,18	1.49	.240	-80,68±0,23	.187	.671
		-80,63±0,19			-80,33±0,15			-80,63±0,29			-80,72±0,20		
	Post	-74,95±0,43	8.24	**.028**	-75,17±0,16	20.7	**.002**	-74,79±0,27	1.46	.244	-74,62±0,21	1.40	.254
		-74,19±0,31			-74,54±0,27			-74,59±0,42			-74,73±0,18		
Gamma	Pre	-86,95±0,08	6.36	**.045**	-86,98±0,08	8.15	**.021**	-86,86±0,12	.112	.742	-86,92±0,16	.270	.611
		-86,72±0,16			-86,76±0,15			-86,84±0,12			-86,96±0,15		
	Post	-84,05±0,11	12.0	**.013**	-83,74±0,20	.003	.961	-83,89±0,21	7.22	**.016**	-83,80±0,22	4.34	***.054***
		-83,48±0,31			-83,75±0,21			-83,57±0,29			-83,59±0,20		

Cluster2_SEM		0-5			5-10			10-20			20-30		
		ON/OFF Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*
Delta	Pre	-69,74±1,16 -69,88±0,24	.059	.816	-70,29±0,70 -70,94±0,51	2.86	.129	-70,53±0,43 -70,63±0,48	.214	.650	-70,52±0,36 -70,80±0,51	1.78	.200
	Post	-70,23±1,01 -69,15±0,88	2.57	.160	-69,65±0,90 -68,98±0,71	1.70	.229	-69,25±0,59 -69,20±0,55	.035	.854	-69,66±0,63 -69,53±0,70	.183	.674
Theta	Pre	-74,31±0,58 -74,85±0,35	2.52	.163	-74,92±0,44 -75,30±0,56	1.43	.266	-75,13±0,18 -75,19±0,43	.160	.694	-75,44±0,22 -74,97±0,37	10.9	**.004**
	Post	-71,92±0,38 -72,30±0,91	.595	.470	-71,47±0,46 -72,32±0,95	3.27	.108	-71,59±0,61 -71,38± 0,84	.374	.549	-71,78±0,71 -71,71±0,71	.047	.832
Alpha	Pre	-77,30±0,14 -77,55±0,26	2.84	.143	-77,85±0,38 -77,79±0,86	.021	.889	-77,39±0,38 -77,65±0,47	1.57	.228	-77,24±0,68 -77,92±0,44	6.30	**.023**
	Post	-74,02±0,53 -73,76±0,62	.402	.549	-73,89±0,49 -74,26±0,34	1.90	.205	-73,91±0,84 -74,09±0,56	.304	.589	-73,890,79 -73,31± 0,73	2.61	.126
Beta	Pre	-80,40±0,23 -80,21±0,19	1.73	.236	-80,39±0,15 -80,36±0,24	.050	.829	-80,64±0,18 -80,32±0,17	15.57	**.001**	-80,47±0,27 -80,53±0,28	.205	.657
	Post	-77,13±0,12 -77,23±0,09	1.91	.216	-77,40±0,38 -77,33±0,43	.074	.792	-77,12± 0,47 -77,19±0,38	.143	.710	-77,08±0,19 -77,25±0,24	2.73	.118
Gamma	Pre	-86,98±0,15 -86,90±0,19	.443	.530	-87,12±0,10 -86,84±0,17	10.0	**.013**	-87,18±0,13 -86,86±0,18	18.75	**.001**	-87,07±0,13 -87,06±0,16	.036	.852
	Post	-86,26±0,21 -86,22± 0,26	.052	.828	-86,36±0,19 -86,44±0,13	.729	.418	-86,31±0,21 -86,15±0,32	1.55	.231	-86,27± 0,26 -86,23±0,23	.175	.681

Cluster3_OL_left		**0-5**			**5-10**			**10-20**			**20-30**		
		ON/OFF Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*
Delta	Pre	-70,02±0,30 -70,32±0,90	.402	.549	-71,09±0,51 -69,92±0,49	13.6	**.006**	-70,58±0,67 -70,95±0,50	1.67	.215	-70,48±0,63 -70,27±0,56	.560	.465
	Post	-71,78±0,89 -71,19±0,54	1.27	.302	-71,68±0,76 -71,05±0,39	2.66	.141	-72,00±0,65 -71,49±0,63	2.92	.107	-71,85±0,90 -71,56±0,59	.640	.435
Theta	Pre	-74,04±0,30 -73,28±0,61	5.02	.066	-74,42±0,87 -73,30±0,68	5.22	***.052***	-74,04±0,46 -74,06±0,58	.009	.926	-74,10±0,39 -73,80±0,65	1.42	.250
	Post	-73,34±0,54 -72,72±0,73	1.83	.224	-73,11±0,96 -72,37±0,85	1.71	.228	-73,34±0,50 -73,17±0,48	.573	.460	-73,33±0,75 -72,51±0,44	8.02	**.012**
Alpha	Pre	-76,74±0,36 -75,77±0,41	12.6	**.012**	-76,61±0,63 -75,94±0,18	5.08	***.054***	-76,68±0,57 -75,94±0,54	7.93	**.012**	-76,45±0,68 -76,16±0,64	.904	.356
	Post	-75,69±0,32 -74,69±0,31	20.0	**.004**	-75,63 0,91 -74,37±0,75	5.76	**.043**	-75,49±0,77 -75,10±0,28	1.97	.179	-75,43±0,50 -74,68±0,51	9.92	**.006**
Beta	Pre	-79,31±0,30 -78,57±0,18	18.2	**.005**	-79,46±0,28 -78,66±0,29	19.6	**.002**	-79,12±0,36 -78,49±0,33	14.8	**.001**	-79,41±0,25 -78,76±0,25	29.7	**<.001**
	Post	-77,37±0,45 -76,28±0,45	11.6	**.014**	-77,03±0,56 -76,27±0,35	6.57	**.034**	-77,26±0,25 -76,76±0,22	21.2	**<.001**	-77,24±0,64 -76,40±0,20	14.5	**.002**
Gamma	Pre	-87,10±0,30 -86,52±0,26	8.71	**.026**	-87,08±0,16 -86,30±0,25	35.4	**<.001**	-87,02±0,15 -86,51±0,22	32.1	**<.001**	-87,23±0,19 -86,76±0,20	25.6	**<.001**
	Post	-85,10±0,41 -84,08±0,37	13.8	**.010**	-84,87± 0,46 -83,91±0,27	16.3	**.004**	-84,89±0,62 -84,35±0,29	5.46	**.033**	-84,33±0,62 -83,99±0,29	2.29	.150

Cluster3_OL_right		**0-5**			**5-10**			**10-20**			**20-30**		
		ON/OFF Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*	**ON/OFF** Mean ± SEM	F	*p*
Delta	Pre	-70,44±1,21 -71,08±0,49	.972	.362	-71,58±0,75 -70,98±0,56	2.08	.187	-71,29±0,94 -71,35±0,62	.025	.877	-71,93±0,41 -71,49±0,85	2.01	.175
	Post	-73,24±0,89 -73,67±0,69	.587	.473	-72,44±1,31 -73,76±1,21	2.72	.138	-73,45±0,84 -73,42±0,75	.007	.935	-73,14±1,00 -73,54±0,57	1.04	.322
Theta	Pre	-75,56±0,33 -73,94±0,22	67.7	**<.001**	-75,90±0,47 -74,10±0,59	28.9	**.001**	-75,34±0,85 -74,35±0,58	8.44	**.010**	-74,82±0,53 -74,66±0,28	.629	.439
	Post	-75,10±0,92 -74,62±0,48	.873	.386	-75,51±0,47 -75,46±0,85	.012	.915	-75,59±0,43 -75,66±0,61	.092	.765	-75,20±0,60 -74,98±0,55	.629	.439
Alpha	Pre	-78,38±0,63 -76,48±0,63	18.1	**.005**	-78,22±0,33 -76,30±0,44	60.3	**<.001**	-77,19±0,63 -76,48±0,54	6.45	**.022**	-77,10±0,67 -76,74±0,61	1.38	.257
	Post	-78,53±0,37 -77,64±0,29	13.9	**.010**	-78,46±0,60 -78,59±0,90	.072	.796	-78,49±0,65 -78,18±0,40	1.41	.253	-78,11±0,59 -77,91± 0,62	.492	.493
Beta	Pre	-80,92±0,16 -78,99±0,22	198	**<.001**	-80,39±0,25 -79,17±0,44	28.2	**.001**	-80,03±0,25 -79,18±0,29	44.4	**<.001**	-80,02±0,19 -79,28±0,39	25.9	**<.001**
	Post	-79,78±0,63 -79,47±0,33	.754	.418	-79,99±0,56 -79,89±0,37	.128	.730	-80,07±0,51 -79,91±0,24	.671	.425	-79,76±0,62 -79,63±0,19	.360	.557
Gamma	Pre	-88,56±0,25 -87,36±0,26	44.6	**.001**	-88,35±0,13 -87,08±0,29	80.2	**<.001**	-87,96±0,17 -87,26±0,18	71.4	**<.001**	-88,05±0,25 -87,44±0,36	17.6	**.001**
	Post	-86,97±0,58 -86,54±0,52	1.25	.306	-87,11±0,42 -86,74±0,29	2.54	.150	-86,90±0,59 -86,80±0,27	.208	.654	-86,31±0,52 -86,45±0,34	.482	.498

Cluster3_SEM		0-5			5-10			10-20			20-30		
		ON/OFF Mean ± SEM	F	*p*	ON/OFF Mean ± SEM	F	*p*	ON/OFF Mean ± SEM	F	*p*	ON/OFF Mean ± SEM	F	*p*
Delta	Pre	-68,04±1,18 -69,65±0,84	4.91	.069	-69,52±0,99 -69,78±0,45	.286	.607	-69,92±0,49 -70,12± 0,60	.618	.443	-70,16±0,78 -69,85±0,76	.731	.405
	Post	-70,18±0,77 -69,74±0,74	.675	.443	-69,88±0,47 -70,11±0,83	.288	.606	-69,96±0,53 -69,96±0,67	.001	.977	-69,80±0,62 -70,27±0,87	1.69	.212
Theta	Pre	-74,50±0,32 -73,92±0,32	6.67	**.042**	-74,69±0,77 -74,23±0,81	.829	.389	-74,78±0,55 -74,60±0,37	.618	.443	-74,64±0,42 -74,62±0,51	.005	.946
	Post	-72,32±0,56 -71,83±0,35	2.16	.192	-71,67±0,80 -72,39±0,66	2.40	.160	-72,45±0,41 -72,20±0,52	1.21	.287	-72,06±1,11 -71,83±0,39	.340	.568
Alpha	Pre	-77,34±0,21 -77,22±0,66	.117	.744	-77,44±0,73 -77,24±0,64	.209	.660	-77,41±0,63 -77,40±0,49	.000	.991	-77,33±0,54 -77,49±0,28	.657	.430
	Post	-75,44±0,75 -75,18±0,61	.300	.603	-74,78± 0,39 -75,42±0,56	4.42	.069	-75,50± 0,88 -75,54±0,66	.012	.914	-74,59±0,87 -74,90±0,48	.916	.353
Beta	Pre	-79,91±0,28 -79,85±0,26	.118	.743	-79,80±0,26 -79,95±0,26	.770	.406	-80,02±0,21 -79,70±0,18	12.0	**.003**	-80,07±0,31 -79,92±0,25	1.36	.261
	Post	-77,68±0,24 -77,42±0,29	1.98	.209	-77,30±0,41 -77,27±0,37	.021	.888	-77,65±0,60 -77,71±0,26	.072	.791	-77,38±0,73 -77,16±0,26	.687	.419
Gamma	Pre	-86,80±0,17 -87,08±0,12	7.04	**.038**	-86,96±0,21 -86,91±0,21	.189	.675	-87,00±0,26 -86,78±0,17	4.44	***.051***	-87,19±0,13 -87,00±0,20	5.63	**.030**
	Post	-85,00±0,46 -84,59±0,36	1.95	.212	-84,58±0,26 -84,50±0,32	.168	.692	-84,78±0,48 -84,67±0,38	.279	.604	-84,14±0,49 -84,43±0,31	2.11	.166

Discussion

As mentioned, in some experiments I attempted to electrically stun cuttlefish (three individuals, *Sepia officinalis*); these attempts resulted in **a.** a strong mantle contraction and dark mottle body colour of the animal, **b.** inking (2 out of 3 cuttlefishes), **c.** animal being motionless without signs of breathing after the stun. I applied a single 2 s electrical stunning pulse (28 or 50Hz, 150-200 V AC). Because of the morphological arrangements of the dorsal side of the mantle - at the level of its anterior extremity overlying the head of the animal - recordings of neural activities were difficult (but possible) in these cases. As mentioned above, all *S. officinalis* stopped breathing immediately after the stun and did not recover. A more detailed description of these experiments is not provided herein. More data with this species are required in future studies.

For the experiments with *O. vulgaris* I noted a series of behavioural indicators to possibly characterize the behavioural responses of the animals during and after the electric shock. The idea was to analyse physiological and appearance (body patterning) changing observable during the different phases of the stunning procedure, as reported in studies conducted on other animals (fish and crustacean, between the others). After accurate analysis of the video recordings before, during and after stunning, it seems that there is no clear correlation between the 'behavioural' response and the parameters used for the electric shock. Most common and shared reaction in almost all the experiments, in particular that using high voltages, is the contraction of the body muscles and consequent darkening and grained texture of the skin. Complete covering of the eyes (by surrounding skin) and stop of ventilation during the electric pulse, have also been observed in most of the cases. Inking during stunning was observed in about 50% of the cases, and this is strictly correlated to the massive muscle's contraction. The strength and the duration of the contraction resulted to be significantly correlated to voltage and current. The four clusters identified after the PCA and clustering analysis corresponded to different responses of the animals to the electrical current flowing across the body, tissues and the nervous system.

Interestingly no evident relationship between the allocation of octopus in clusters and the electrical parameters set for stunning was found. This confirms that the response of the animals related to stunning parameters was not directly linked to a given parameter. Biological/behavioral indicators appeared the responses differentiating the agglomeration of

individuals in clusters. I further explored differences linked to the neural signals recorded pre- and post-stunning.

My results on neural traces detected after visual stimulation (blue light, VEPs) revealed visually evoked potentials that after the electrical stunning appeared unexpected.

My stunning parameters did not induce silencing of neural traces in *O. vulgaris* (contrary to what occurred in *Sepia officinalis*, as mentioned above). However, it should be taken in consideration that I did not restart recording of neural activities immediately after the stunning event, but only after a while, thus there is a time-lag during which response of the neural system are not known in my case: i.e. for at least 15 seconds after the stunning. In fact, in order to detect neural signals because of technicalities (e.g., repositioning of the electrodes and/or reconnecting them to the electrophysiological setup) the time for reinstating recordings varied from half a minute to some minutes after the stunning, also waiting for the regression of the body-contraction observed.

Considering the characteristics of the experimental setup, it was not possible to record neural responses (baseline and to stimulations of any type) during the stunning, as this induces the risk of damages at the entire electrophysiological setup. The use of subcutaneous electrodes in correspondence of the three brain structures allowed to start recording again neural traces after about 20 seconds from the stunning, giving the chance to observe evoked responses closer in time to the event.

In any case, I have a hole in our knowledge of what happens in the animal (and nervous system) immediately during and after the electrical stunning event.

On the other hand, I did not explore a double-stun approach, as reported to be effective for salmons, for example.

In any case, we observed an increase of the amplitude of the neural signals evoked by stimulation with blue LED; this occurred in more than 50% of the experiments. I have no data at the moment to support any hypothesis for assessing why this change in amplitude (increase) did not occur in every octopus.

As showed above, characteristics of the neural signals are bigger in amplitude, compared to the neural signal recorded before electrical stunning; amplitude that is about double when compared to the signal recorded from the same animals as benchmark, before stunning. The amplitude, however, progresses towards a gradual and fast decrease during the first minutes after stunning.

This increase in signal amplitude maybe a sign of short-term plasticity/neuromodulatory activation, a novel finding to the best of my knowledge.

When looking at the ERPs plots computed from signals detected from areas proximal of the main brain structures (i.e. *O. vulgaris* optic lobes and supraoesophageal mass), it is evident that the impact of the stunning on the nervous system is "high" in all the structures. Activity recorded after the shock resulted very 'noisy' and chaotic and it is not possible to clearly define general characteristics (i.e. shape and delay, among the others) of the potentials evoked by stimulation. These 'noisy' activities could reflect some sort of epileptic like activity, which in turn prevented synchronized neuronal responses to external stimuli. In this case, it seems that the stunning effectively created a chaotic neural state starting from an "orderly" more natural status.

It is interesting to report that Hjelmstedt and colleagues (2025) showed that when electro-stunning of 1 s (current density of 1,3 A_{rms}/dm^2) is applied to a catfish (*Ictalurus punctatus*) an increase of brain activity was observed; this correspond to an epileptic-like insult indicating loss of consciousness. Moreover, the mean duration of the insult resulted to be of 31 s and any fish showed an isoelectric trace following the insult; however, all of them recovered VERs and ventilation within 2 minutes after stunning episode.

My findings suggest that the stunning may have impaired somehow functioning of the octopus' brain. We may interpret the signs of erratic neural activity corresponding to either a massive short term neuromodulatory compulsive activity or something linked to a sort of epileptic like (seizure) activity.

The analysis of the resting states (light ON/OFF) also is novel to the best of my knowledge. This approach is widely used in studies with humans and allow to record changes in electrical activity patterns and oscillations, with the aim to identify possible alteration of the normal computation

capacities of the brain regions involved in high cognitive processes. Resting states are representative of neural activity that is generated within the brain in the absence of any specific stimuli or task and represents a measure of the brain's intrinsic activity.

Adopting this approach, we tried to investigate the effect of the electroconvulsive shock on the neural properties at the level of the main areas of the so-called central nervous system of octopus, namely optic lobes and supraoesophageal mass; structures considered to play important role in processing, elaborating sensory/motor information and modulating the resulting responses (Young, 1964; 1991a; 1995; Ponte et al., 2022).

When looking at the frequency patterns characterizing the firsts 30 seconds in the two conditions (light ON/OFF) before the stunning, optic lobes show similar power density for all the frequency bands while SEM resulted to have a higher power density for the lower frequencies (see Figures 44-46, and Table 6). After the stun, there is a change in the neural pattern for all the tissues and a general increase for all the frequency bands, except the delta band. As previously reported for VEPs, also in the case of the spontaneous neural activity a more 'chaotic' trend was observed, resulting in a higher variability in the frequency power for all the frequencies analysed. The same analysis conducted on the animals grouped in the clusters (see above), confirmed that the stunning evoked different neural responses which are significantly different between the groups of animals (i.e. clusters). Therefore, we could assume that it is possible to identify grouping of *O. vulgaris* also analysing the neural responses from areas of the animal proximal to the optic lobes and the supraoesophageal mass, i.e. the brain.

It is noteworthy to remind that we utilized a semi-random approach in setting up the electrical parameters utilized for inducing the stunning. As referred elsewhere in this Thesis (see Supplementary Info), the original idea was to use 50 Hz (as for most of the fish species) and then increase voltage; this approach resulted effective in the cuttlefish, despite the limited number of individuals utilized. Based on some data referred to impedance of the tissues, we decided to adjust the frequency and the voltage of the stun, but in order to limit the number of individuals and to explore other aspects we decided to follow a not-systematic approach. This should be planned in the future, maybe also using DC or different approaches (see below). In any case, I

was unable to find a clear correlation between the electrical parameters set for the electrical stun and the response observed at the level of the neural system in octopus.

General Discussion

For the sake of this PhD project, I searched for additional experimental evidence in support of the possibility of examining properties and conditions at the basis of consciousness in cephalopod molluscs. As mentioned above, I focused my attention to some neurophysiological indicators of signatures of conscious states in these animals.

Considering minimum 'requirements' for the search of hallmarks of consciousness I presented in these above pages a short overview of evidence postulating the existence of **functional analogues of cortex and thalamus** (i.e. subvertical and dorsal basal lobe, part of octopus supra-oesophageal mass; for review see also Shigeno et al., 2018). Based on available knowledge I also provided a short list of possible studies to be carried out in the future to eventually support this hypothesis.

Butler (2008) reviews that the dorsal thalamic nuclei receive GABAergic inputs of different sources, in particular from thalamic reticular nucleus (TRN) which plays a crucial role in the regulation of thalamic neuronal activity in mammals. TRN GABAergic cells have also been described in other structures in the brain of different species (i.e. reptiles, birds) thus supporting the working hypothesis that GABA containing cell populations is ancestral and could be a conserved characteristic of the thalamic-neural system. GABAergic cells have been identified in various region of the octopus brain, and particularly abundant at various locations on the supraoesophageal mass; relevant to this is the findings of GABAergic cells and fibers at the level of the dorsal basal and vertical lobe system (Ponte, 2012; Ponte and Fiorito, 2015).

In order to explore neural signatures, I **developed methods to record in live, but sedated animals, neural activities**. Under my experimental settings, I found that mild sedation using mix ($MgCl_2$ and EtOH) effectively allows to record *in vivo* neural signals from nerves and brain areas from the live animal, and to reliably link neural traces to evoked stimulation (mechanical or electrical). My experiments confirm a previous finding for the use of mix as "anaesthetic" in octopus; a condition that does not impairs induction and detection of long-term potentiation (Hochner et al., 2003; Shomrat et al., 2008). Under my experimental conditions, I was unable to record any signal correlated to mechanical stimulations (von Frey filaments) at the level of the

higher brain centres (i.e. supraoesophageal mass). This may be due to threshold filtering in the higher centers of the nervous system, and/or to the general organization of the brain.

In general, my findings allowed to: ***i.*** identify neural activities from the peripheral nervous system of octopus correlated to mechanical and electrical stimulations, analogous to those reported in other published papers; ***ii.*** detect neural electrical activities, both evoked and spontaneous, in sedated animals confirming the reliability of the use of a mix solution ($MgCl_2$ and EtOH) at low concentration to record from peripheral and central nervous system, without interfering with general neural functions. In my experimental settings, use of the mix solution allowed to record neural traces for a longer period of time, in comparison with the previous studies. Recordings from pallial and brachial nerve cords (PNS) highlighted patterns of evoked responses after mechanical and/or electrical stimulation, confirming that it is possible to record neural activity of large amplitude in sedated (mild) octopus; this also parallel findings from Shomrat and colleagues (2008) detecting LTP in the same species.

Recording at the level of the arm Zullo and colleagues reported neural responses to stimuli of the same intensity but statistically larger in DTP than in PTD (Zullo et al., 2011). This fits to my data, although I did not stimulate suckers, suggesting a larger number of sensory receptors in the distal part of the arm compared to the more proximal one. Furthermore, recordings of evoked potentials at the level of arms confirmed findings from Kuuspalu et al. (2022), evidencing the lack of direct neural connections between adjacent arms (in my case right arms R1 and R2).

Spontaneous neural activities (LFPs) detected from the dorsal part of supraoesophageal mass resembled those reported by Brown in 2006. In my experiments, neural recordings show no correlation with any kind of stimulations (mechanical and electrical). In addition, and in contrast to the original study, LFPs were to last >500 ms evidencing that these could be result of the sum of potentials from a bunch of neurons that synchronously activated.

The setting up of a viable biological preparation to monitor neural activities and signatures in a live animal without any impairment and/or any implant is unprecedented, to the best of my knowledge.

It is worth to mention that a recent, very interesting and innovative study, allowed continuous acquisition of neural traces *in vivo* from different locations in the octopus brain in *O. laqueus*. However, this required the amputation of all the arms of the animal in order to make the octopus immobilized and avoid any possible self-removal of the Neuropixels recordings' probes (Pophale et al., 2023).

The control of mild sedation (sensu Gleadall, 2013) and keeping dissolved oxygen adequate to the animal, without any additional interference, left the vital parameters stable over time (more than one hour, and much longer in several instances), and octopuses that stopped voluntarily respiratory events only in a very few cases.

In the second part of this PhD project, I developed minimally invasive approaches to acquire **EEG-like signatures in live octopus, extra brain for the first time**. I used commercially available plate and wire electrodes and was able to detect spontaneous and evoked neural signals reliably after visual stimuli. The two minimally invasive methods used in this project (plate and wire electrodes) resulted to be reliable in collecting evoked responses by blue LEDs and spontaneous neural activity. For example, for VEPs the coefficient of variation (CoVs) of N1 amplitude (first negative peak from the beginning of the neural response) resulted to be of 180% with plate electrodes and 62% with wire electrodes. The huge variation of signals detected by plate electrodes could be assumed to be linked to the larger diameter of the electrodes and related to neural activities collected in correspondence of a larger area. Wires should assure a more specific location of data-collection, due to the small dimension of the non-insulated area; however, it is fundamental for the reliability of the method to position the wires in the same position as much as possible. In my experiments attention was paid to locate wire electrodes in standard positions on the head of the animals.

Gutnick et al. (2023) recorded brain activity in freely moving octopuses using an implanted datalogger; Authors identified neural signatures using an array of four-electrodes inserted below the surface of the vertical-medial superior frontal lobes (SEM). The signals detected included frequency oscillations around 1.5-3 Hz and slow electrical transients lasting 15-45 seconds, and individual transients with unique rise and decay times. In addition, Pophale et al. (2023) recorded

neural activity during quiet and active sleep states in freely behaving octopuses using Neuropixel probes inserted across nine regions in the supraoesophageal mass. Authors observed rhythmic neural activity during active sleep, while during quiet sleep the brain was relatively inactive, apart from occasional 12-18 Hz oscillations. Finally, Pungor et al. (2023) developed a two-photon calcium imaging technique to study visually evoked responses in *Octopus bimaculoides*. In this case Authors report retinotopic response organization, ON/OFF pathways, and size-selective processing at various levels of the optic lobe in octopus.

My findings supplement the above recent data. Apart from the methodological innovation (non-minimally-invasive extra brain neural recording approaches) in live animals (mild sedation), I was able to detect frequency bands in the electrical activity recorded on the head of the animal at the level of areas proximal to the optic lobes and the octopus supraoesophageal mass.

Moreover, Pophale and colleagues (2023) reported a quiet state (QS) characterized by a general silent electrical activity interrupted by spontaneous oscillatory activities. This is something I observed during neural recordings in correspondence of the SEM and OLs; I detected various oscillatory patterns (data not shown) which seemed not to be correlated to any stimulation or event. I was unable to explore raw electrophysiological data from Pophale and colleagues to further investigate this possibility, and no frequency analysis of the 'silent' baseline period (QS) is available in the original study to allow a correlative inspection with my data.

In humans, brain waves are generally grouped in five categories based on Frequency (Hz) of the neural signal: Delta (0.5-4 Hz), Theta (4-8 Hz), Alpha (8-12 Hz), Beta (12-30 Hz) and Gamma (> 30 Hz). Frequency bands differ in amplitude and duration of the waves and characterization of predominant brain wave during a particular task or moment is widely used to define mental states. As an example, Delta frequencies are predominant during deep sleep and recovery stages, while Alpha band is typical of relaxed wakefulness state, during no particular stimulation or attention to outer world. In any case, all band waves can be detected during electrophysiological recording from the CNS, and traces collected in different conditions could be characterized by a predominant frequency range, while the other frequencies can be present at trace level.

Spontaneous fluctuations of electrical activities in the brain are generally observed during a resting-state EEG, when a person stay in a relaxed state with eyes closed. Resting state activity is defined as a series of spontaneous (intrinsic) neural patterns and oscillations that can be collected

without imposed stimulations (e.g., Snyder and Raichle, 2012). Resting state studies during the past few years have shown that properties of endogenous activity are modulated by the state of the eyes (e.g., McAvoy et al., 2008) and by concurrent sensory stimulation (e.g., Hampson et al., 2004), among others.

Despite differences my findings from recording from octopus brain offer unprecedented insights and suggestions around the evolution of "brain activity oscillations", for example, from simple forms and static to complex forms and dynamic in a resting state. We are not sure if these questions have ever been studied in any detail. I have been able to detect EEG signatures in octopus, including electrical brain activity, compound field potentials, and evoked potentials, widespread brain activity.

Studying the brain cognitive functions in freely moving animals has been a challenge in the neuroscience field. Aquatic animals are especially difficult to deal with; most cognitive studies of brain are in stationary animals with their head fixed.

We are very far to record neural correlates from a live octopus trained to move a given arm or performing a cognitive task, extra brain, but at least we can start to increase our knowledge on the characteristics of the neural activities in live octopus; something I only marginally surfed here.

Interestingly enough, I was able to detect neural signatures (spontaneous and evoked) before and after (not in the immediate temporal proximity) electrical stunning.

As mentioned above, my stunning parameters did not induce silencing of neural traces in *O. vulgaris* (contrary to what occurred in *Sepia officinalis*). It is also true that in order to detect neural signals I progressed to record sometime after the stunning event and that because of technicalities (e.g., repositioning of the electrodes and/or reconnecting them to the electrophysiological setup) the time for reinstating recordings varied from tens seconds to some minutes after the stunning. Despite the fact that in octopus electrical stunning – with parameters and conditions tested till now – resulted not effective in suppressing neural traces (in more than 50% of the animals), I was able to detect a significant alteration in the brain frequencies oscillations after stunning.

This confirms that a sort of epileptic neural patterning occurs after brain “electrical stimulation”. For the animals that apparently did not provide evidence for evoked responses after the stunning, we cannot rule out whether the absence of the response is determined by the stunning itself which ‘shut down’ the nervous system, or if other impaired physiological conditions intervened, causing the disruption of the neural functioning (for example, anoxia).

Unfortunately, and because of technical issues, I have been unable to include in this Thesis data from experiments where I tested gene expression and neuromodulator concentrations in brain tissues (and control) from animals (control, sham and after stunning). I can anticipate that a significant change in gene expression occur (e.g., stress-related and neuromodulators such as GABA, dopamine depending on condition tested) after stunning, but I hope to be able to refer this in a manuscript in a future. Molecular correlates are very interesting to explore for addressing many fundamental questions.

It is also interesting to recall that after stunning, some animals show clear significant increase in amplitude of the ERP recorded. This did not occur in all animals, but in a large quota of my sample. Future work is needed to understand this different response of the neural system, and of the possible biological machinery involved when this increase in amplitude is seen.

When looking at the resting states, the changes in light condition (ON/OFF conditions) during ‘eyes open’ octopus provided a general increase of frequency band power when light is on. In particular, I observed an increase in the frequency ranges 1-2,5 Hz and 4-9 Hz, which is confirmed after electrical stunning.

Changes in the frequency power observed in animals differed from the human studies on resting state activity in two cases: during eyes open, light-on condition shows a reduction of power for almost all the frequency bands in humans, while in octopus we have an inverse situation (decrease in power during light off); in humans alpha wave band (8-12 Hz) is the one that mostly increase in power when light is turned off, but under my experimental condition in octopus we can observe an increase in delta (0,5-4 Hz) and theta (4-8 Hz) waves.

For the effectiveness of the electrical stunning, some comments must be made. First, it is interesting to recall the difference of the effect of the stunning in the two species tested (cuttlefish resulted effective, octopus not) under my experimental conditions. Second, a diversity of methods and parameters is reported to be required for stunning different fish species effectively. I did not have the chance to explore, for example, double stun (high frequency pulse, followed by 30 seconds low frequency low voltage) mostly because of time and animals' availability; I was also unable to explore the effect of electronarcosis in octopus.

We tested the combination of different electrical parameters (frequency and voltage) based on various studies on wet electrical stunning in fish. Most of the Authors reported stunning condition and electrical parameters to effectively stun the specimens strictly correlated to the anatomical and neural characteristics of the single species; the resulting picture is that it is not possible to identify electric shock parameters applicable to all the species.

Considering an animal welfare perspective, the European legislation clearly requests the use of a first method which instantaneously bring the animal in a status of insensibility and unconsciousness (e.g., stunning), followed by a second method - confirmation of death - that must be conducted as soon as possible to avoid the animal to recover conscious status.

In our case, as one of our main aims was to validate the method exploring the efficacy and the effects of the stunning on neural traces, we mainly focused on the properties of the neural signals through analysis of the visually evoked potentials and resting state activities. Therefore, we did not integrate a confirmation of death in the stunning procedure as we looked at changing in behavioural, physiological and neural parameters after the electrical stunning. In addition, as reported before, we conducted a preliminary molecular analysis (not included in this thesis), to look at changes in gene expression of genes related to neurotransmission and oxidative stress, between the other.

We do not exactly know what happened in the period of time that goes from the end of the stunning and the sacrifice of the animal (carried out using anesthetic overdose followed by transection of dorsal aorta, as required by the current legislation), so we cannot exclude that some sort of recovery was experienced by the animals in some cases. Also, we mainly focused on the analysis of the neural signals just after the electrical stunning (traces analysed those within

5-10 min from the stun) so we do not know the properties of the spontaneous and evoked activity after the given time.

However, we observed an 'epileptic-like' response of the nervous system that is reported to be a state of loss of consciousness.

We observed in some animals an increased amplitude and power density of the neural traces, which is typically observed during seizure in humans and other animals. Further analysis of the neural traces collected after stunning are needed to identify the 'long term' effects of the electric shock on the brain. It will be fundamental to clarify if the neural activity return in the pre-stun condition or if brain activity is shut out completely and it is not recovered.

Du Plooy et al. (2023) refer to the use of EEG for a reliable measure of brain activity and a tool to determine changes in frequency band power in Nile crocodiles subjected to electrical stunning. In this case, the analysis of EEG traces revealed that a number of animals (50%) a decrease of alpha (8-12 Hz) band power and an increase of delta (0-4 Hz) band power; a finding that the Authors pose as indicator of loss of consciousness, together with a tonic-clonic wave patterns in electroencephalogram recordings. Behavioural indicators defined in the study seemed to be not completely reliable to assess status of unconsciousness in crocodiles. Neil et al. (2024) tested the efficacy of electrical stunning in two crustacean species (*Carcinus maenas* and *Homarus gammarus*), using behavioural, physiological and neural indicators to define loss of sensibility. In this study animals stunned with commercially available device (Crustastun™; stunning cycle of 110 V, 2–5 Amp electrical charge for 5 or 10 s) resulted with a total loss of neural activity (spontaneous and evoked) during the entire time of the experiments. Cardiac activity, instead, resulted to stop for the 1-2 minutes after the stun but was quickly recovered in all the cases. In this study animals were considered fully insensible for a long period of time, which allowed for a second method to effectively kill the animals.

In lobster and crayfish Fregin and Bickmeyer (2016) shown that electrical stunning induced epileptic like seizures, paralysing the animals, but leads to a reversible decline of nerve system activity after seizure.

In fish, electrical stunning is reported to be efficient (loss of insensibility and unconscious status) when a series of behavioural signs are observed. For salmon and trout, for example, reflexes such as eye roll, breathing (movement of the operculum), swimming, equilibrium and escape

responses to stimulation are recognised as indicators of the effects of stunning (e.g., Kestin et al., 2002). However, the lack of movement and clinical reflexes does not necessarily mean that the animal is not sensible to pain or distress (Robb et al., 2000). In fact, behavioural indicators of consciousness in fish need to be correlated to changes of EEG signal characteristics, which can vary in terms of both amplitude and frequency.

As a way of a coda, I would like to summarize that I attempted to contribute to the search of neural hallmarks of consciousness in cephalopods. Through the development of minimally invasive electrophysiological techniques, I was able to acquire EEG signatures for both spontaneous and evoked neural signals from live octopuses, detecting neural traces and their amplitude, frequency domain and oscillations. As mentioned, this approach is unprecedented, to the best of my knowledge. The identification of mammalian like EEG signatures and widespread brain activity considering frequencies of neural signatures and the corresponding frequency-bands of EEG signal detected in species far from cephalopods molluscs open the avenue for future studies.

Finally, I do not know if I was able to contribute to identify elements/features of the *first-order or primary consciousness* in octopus (Edelman, 1989) but attempted to put some ticks to the checklist of neural hallmarks (at least partially), as suggested by Edelman and colleagues (Edelman et al., 2005; Edelman and Seth, 2009; see also Ponte et al., 2022).

Many recent accounts overviewed elements and domain of consciousness in invertebrates (Birch, 2022; e.g. for insects see also Paoli et al., 2023) – and I am not deliberately surfing the literature at this stage - but only a few contributed with new data. I hope my work can be seen in this view.

These neurophysiological observations provide a foundation for exploring the possibility of consciousness in cephalopods. Exploring further these analogues could offer valuable insights into the evolution and nature of consciousness across diverse animal taxa.

Future investigations

First at all, in support to the anatomical and cytoarchitectonical investigation of the analogies of the thalamo-cortical system in cephalopods, we believe important to include the inspection of the connectome-organization of the central nervous system (maybe with a comparative outlook among cephalopod taxa) and explore further similarities in neurotransmission and neural organization in cephalopod neural structures which are possibly though to be seat of conscious state (e.g., subvertical, dorsal basal lobes) as suggested in this Thesis. Findings of the high neural structures and processes could give new insight and suggestion to define the 'Global Workspace' (for review e.g., Zacks and Jablonka, 2023) also in cephalopod species.

Preliminary results obtained in this work give a good starting point to assess the effectiveness of the electrical stunning in cephalopod species. As this is the first time that a stunning method is tested on these animals, to the best of my knowledge, it is clear that further and more standardized investigation are needed in order to understand if the method could be considered reliable and efficient as humane slaughtering. Interesting would be to test the effect of a two-stages electrical stun (already tested on few fish species) which is reported to be more efficient in stun the animal: a brief high voltage/high frequency electric field followed by a longer low voltage/low frequency stun for inducing a no-recovery. Moreover, it would be useful to optimize the new electrophysiological approach to better identify the changing in frequency domain of neural activity recorded from the central brain, reducing also the time between the stunning and the starting of the recording.

Fundamental will be to highlight the biological/molecular correlates of the animals' responses to the stunning procedure. The preliminary investigation on the variation of selected genes, caused by stunning carried out during the last year of the PhD, is a first attempt to understand how the electric shock could interfere with neuromodulation and gene expression. Furthermore, other tissue samples collected during the experiments will serve for different analysis, such as high-pressure liquid chromatography (HPLC), that for lack of time I did not explore; this will inform and possibly quantify selected molecules (biogenic amines, neurotransmitters, hormones) which could be affected by the electric shock.

Finally, my minimally invasive approaches allowed to record for the first time from live (mild sedated) animals. This approach (e.g., subcutaneous wire electrodes) maybe complemented by un-tethered high speed data-transfer device and/or conversion (e.g., convert neural signals into sound, Destexhe and Foubert, 2022) of light impulses, in a way that will allow propagation of data underwater.

Despite futuristic, all the above may reveal a new endeavour in the research on consciousness in cephalopods, as representatives of distant taxa from mammalian clade.

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Supplementary Information to M. De Luca PhD Thesis

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Supplementary Info – STAR Methods

In order to facilitate readability through the different chapters of this Thesis, I preferred to collate the information about animals, chemicals, equipment and methodological details in a single section, and as Supplementary Information. This approach does not limit the potential of submitting separate manuscripts for publication, based on the individual chapters (and/or their parts), as I – and my Supervisors – aim in the next future, and of course based on the outcomes of this PhD.

In preparing this section, I inspired myself to guidelines available for the so-called STAR Methods, i.e. structured, transparent, accessible reporting initiated by iScience. This allows a reporting of methods (and materials) with sufficient detail that allow possible accurate description also in cases of complex methodology. As indicated in the launch Editorial (Marcus, 2016), an accurate «how to […] informs the interpretations and robustness of the scientific claims and provides the foothold for other scientists to build upon those findings. […] Recent calls […] by society, the research community, and funding agencies to improve rigor and reproducibility in science clearly point to the need to take a new approach to communicating not just the "what" but the "how" of science» (Marcus, 2016). The initiative promoted by scientific journals of Cell Press aims at facilitating clear and complete description of how the experiments have been carried out. In adopting such a template, I exercised myself in providing the greatest possible detail of methods and approaches adopted during the last three years; failures in achieving this goal are my fault.

I was expecting to recall and reiterate several methodological details in the various chapters of this Thesis; this bring me to the perception that individual methods sections may result difficult to comprehend, repeating several times information thus impairing (possibly) an overall picture of the 'storyboard' that is at the core of the experiments carried out for the sake of this PhD project. The "STAR Methods: Structured, Transparent, Accessible Reporting" approach has been selected to help me in emphasizing the continuous efforts I (and my Supervisors) pursued in the search of neural substrates and indicators of conscious states in cephalopods (see also Reporting, below). With all limitations linked to a slow stepwise proceeding, also possibly linked to the limitation of live animals for this study, my original ambitious aim has been redesigned for searching the goal of promoting future endeavours further exploring similar research questions.

In following pages this Supplementary Information section has been structured as STAR Methods also in the hope to facilitate a transparent reporting of the experiments and methodological details. Whenever possible, I followed the original guidelines to STAR Methods. I utilized tabularized overviews as much as possible, complementing the text. However, in applying STAR Methods 'approach' I deliberately changed the original instructions, by: **a.** including references to previous studies (in the original recommendation, the methods text should avoid to refer to other papers to understand how procedures were performed) whenever appropriate, but including brief mentioning of a procedure/protocol and any change I applied; **b.** using figures (not considered essential in the original guide to authors) and tables, apart from the 'Resource Table'; **c.** use additional headings to facilitate readability.

I hope that this approach, will help the overall concept of this Thesis and will help the much-desired follow-up of this fascinating study.

Experimental Organisms

The original aim of this PhD project was to use different species of coleoid cephalopods, namely *Sepia officinalis* and *Octopus vulgaris*; with squid as potential for further trials. Here we have been oriented to a large use of octopus as experimental target in this PhD project; this is linked to the circum-annual availability associated with reliability of artisanal fishermen operating in the Bay of Naples (Italy), where the experiments have been carried out (i.e. Department of Biology and Evolution of Marine Organisms, Stazione Zoologica Anton Dohrn, Naples, Italy). *S. officinalis* has been included in a few experiments assessing spontaneous and evoked neural correlates and testing the efficacy of electrical and mechanical stunning. For simplicity these results are not included in this Thesis; possible future studies will provide an overall picture.

Details about the current taxonomical position of the species are available through WORMS (World Register of Marine Species). The common octopus is one of the most representative species for cephalopods. *O. vulgaris* is an iconic species that received a great attention in several studies on different aspects of its biology, physiology and behavior, including learning (a review on these aspects is not included in this Thesis). The species is distributed in marine coastal waters. Details about the biology of the species can be traced into different sources and are not included herein.

S. officinalis and *Octopus vulgaris* are species complex with overall almost worldwide distribution from tropical to temperate waters. The recent descriptions of various forms inhabiting different marine geographical areas (Gleadall, 2016; Avendaño et al., 2020; Rosa et al., 2024), confirmed the original view by Mangold, that the “true” *O. vulgaris* is not a world-wide distributed species, but occurring only in the Eastern Atlantic Ocean and the Mediterranean Sea (Mangold, 1997). The question whether different morphotypes are living in the Atlantic Ocean and even in the Mediterranean Sea is continuing to raise increasing scientific interest, attempting to suggest a potential diversity not only for the biological specimens, but also for possible physiological and behavioral adaptations, associated to the marked biological plasticity (including predatory behaviour, adaptation and geographical differences in body patterning) that the species reveal. *O. vulgaris* is the most famous and best studied of all octopus species. This is largely due to the initiative of Professor J. Z. Young who, since 1947, carried out at the Stazione Zoologica A. Dohrn of Napoli (Italy) a systematic analysis on the relationship between neural structures and behaviour in this animal: a study mainly centred on the extraordinary learning capabilities of this

cephalopod mollusc (Sanders, 1975; Wells, 1978). Genome resources for this species are now available (Zarrella et al., 2019; Destanović et al., 2023).

O. vulgaris (see Key Resources in this Supplementary Info) of both sexes were caught by local artisanal fishermen in the Gulf of Naples (Tyrrhenian Sea, Italy). All the animals were transported within half an hour to the lab according to current FELASA recommendations (Sykes et al., 2023) and were humanely killed at the end of the experiment, within the same day.

In the laboratory animals were hold in plastic buckets filled with fresh oxygenated seawater at a temperature equivalent to the seawater in the Bay of Naples at the time of sampling from the field.

All the observations and experiments started within about 15 min from the arrival in the laboratory. Animals were constantly monitored for any sign of distress, including monitoring of indicators of welfare (Fiorito et al., 2015a).

Ethical statement

This study was conducted in respect of principles stated in the Directive 2010/63/EU.

On the basis of the current regulatory framework, all the experiments with live cephalopods require authorization from the National Competent Authority according to the above-mentioned Directive and the National transposition of the EU Directive (in Italy since March 2014). As defined in the Directive 2010/63/EU, any procedure carried out on live animals refer to: “any use, invasive or non-invasive, of an animal for experimental or other scientific purposes, with known or unknown outcome, or educational purposes, which may cause the animal a level of pain, suffering, distress or lasting harm equivalent to, or higher than, that caused by the introduction of a needle in accordance with good veterinary practice”.

All procedures adopted during this project were performed by this PhD candidate after FELASA (functions a, c, d) certification, and under supervision of Drs G. Ponte and G. Fiorito[1]. In all instances we followed practice and adopted methods to warrant animal welfare and minimize

[1] Department of Biology and Evolution, Stazione Zoologica Anton Dohrn, Napoli (Italy), and Association Cephalopod Research-ETS (Italy)

pain, suffering and distress of the octopuses involved in the experiments (Andrews et al., 2013; Fiorito et al., 2015a).

The use of data from live *O. vulgaris* applied in this study is considered compliant with the some of the 3Rs principles (Replacement, Reduction, Refinement; https://www.nc3rs.org.uk/the-3rs) to cephalopods (Fiorito et al., 2014; Ponte et al., 2019).

Ethical clearance for this project has been issued by competent AWB. Because animals included in this study have not been exposed to any observation and study apart from the final fate of humanely killed within two/three hours from capture, this study is considered out of the scope of the Directive 2010/63/EU.

All experiments carried out with live animals in this study are also classified as 'non-recovery'; all animals were killed within the same day and immediately after the conclusion of each individual experiment.

Humane Killing

Animals were humanely killed following principles detailed in Annex IV of Directive 2010/63/EU as described in Fiorito et al. (2015). In brief, octopuses were immersed in freshly made 3.5% magnesium chloride hexahydrate (Sigma Aldrich, CAS Number: 7791-18-6) dissolved in sea water. After 30min immersion, the animals were unresponsive to handling and a noxious mechanical stimulus, and ventilation completely stopped (after 10 min).
Killing was completed by transection of the dorsal aorta.

Following Annex IV of the Directive 2010/63/EU (see also update from March 2024) overdose in anaesthetic solution of $MgCl_2$ ($6H_2O$) at 3.5% dissolved in seawater is the only approved method to successfully suppress cephalopod species. Confirmation of death in cephalopod can be done through exsanguination or destruction of the brain. I adopted transection of the dorsal aorta as method of confirmation of death, in my experiments; this to facilitating sampling of tissues for further experiments (not included at this time in the Thesis).

Electrophysiological methods

Recording from Peripheral Nervous System

A minimally invasive procedure was used to record efferent and afferent neural activity from/to the peripheral nervous system (PNS) which is directly connected to the central nervous system (CNS) in octopus. In cephalopods PNS is represented by ***i.*** two pallial nerves, nervous structures that leave the central region of the Palliovisceral lobe (SUB) and irradiate to the two side of the mantle, each of them ending in a structure, the stellate ganglion (see below), ***ii.*** eight brachial nerves originating from the prebrachial lobe of the SUB and progressing into each arm, contributing to sensory and motor control.

Once sedation was reached (see Supplementary Info: 'Sedation'), the octopus was transferred in a plastic dish with mix solution and prepared for the electrophysiological recordings. Nerve recordings were performed with silver wire hook electrodes (see below for details) that were connected via an ultra-low noise Differential Preamplifier (Model MA103) to a 4-channel amplifier/signal conditioner. Signals were amplified (2000x – 10000x) and filtered (lowcut 300 Hz, highcut 1.0-3.0 kHz). Signals were digitized at a sampling rate of 12.5 kHz using the Micro 1401-4 acquisition unit (CED, Cambridge, UK) controlled by Spike 2 v8.1 software.

For nerve recordings monopolar hook electrodes were use. The electrodes were fabricated from Teflon coated silver wire (0.4 mm). The insulating Teflon coating was scraped off the end of the wire to a length of approx. 2 mm. The wire was passed through the 18 mm long section of the end of an Eppendorf pipette tip (10 µl). The large diameter of the section pointed towards the tip of the wire. The pipette tip was filled with Vaseline. The end of the wire was bent into a hook onto which the dissected nerve was placed. The hook and nerve were now pulled into the pipette tip, thus isolating the recording site from the surrounding ASW. The electrode was connected to the non-inverting input of the pre-amplifier. The end of the electrode connected to the inverting input was positioned less than 2 cm from the recording site in the ASW.

In some experiments hook electrodes were used for electrical stimulation of a nerve. The electrodes were fabricated as described above. The electrodes were connected to a Stimulus Isolation Unit (MI 401) that received input from a pulse generator (MS 501; both instruments from Electronics workshop, Zoological Institute, Cologne, Germany). Rectangular current pulses of 10 ms duration and various amplitudes were used.

Pallial nerve cords

In this case the animal was positioned in the plastic dish immersed in mix solution and exposing ventral mantle side, the mantle was slightly overturned to expose the nerve and the stellate ganglion nearby. With the help of a scalpel an incision was made on the internal side of the mantle by cutting the skin, muscle and connective tissue in order to expose a small section of the pallial nerves (about 0.5 cm). The nerve was then hooked onto the electrode as described above, about one centimetre distant from the stellate ganglion. Thereafter, the mantle was flipped back and the animal was positioned showing dorsal side (Fig. S.2). The mix solution was diluted (50%) with fresh seawater to allow the animal to stay in a lightly sedated state during the entire recording procedure.

Electrical activities from pallial nerves were collected, during mechanical stimulation of the mantle using both forceps to pinch the skin, and von Frey filaments (Bioseb Inc., France) to apply pressure stimuli of defined strengths.

Pressure stimuli were applied to six spots on the mantle (see Figure S.1) located ipsilateral and contralateral to the recordings site and in the mantle midline. Spots from 1 to 4 are in correspondence of the so called “long mantle papillae” (Packard and Sanders, 1971; Borrelli et al. 2006) which are sensitive to mechanical stimulation in comparison with other mantle regions; spot 5 corresponded to a region behind the head, while spot 6 was selected as a location at theposterior end of the mantle (Figure S.1).

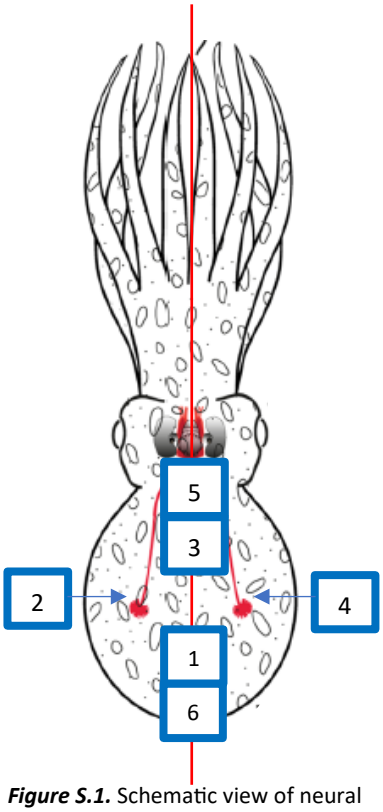


Figure S.1. Schematic view of neural system of an octopus and representation of the six spots mechanically stimulated. (Modified from Imperadore et al., 2019)

Pallial nerve recordings were generally carried out after 20-25 minutes after sedation, and 5 minutes after the 50% dilution of mix solution with fresh seawater (see ‘Sedation’ for more information on sedation process and animal preparation before electrophysiological recording).

Recording from arm nerve cords

To record neural signals from arm nerve cords, I applied a similar approach as previously described for pallial nerves. Before the electrodes were attached, the animals were treated as described above. One or two arms (R1 and/or R2) were positioned on an elevated plastic plate to facilitate preparation of the nerve and application of the electrodes. Usually hooked silver wire electrode were anchored to dissected and de-sheathed arm nerve cord at about 7 cm from the commissural fibres - aganglionic sectionof the axial nerves (Fig. S.3). Different regions of the arm (distal, proximal) and suckers were mechanically stimulated using forceps and von Frey filaments.

Figure S.2. *Octopus vulgaris* in mix solution, during electrophysiological recording from both pallial nerves

Figure S.3. Silver teflon-coated hook electrode anchored to isolated arm nerve cord during electrophysiological recording.

Recording from the octopus brain

An invasive brain recording method, analogue to the procedure carried out by Brown and colleagues (2006) was applied with the aim to record spontaneous and evoked electrical activity coming from supraesophageal mass (SEM) in sedated *O. vulgaris*, using two tungsten electrodes positioned on the dorsal surface of the brain, at the level of the vertical lobe (VL) and/or superior frontal lobe (SFL).

Once sedated, the animal was transferred to the Faraday cage into a plastic dish containing mix solution. The dorsal side of the head was outside the mix solution to allow surgery and facilitate electrodes implantation. For placing the electrodes, a small hole was cut into the head right above the VL (between the eyes) of the animal using scissors and scalpel. Skin, muscles and cartilaginous skull layers were gently cut. To guide and hold the electrodes, two holes with a diameter of 1.5 mm were drilled close to each other in the center of a 3x3 mm plastic plate (made from the lid of a Petri dish). To further stabilize the electrodes, two 5 mm long glass pipettes (micro electrode glass) with the same diameter were inserted into these holes and fixed with superglue. The plastic plate was placed on the cranial cartilage above the incision. The soft tissue

was then fixed to the glass tubes with tissue glue (Histoacryl, B.Braun). The two tungsten electrodes were now inserted through the glass tubes and placed on VL and SFL, without penetrating the brain masses. The technical procedure for recording brain signals was the same as for nerve recordings. Electrical signals were amplified (gain 5000-10000 x) and filtered (lowcut 300 Hz, highcut 1.0-3.0 kHz). Signals were digitized at a sampling rate of 2,5 kHz (Micro 1401-4 acquisition unit, CED, Cambridge, UK) controlled by Spike 2 v8.1 software (CED, Cambridge, UK). A ground electrode was immersed in the mix solution to avoid electrical noise interferences during recording.

Background spontaneous activities were recorded and any movement possibly creating artifacts during recording of neural signals - such as movement artifact due to ventilation (opening and closing of the mantle) or involuntary muscles and skin movement - was identified and noted, thus to discriminate from the following analysis.
Evoked potentials were induced using both mechanical and electrical stimulation of mantle, arms and suckers. Mechanical stimulation was conducted using von Frey filaments; brief electrical shocks were applied using hook electrodes anchored to peripheral nerves (as described above).

Minimally invasive electrophysiological methods

Novel minimally invasive electrophysiological approaches were developed for the purpose of this project.

Brain electrical activity was collected with two types of electrodes, ***i.*** ECG Ag/AgCl plate electrodes with sticky electroconductive gel placed on the head and ***ii.*** Teflon-coated silver wires inserted under the skin of the head in correspondence of target brain areas (see below and main text for details).
Electrical signals were amplified (gain 5000 x) and filtered (lowcut 1.0 Hz, highcut 1.0 kHz; Ultra-low noise Differential Preamplifier, Model MA103 and 4-channel amplifier/signal conditioner, Model MA102 Electronics workshop, Zoological Institute, Cologne). Signals were digitized at a sampling rate of 2,5 kHz (Micro 1401-4 acquisition unit, CED, Cambridge, UK) controlled by Spike 2 ver.10.11 software (CED, Cambridge, UK).

A video camera (Microsoft, LifeCam studio, 1080p HD, 30 FPS) was used and connected to Spike2 software for videorecording the animals for the entire duration of the experiments to control animal movements and electrode positions.

Stimulation

During recordings, mechanosensory and visual stimuli were applied for analysing their effects on brain activity. Mechanical stimulation was performed with both forceps and von Frey filaments on different parts of the mantle, arms and suckers. Pinches and pressure stimulations were given throughout the entire experiment, to observe changes in the neural responses when the animal reached different sedation levels.

Visual stimuli were applied to induce Visually Evoked Potentials (VEPs) from the octopus visual system. In this case a blue LED (Cree® 5mm Round LED, λ=465-480 nm) was directed to one of the eyes during electrophysiological recordings, positioned at a distance of 2 or 0.5 cm from eye. Using a universal stimulus isolator and Spike2 software, light amplitude, duration and number of the pulses (train) were adjusted such that responses from the brain were reliably evoked. Damage to the visual system was avoided by setting light intensity in the range of 127,0-472,3 $\mu W/cm^2$. This proved to be optimal under my experimental conditions because it allowed the reliable recording of neural signals with a good signal-to-noise ratio in the vast majority of octopuses utilized. All the stimulations were conducted using trains of usually 10 short rectangular stimuli (0.1s duration, 0.2Hz) and a single long rectangular stimulus of 2s.

Use of Ag/AgCl plate electrodes

The experiments were carried out on *O. vulgaris* in a light sedated state (see also 'Sedation'). Ag/AgCl plate electrodes (8mm diameter; Kendall, Medi-Trace 200 Foam Electrodes, USA) with sticky electroconductive gel were used. The sticky foam substrate surrounding the electrodes was cut away to minimize the size of the conductive surface. To enable a connection to the amplifier, a silver wire was soldered to each electrode. Non-inverting electrodes were positioned on the external surface (skin) of animals' head, in locations corresponding to supraoesophageal mass (SEM) and/or optic lobes (OLs) (Figure S.4). Inverting electrodes were positioned in the transition region of head and arms to minimize pick-up of brain signals. A third electrode

connected to the ground input of the pre-amplifier was positioned in the transition region of head and mantle. For a small group of animals, pallial or brachial nerve recordings were included in the experiment, to explore eventual electrical signal transmission from the peripheral to the central nervous system (data not shown).

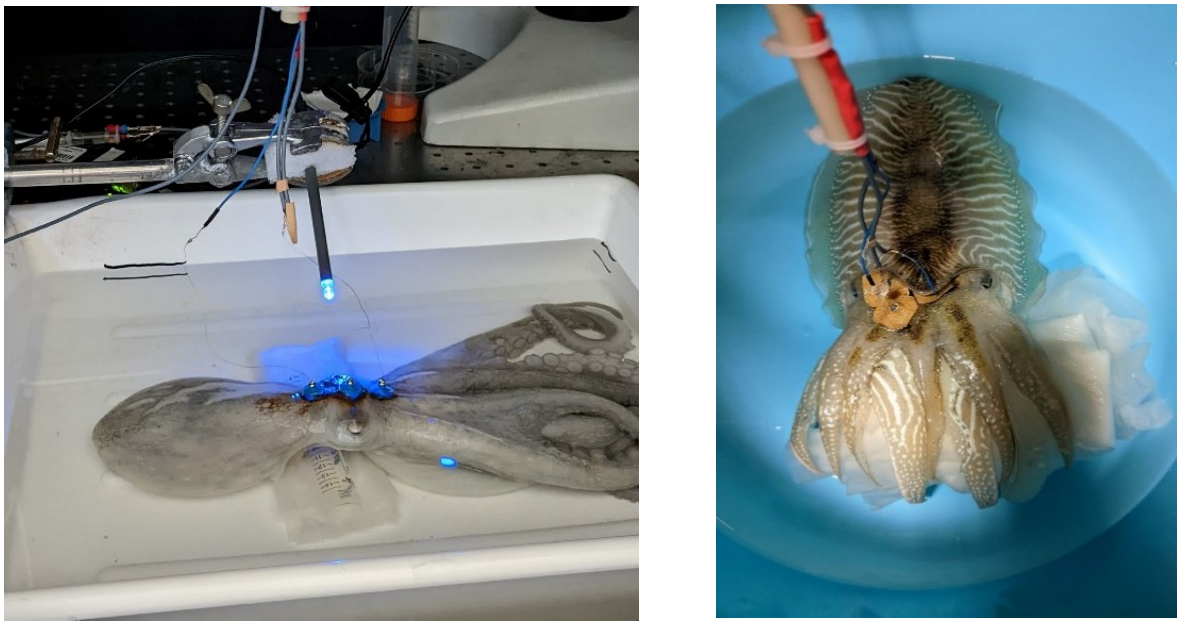


Figure S.4. O. vulgaris (Left) and S. officinalis (right) preparations during electrophysiological recording using Ag/AgCl plate electrodes in correspondence of optic lobes and supraoesophageal mass.

During visual stimulation, plate electrodes were on different regions on the head of the animal. The non-inverting electrodes were placed at three different locations on the head. The locations corresponded to the closest parts of the brain below, i.e. the two optical lobes, ipsilateral and contralateral to the illuminated eye and the supraesophageal mass. These different electrodes arrangements were considered useful to clarify which structure of the CNS was responsible for the VEPs elicited by LED stimulation. Areas of the skin with electrodes were not covered by the mix solution because the sticky conductive gel of electrodes is water soluble.

Use of Ag/AgCl wire electrodes

In order to further refine recordings of neural signals, I utilized another approach using subcutaneous wire electrodes (Figure S.5). The general idea was to ***i.*** reduce the signal recording

area to be more precise, limiting the collection of electrical signals to a more limited area, ***ii.*** be closer to the central brain structures and ***iii.*** try to remove possible artefacts due to superficial skin movements.

Teflon-coated silver wires (0.4mm diameter; World Precision Instruments, USA) were used for this set of experiments. The Teflon coating was removed, and silver wires were chlorided through immersion in common bleach (<5% NaClO) for at least 1h to improve their performance and reliability in recording bioelectrical signals. Ag/AgCl electrodes were isolated using nail polish, except for the last 1.5/2 mm at the tip of the wires, to allow electrical signal detection.

Three pairs of electrodes were used. Each pair shared a common inverting electrode that was placed under the skin in the transition region of the head and arms. A fifth electrode that was connected to the ground input of both pre-amplifiers was placed under the skin in the transition region of head and mantle. The three non-inverting electrodes were implanted in correspondence of the SEM and both OLs. Moreover, to improve the implantation of the electrodes under the skin, wires were passed through syringe needles and bent at the tip to allow better anchoring in the underlying muscles tissue. The syringe needles with the wire were pushed under the skin and pulled back. This procedure left the wire anchored in the muscle layer under the skin.

Position of the electrodes remained the same for the entire duration of the experiment, but it should be taken in consideration that involuntary movements of muscles and skin tissues could indirectly modify the initial position of the electrodes.

During electrical recordings, spontaneous and evoked bioelectrical signals were collected.

In this case, only the visual system was stimulated and tested using two different methods: ***i.*** blue LED was pointed to the eye at 2 or 0.5 cm using short (0,1 s) and long stimuli (2 s); ***ii.*** diffused white light (room light) was switched ON/OFF 10 times for 30 s each. LED stimulation, as previously described, has been involved to collect VEPs from the CNS, while periods of diffuse light ON/OFF were thought useful to record changes in background neural activity and bioelectrical oscillations (resting state).

Spontaneous raw signals were firstly collected (no stimulations were conducted on the animal), then alternating light ON/OFF periods were tested to identify general changes in frequency band domain before and after visual stimulation and in different conditions of light. LED stimulation to the eye was conducted in different periods from the beginning to the end of the experiment.

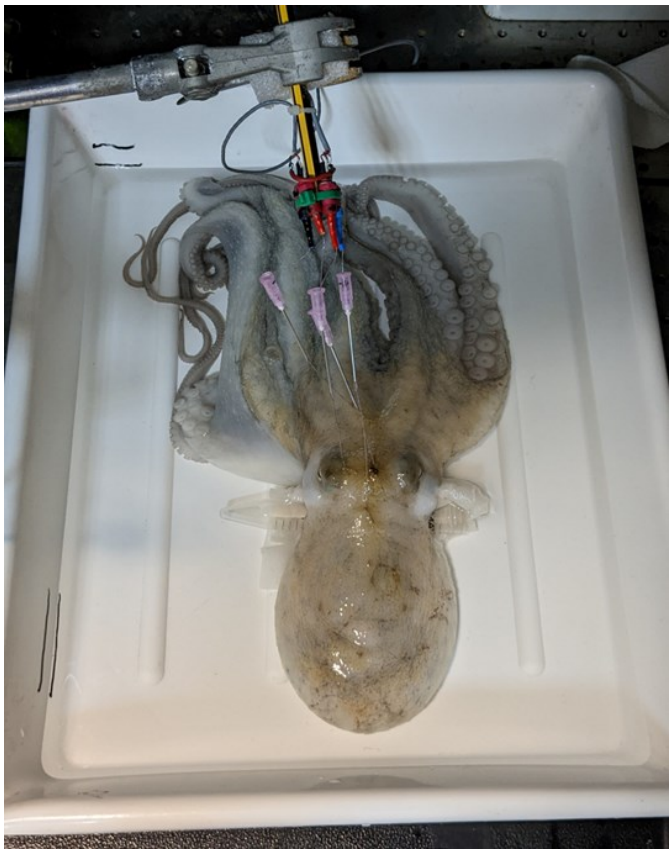


Figure S.5. *O. vulgaris* specimen preparation for electrophysiological recordings using Ag/AgCl wire electrodes in correspondence of optic lobe and supraoesophageal mass.

Electrical Stunning

Stunning apparatus

Two different stunning machines were utilized through this study. The first was similar to the standard apparatus utilized for fish (mainly salmons; Optimar AS - Norway). The apparatus is composed of a stainless-steel cabinet that could delivers sinusoidal waveform (fixed at 50 Hz) of up to 300V, for variable durations from 1 to 10 seconds. The use of these parameters derived by recommendations by Dr Endre Grimsbø (UiT- The Arctic University of Norway, co-supervisor for this PhD). Similar approaches have been applied in various fish species (Lambooij et al., 2002; Lambooij et al., 2008; Grimsbø, 2016; Grimsbø et al., 2016).

The stun machine was connected to a stainless-steel module via two cables, to allow current flow from the main source through the animal. Current flowing through all the system was measured using an industrial ScopeMeter (Fluke 123) and a multimeter (Agilent U1193A).

After some experimental trials, a preliminary results suggested that a fixed frequency at 50Hz sine wave was probably not an optimal parameter for electrical stunning of octopus. A stunning unit was built in purpose (Fig. S.6) with adjustable frequency, voltage and duration of the electric shock, by Dipl.-Ing. Michael Dübbert (University of Cologne, Institute of Zoology, Neuroscience Electronics Lab - Germany). The unit is designed to operate as a high power and high voltage linear amplifier in the range of 10Hz-5kHz (sine waves) and a voltage up to 620V peak-peak. This machine has an internal unit for stimulus duration, frequency and amplitude, but it could be easily controlled by an external source, such as the Micro 1401-4 acquisition unit (CED, Cambridge, UK) controlled by Spike 2 v8.1/10.11 software (CED, Cambridge, UK), both already used for the electrophysiological recordings.

Figure S.6. Electrical stunning unit used during stunning experiments.

The stunning unit was connected to electrodes fixed in a plastic tube via electric cables (length=1m, diameter=3 mm) and plugged to adapters connecting the electrodes (see following paragraph Stunning conditions).

Electrical parameters for the stun were adjusted directly on the machine or, in some cases, set up from the graphical editor for stimuli of the software Spike2 (ver.10.1). Effective voltage and current passing through the system during stunning, were measured by the inbuilt voltmeter and current meter.

The stunning machines tested during this study were thought to be first prototypes able to stun the animals using efficient electrical parameters and considered safe and feasible to use on boat by fisherman, both in artisanal and industrial fishery practices.

Stunning conditions

For stunning experiments, a sedated animal was positioned in the plastic tube constructed for the purpose of these experiments. This was designed as a 60 cm PVC tube closed at the two extremities with plexiglass plates including stainless-steel electrodes (3 cm width) screwed on

both plates. Two 'filters' were positioned inside the tube in order to avoid direct contact of the animal with the electrodes.

Efficacy of the electrical stunning was tested as follows. One electrode is placed on each extremity of the tube, Antero-Posterior to the animal. Because both electrodes are covered by seawater, this condition was named **wet**-condition. In other circumstances, **semi-wet: 'head-to-mantle**' (one electrode on one extremity of the tube, posterior to the animal, and the second electrode coming from above and in contact with the head of the animal, in correspondence of central brain) or **Semi-wet: 'head-to-body'** (two electrodes A-P to the animal and one in contact with the head) have been utilized.

Please note that the great majority of the results illustrated in this Thesis refer to "wet" condition. In all these configurations the animals were positioned in the tube and almost totally immersed in seawater, except for the dorsal side of the head, allowing implantation of electrodes and avoiding short circuit between the recording electrodes. Changing the position of the stunning electrodes was thought to be useful to understand how current flow through different regions of the animal's body affects the stunning of the animal.

All the data analyzed and reported in the Result section, refer to animals tested under Wet condition. Experiments under Semi wet sub conditions, 'head-to-mantle' and 'head-to-body', where excluded from the main analysis.

Figure S.7. Animal preparation during stunning experiments, in condition of 'Wet' (previous page) and semi-wet stunning (lower image).

Electrical parameters

Electrical parameters tested during the experiments were selected taking into consideration various approaches from the literature. As there is no evidence on the use of electrical stunning in octopus, and cephalopods in general, different approaches were used to find the optimal parameters essentials for an effective and instantaneous stunning.

I followed an erratic approach taking in consideration studies on fish, which were confirmed by some attempts on cuttlefish (data not shown). Attempts in octopus resulted to be not efficient (see main text for details). In general, setting up of electrical parameters followed two main paths: ***i.*** increase of frequency (Hz) and/or voltage (V) in order to achieve a maximal stunning effect, as in the case of some fish species; ***ii.*** inspiration by studies of Maldonado (1968; 1969) in octopus, where electroconvulsive shocks revealed their efficacy in inducing a sort of temporary electronarcosis.

It is important to consider that evidence available nowadays show that every species has own effective electrical parameters for stunning, mainly due to their differences in biological and physiological characteristics.

Experimental procedures - generalities

Animals were transferred in sedation mix after induction and observed for a number of behavioral and physiological parameters assessed periodically (see also 'Sedation'). After sedative induction *O. vulgaris* was positioned in a plastic tube containing SW, leaving the dorsal side of the head outside the water to allow the implantation of electrodes for electrophysiological recordings.

For the detection of changes in brain activity that may indicate a stun induced by the electroshock, the minimally invasive electrophysiological recording method has been used before, during and after stun, to obtain electrophysiological data on neural activities corresponding to the different status of the animals. Ag/AgCl hook electrodes were implanted under the skin in correspondence of both optic lobes and SEM to record spontaneous and evoked electrical activities from the central nervous system during the entire procedure. Furthermore, indicators for behavior and appearance were noted during all the phases of the stunning experiment to underline possible correlation between behavioral/apparent patterns and differences in the CNS' electrical signals caused by electric shock.

VEPs were elicited by visual stimulation as described in section 'Ag/AgCl wire electrodes' (K=5 consecutive pulses, duration 0.1s/pulse, inter-pulse interval of 5s; M=Single pulse of 2s). Intensity of light stimuli was generally adjusted for every animal depending on the amplitude of the evoked response recorded, from 0.5 mA to 1.0 mA. Baseline electrical activity and VEPs were recorded just before and after (between 20-40 seconds) electrical stunning.

To prevent damages to the electrophysiology setup, electrodes were disconnected from the amplifier by an electric switch 15 seconds before the delivery of the electric shock and reconnected after 20-40 seconds from the stun. All the stunning experiments were filmed with a camera, to allow later observations on behavioral and physiological reactions of animals before, during and after the electric shock.

After stun, VEPs and spontaneous responses were noted for following analysis to look at changes in patterns and oscillation of electrical signals from octopus brain. Behavioral and physiological indicators were observed and recorded for a maximum of 15 minutes after the stun. At the end of the procedure, electrodes were removed, and the octopus was transferred in the anesthetic bath for anaesthesia overdose (see 'Humane killing').

Sampling from animals

After confirmation of death *O. vulgaris* were positioned on seawater ice bed for dissection to collect morphological data for the animals and collect tissues for other and future investigations.

Samples of nervous tissues were collected for gene expression and HPLC detection of level of neuromodulators. I collected supraoesophageal (SEM) and suboesophageal (SUB) masses, and the two optic lobes. In addition, stellate ganglia were also collected.

Part of the samples were thought to be used for HPLC (High Pressure Liquid Chromatography) analysis, in order to identify selected molecules (biogenic amines, neurotransmitters) which could be responsible for the observed changing in bioelectrical responses after electrical stunning procedure. Another part of the samples was utilized in molecular biology experiments to look at changes in expression of genes (neuromodulation and/or stress) possibly induced by experimental conditions and stunning. In order to optimize samples' use, neural tissues of SEM and SUB were split in half (sagittal section) resulting in left and right masses, and intended to be used in gene expression and HPLC experiments. Each OLs and stellate ganglia were also separated between the two procedures. Left and right sides of the nervous tissues were randomly chosen between the animals, in order to have a representative number of each side for the following analysis.

For the sake of timing the experiments included in this Thesis do not incorporate findings after HPLC experiments; data from gene expression study are only reported as preliminary observations. All samples were stored at -80°C until use for further processing.

Data analysis

Statistical analysis were run using SPSS (PAWS Statistics 18, rel. 18.0.0, 2009; SPSS Inc) according to Zar (1999) and Siegel and Castellan (1988). Whenever required, One-way or Repeated measure ANOVAs followed by Bonferroni multiple comparisons test were used. I also apply Mann–Whitney U test or Wilcoxon signed-rank test for non-parametric statistics.

In the case of multivariate data, a Principal Component Analysis (PCA) followed by hierarchical clustering (Ward's method) was applied (see also main text for details). PCA was utilized to identify patterns of changes and correlations between expression levels of descriptors of *O. vulgaris* states before, during and after stunning. The computational strategy adopted followed Zar (1999), Abdi and Williams (2010). Varimax rotation with Kaiser normalization was applied to obtain a Rotated Component Matrix, and factor scores (regression) calculated. The hierarchical cluster analysis follow Wishart (1987) using Ward's (1963) method. It is considered a very efficient method even though it tends to produce clusters of small size (Ward, 1963; Wishart, 1987; Everitt et al., 2001).

Whenever possible, basic descriptive statistical functions implemented in Spike2 (CED) were utilized (e.g., waveform analyses, averaging, power spectra and correlations; event analyses; data features, triggers and features in evoked, spontaneous activity; time points, data values, curve area, slope, max/minimum, mean, modulus and peak to peak; time and amplitude measurements).

In other instances, standard digital signal processing methods were executed with customer-made MATLAB algorithms (courtesy of Dr Weimin Zheng, Naval Health Research Center, San Diego, USA). Grand average ERP (event related potentials) plotting and statistics have been applied also following Delorme et al. (2015).

For both resting state EEG and light (visual)-evoked potentials (VEPs), the signals were cleaned by removing artifacts originated from power-line noises with a notch-filter centred at 50 Hz (49 to 51Hz stop-band, Order 4 IIR Butter filter). The EEG signals were then bandpass (1 to 100Hz) filtered to remove high-frequency components (Order 4 Butter filter, Zero-phase shift, MATLAB filtfilt function) outside the normal EEG frequency range. After cleaning, the resting state EEG signals were epoched to light-off and light-on segments. The averaged EEG amplitude and spectral band powers [delta (0.5–4 Hz), theta (4–8 Hz), alpha (8–12 Hz), beta (12–30 Hz), gamma (30–100 Hz)] were calculated and compared between light-off and light-on conditions.

The VEPs were epoched into segments of 1.5 seconds (0.5 seconds before and 1.5 seconds after light pulse onset). The peak amplitude and peak latency of the grand average VEP waveforms across animals evoked by each light pulse of the light pulse trains (0.1 second pulse duration and 5.o second inter-pulse interval) were measured. Clusters of VEP waveforms were visually

identified in the VEP peak time and peak amplitude space and verified with k-mean cluster algorithms. The same analysis on VEPs were performed in both before and after electrically stunning the animals.

Bar charts or box and whisker plots were utilized to plot data. I utilized SPSS graphical output standards or any other graphical software. In the cases of box and whisker plots, boxes represented the interquartile range, 25th-75th percentiles, bars within boxes the median values, whiskers the 10th and 90th percentiles, and outliers are marked.

Statistical differences were considered significant at $p < 0.05$.

Key resources

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Brain -Supraoesophageal mass, Suboesophageal mass, and Optic lobe - and Stellate ganglia	*O.vulgaris* collected from the Bay of Naples (Mediterranean Sea, Italy)	N = 18
Chemicals, peptides, and recombinant proteins		
Magnesium chloride, $MgCl_26H_2O$	Sigma-Aldrich, Merck	CAS No.: 7791-18-6
Ethanol 96°	Carlo Erba	Product code: 414632 CASNo: 64-17-5
RNAlater solution[2]	Invitrogen- Thermo Fisher Scientific	AM7021
PureLink™ RNA Mini Kit[2]	Invitrogen - Thermo Fisher Scientific	12183018A
Turbo DNA-free kit[2]	Thermo Fisher Scientific	AM1907
kit High Capacity cDNA Reverse Transcription[2]	Invitrogen	4368814
O'RangeRuler 20 bp DNA Ladder, ready-to-use[2]	Thermo Fisher Scientific	SM1323
GeneRuler 100 bp DNA[2]	Thermo Fisher Scientific	SM0241
Power Track SYBR Green Master Mix[2]	Applied biosystem, Thermo Fisher Scientific	A46109
Experimental models: Organisms/strains		
O. vulgaris pallial nerves recording	N=9 (4M and 5F) weight range = 280 – 568 g DML mean = 118,5 mm	
O. vulgaris brachial nerves recording	N=10 (4M and 6F) weight range = 326 – 564 g DML mean = 116,6 mm	
O. vulgaris tungsten electrodes (brain) recording	N=8 (5M and 3F) weight range = 340-520 g DML mean = 117 mm	
O.vulgaris plate electrodes, NOT stunning	N=16 (8M and 8F) weight range=248-1556 g Weight mean=575 g DML mean = 118 mm	A.1., A.2., A.5, A.6., A.7., A8., A.9., A. 10., A.22., A.25., A.26., A.27., A.28., A. 29., A.30., A.31.

[2] Gene expression experiments have been included as part of this PhD project but not referred in this Thesis for a matter of timing

REAGENT or RESOURCE	SOURCE	IDENTIFIER
O.vulgaris plate electrodes and stunning	N= 39 (14M and 25F) weight range=140-1070 g Weight mean = 497g DML mean = 108 mm	A.3., A.4., A.11., A.12., A.13, A.14, A.15, A.16., A.17., A.18., A.19., A.20., A.21., A.23., A.24., A.32., A.33., A.34., A.35., A.36., A.37., A.38., A.39., A.40., A.41., A.42., A.43., A.44., A.45., A.46., a.47., A.47.,A.49., A.50., A.51., A.52., A.53., A.54., A.55.
O.vulgaris wire electrodes, NOT stunning	N= 8 (5M and 3F) weight range=212-688 g weight mean = 489 g DML mean = 127 mm	A.56., A.57., A.63., A.64., A.65, A.69., A.70., A.71.
O.vulgaris wire electrodes and stunning	N= 24 (10M and 14F) weight range=208- 116g weight mean = 521 g DML mean = 123 mm	A.58., A.59., A.60., A.61., A.62., A.66., A.67., A.68., A.72., A.73., A.74., A.75., A.76., A.77., A.78., A.79., A.80., A.81., A.82., A.83., A.84., A.85., A.86., A.87
Oligonucleotides		
Primers see Gene Expression	Gene expression experiments have been included as part of this PhD project but not referred in this Thesis for a matter of timing	NA
Software and algorithms		
Spike2 v8.1 software	Cambridge Electronic Design limited	
Spike2 v10.1 software	Cambridge Electronic Design limited	
Matlab R2023a	MathWorks	
Jamovi 2.6.13		
Other		
Differential preamplifier	Elektroniklabor, Zoologie, University of Cologne	MA 103
4-channel amplifier/signal conditioner	Elektroniklabor, Zoologie, University of Cologne	MA102
Data acquisition unit	Cambridge Electronic Design limited	Micro 1401-3
Electrical Cabinet for Stunning (fish) 10Hz-5kHz, 620 Vpp Max, 8App Max)	Optimar AS Norway	HSGG8537
Industrial ScopeMeter® Hand-Held Oscilloscope (20 MHz)	Fluke	123B

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Power Signal Generator for electrical stunning	Neuroscience electronic Lab., University of Cologne	510
Micropipette Puller Sutter	Sutter Instrument Co.	P-97
SimpliAmpTM Thermal Cycler[3]	Applied Biosystems - Thermo Fisher Scientific	A24812
Electrophoresis Power Supply[3]	Biorad	PowerPac 300
QuantStudio™ 5 Real-Time PCR System for Human Identification, 96-well, 0.2 mL[3]	Thermo Fisher Scientific	A34322
USB WEBCAM, 1080P HD, 30FPS	Microsoft LifeCam Studio	
Cree® 5-mm Round LED, λ: 465-480 nm	Cree LED	
Teflon-coated silver wire electrodes, 0.38 mm diameter	World Precision Instruments	AGT1510
Von Frey Filaments	Bioseb	BIO-VF-M
Borosilicate Standard Wall with Filament	Clark Electromedical Instruments	GC100F-15
Medi-trace 200 Series Foam Electrodes, 8mm	Kendall™	F5327
Filters Millipore 0.22 um for syringe	TPP Techno Plastic Products AG	99722

[3] Gene expression experiments have been included as part of this PhD project but not referred in this Thesis for a matter of timing

Reporting and considerations around sampling from the wild animals

During the path of this PhD project, I collected a lot of data and notes and only a part is effectively included in this Thesis; most of the notes, serve as identifying problems, technical information and so on. Nevertheless, I tried in this work to consider all possible data and information that maybe traced eventually as a potential source of sampling bias, even more relevant considering that I am based on wildlife species. Other than "STAR Methods: Structured, Transparent, Accessible Reporting" approach I also have been guided by other recommendations in collating data and results for these experiments. As mentioned, in this Thesis I reported only data in support of the description of the study I am presenting as outcome of my PhD project. However, it is intention from us to collate the entire set of data and to make them available after the final publication of the results of this Thesis.

I also considered as guidance:

i. the STRANGE framework providing indication to avoid/limiting sampling bias and considering issues during the design, conduct, reporting and interpretation of studies.

ii. ARRIVE Guidelines (and checklist) that should be considered always when reporting (i.e. at the basis for scientific publications) data collected from observations and/or experiments on live animals (Kilkenny et al., 2010; Percie du Sert et al., 2020).

In laboratory animal science – a field relevant to cephalopods at least since the inclusion of these invertebrates in a regulatory framework (Directive 2010/63/EU), but possibly even earlier - and in general for any instances of reporting studies including live animals, resources such as the ARRIVE and PREPARE (Smith et al., 2018) are instrumental guidance documents helping researchers in planning (ARRIVE and PREPARE) and transparently reporting (ARRIVE) animal studies. Many leading scientific journals recommend to follow the ARRIVE guidelines for reporting.

A clear and transparent reporting of study design and its implementation offer the advantage to others to being able to replicate (or adapt the methods) thus to and evaluate the reliability of the findings. Despite the fact that I did not use animals that originate from laboratory establishments, we follow similar principles, considering the importance of transparency in wildlife research

reporting. Comprehensive and open reporting in wildlife studies also enhances our understanding of how variations in study design may influence animal behavior, welfare etc. This knowledge is nowadays considered instrumental to refine future research, improving both animal welfare and data quality.

The ARRIVE guidelines specify the critical information that should be included in manuscripts describing animal research. Compliance with these guidelines does not mandate specific procedures or study designs but instead requires sufficient detail about the methods and designs employed. Although the examples in the ARRIVE explanation and elaboration document are primarily focused on preclinical research, most of the guidelines apply broadly to all areas of animal research. Key considerations include sample size, inclusion and exclusion criteria, randomization, and blinding.

Here I attempted to follow these principles, as much as possible.

The animals utilized in this study are formally (and in practical terms) from wild and not laboratory-animals *per se*. Sampling of them has been based on a clear and continuous trust between experimenters and local artisanal fishermen. We attempted to select animals only from fishermen that realized that these animals – captured for human consumption – will end their life within few hours and for different purposes. According to current regulations, projects involving wildlife may require a license/ethical clearance under the provisions of the various rules, legislation, and national and worldwide recommendations. This study obtained licenses for sampling from the wild and ethical clearance for the use of wild animals for research purposes, are required by current legislations.

In addition, all people - and other external parties - undertaking project that involve taking animals and tissue samples from were requested to satisfy the competency requirements. This is to ensure that personnel involved have the necessary knowledge and experience to minimize the potential impacts of handling and sampling on the welfare of the animals.

Considerations around the welfare assessment of animals

Minimizing animal suffering through the refinement of procedures is a key aspect of scientific practice. It is not only essential for ethical reasons but also mandated by legislation in certain countries. To effectively reduce animal suffering, humane endpoint should be identified *a priori,* and the indicators utilized to promptly apply appropriate interventions, such as administering analgesia, revising procedures, or humanely euthanizing the animal.

In application of the principle of the 3Rs, effective refinement relies on accurately assessing animal welfare and detecting signs of pain or distress as quickly as possible to mitigate suffering. While some indicators of animal distress are relatively straightforward to recognize and evaluate, in many instances research is still inadequate; welfare assessment should be based on judgments and opinions and be applied through interaction of competent people.

In this study I utilized as guidance documents introduced via an active collaboration between some of my mentors, CephRes-ETS and FELASA (Fiorito et al., 2014; Fiorito et al., 2015b) and an internal policy has been applied.

Despite the fact that electrical stunning created “important” effects on the animals, we attempted to limit their further suffering, extending experiments within about 30 min after the first stun and always evaluating for further reactions that may provide signs of extended pain, suffering, and lasting harm. We accurately monitored animals and applied humane end-point whenever the case.

This approach was followed in all circumstances.

In considering the internal policy I have been educated, also via FELASA course and by my previous experience with the Association Cephalopod Research-ETS, to welfare assessment for each animal I utilized. In applying it we followed the following general principles: **A team approach** (sharing experiences and information about levels of harms provided by the welfare assessment is useful in developing and implementing refinements, as well as informing the harm/benefit judgements that ethics committees may advice), **Selection of appropriate welfare indicators** (in this study I included and noted a series of variables/parameters along the entire

duration of my experiments), **Full recognition of all potential adverse effects from all sources, Consistency for all species**.

A baseline standard of good welfare should first be defined/identified to act as the point of reference for the species to be used in the study. A more useful reference point for the welfare assessment protocol is to define a hypothetical 'ideal' level of welfare. This can be defined as: the state of being in animals when their nutritional, environmental, health, behavioral and mental needs are met (consider five freedoms principle). Following Hawkins et al. (2021; for reference see Fiorito et al., 2015) there are three key components to be considered, as schematized below:

Component	Characteristics	Examples of indicators
Physical state	Good level of physiological fitness, with no physical disabilities that either cause discomfort or pain, or that have an impact on physical function that could cause distress	Indicators relating to the observable physical condition of the animal, e.g., body weight, state of the coat/skin integrity, posture, lameness and excessive attention to surgical sites/wounds
Physiological, biochemical state	Levels of stress and distress do not exceed those that would occur during the course of normal life/experience of the animal. fIf parameters such as heart/respiratory rate or blood pressure were to be measured; they would not be expected to indicate significant stress.	Physiological parameters such as heart/respiratory rate, levels of stress hormones such as corticosteroids or genes involved in the stress pathway
Psychological state	The animal displays an 'appropriate' range of behaviors, according to what is known about the species (and strain, if relevant)	Changes in behavior such as increased aggression, withdrawal, stereotypies, etc

It is preferable to use a combination of indicators from each of the categories listed in the table above to overcome difficulties with interpretation and to provide a more detailed and complete picture of an animal's welfare.

I have been educated to always control for more than one state of the animal. Independently for some experiments where temporary discomfort was applied to animals, I always provided with the higher attention to animal welfare, within the limitations imposed by this study. No additional unnecessary suffering was induced to animals for the sake of this project. As mentioned, **all the procedures applied in this study belongs to non-recovery classification of severity**.

In January 2013 the Directive 2010/63/EU came into force in Member States of the European Union, regulating the use of animals for scientific research and educational purposes. At the date, all 28 EU Member States have transposed the Directive into national legislation. Among several innovations in this revision of EU legislation on the use of "laboratory animals," the most remarkable change is arguably the inclusion for the first time of a taxon of invertebrates in the list of regulated species.

All "live cephalopods" (e.g., nautiloids, cuttlefish, squid, and octopus) were for the first time given equal footing with all vertebrates. From hatching to death, all species belonging to the class Cephalopoda are the sole representatives of the invertebrates that (till now) are included in the Directive. Despite being limited in number – when compared to numerosity of fish species -, with about 800 species described to date, cephalopods are an astonishing example of diversity of forms and functions, even if the taxon has a lifestyle linked exclusively to marine environment.

Despite some remarkable analogies (e.g., Packard, 1972; Shigeno et al., 2018; Edelman & Seth, 2009; Albertin & Simakov, 2020), cephalopods belong to the clade of Protostomia (Lophotrochozoa) – thus very distant from vertebrates – and evolved behavioural repertoire and morphological complexities unparalleled among invertebrates.

What does this mean in this context? It means that for the first time an entire class of animals (i.e. Cephalopoda) has been specifically regulated and, as outcome, it is for the first time ever that a class of invertebrates is regulated. This also implies that in all EU Member States, procedures exceeding the threshold for induction of pain, suffering, distress, or lasting harm carried out on cephalopods are regulated in an identical way to any vertebrate "laboratory"

species. It is noteworthy to recall that according to the Directive 2010/63/EU, the threshold is indicated as “any procedure which may cause pain, suffering, distress or lasting harm equivalent to, or higher than that caused by the insertion of a hypodermic needle in accordance with good veterinary practice.”

It should be noted that this covers both invasive and non-invasive procedures, and therefore studies involving behavioural interventions may reach this threshold. Finally, considerations about “severity” and humane end points in procedures should be taken into appropriate account when planning experiments with living cephalopods.

This is a paramount change in the consideration of cephalopods and invertebrates in any research context. The application of a regulatory framework to cephalopods corresponds to a significant turning point in policies requiring accountability from those investigating cephalopod biology, including aspects of aquaculture research (reviewed in Di Cristina et al., 2015). Complying with the legislation provides a challenge for anyone working on these molluscan species. It also impacts on those working outside the EU as, for example, scientific journals and funding agencies are adopting some of the principles of the Directive 2010/63/EU, including the application of the “3Rs” (Replacement, Reduction, Refinement) to cephalopod research (Fiorito et al. 2014; 2015).

Furthermore, there are policies that also affect cephalopod “world”, such as: ***i.*** the revision of the current EU Common Fisheries Policies; ***ii.*** the inclusion of several species into the revision of the IUCN Red List; **iii.** the explicit mention of these animals in the Marine Strategy Framework Directive; ***iv.*** the requirement (even not technically applicable to the taxon) that animals should be stun before death for human consumption.

Fiorito et al. (2015) provide a guidance list of welfare indicators and suggest a gradation of responses (see table 5 in Fiorito et al., 2015) that may be useful to assess the impact of captive holding and procedures on cephalopod species in a research context. I utilized this guidance in my study. In particular, I attempted to always monitor body patterning (as in the extreme conditions it may reveal signs of discomfort), respiration, any postural and locomotory reactions that maybe indicators of PSDLH (Fiorito et al., 2015a; Ponte et al., 2022).

Welfare considerations for cephalopods is not strictly linked to a scientific research context but it is also related to fishery and aquaculture. While cephalopods are still not a volume for

aquaculture, they are an important fishery resource, commanding a significant effort, particularly in areas where they are a local traditional marine product (either for local consumption or shipment to other markets). This means that a disproportionately large number of animals are killed in fisheries. Traditional killing methods such as the destruction of the individual's brain are possibly considered relatively benign to the animals, provided they are performed by skilled individuals, but higher volume enterprises tend to deal with groups rather than individual animals, with a potentially deleterious side effect for animal welfare. Putting these animals together in large buckets and let them to die after long asphyxia is the general praxis. It is noteworthy to remind that a recent initiative has been taken under the auspices of FELASA (Federation for Laboratory Animal Science Associations) and of a non-for-profit international organization, to educate fishers, thus to improve not only quality of samples collected for scientific purposes, but also quality of flesh as food for humans (Sykes et al., 2023). This culture of care it is not intended exclusively to decrease animal stress but fostering a grow of an attitude for the economical, rather than the emotional, perspective.

In scientific research, the 3Rs principles are now an integral part of the legal regulatory framework and should be considered not only for the use of vertebrates; here have been adopted to cephalopod molluscs in a scientific research context. As reiterated above, this is linked to any 'procedure' with the potential to cause pain, suffering, distress or lasting harm. Another required consideration relates to the likely benefits of the research, to humans, animals or the environment: these should be weighed against the likely harms to the animals involved.

The 3Rs and harm/benefit assessment are therefore relevant also to wildlife research that is not directly regulated by national and EU legislation, but which, nonetheless, has the potential to compromise the welfare of the study or non-study species. The 3Rs should always be considered as part of the design and conduct of wildlife studies (e.g., Zemanova, 2020).

Guidance for scientists working with wild animals should always assess potential sources of harm to study and non-study species and how these will be eliminated or minimized should form part of all wildlife research proposals and practice. Any negative impact on animal welfare can be reduced by careful planning, robust experimental design and choosing the least invasive approach for the species in question. Considerations should be given to (possibly relevant to this

PhD): Capture/trapping, Handling and restraint, Attaching/implanting tracking devices, Surgical interventions of any sort, Sample collection, Methods for measuring body weight, respiration rate, heart rate, etc., Euthanasia, Transport, Frequency and duration of human observation.

As much as possible I applied 3Rs to this study. In my experiments octopus did not show any escape reaction, or sign of discomfort during experiments (data not shown). In all cases, information about the specimen, its appearance and behaviour were noted; body weight, sex and basic morphological variables were registered for every animal at the end of the experiments, after humane killing.

Supplementary Info - Sedation

All the animals involved in this PhD project were kept under sedation state during the entire experimental procedure. Animals were humanely killed (for details, see Supplementary Info: STAR Methods) at the end of the experiment.

In achieving this approach I followed recommendations by Drs G. Ponte and G. Fiorito based on previous works and unpublished studies (e.g., Sirabella, 2006; Grimaldi et al., 2007; Pagano et al., 2011; Valentino, 2011; Andrews et al., 2013; Gleadall, 2013; Lopes et al., 2017; Pugliese, 2017).

Despite some recent attempts (Mooney et al., 2010; Polese et al., 2014; Butler-Struben et al., 2018; Roumbedakis et al., 2020; Sprecher et al., 2022; Di Cosmo et al., 2023), a systematic research is still required for the development of methods to sedate and anaesthetize cephalopods. Classic studies (Lo Bianco, 1909; Grimpe, 1928; Young, 1971) provided guidance on the parameters to be utilized to achieve some sort of surgical anaesthesia and distinguish from various levels/criteria. A description of these attempts is already available in Grimpe (1928; for a translation and review see De Sio et al., 2020), and a summary of events occurring during anaesthesia with octopus, for example, are found in the classic contribution by Young (1971).

General anaesthesia is required to perform surgical or investigative procedures including those required for veterinary and research purposes. In the majority of vertebrate species, techniques for general anaesthesia are well-developed, with physiological and cellular effects of the agents used for inducing anaesthesia are relatively well-understood. However, this requires a species-specific approach as expected by the diversity in physiology when considering taxa (for fish e.g., Sneddon, 2012; Readman et al., 2017). The potential anaesthetic properties of over various tens of agents have been explored in cephalopods (for review see for example: Fiorito et al., 2015), however few studies regarding the physiological effects of these agents on the animals are available and/or complete. For example, neurophysiological responses to anaesthesia have been explored by Mooney et al. (2010) in squids, and Butler-Struben et al. (2018), in cuttlefish and octopus; these are the sole studies to have attempted to correlate neural signals during anaesthesia in cephalopods, to the best of my knowledge.

As mentioned in various instances, the inclusion of cephalopods in Directive 2010/63/EU regulating the use of animals in scientific research has prompted reconsideration of the criteria for general anaesthesia in cephalopods (see also main text).

Until the early 1960s urethane (i.e., ethyl carbamate) was the most widely used anaesthetic agent for surgery (e.g., brain lesions) in octopus and other cephalopods (Young, 1971; Gleadall, 2013). The substance was abandoned because of its elevated carcinogenicity.

As a substitute ethanol and magnesium chloride have been utilized in various formulations to anaesthetize species representative of the three major orders of cephalopods (review in Fiorito et al., 2015).

The criteria commonly used for assessing general anaesthesia in cephalopods (Valentino, 2011; Andrews et al., 2013; Gleadall, 2013; Butler-Struben et al., 2018) have been identified in: ***a.*** skin pallor (mantle and arms), ***b.*** loss of texture, ***c.*** loss of arm muscle tone and sucker adhesiveness, ***d.*** loss of the righting reflex, ***e.*** absence of a response to a noxious mechanical stimulus applied to the arms or mantle, and **f.** marked suppression of ventilation.

Furthermore, under anaesthesia the systemic heart rate is reported as very low (using $MgCl_2$) or slow in *O. vulgaris* anesthetized with a mixture of magnesium chloride and ethanol (Grimaldi et al., 2013). It is worth to report here that according to J.Z. Young, the «heartbeat stops» (using urethane as an anaesthetic agent), thus allowing surgery intervention «… simpler because of absence of bleeding, but limits its duration to 15–20 min» (Young, 1971, p. 642).

Marked suppression of heart rate is associated with a fall in cardiac output and blood pressure, reduced tissue perfusion and ischemia, with the brain likely to be particularly vulnerable.

A profound fall in blood pressure may contribute to central nervous system depression including suppression of ventilation driven from the posterior sub-oesophageal lobes further exacerbating the ischaemia. Cardiovascular effects of anaesthetic agents could contribute to loss of consciousness, by significantly reduce brain perfusion (Pugliese et al., 2016; Pugliese, 2017).

For my experiments, I applied conditions under which *O. vulgaris* and *S. officinalis* (data not shown for cuttlefish) are kept[4] to a status of sedation that did not impair physiological states of the animals.

After Gleadall (2013) we adopted the following definitions and criteria.

Light sedation (*stage 1*): a status during which mantle contractions linked to breathing are depressed from normal. Visual response impaired (failed or dulled reaction to a moving shape and/or pupil reaction to light).

Deep sedation (*stage 2*): pinching the skin above the eyes produces no reaction in the animal.

In the above definitions, stages of anaesthesia are identified following McFarland and Klontz (1969) in fishes and adapted to cephalopods by Andrews et al. (2013) and Gleadall (2013). It is noteworthy to mention that decreased breathing rate is noted by Gleadall (2013, see table 1) to occur only in cases of deep sedation. As mentioned, cessation of mantle contractions (breathing rate) is a clear sign of anaesthesia but its occurrence in relation to other measures of anaesthesia shows wide individual variation (Young, 1971; see also Valentino, 2011).

In my experimental conditions, animals were first immersed in a 3 L mixture of 1.1% $MgCl_2\,6H_2O$ (M=203,3 g/mol; Sigma-Aldrich, Germany) and 1% EtOH 96% (Carlo Erba reagents) in seawater (hereunder and in the Thesis generically referred as 'mix').
Initial exposure (induction) was limited to 25 min, a time considered sufficient to reduce animals' responsiveness in a significant way. At this stage, animals were moved to the electrophysiological setup and the experiments started after less than 10 minutes. Animals were kept always partially covered by the anaesthetic solution in seawater (only the head was kept out of water to prevent short circuit between electrodes). Under these circumstances main physiological responses of the animals remained adequate for the purposes of these experiments, as shown by previous studies (Shomrat et al., 2008; Pagano et al., 2011).

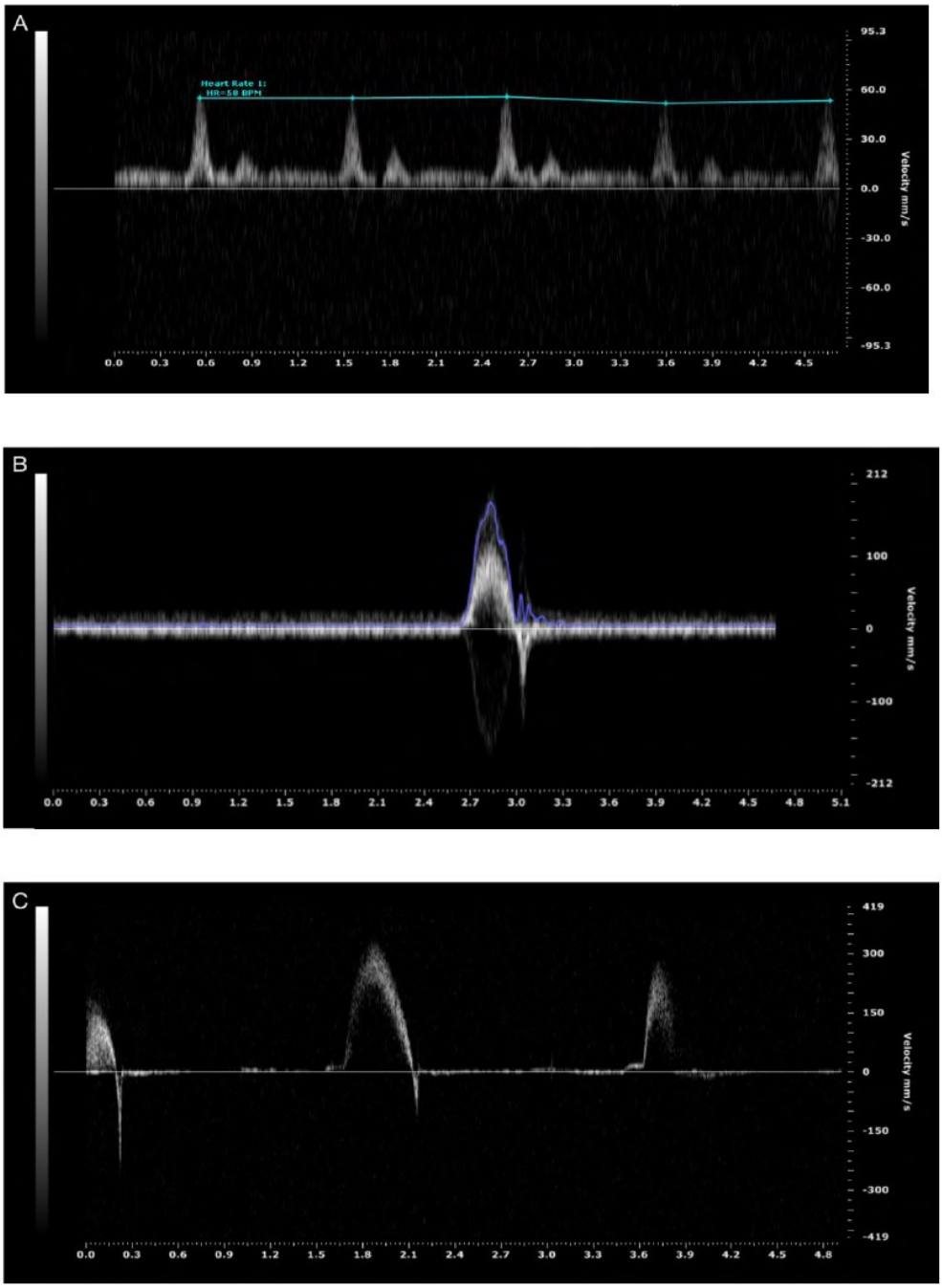
After the initial phase the experiments continued and I progressively reduced the concentration of the mix by diluting it with seawater. The initial dilution was generally applied at around 15 min

[4] After initial anaesthesia utilized to prepare the animal to the acquisition of neural signals (e.g., implanting electrodes)

from the starting of the experiments (in the electrophysiological setup). By 20-25 min from the beginning of the experiment I estimated that at least 40% of the mix solution was diluted to about 50% of the original concentration. This also allow to keep the seawater in the tube (or basin) holding the animal oxygenated.

In my experimental conditions sedated *O. vulgaris* continued to show breathing rates comparatively uniform and stable (BR: range 2-20 min^{-1}; Mean ± SEM = 8.5 ± 3.4 mantle contractions ^{x} min^{-1}). Any changes of breathing rates have been noted and referred in the main text whenever relevant. The above breathing performance of *O. vulgaris* appear in line with what reported in a longitudinal behavioural study with a larger sample of live octopuses (about 10 mantle contractions/min for days; see Borrelli, 2007).

Furthermore, in a subset of animals utilized only to assess physiological parameters/states under these experimental conditions, ultrasonography was used to assess respiration and cardiovascular performance of octopus. Following Pugliese (2017) I applied ultrasound and echocardiography principles to monitor *in vivo* the heart in *Octopus vulgaris*. The cardiovascular performance of animals was accessed using the Vevo3100 Imaging System (VisualSonics, The Netherlands & Canada). Measurements were performed during induction and in a sedation state using mix solution ($MgCl_2$ alone at the recommended concentration for humane killing was used as comparison). Sonographic images of live animals were obtained using hand-hold VEVO transducers directly immersed in seawater solution. Transducers were kept by hand at about 2 cm distance from the octopus mantle and positioned perpendicularly to its body. Sonographic examination allowed to reveal the systemic and branchial hearts in Colour Doppler and pulsed-wave Doppler (PWD) modes, for anatomical identification and blood flow quantification as originally shown by Pugliese (2017). The sonographic examination we performed allowed us to assess octopus' heart rate. In our conditions, the heart rate resulted to be about 60 beats per minute (bpm; Supplementary Fig. A2-1), with a reduction in the octopuses' heart rate observed after the first minutes of anaesthesia, with both magnesium chloride and mix. In the latter case, the heart rate appeared reduced to 12bpm after 10 minutes (Supplementary Fig. A2-1B).



Supplementary Figure A2-1. Changes in heart rate of *Octopus vulgaris* in response to anaesthesia. **A**: Representative pulsed-wave Doppler (PWD) signals acquired from an un-anaesthetized octopus; **B:** PWD from an octopus anaesthetized with 1.12% magnesium chloride and 1.0% ethanol (Mix) after 10 minutes, and (**C**) from an octopus recovering in sea water (5 min) after anaesthesia with 3.5% magnesium chloride (original induction 25 min). Courtesy of Association Cephalopod Research-ETS.

Magnesium chloride and Ethanol, used separately and in higher concentration, are considered to be the best anaesthetic agents for cephalopod species nowadays, as different studies report no aversive reactions, achievement of stable anaesthetic level and a pretty fast recovery without

trauma to the animals (Messenger et al., 1985; Fiorito et al., 2015a). Here we adopted the use of a mix solution and kept the animal sedated with lower concentration of each agent, to reach a stable light sedation, as defined above and by Gleadall (2013), which is considered to avoid (or limit as much as possible) pain, suffering or distress to the animal during the entire duration of the experiments.

Animals monitoring during the experimental procedures was an important achievement during this PhD and allowed to promptly intervene if any recovery from sedation or adverse reactions were recognized, thus to prevent suffering exceeding the threshold set.

Considerations around animal sentience and slaughtering

The existence of sentience (and primary consciousness) is a requisite for considering the selected organism to require an adequate attention at least when dealing with it for scientific purposes. It is noteworthy to remind that – according to the legislation implemented in European countries, such an attention should be given to animals that are also used for human consumption. We refer here to Council Regulation (EC) 1099/2009 on the protection of animals at the time of killing, stating that "Killing animals may induce pain, distress, fear or other forms of suffering to the animals even under the best available technical conditions. […] Business operators or any person involved in the killing of animals should take the necessary measures to avoid pain and minimise the distress and suffering of animals during the slaughtering or killing process, …". According to the European legislation on the slaughter of farmed species (EC Reg. 1099/2009) and the recommendations of the WOAH and EFSA, animals must be subjected to a stunning process before being slaughtered to become unconscious and insensible. In the most cases for fish (and cephalopods) slaughter is practiced via asphyxia in air or the thermal shock method, i.e. putting animals on some ice. These procedures are considered non-humane methods of slaughtering by WOAH and EFSA. For example, the thermal shock does not stun the animal but produces only apparent sedation, leading the conscious animal to death by asphyxia. In other circumstances slaughter is achieved via electrical narcosis and the percussion method, followed by gill-cutting (for fish, or brain centers destruction) which, if carried out correctly, can induce a state of instantaneous unconsciousness before slaughtering (EFSA, 2004). Specifically, the electrical method induces unconsciousness, but its efficacy can vary considerably according to the parameters set (mainly voltage, current, frequency and time of application) for the given species. The general principle of electric stunning (also referred eventually as electronarcosis) is to pass sufficient current through the brain to cause an epileptic-like status in the animal. This induces an immediate unconsciousness and insensibility to pain. If the current flows for long enough the animal will die of anoxia before the brain is able to recover sensibility. The electric current also causes spasms in the muscles which can, under some circumstances, result in haemorrhages and other carcass damage, as reported in fish. Thus, stunning conditions need to be carefully designed to ensure that the process causes neither pain nor carcass/animal/tissue damage and that recovery before confirmation of death is not possible.

These conditions are known to vary widely between different species.

As reviewed by the Humane Slaughtering Association (HSA), “an electric stun must cause unconsciousness within few seconds of application and the unconsciousness must last long enough to ensure that the animal does not regain consciousness before dying”. In the context of fisheries and aquaculture, electrical stunning systems have been developed which stun animal whilst they remain in water, or other systems where the animal is out of water and the electrodes make direct contact with the animal ('dry' or 'semi-dry' systems). Stunning fish in water reduces the stress of exposure to air and reduces the possible mechanical damage to the skin. The other two approaches are reported to be more effective with minor damage to animals. In some instances, electrical stunning is in combination of a percussive stun (i.e. administering a blow to the skull of the fish, with the animal remaining unconscious until death). For electrical stunning, the systems developed apply DC or AC using electrical parameters set that are considered appropriate for the animals (voltage and frequency). The current effectively passing through the animal depends by the relative conductivity of the body/tissues and seawater. Just as a reminder, water has conductivity in the range 50-700µS/cm (reported between 0 and 1500 µS/cm), while seawater of about 50,000µS/cm (for seawater consider also other factors affecting conductivity; see Tyler et al., 2017).

As for the frequency, it is the parameter that determine the effect of the current on the animal. The electrical current of fishes electrically stunned in water passes around the animal and through the body. Depending on the species, frequencies close to 50Hz have a greater effect on both the animal brain and muscle than higher frequencies, resulting in an effective stun. However, in some species experimental data suggest higher frequencies (e.g., for salmons: Grimsbø et al., 2016) and the decision is based also on the known effects on muscle tissues. For several fish species a pulse at higher frequency induces immediate insensibility whilst also minimizing carcass damage. As reviewed by the HSA, depending on the species a two-stage electrical stunning/killing method is necessary; as reported: an initial stun (electric field = 2.5V/cm, 1000Hz) followed by an electric field of 0.5-1.0V/cm with a frequency of 50Hz (30-60 s) during which the animal is kept till to induce permanent insensibility. The practical advantage of this method is to reduce the power requirements of the stunning machine in comparison with single-stage systems.

As reported in the guidance documents from HSA (Humane Slaughtering Association, UK), it is important to note that fish stunned for a brief period of time can quickly regain full consciousness; it is therefore important that the animals are moved into the low-voltage maintenance stun electric field within a few seconds. The introduction of a pause between the two stages can reduce the effectiveness of the stun but is necessary in continuous flow systems to prevent interference between the two electric fields. A single step stunning has been reported to be efficacy in salmons and few other fish species.

In my experiments with stunning, despite some attempts using dry-stunning approaches (data not shown), I preferred to adopt a 'wet' condition (i.e. most of the animal immersed in seawater in the container, leaving the head region outside for the installation of electrodes for neurophysiological measurements) using a 0.22-3.63V/cm with variable frequencies (see main text and e.g., Figure 27 for details). This may represent an intermediate approach and a condition possibly easy to keep in mind for possibly extending this approach to fishery.

To achieve humane stunning with a two-stage method it is necessary to ensure that: **a.** Insensibility is established rapidly; **b.** an irrecoverable stun is achieved using a low-voltage maintenance stun.

I did not explore such a method in my study.

In my experiments stunning cuttlefish with a single stun resulted in animals stop breathing without any sign of recovery – thus to be considered effective potentially without requiring further method confirming the death.

One of the key aspects of evaluating the welfare of animals slaughtered is to assess their state of unconsciousness or death after the application of the stunning/killing method. The scientific requirement for validating the effectiveness of the stunning method in each species is based on the establishment of unconsciousness or death by EEG, i.e. search for the neural correlates and

evaluate a significant reduction of neural activities after the stunning. This of course if such a validation does not exist for the species of interest. Due to evident practical problems, the evaluation of the reflexes, spontaneous behaviour or response to external stimuli (as recommended by EFSA and WOAH) maybe a valid substitution. Breathing and coordinated movements are the most frequently (and easy) assessed indicators for various species; response to external stimuli and "righting ability" is also assessed in some cases. In a review, the distribution of the use of indicators to assess fish unconsciousness differentiated by the stunning methods has been shown by Clemente et al. (2023). Indicators used by fish-slaughter facilities to assess unconsciousness according to the stunning/killing method used is shown in figure 4 in Clemente et al. (2023). According to Authors methods considered non-humane are still utilized for slaughtering of fish (e.g., sea bass, sea bream). In most cases, however, there is lack of scientific data and validated indicators that do not allow to obtain a clear picture of the welfare condition at slaughter for many species.

Sentience in cephalopods

The inclusion of cephalopods in the Directive 2010/63/EU has been followed by an increased interest for their sentience here considered as the capacity to have feelings (i.e. pain, pleasure, hunger, thirst, warmth, joy, comfort and excitement). It is not simply the capacity to feel pain, but feelings of pain, distress or harm, broadly understood.

Drawing on over 300 scientific studies, Birch et al. (2021) evaluated the evidence of sentience in the cephalopod molluscs and the decapod crustaceans, and the potential welfare implications of current commercial practices involving these animals. The document is presented around a framework for evaluating scientific evidence of sentience, and proposed the assessment around eight criteria: **1.** possession of nociceptors, **2.** possession of integrative brain regions, **3.** connections between nociceptors and integrative brain regions, **4.** responses affected by potential local anaesthetics or analgesics, **5.** motivational trade-offs that show a balancing of threat against opportunity for reward, **6.** flexible self-protective behaviours in response to injury and threat, **7.** associative learning that goes beyond habituation and sensitisation, and **8.** behaviour that shows the animal values local anaesthetics or analgesics when injured.

No single criterion provides conclusive evidence of sentience by itself, it is the combination of evidence matching various criteria that allow recognition of sentience in these animals. Based on these assumptions and the commented evidence found in scientific literature, Authors propose an organization scheme (confidence level of matching a given criterion, VH: very high; H: high, M: medium, L: low, VL: very low; Birch et al., 2021) where different taxa of cephalopods are coded, resulting with octopus likely to achieve the highest level of confidence in the greatest number of criteria. Octopuses match at least four criteria at very high level of confidence, three at a high level, leaving only one classified at medium level of confidence. There is no doubt that particular care should be given to these animals when considering using for research purposes (and maybe in general).

Considerations around condition factor/index and bioimpedance

Condition Index

The relationship growth in length versus growth in weight (**condition index**) is measure of collinearity utilized as an indicator of animal 'health', or better say to show the distribution of animal growth in a population/sample. There us accumulating knowledge that consider the animal growth to length ration dependent on a particular geographic area of origin, gender, seasonal variations, to mention some. The condition index is a simple measurement that can be used to provide important biological information that can then be used to make assumptions and/or management decisions. Length-weight relationships are available for a series of fish species in various contexts (e.g., Lloret et al., 2002). In comparison, relatively few studies have been conducted on cephalopods. A recent study by Önsoy and Salman (2022) revealed isometry in eight species including teuthids, sepiids and octopods, and positive allometry was found in two species of squid; other species showed negative allometry. For cephalopods different body structure and growth type (in respect to fish), possibly differences in length measurement methods applied, different body parts that might have different growth rates, and preservation methods that could affect the body shape and weight in soft bodied animals may have affected the study. To the best of my knowledge, this relationship has been also explored in *O. vulgaris* (Gestal et al., 2002) and related to parasite load, but also to a large scale study in the Bay of Naples (Italy; G. Ponte, personal communication).

I matched my sampling to the database available to OctoLab and found no significant deviances for animals I utilized in my study; a correlation between condition index of my animals and performance (i.e. amplitude or latency in neural recordings) have been not explored; it maybe a further way to explore the quite large interindividual variability I observed in my data (data not shown); a possible consequence in support of the well documented interindividual behavioural variability of this species (Borrelli, 2007; Borrelli et al., 2020; De Luca, 2020; Dissegna et al., 2023) that should be explored.

Bio-Impedance

Following the Glossary available from European Commission, **Bioimpedance** is the response of a living organism to an externally applied electric current. It is a measure of the opposition to the flow of that electric current through the tissues, the opposite of electrical conductivity. Measurement of bioimpedance in humans and animals – also referred to as bioelectrical impedance analysis – has proved useful as a non-invasive method for measuring as blood flow and body composition.

The flow of electricity through an object is known as the current (measured in amps). The driving force (electrical pressure) behind the flow of current is known as the voltage (measured in volts). Voltage may also be referred to as the potential difference. The property of a material that limits current flow is known as resistance (R), measured in ohms. This resistance is called impedance.

Following Ohm's Law: Current (I) = Voltage (V) / Resistance (R). The larger the cross section of the conductor, the lower its resistance. Impedance is the volt-to-current ratio, it can be obtained by reading a voltage when a constant amplitude current is applied. Resistance is how much the resistor opposes and limits the current flow. Reactance includes the capacitator and how well it opposes an AC. Different tissue types have varying resistances to electrical current, and this impacts impedance. Muscle for example is highly conductive, whereas fat is not. The cell membrane behaves differently when exposed to low and high frequencies. At low frequencies, the current will pass around the cell. At high frequencies the current will pass through the cell. Therefore, impedance measurements at low frequencies are measurements of extracellular fluid impedance, and at high frequencies are measurements of both intra and extracellular fluid impedance. Relaxation phenomena dictate the electrical properties of tissue across the total frequency range. There are three different dispersion ranges: alpha, beta, and gamma. In the context of bioimpedance, the alpha and beta ranges are particularly relevant. The alpha dispersion range is at very low frequencies (below 10kHz). This range reflects the polarization of cell membranes and the activity of ion channels, which are critical for nerve stimulation. The beta dispersion range (approximately 10kHz to 100MHz) demonstrates the influence of the intracellular and extracellular spaces on the capacitive charge of cell membranes. This range includes effects from both the cytoplasmic and extracellular matrix components.

Bioimpedance has been considered an important information able to guide in selecting parameters (mostly frequency) for an effective electrical stunning of a given species.

At the beginning of this PhD project, we attempted to measure the impedance of *S. officinalis* and of *O. vulgaris*. Guided by Dr Endre Grimsbø (The Arctic University of Norway, my second Supervisor) we calculated in a series of experiments maximum impedance vs frequency in the two species.

Previous research on salmon resulted to identify an optimal frequency at around 100Hz for this species. However, it is expected that variation in physiology and fat content will have an impact on the optimal frequency and also the electrical impedance. In these studies, under influence of α- and β-dispersion, the impedance decreased with increasing frequency from 100 Hz, and reached 0.078 ± 0.03 kΩ at 1 MHz. Individual variations around peak impedance were observed when the frequency ranged from 70 to 100 Hz. For all fish was a decrease in impedance at 900 Hz, indicating electrical resistance change at this frequency. The impedance of each single fish was independent of the fish size as no significant correlation between maximum impedance and weight were found at 0.5 V (Grimsbø et al., 2016). The maximum average impedance value at 100 Hz (see figure 2 and table 1 in the above-mentioned study) corresponded with the maximum cell membrane capacitance below the α-dispersion frequency range. A rise in the applied current into the intracellular space above 100 Hz are known to correspond with reduced electrical cell membrane impedance. To release the action potential in brain nerve cells, the electro-stunning current is dependent on the voltage difference across the nerve cell membranes, which require a high impedance level.

In the practice, frequencies below 100 Hz have been successfully applied to salmon for effective stunning.

During the experiments carried out with cephalopods (details not shown) impedance vs frequency measurements resulted to account for 31 ± 11 Hz (N = 9, *S. officinalis*). Preliminary measurements in O. vulgaris provided similar values (28 ± 11 Hz); however, maximum impedance vs frequency curves in octopus indicated a very low frequency values corresponding to 10.1 ± 0.3 Hz (N = 10). These data in octopus raised a series of doubts around the effects of the anaesthetic solution (originally tested either after killing in ethanol or magnesium chloride), the possible impact of the electrode configuration (dry-stunning condition) with doubts of the effect of big contact capacitance dominating the measurements.

In my experiments, the choice was to not consider frequency information as derived by these preliminary studies. Further measurements have not been implemented being also highly variable based on equipment availability. Studies of bioimpedance in cephalopods is required in

the future for completeness.

Quality Index

The Quality Index Method (QIM) is a widely recognized approach for assessing fish tissues freshness. It uses a structured sensory grading scale to evaluate freshness and predict the remaining shelf-life for specific species or products. Bioimpedance is increasingly being utilized as a method in this context. However, oversimplified applications of QIM risk undermining its credibility despite its significant potential. The QIM methodology requires a balance between the number of fish samples and the sensory attributes related to spoilage that need to be evaluated. These attributes help determine whether quality standards are met. Nevertheless, the assumptions inherent in the method can affect the accuracy of shelf-life predictions. Addressing the variability linked to assessors, products, and data structuring for statistical analysis could enhance the method's predictive reliability. However, this might add complexity, making it less suitable for industry needs, where simplicity and rapid application are priorities.

The rationale behind is that consumers' demands for foods with high nutritional value or certain specific sensory properties (e.g., appearance) are a result of consciousness of the impact of food products on their health, pleasure, or preference. All seafood products are associated to highly perishable products, mostly the ones that are to be sold as fresh products. Degradation of fish (and cephalopods) products is related to three main post-mortem processes, responsible for their main sensory changes: oxidation, microbial degradation, and autolysis. The knowledge of the evolution of various descriptors and properties associated with the spoilage process, allows the evaluation of optimal condition (after slaughter, if any), as well the estimation of its capability to retain those sets of characteristics through time.

QI are assessed through a series of different measures, including bioimpedance (Yuan et al., 2018; Champion et al., 2020) despite some possible requirements of reliability of methods and data (Hassoun and Karoui, 2017). Some methods have common drawbacks such as being destructive and labour-intensive. Also, no single index can encompass all the complex changes occurring during spoilage. However, they can complement each other and deliver acceptable estimations. Independently of the chosen method and the reasons behind it, sensory evaluations

will remain a key factor since sensory clues are the main parameters. Therefore, any chemical or instrumental analysis that is developed or used, must agree with “sensory” results.

The reason I am mentioning this here is because one possible scenario to use bioimpedance on cephalopod tissues, it will be to assess quality of tissues/freshness after a given stunning or treatment.

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