UNIVERSITY OF NAPLES FEDERICO II

PhD THESIS IN BIOLOGY

OCTOPUS NEUROETHOLOGY (*Octopus vulgaris*): OCTOPUS SENSES & RNA EDITING MECHANISMS

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ABSTRACT

Octopuses, particularly the common octopus (*Octopus vulgaris*), exhibit intriguing sensory capabilities and intricate RNA editing mechanisms. This Ph.D. thesis aims to explore the neuroethology of *O. vulgaris*, specifically focusing on evidence for smell-by-touch behavior and molecular evidence for olfactory receptor genes in arm suckers, as well as photoreceptor genes in the optic lobe. Furthermore, it investigates the RNA editing mechanism of Egr1 (Early Growth Response 1) and its implications for protein diversification in *O. vulgaris*.

The investigation of photoreceptor genes and their localization was conducted using molecular techniques and WMISH in the optic lobe of *O. vulgaris*. Molecular approaches identify the expression and localization of various photoreceptors, including Retina Rhodopsin, Retinochrome, Melanopsin Rhodopsin, Rhabdomeric, and Rhodopsin kinase in the optic lobes. These findings suggest distinct roles for these photoreceptors in light perception and visual processing, contributing to our understanding of cephalopod vision and octopus' vision.

To investigate *O. vulgaris*' sensory systems, including smell-by-touch behavior and olfactory receptor genes, a comprehensive approach has been employed. Molecular techniques and behavioral experiments have been used to examine the evidence supporting the detection of odors through tactile contact by octopus's suckers. The respective genes have been localized using the whole-mount *in-situ* hybridization (WMISH) technique, indicating the presence of a "smell by touch" behavior. The expression of selected G-protein-coupled receptors (GPCRs) in the suckers of octopuses provides direct evidence of the presence of olfactory receptors in contact chemoreception, among other varieties of GPCRs. Behavioral experiments have revealed that octopuses can recognize water-insoluble compounds and distinguish them, showing clear interest. The time spent analyzing the compounds has been quantified using BORIS software.

The process of effectively anesthetizing invertebrates, particularly *O. vulgaris*, during animal experiments presents a significant challenge, with the goal of reducing stress on these animals while minimizing the use of anesthetics. The research explores the limitations of various substances used for anesthesia and underscores the need to achieve muscle relaxation, pain relief, unconsciousness, and amnesia with a single substance. In this study, it is demonstrated that the clinical anesthetic isoflurane (1%), commonly used by veterinarians, can rapidly and efficiently anesthetize octopuses when employed in combination with 1% magnesium chloride, which serves as a muscle relaxant. Recovery is similarly swift, and the entire process, from anesthetization to recovery, is completed in 30 to 35 minutes, thus minimizing animal stress. This research development showcases the effectiveness of this approach in reducing stress on the animals and reducing the quantity of anesthetics required, making a valuable contribution to the field of invertebrate anesthesia.

In addition to sensory systems, the last chapter investigated the RNA editing mechanism of Egr1 in *O. vulgaris*. By employing molecular techniques and bioinformatic analyses, the RNA editing events within the Egr1 gene were examined. RNA editing mechanisms, particularly in the Egr1 gene, were explored in *O. vulgaris*. RNA editing was found to be prevalent in cephalopods, with a high percentage of editing events occurring in the nervous system. The investigation compared A-to-G(I) RNA editing levels in the Egr1 gene between wild and controlled *O. vulgaris*, revealing variations likely due to different environmental conditions and stressors. Although the modifications in the Egr1 gene does not result in changes to the amino acid sequence, further research is needed to understand the timing, duration, and impact of RNA editing in cephalopods. Understanding these specific editing patterns and their functional consequences will shed light on the regulation of neural plasticity and adaptive responses in *O. vulgaris*.

Overall, this Ph.D. thesis provides comprehensive insights into *O. vulgaris*' sensory capabilities and RNA editing mechanisms. The investigation of photoreceptor genes in the optic lobe enhances our understanding of cephalopod vision and its adaptation to visual surroundings. The evidence for smell-by-touch behavior and GPCRs in arm suckers highlights the multi-modal sensory system of octopuses. The use of clinical anesthetics provides a standardized method for efficiently anesthetizing octopuses, reducing the entire process from anesthesia to recovery and minimizing animal stress. Furthermore, the exploration of RNA editing mechanisms and the Egr1 gene sheds light on the regulatory processes underlying neural plasticity in *O. vulgaris*. The findings from this research contribute to our broader understanding of cephalopod neuroethology and have implications for sensory biology, neurobiology, and potential applications in therapeutic strategies and artificial visual systems.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 CEPHALOPODS

Cephalopods are considered an advanced class of molluscs and are categorized into two subclasses namely; nautiloids and coleoids (Boyle, 1986; Amodio et al., 2020). The nautilus belongs to the subclass nautiloids and is reported as the most ancient member in the class. Currently, there are currently fewer than ten species distributed worldwide. Coleoids include octopuses, squid and cuttlefish and nearly there are more than 800 species reported from tropical to polar regions. In addition, they are also known as ravenous predators with a broad range of prey and various hunting strategies (Amodio et al., 2020).

Nevertheless, cephalopods differentiated from other molluscs by having some unique characteristics such as an external shell which use for buoyancy in addition to protection and is absent in coleoids; a mobile funnel that facilitates jet-propulsive movements; and sets of flexible appendages. In addition, they have camera eyes with acute vision, dynamic skin to camouflage and ink sacks to avoid predators. Cognitive sophistication is the most remarkable feature of cephalopods and they have the most advanced nervous system among invertebrates, which provides them with many behavioral activities such as foraging, tool use, avoiding predators and mating activities (Borrelli & Fiorito, 2008; Deryckere & Seuntjens, 2018; Shigeno et al., 2018; Amodio et al., 2020).

1.2 CEPHALOPOD COGNITION

The brain of cephalopods originates from the ancestor of the molluscan nearly 550 million years ago. In addition, brain structure and specialization are considered as indicators of the intelligence of the animal. However, when compared with the cephalopods' and vertebrates' brains anatomies with the novel approaches that revealed specific centers may be functionally

equal to the neural system of vertebrates such as pallium, basal ganglia, midbrain and spinal cord. Mainly, the brain of cephalopods consists of three distinctive parts: the supraesophageal mass, the subesophageal mass, and a pair of optic lobes. The coleoids' brain is arranged centrally around the esophagus of the animal and it is further differentiated into both sub and supraesophageal masses that differentiate into several distinct lobes. The central parts of the brain are considered as most differentiated part and most developed area (Borrelli & Fiorito, 2008; Amodio et al., 2020). The sensory inputs and the motor outputs are directly linked with the subesophageal mass, while the supraesophageal mass acts as an intermediate center linked with regulatory interneurons. The main connection to the central brain with the peripheral network or nerve trunks that help regulate the arms, viscera, and other parts of the animal's body (Shigeno et al., 2018; Amodio et al., 2020). Nevertheless, the nervous system found in the arms of the cephalopod exhibits autonomy behaviour that has the ability to break off and move independently for a short time of periods without interfering with central nervous system (Mouritsen & Styrbæk, 2021).

Therefore, this arrangement provides the cephalopods' brain with higher centralization compared with other mollusks and invertebrates (Borrelli & Fiorito, 2008; Deryckere & Seuntjens, 2018). Besides, frontal and vertical lobes are involved with the tactile and visual cognition of the cephalopods. In addition, the presence of neuronal numbers, relative size, specific cells, reverberating and parallel circuits, and long-term potentiation has indicated the synaptic memory formation of cephalopods brain (Amodio et al., 2020).

The complexity of the cephalopods' brain provides the main reason for their complex behaviors (Deryckere & Seuntjens, 2018). Research has shown that there is evidence of two types of cognitive sophistication that are relatively independent in cephalopods. The first investigation studies general learning mechanisms and memory, while the second investigates the flexibility of behaviors in ecologically relevant situations. Therefore, as both lines of

investigation provide evidence of the behavioral flexibility of these animals, there is an ability to readily switch to alternative strategies for problem-solving. These types of behavioral flexibility are referred to as an index of cognitive sophistication or denoted as intelligence (Amodio et al., 2020).

The large body size of cephalopods does not hinder their learning abilities, as they have demonstrated the capacity to adapt their behavior based on past experiences. Several studies have shown that coleoids possess general learning abilities related to spatial domain, memory-based navigation, and social learning behaviors (Amodio et al., 2020). In addition, cephalopods exhibit remarkable flexibility in their behavior during reproduction and predatory avoidance. Research on the learning mechanisms and memory capabilities of cephalopods provides evidence of their ability to learn and display various adaptive responses in different contexts (Amodio et al., 2020). There is evidence that suggests cephalopods' nervous system has evolved over time. It is believed that they may have started with a simple nervous system similar to the nerve net, and then later developed and expanded to a larger size with adult neurogenesis. This increase in the number of neurons is thought to have facilitated the cognitive capacity and ability of cephalopods to problem-solve. Additionally, the presence of 500 million nerve cells is also believed to be the main reason why cephalopods have more complex behaviors compared to other invertebrates and vertebrates (Deryckere & Seuntjens, 2018).

Most of the studies have conducted experiments including anatomical investigations of the nervous system with histological analysis of brains, which have revealed that the circuit is dedicated to visual and tactile learning activities. Therefore, these findings have proved that the learning and memory of cephalopods were achieved by a “series of matrices of intersecting axes, which find associations between the signals of input events and their consequences” (Borrelli & Fiorito, 2008). Based on the experiments conducted to date, different types of learning processes in main coleoids have been reported, such as associative learning, sensation,

habituation, reversal learning, avoidance learning, and discrimination of stimuli based on sight, tactile features, taste, and smell (Hanlon & Shashar, 2003; Borrelli & Fiorito, 2008; Amodio et al., 2020; Maselli et al., 2020a; van Giesen et al., 2020).

1.3 OCTOPUS LEARNING AND BEHAVIOR

Octopuses are considered the most intelligent invertebrates, thanks to their brain structure and neuron connectivity. Their brains have the same basic structure as other cephalopods, and the ratio of brain size to body size is similar to that of fish and birds, indicating their high intelligence (Mouritsen & Styrbæk, 2021). The arms of octopuses contain 500 million neurons, which is two-thirds of the total number, allowing for autonomously generated, stereotyped movements (Borrelli & Fiorito, 2008; Deryckere & Seuntjens, 2018; Mouritsen & Styrbæk, 2021). Research has shown that the supraesophageal mass of the octopus brain controls behavior and sensory processes, as well as motor commands and coordination to higher motor centers, called basal lobes. The subesophageal mass coordinates the control of particular effectors through intermediate and lower motor centers (Borrelli & Fiorito, 2008). Interestingly, octopuses lack a neocortex, which is responsible for intelligence in many other animals. Instead, their brains have many folds and complex morphological structures that contribute to their high level of intelligence (Figure 1.1) (Mouritsen & Styrbæk, 2021).

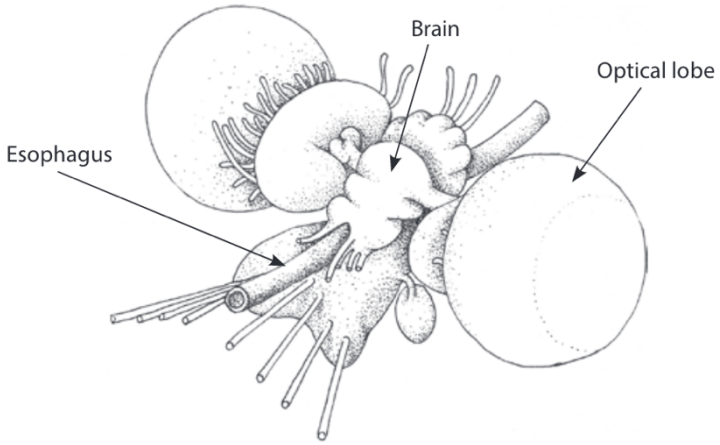

Figure 1. 1. The illustration of Octopus brain with Optic lobes (Mouritsen & Styrbæk, 2021).

From a behavioral perspective, the behavioral flexibility of the coleoids is unique, and octopuses face various regular natural problems in complex ways during feeding and survival in their habitats (Amodio et al., 2020). One of the main hypotheses of octopus behavior is that it occurs due to the animal's response to associative learning. In this approach, the animal receives sensory inputs that go to the vertical lobe, which response to the tactile system based on positive and negative signals. Then, the system is tuned by the sense of lowering and raising the level of the responses based on previous experiences. According to this hypothesis, new information is added to the long-term cumulative experiences, whereas short-term memory helps with fluctuations and adaptive behaviors (Wells, 1978). Therefore, several types and forms of associative learning protocols have been used in different aspects, such as problem-solving tasks, passive avoidance, and habituation tests. Most of the protocols used show the octopuses' behavioral flexibility in experiments. The *Octopus vulgaris* is considered the most commonly used animal with the above protocols, including problem-solving and social learning experiments, and is capable of learning things by modifying behavioral responses (Borrelli & Fiorito, 2008; Di Cosmo et al., 2018a; 2018b; Amodio et al., 2020; Maselli et al., 2020b).

Moreover, the animal's learning capabilities have shown the development of neural circuitry that plays a vital role in processing behavioral responses and learning capabilities. Further, *O. vulgaris*'s behavioral flexibility in terms of learning capabilities and capacity corresponds to ecological plasticity. One of the main reasons for this is their higher versatility of foraging, which gave a change of lifestyle and adaptability in water columns moving vertically and horizontally (Borrelli & Fiorito, 2008; Di Cosmo et al., 2018a; 2018b; Amodio et al., 2020; Maselli et al., 2020b). Nevertheless, the complex behavior of octopuses shows that they can learn by observing other octopuses (Shomrat & Nesher, 2021). Furthermore, octopuses have shown this learning complexity by opening jars associated with food rewards

with a discriminate approach and the ability to pull an L-shaped container through the hall with a barrier (Mather & Dickel, 2017).

1.4 SENSORY SYSTEMS IN OCTOPUS

Cephalopods face challenging conditions in their habitats due to environmental changes. To overcome these challenges, they have evolved excellent tools through their sensory capabilities. Therefore, they have a well-developed sense organ that is sophisticated compared to other invertebrates (Di Cosmo & Polese, 2017). Octopuses, along with other cephalopods, have the ability to camouflage themselves using visual cues and can discriminate between colors using photoreceptors. They can also use chemical cues, either alone or together with visual signals, to detect ecological changes. Cephalopods have remarkable sensory structures, including chemical sensory epithelia (buccal lips and mouth), sensory neurons scattered on the body surface, olfactory organs, and arm suckers, which help them find mating partners, avoid predators, and detect prey (Di Cosmo & Polese, 2017; Young, 1960; Young, 1972).

In addition, Recent studies have revealed that octopuses can achieve distal chemoreception either by contact or by olfactory organs and gustatory systems, respectively (Di Cosmo & Polese, 2017; Maselli et al., 2020b; van Giesen et al., 2020). These structures have enabled cephalopods to sense their surroundings and navigate their environment, despite the challenges they face.

The usual sensory inputs of the octopus are directly linked to lower centers called the suboesophageal mass, while other sections help with intermediate and higher centers with regulatory interneurons (Amodio et al., 2020). Octopuses achieve these senses mainly through their eyes, suckers, and skin. The visual information is primarily processed by the lateral superior frontal lobes, median superior frontal lobe, vertical lobe, and sub-vertical lobe, acting as a network. The optic lobe acts as the main system to process the visual inputs, with the active involvement of the supraesophageal center. Suckers are mainly located in octopuses' arms and

are controlled by their individual nervous systems. In addition, arms behave independently of each other. Suckers, as a multisensory model, play a vital role in the well-developed nervous system, hence they help to receive sensory perception from the surrounding environment with the help of special receptors. Sensory systems in the skin help octopuses in various ways, such as seeing, tasting, and smelling. This has been proven by many researchers who have found many specific types of proteins such as opsins. The olfactory organ is positioned behind the optic lobe; it helps to detect odors, whereas chemoreceptors help to taste objects by touching them (Polese et al., 2016; Di Cosmo & Polese, 2017; Mollo et al., 2017; Di Cosmo et al., 2018b; Shigeno et al., 2018; Tarvin, 2020; van Giesen et al., 2020; Mouritsen & Styrbæk, 2021; Buresch et al., 2022).

In recent research, octopuses have emerged as intriguing subjects due to their unique sensory system (Di Cosmo & Polese, 2017; Young, 1960; Young, 1972). While invertebrate welfare was often overlooked in the past, the growing understanding of cephalopods, including octopuses, has prompted a shift in perspective. It is now recognized that these creatures may possess sentience and the capacity to experience pain and suffering. This realization has led to a call for more respectful treatment of invertebrates during laboratory experiments, with a specific focus on cephalopods. Anesthesia is a critical consideration in caring for octopuses, given their distinct sensory system. An ideal anesthetic for these animals should not only induce muscle relaxation but also provide pain relief, anesthesia, and amnesia. However, achieving these goals with a single substance is challenging, often requiring the use of multiple drugs. Unlike human patients in clinical settings, octopuses' sensory experience during anesthesia is complex, necessitating tailored approaches to minimize stress and pain during physiological experiments involving them (De Lisa et al., 2012; Polese et al., 2014; Winlow & Polese, 2014).

To address the unique sensory system of octopuses, researchers have explored the use of magnesium chloride as an anesthetic agent. While magnesium chloride primarily acts as a muscle relaxant, its impact on octopuses' sensory perception has been a topic of discussion. It competes with calcium ions, inhibiting neurotransmitter release in the peripheral nervous system, but typically does not affect the central nervous system. In efforts to better align with octopuses' sensory needs, researchers have considered magnesium chloride as both a pre-anaesthetic agent and an adjunct to anesthesia. This approach aims to reduce the time required for successful anesthetization and promote faster recovery. Notably, the use of isoflurane as an anesthetic for Octopus vulgaris has proven successful, offering direct access to both the peripheral and central nervous systems when administered through the respiratory system. This method has significantly reduced anesthetization time and promoted faster recovery, all while taking into account the unique sensory system of octopuses. Hence, understanding and accommodating the sensory system of octopuses is essential for promoting their welfare in laboratory experiments. By focusing on their sensory needs, researchers can reduce stress and pain, ultimately improving the treatment of these fascinating creatures (De Lisa et al., 2012; Di Cosmo et al., 2023; Polese et al., 2014; Winlow & Polese, 2014).

1.5 POST-TRANSCRIPTIONAL RNA EDITING MECHANISM IN OCTOPUS

The central dogma concept describes the process of transcribing DNA nucleotide sequences into protein through messenger RNA. However, during this transcription process, changes can occur in the nucleotide sequence of mRNA without altering the original DNA sequence. This process is known as RNA editing and is carried out by the enzyme ADAR (Adenosine Deaminase Acting on RNA). During A-to-I(G) RNA editing, adenosine is deaminated into inosine (I), which then behaves as guanine (G). This is the most common type of RNA editing found in metazoans. A-to-I RNA editing usually occurs within noncoding regions, but some

animal groups, such as coleoids, exhibit this mechanism in their coding regions, specifically in the nervous system (Garrett & Rosenthal, 2012a; Alon et al., 2015; Liscovitch-Brauer et al., 2017; Di Cosmo et al., 2018b).

The studies of the octopuses on RNA editing have revealed that A-to-G RNA editing also occurs in the coding regions, that are non-synonymous. Hence this can result in a change of amino acid of the proteins known as protein recoding. In addition, the A-to-G RNA editing process has been identified as important in octopuses due to the higher editing levels found within the region of conserved sites in higher proportions non-synonymously (Rosenthal & Seeburg, 2012; Alon et al., 2015; Rangan & Reck-Peterson, 2023).

Many studies have been conducted on A-to-G RNA editing, with a focus on its role in temperature adaptation through K+ channel kinetics. However, it is still unclear whether octopuses use this mechanism for acclimatization in the short or long term (Garrett & Rosenthal, 2012a; Garrett & Rosenthal, 2012b). Moreover, the A-to-G RNA editing process in octopuses is not well understood, and there is no clear evidence regarding how they utilize and regulate this mechanism (Garrett & Rosenthal, 2012b). Additionally, there may be other unexplored mechanisms underlying A-to-G RNA editing that have yet to be studied. Therefore, this study aims to investigate the RNA editing mechanism in *O. vulgaris* under different behavioral and stress conditions, such as exposure to different foods and environmental parameters, in order to better understand how RNA editing affects them.

1.6 AIM OF THE THESIS

The overall aims of this thesis are to examine the senses and RNA editing mechanisms of *O. vulgaris* to understand the molecular bases and behavioural approaches. The specific objectives of this research are mentioned below:

1. To examine and localize the photoreceptor molecules in the optic lobe.

2. To investigate the evidence for smell-by-touch behaviour and molecular evidence for the presence of G-protein-coupled receptors (GPCRs) involved in arm suckers of *O. vulgaris*.
3. To developed an upgrade of *O. vulgaris* anaesthesia protocol as a contribute to animal welfare.
4. To examine the RNA editing mechanism involved with selected genes.

1.7 THESIS STRUCTURE

The main body of this thesis consists of five (5) research chapters and the first chapter of this thesis indicates a general introduction to the octopus neuroethology (*O. vulgaris*) approach with octopuses' senses and RNA editing mechanisms. It elaborates on the overall general senses and their role in function in cephalopods and octopuses. In addition, the second chapter provides a comprehensive investigation of localisation and mapping for photoreceptor genes in the optic lobe with its function as an extraocular photoreceptor responsible for photoreception and its transduction. The third chapter presents the neuroethological and molecular evidence to prove smell-by-touch behaviour including demonstrating the presence of GPCRs with gene localizations using WMISH techniques, real-time qPCR, bioinformatics and behavioural approaches. The fourth chapter involves the development and upgrade of the *O. vulgaris* anaesthesia protocol, contributing to animal welfare by reducing stress on cephalopods used in laboratory experiments. Further, the fifth chapter focuses on the RNA editing mechanisms with given special reference to the olfactory genes and early growth genes to understand the involvement of this post-transcriptional process with different conditions. In each chapter, overall findings are summarized and highlighted the border implications together with promising future directions of research.

CHAPTER 2

PHOTORECEPTOR GENES IN THE OPTIC LOBE OF *O. vulgaris*

2.1 INTRODUCTION

The optic lobes of cephalopods are the largest part of the brain in the central nervous system. They serve as the central hub for coding visual inputs, storing and responding to produce motor responses. Additionally, these lobes play a role in regulating body skin patterns and locomotive behaviors (Hara & Hara, 1980; Young, 1997; Kingston et al., 2015b; Liu et al., 2017; Bonadè et al., 2020; Songco-Casey et al., 2022). As the primary system for visual analysis, the pair of optic lobes receives visual inputs from the photoreceptors in the retina and transfers complex visual information to the central ganglia through multiple synaptic interactions (Hara & Hara, 1980; Saidel, 1982; Young, 1997; Liu et al., 2017; Williamson & Chrachri, 2004; Songco-Casey et al., 2022). This process involves the top-down detection of visual inputs by the eye (Kingston et al., 2015b).

The optic lobes consisted with mainly outer cortex and medulla. Williamson & Chrachri (2004) (Williamson & Chrachri, 2004) and Songco-Casey et al. (2022) (Songco-Casey et al., 2022) have provided detailed explanations of the anatomical organization and neuronal morphologies of the optic lobes. Numerous studies have confirmed that the cortex in the optic lobes is primarily involved in visual information processing, while the medulla is involved in coordinating motor commands during dynamic body patterning (Williamson & Chrachri, 2004; Liu et al., 2017; Songco-Casey et al., 2022). Moreover, studies on the ontogenetic changes that occur in the optic lobes of cephalopods have revealed neuronal adaptations throughout evolution. These adaptations are crucial for different visual behaviors and early visual experiences in embryos (Liu et al., 2017; Napoli et al., 2022; Katz & Lyons, 2023).

The presence of photoreceptors plays a vital role in phototransduction processing in the microvillar photoreceptor membrane in all vertebrates, including cephalopods (Paulsen et al., 1983). In cephalopods, photoreceptors are distributed throughout the retina, with their nuclei located at the posterior part. Axons originating from the plexiform layer behind this nuclear layer exit the eye and directly synapse with the optic lobe (Koenig et al., 2016).

The detection of light is crucial for cephalopods to compete and survive in their habitat. This is achieved through highly organized structures such as retinas, which consist of thousands of receptors, including photoreceptors and supporting cells (Williamson & Chrachri, 2004; Kingston & Cronin, 2016; Songco-Casey et al., 2022). However, certain cephalopods, such as squid and cuttlefish, have photoreceptors located outside of the central nervous system. Their skin acts as a source of light detection, and they change their skin patterns using visual opsins (Kingston et al., 2015b).

Cephalopods possess both extraocular and retinal photoreceptors. The extraocular photoreceptors function as non-visual receptors primarily responsible for light detection, while the retinal photoreceptors process light for image formation and motion vision. Unlike retinal photoreceptors, extraocular photoreceptors do not contribute to image analysis. It is believed that all extraocular photoreceptors detect light using rhodopsin, which is identical to the form found in cephalopod eyes. In addition to rhodopsin, there are other photoreceptors involved in visual phototransduction, such as retinochrome, Gqα, sTRP transcripts, arrestin, rhodopsin kinase, cryptochromes, and retinochrome (Hara & Hara, 1980; Kingston, Kuzirian, et al., 2015; Kingston et al., 2015b; Kingston & Cronin, 2016; Bonadè et al., 2020).

Since vision is crucial for animals, most studies have primarily focused on retinal photoreceptors and the associated opsins and cryptochromes. Kingston & Cronin (2016) have compiled a comprehensive list of extraocular opsins found in various animals. In cephalopods, Bonadè et al. (2020) discovered four types of photoreceptor gene families: canonical r-opsins,

non-canonical r-opsins, xenopsins, and retinochromes. However, it is evident that cephalopods exhibit limited opsin diversity compared to other animals, expressing only a single type of rhodopsin protein in their retina. This unique characteristic suggests the presence of extraordinary mechanisms in cephalopods that enable the existence of extraocular photoreceptors. Furthermore, the presence of these extraocular photoreceptors enables cephalopods to adapt their skin patterns to match their surrounding habitats. In cephalopods, rhodopsins (specifically r-opsins) and retinochrome are predominantly identified in the retina, utilizing the same phototransduction pathway. These phototransduction genes are also found in cephalopods' light organs, where they exhibit light sensitivity. Cephalopods' photoreceptors can be categorized into two groups: nervous system photoreceptors and putative dermal photoreceptors. Rhodopsin has been observed in dermal photoreceptors, and it operates through a similar phototransduction pathway as retinal photoreceptors. Moreover, as previously mentioned, various genes involved in visual phototransduction, including visual or retinal rhodopsins, retinochrome, rhodopsin kinase, arrestin, sTRP transcripts, cryptochromes, and Gqα, have been detected and identified in cephalopods' skin, confirming their capability to function similarly to retinal phototransduction (Hara & Hara, 1980; Kingston et al., 2015a; Kingston & Cronin, 2016; Bonadè et al., 2020).

The Opsin family is one of the primary gene families associated with photoreceptors, belonging to the G protein-coupled receptors (GPCR) family, and is present in eumetazoans (Kingston & Cronin, 2016; Bonadè et al., 2020; Roberts et al., 2022). A recent analysis of the opsin family has revealed that cephalopods possess only four out of the nine known types of opsins, indicating a limited diversity of opsins in these organisms. Rhodopsins play a significant role in vision in cephalopods and are also involved in the vision processes of most protostomians, along with retinochromes (Kingston & Cronin, 2016; Bonadè et al., 2020). However, retinochromes can convert retinal back to its original position after primarily

interacting with r-opsins as a mediator (Bonadè et al., 2020). Cryptochromes are capable of detecting light in the blue and UV range, aiding the vision of cephalopods. Additionally, they can have non-visual light sensing functions in cephalopods, such as magnetoreception, compass navigation, and circadian rhythm regulation (Bonadè et al., 2020). Arrestins play a major role in regulating signal transduction by interacting with GPCRs, but they are predominantly found in the eyes of cephalopods. Xenopsins have been identified in cephalopods and are associated with ciliary photoreceptors, although their functional aspects have not been fully explored. They are present in adult animals and play a vital role in vision in the optic lobes, occasionally expressed during development (Bonadè et al., 2020; Roberts et al., 2022). Furthermore, xenopsins are co-expressed with r-opsins and exhibit features of both rhabdomeric and ciliary photoreceptors (Roberts et al., 2022). Melanopsin, another type of vertebrate opsin, is found in various animal species, including cephalopods. In cephalopods, this melanopsin is considered to have multimodal functionality, providing both mechanical and photosensitive information. Other photoreceptors, such as rhodopsin, retinochrome, Gqα, and sTRP transcripts, are involved in phototransduction processes, and all of these photoreceptors, except for sTRP transcripts, are present in chromatophore organs, contributing to signaling processes and camouflage (Kingston et al., 2015b; Kingston & Cronin, 2016). Rhabdomeric photoreceptors are present in protostome eyes and utilize a Gq-type signal transduction pathway to depolarize light. Along with ciliary photoreceptors, rhabdomeric photoreceptors have been recognized as being involved in the vision of lophotrochozoans (Roberts et al., 2022).

The presence of photoreceptors in the octopus has been extensively studied. Genome analysis of *O. bimaculoides* has revealed the presence of rhodopsin, rhabdomeric opsin, peropsin, and retinochrome (Albertin et al., 2015; Al-Soudy et al., 2021). Additionally, studies have localized photoreceptors, including extraocular photoreceptors, in octopus species such

as *O. vulgaris* (Al-Soudy et al., 2021). Despite numerous studies focusing on the visual systems of octopus species, little attention has been given to localizing adult species, particularly in the central nervous system, and identifying the distribution and function of photoreceptors (Bonadè et al., 2020; Songco-Casey et al., 2022). Therefore, the optic lobe serves as the primary focus for locating photoreceptors and understanding their structure and function, as it plays a crucial role in light sensing and coordination. The aim of this study is to map the photoreceptors in the optic lobe and elucidate their functional importance in light sensing and coordination.

2.2 MATERIAL AND METHODS

2.2.1 ANIMAL ACQUISITION

Adult specimens of *O. vulgaris* (with a body weight of approximately 800g) (n=3) were collected from the Bay of Naples in Italy and transported to the Professor Anna Di Cosmo's cephalopod facility at the Department of Biology, University of Naples Federico II, Naples, Italy. The adult specimens of *O. vulgaris* were then anesthetized using isoflurane insufflation and subsequently dissected under sterile conditions (Polese et al., 2014)

2.2.2. TISSUE COLLECTION

The optic lobes were collected from the sacrificed animals to investigate the gene expression of selected photoreceptors, namely Retina Rhodopsin, Retinochrome, Melanopsin-A-like, Rhabdomeric, and Rhodopsin kinase. Out of the two optic lobes present in each animal, one optic lobe sample was preserved in RNA Later and stored in a -80°C freezer until total RNA extraction. The remaining optic lobes from the specimens were preserved for Whole mount *in-situ* hybridization (WMISH).

All research conducted was in compliance with European Directive 2010/63 EU L276, the Italian DL. 4/03/2014, no. 26, and followed the ethical principles of Reduction, Refinement, and Replacement (Project n° 608/2016-PR-17/06/2016; protocol n° DGSAF 0022292-P-03/10/2017).

The optic lobes preserved for WMISH were collected without any damage, and cross sections were made from the middle part of the optic lobes of *O. vulgaris* to visualize the general morphology and cell organization in the different distinct zones. Following the cross-sectioning, the optic lobes were fixed overnight at 4°C using 4% PFA (4% Para-formaldehyde in PBS, pH 7.4).

Subsequently, the fixed tissues were dehydrated using a graded series of methanol (MeOH) with 1X PBST (phosphate buffered saline with 0.1% Tween-20) for 15 minutes each.

The dehydration process involved successive immersion in 25% MeOH, 50% MeOH, 75% MeOH, and finally 100% MeOH. After dehydration, the tissues were stored in 100% methanol PBST at -20°C freezer until further use.

2.2.3 RNA EXTRACTION AND CONFIRMATION OF GENES EXPRESSION

Total RNA was extracted from the optic lobes using the RNeasy minikit (Qiagen USA) following the manufacturer's instructions. The extracted total RNA was stored in a -80°C freezer for future experiments.

Furthermore, cDNA (complementary DNA) was synthesized from the total RNA extracted from the tissue using the QuantiTect® Reverse Transcription Kit (Qiagen, USA) according to the manufacturer's instructions.

To design the primers for the Retina Rhodopsin, Retinochrome, Melanopsin-A-like, Rhabdomeric, and Rhodopsin kinase genes, the sequences available for *O. bimaculoides* were acquired from the National Center for Biotechnology Information (NCBI) database, based on the available genomes (Albertin et al., 2015) (Table 2.1). Geneious Prime® 2021.1.1 (https://www.geneious.com) was used for this purpose. The validation of the primers, as well as the photoreceptors genes in optic lobe, was performed through Polymerase Chain Reaction (PCR) reactions and sequencing approaches using the synthesized cDNA.

The PCRs were performed using the following mixture in a final volume of 20 µL for both species: 100 ng of synthesized cDNA template, 0.2 µL of Pfu DNA polymerase (Thermo Scientific, Waltham, MA, USA), 4 µL of 4X Tris buffer with MgCl2, 1.6 µL of dNTPs (each dNTP at 2.5 µM), and 0.2 µL of each primer (at a concentration of 50 µM). The PCR cycling parameters consisted of an initial denaturing step at 98°C for 3 minutes, followed by 35 cycles of 10 seconds at 98°C, 30 seconds at 55-63°C, and 1 minute at 72°C. The amplification products obtained from the PCR were used to isolate the fragments corresponding to the desired

base pair sizes of the photoreceptor primers. The isolated gel fragments were purified using a QIAquick Gel Extraction Kit (Qiagen, USA) and subsequently sequenced.

Table 2. 1. Designed Primers for selected photoreceptor genes in *O. vulgaris*

Gene name	**Primer Sequence**	**NCBI Accession**
Retina Rhodopsin	F: CGTCTTCAACTGGGGAGCAT	**XM_014927502.1**
	R: GGCCCAAACTGTGCAAGAAG	
Retinochrome	F: GATGGGGCGAGTATGGACTG	**XM_014915229.1**
	R: TGCTTTAGGAATGCTGCGGA	
Melanopsin-A-like	F: TTCCGTAATCTGGAACCCGC	**XM_014918551.1**
	R: TAAGCGTCGGCTGAAACGAT	
Rhabdomeric Rhodopsin	F: TCCTTTATTCGGATGGGGCG	**XM_014916694.1**
	R: GCCAATGACCGCACAGTTTT	
Rhodopsin kinase	F: AGGCCACAGTCCATTTAGGC	**XM_014918773.1**
	R: AGATCTCTCCTTCCACAATCACA	

The sequenced samples were assembled using Geneious Prime® 2021.1.1 (https://www.geneious.com) software, and the identity of the fragments was confirmed by performing GenBank BLASTn and BLASTx (BLAST, basic local alignment search tool) searches.

2.2.4 SYNTHESIS OF *IN-SITU* HYBRIDIZATION PROBES FOR WMISH

For WMISH, digoxigenin (DIG)-labeled single-stranded RNA probes were synthesized using the PCR standard method, as described in Al-Soudy et al. (2021). The specific primer set (Table 2.2) was designed to incorporate an inserted T7 RNA polymerase promoter sequence. The PCR products obtained were then used to synthesize DIG-labeled single-stranded RNA probes using the DIG RNA Labelling Kit (Roche:11175025910).

In the WMISH procedure, Nitro Blue Tetrazolium chloride/5-Bromo-4-Chloro-3-Indolyl Phosphate (NBT/BCIP) was used as the alkaline phosphatase substrate for the detection of single mRNA.

Table 2. 2. Designed Primers for antisense RNA probes for WMISH to localize photoreceptors

Primer name	Primer sequence
In-situ hybridization antisense probe in *O. vulgaris*	
Retina Rhodopsin	F: **CGTCTTCAACTGGGGAGCAT**
	R+T7:**TAATACGACTCACTATAGGGAGAGGCCCAAACTGTGCAAGAAG**
Retinochrome	F: **GATGGGGCGAGTATGGACTG**
	R+T7:**TAATACGACTCACTATAGGGAGATGCTTTAGGAATGCTGCGGA**
Melanopsin-A-like	F: **TTCCGTAATCTGGAACCCGC**
	R+T7:**TAATACGACTCACTATAGGGAGATAAGCGTCGGCTGAAACGAT**
Rhabdomeric Rhodopsin	F: **TCCTTTATTCGGATGGGGCG**
	R+T7:**TAATACGACTCACTATAGGGAGAGCCAATGACCGCACAGTTTT**
Rhodopsin kinase	F: **AGGCCACAGTCCATTTAGGC**
	R+T7:**TAATACGACTCACTATAGGGAGAAGATCTCTCCTTCCACAATCACA**

The PCRs were conducted using the aforementioned conditions, utilizing cDNA as a template along with primers designed with T7 sequences. The resulting PCR products were isolated using a 1.5% agarose gel and subsequently purified to serve as templates for in vitro transcription reactions. The synthesis of DIG-labeled single-stranded RNA probes was carried out using the DIG-RNA Labelling Kit (SP6/T7) from Roche Applied Sciences (Laval, QC, Canada), following the manufacturer's instructions.

The synthesized RNA probes were purified using the RNeasy MinElute Cleanup Kit (Qiagen, GmbH Valencia, CA, USA). To estimate the concentration, one microliter of the purified probes was visualized on a 1.5% agarose gel, as described by (Al-Soudy et al., 2021).

2.2.5 WHOLE-MOUNT *IN-SITU* HYBRIDIZATION

The WMISH approach was utilized to visualize and localize the target photoreceptor genes in the preserved optic lobes of *O. vulgaris*. The optic lobe sections, which had been stored in 100% MeOH in 1X PBST as described previously, were brought to room temperature and

sorted based on the types of suckers into multi-well culture plates (Thermo Fisher Scientific Inc., USA).

To initiate the WMISH procedure, all selected tissues were rehydrated using a descending series of methanol in 1X PBST. This involved sequential incubation in 75%, 50%, and 25% MeOH in 1X PBST for 15 minutes each at room temperature (RT). The rehydration process was repeated twice using 100% 1X PBST for 10 minutes each, with gentle shaking on a shaker. Subsequently, the tissues underwent incubation and digestion processes. A Proteinase-K reagent mixture, prepared at a concentration of 20 µg/ml in 1X PBST, was used for incubation at 37°C for 20 minutes. This step facilitated the digestion of proteins. Following digestion, the tissues underwent a post-fixation process in 4% PFA for 20 minutes at RT. Subsequently, the suckers were washed three times in 1X PBST for 5 minutes each to remove any residual reagents and ensure proper sample preparation (Al-Soudy et al., 2021).

After the post-fixation process, the tissues were subjected to a pre-hybridization step lasting 2 hours. The pre-hybridization solution was prepared by combining the following components: 50% formamide (Sigma, USA), 5X saline-sodium citrate (SSC), 1X Denhardt's solution (Sigma, USA), 500 mg/ml yeast tRNA (Roche, Germany), and 500 mg/ml salmon sperm DNA (source to be specified), as outlined in the method provided by (Al-Soudy et al., 2021). For the hybridization step, a fresh hybridization solution was prepared by adding 10 µL of heparin (100 mg/mL, H3149, Sigma, USA) to 20 mL of pre-hybridization solution. The amount of heparin added was adjusted based on the number of optic lobe sections used. The RNA probes, synthesized at a final concentration of 1-2 µg/mL, were then added to the hybridization solution. To denature the probes, the hybridization solution with the RNA probes was heated to 80°C for 10 minutes and immediately placed on ice for 5 minutes. The denatured hybridization solution containing the RNA probes was then added to the tissues, and the samples were allowed to hybridize overnight at a temperature range of 62-65 °C. Prior to

performing the hybridization, the multi-well container and the hybridization solution were pre-warmed to the designated hybridization temperature, as outlined in the method described by (Al-Soudy et al., 2021).

After the overnight hybridization process, post-hybridization washes were performed on the optic lobe sections. Initially, the sections were washed twice with a 2X sodium chloride sodium citrate solution containing 0.1% Tween-20 (SSCT) at RT. Next, the sections were washed with 5X SSCT-50% formamide (v/v) at RT for 5 minutes. Subsequently, the suckers were incubated with RNase A (Invitrogen 12091021) at a concentration of 20 µg/ml for 15 minutes at 37°C. This step helped to remove any non-specifically bound RNA. After the RNase A treatment, the arm suckers were washed again with 2X SSCT for 10 minutes. This was followed by subsequent washes with 2X SSCT-50% formamide (v/v), 2X SSCT, 0.2X SSCT, and finally, 1X PBST. Each wash solution was used for a 15-minute duration. These post-hybridization wash steps were essential for removing unbound or non-specifically bound molecules, ensuring the specificity and accuracy of the WMISH results (Al-Soudy et al., 2021).

Following the post-hybridization washes, the optic lobe sections were incubated with a 1X blocking buffer solution (Roche Applied Science 11096176001) in 1X PBST for 1 hour at RT. This blocking step helped to reduce non-specific binding of the detection antibody. After the blocking step, the tissues were incubated overnight at 4°C on a rocker with an Anti-Digoxigenin-AP antibody. The antibody was diluted to a concentration of 1:2500 in the blocking buffer solution (Roche 11093274910). This antibody specifically binds to the DIG-labeled RNA probes, enabling the detection of the target genes. The overnight incubation allowed for sufficient antibody binding and subsequent signal detection during the visualization step of the WMISH protocol. The gentle rocking motion ensured uniform antibody distribution across the tissue sections (Al-Soudy et al., 2021).

After labeling with the Anti-Digoxigenin-AP antibody, the coloration step was performed on the optic lobe sections. To prepare for coloration, the sections were washed five times in 1X PBST for 25 minutes each to remove any unbound antibody and ensure clean background staining. Following the washes, the sections were equilibrated in an alkaline phosphatase buffer (AP) containing 100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris (pH 9.5), and 0.1% Tween-20 at RT. This step provided optimal conditions for the color reaction. The color reaction was initiated by adding NBT/BCIP stain solution (11681451001, Roche, Germany) to the sections. The stain solution was diluted 1:200 in the AP buffer. NBT/BCIP is an alkaline phosphatase substrate that produces a colorimetric signal upon reaction with the enzyme.

The color reactions were performed in a light-resistant environment to prevent any interference or degradation of the staining. The sections were incubated in the staining solution until a sufficient intensity of color development was achieved, indicating the presence of the target genes. Following the coloration reaction, the optic lobe sections were subjected to a dehydration process using ascending ethanol concentrations in 1X PBS. This step aimed to minimize background staining and enhance the specific signal. Subsequently, the sections were rehydrated in 1X PBS to restore their original hydration state.

The whole-mount optic lobes sections, along with the anti-DIG-labeled probes, were observed using a Carl Zeiss Stemi 305 stereomicroscope equipped with an Axiocam ERc 5s camera. ZEISS ZEN lite Software at UNINA was utilized for image acquisition and analysis. By including control samples and conducting control experiments, the staining process ensured accurate and reliable results. This approach allowed for the specific visualization and localization of the target photoreceptor genes in the optic lobe sections of *O. vulgaris*.

2.3 RESULTS

2.3.1 PHOTORECEPTOR GENES CONFORMATION IN THE OPTIC LOBE

The primer combinations and gene confirmation in the optic lobe tissues were achieved by conducting PCR reactions using prepared cDNA as the template. The cDNA was synthesized from RNA extracted from *O. vulgaris* optic lobe tissues. Gel electrophoresis analysis of the PCR results provided evidence for the presence of all the targeted photoreceptor genes, including Retina Rhodopsin, Retinochrome, Melanopsin Rhodopsin, Rhabdomeric, and Rhodopsin kinase, thereby confirming their expression in the optic lobes (Figure 2.1).

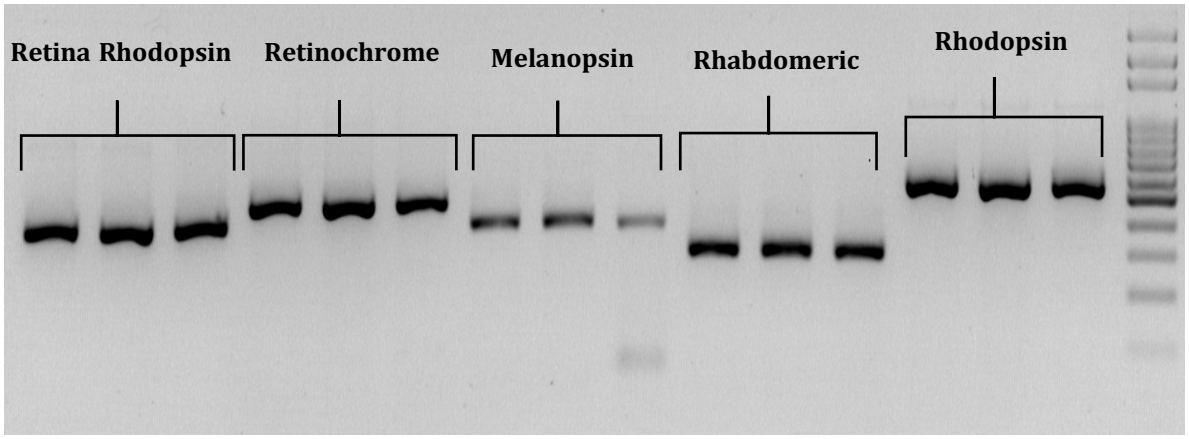

Figure 2. 1. Expression evidence of photoreceptor genes in the optic lobe of *O. vulgaris*.

2.3.2 LOCALIZATION OF PHOTORECEPTORS

To localize photoreceptors in the optic lobes of *O. vulgaris*, we employed the whole-mount *in-situ* hybridization (WMISH) technique using Digoxigenin-labelled antisense probes. This approach enabled us to visualize the spatial distribution of photoreceptors within the optic lobes. Our findings demonstrated the presence of a substantial number of positive neural cells scattered throughout the inner medulla. Moreover, the outer layer displayed a dense concentration of intensely positive cells, indicating regions of heightened staining within the optic lobes. These results provide valuable insights into the localization patterns of

photoreceptors, shedding light on their distribution and potential functional significance within the *O. vulgaris* optic lobes.

2.3.2.1 EXPRESSION OF RETINA RHODOPSIN

The expression pattern of Retina Rhodopsin transcripts in the optic lobe of *O. vulgaris* exhibited a high degree of localization, predominantly observed in two distinct regions: the outer cortex (O.C) and the radial column zone (R.Z). Specifically, within the optic lobe, the expression of Retina Rhodopsin transcripts appeared to be scattered, primarily within the radial column zone (R.Z). Notably, no positively stained neuron cells were detected in the optic tract region (OT) and the central tangential zone (T.Z). These findings provide valuable insights into the spatial distribution of Retina Rhodopsin expression within the *O. vulgaris* optic lobe, highlighting the importance of the outer cortex and radial column zone in its localization. (Figure 2.2).

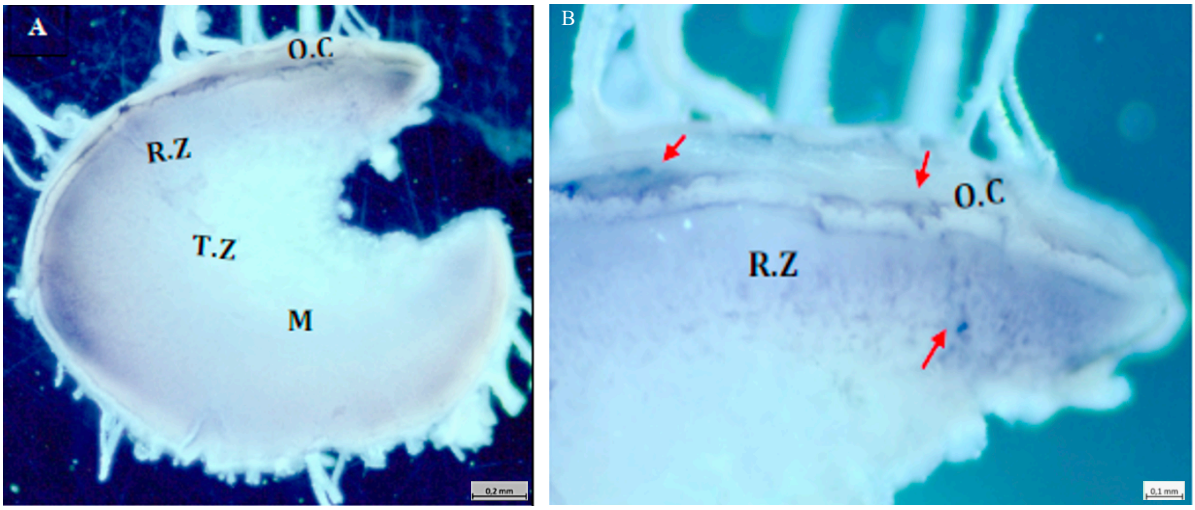

Figure 2. 2. Expression of retina rhodopsin transcripts in in the optic lobe of *O. vulgaris*. (OC: outer cortex, OT: optic tract region, R.Z: radial column zone, T.Z: central tangential zone)

2.3.2.2 EXPRESSION OF RETINOCHROME

The expression analysis of Retinochrome transcripts in the optic lobe of *O. vulgaris* revealed their presence in both the outer regions and the outer cortex (OC). Remarkably, positive signals of retinochrome were also observed in the optic tract region (OT) of the optic lobe. While some

expression of retinochrome was detected in the radial column zone (R.Z) of the optic lobe, no positive signals were observed in the central tangential zone of the medulla (M) within the optic lobe (T.Z). These findings highlight the specific localization of retinochrome expression within different regions of the *O. vulgaris* optic lobe, underscoring its potential functional roles in these areas. (Figure 2.3).

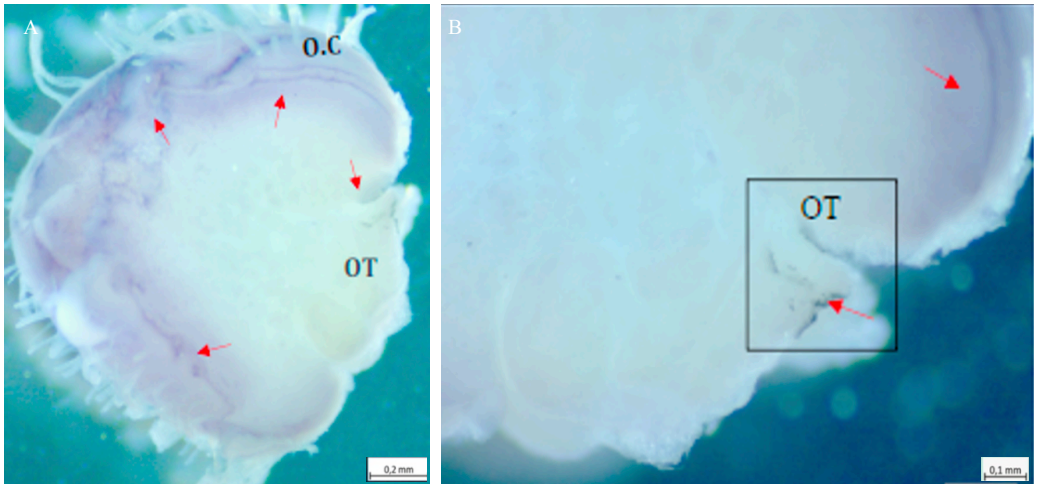

Figure 2. 3. Expression of retinochrome transcripts in the optic lobe of *O. vulgaris*. (OC: outer cortex, OT: optic tract region)

2.3.3.3 EXPRESSION OF RHABDOMERIC

The expression analysis of rhabdomeric photoreceptor transcripts in the optic lobe of *O. vulgaris* demonstrated highly localized signals of expression in the cortex and the radial column zone (R.Z) of the medulla. However, no positive transcript signals were detected in the inner medulla, particularly in the central tangential zone (T.Z), or the optic tract region of the optic lobe. These findings indicate a specific distribution pattern of rhabdomeric photoreceptor expression within the *O. vulgaris* optic lobe, suggesting potential functional roles of these photoreceptors in the cortex and radial column zone while being absent or minimal in the inner medulla and optic tract region. (Figure 2.4).

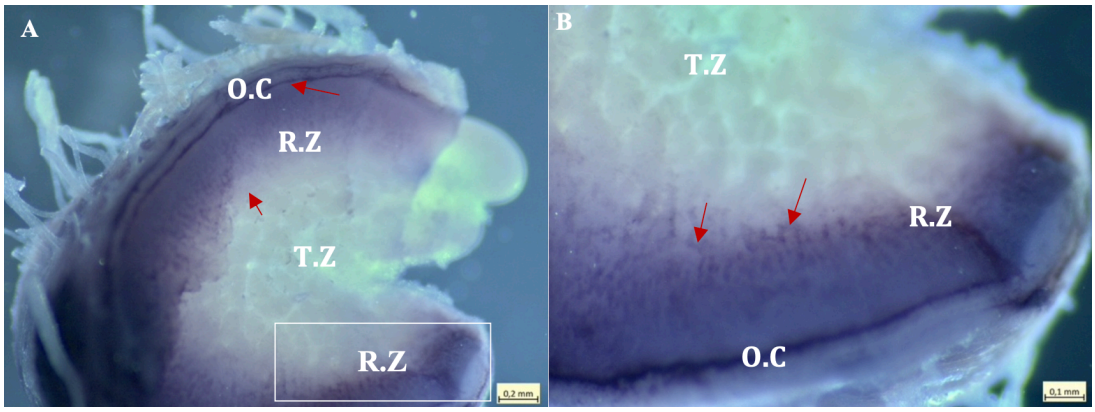

Figure 2. 4. Expression of rhabdomeric transcripts in the optic lobe of *O. vulgaris*.

(OC: outer cortex, OT: optic tract region, R.Z: radial column zone, T.Z: central tangential zone)

2.3.3.4 EXPRESSION OF MELANOPSIN

The expression analysis of melanopsin transcripts in the optic lobes of *O. vulgaris* revealed a high level of expression in the optical tract (OT) region. Furthermore, these transcripts were predominantly distributed within the radial column zone (R.Z) of the medulla in the optic lobes. These findings indicate a specific and localized expression pattern of melanopsin in these regions, suggesting its potential role in light perception and visual processing in *O. vulgaris*. The enrichment of melanopsin transcripts in the optical tract region and the radial column zone highlights their importance in mediating light-related functions in the optic lobes of this species. (Figure 2.5).

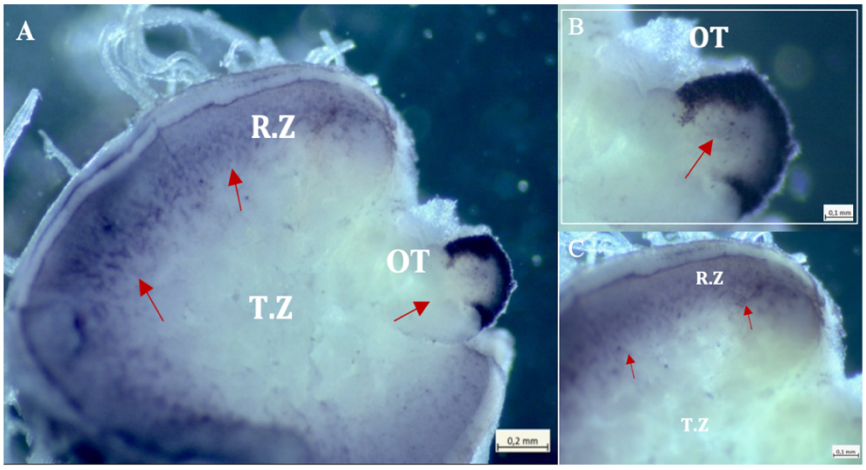

Figure 2. 5. Expression of melanopsin transcripts in the optic lobe of *O. vulgaris*

(OT: optic tract region, R.Z: radial column zone, T.Z: central tangential zone)

2.3.3.5 EXPRESSION OF RHODOPSIN KINASE

The expression analysis of the rhodopsin kinase gene and its localization in the optic lobes of *O. vulgaris* demonstrated scattered positive signals within the radial column zone of the medulla (R.Z) and the inner medulla, particularly in the central tangential zone (T.Z) (Figure 3.6). Notably, the outer layer of the optic lobe exhibited intense positive signals, indicating it as the most prominent area of expression. Additionally, the expression of rhodopsin kinase was localized in the optic tract region of the optic lobe in *O. vulgaris*. These findings provide insights into the distribution and localization of rhodopsin kinase in the *O. vulgaris* optic lobes, suggesting its potential involvement in regulating phototransduction and visual signal processing in these regions (Figure 2.6).

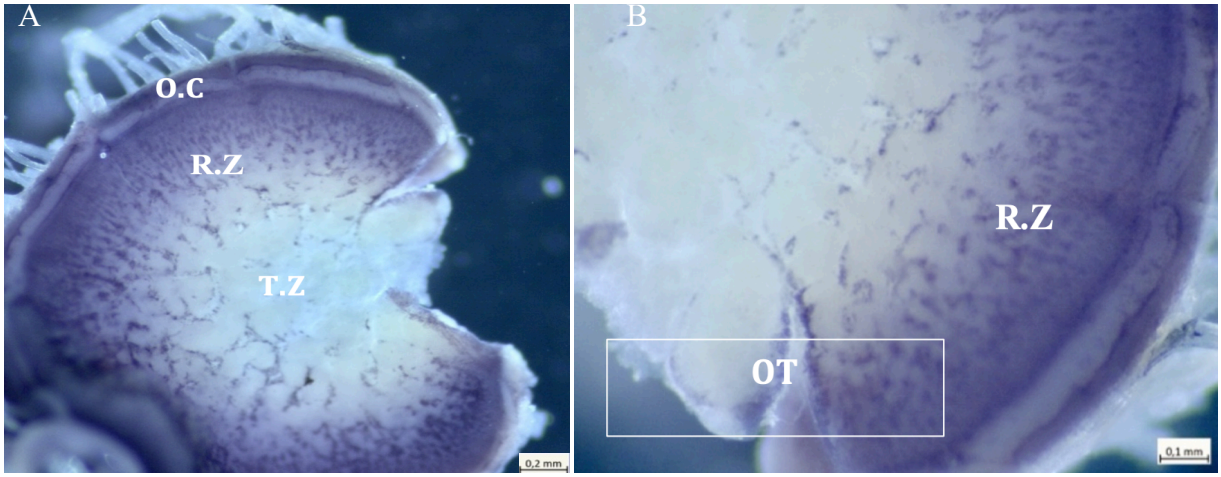

Figure 2. 6. Expression of rhodopsin kinase in the optic lobe of *O. vulgaris*.

(OC: outer cortex, OT: optic tract region, R.Z: radial column zone, T.Z: central tangential zone)

2.4 DISCUSSION

Cephalopods exhibit extraordinary photoreceptor structures that contribute to their exceptional visual capabilities. In contrast to vertebrates, the photoreceptors in cephalopods are directly exposed to incoming light without any intermediate layers. This unique arrangement results in a reversed order of retinal layers compared to vertebrates, with the photoreceptor cells facing the light source directly. The retina of cephalopods is richly pigmented, enabling efficient absorption of light. These remarkable creatures possess well-developed eyes that include lids, cornea, iris, pupil, lens, and retina. The lens can be adjusted by ciliary muscles to focus incoming light precisely onto the photoreceptors. Positioned at the forefront of the retina, the photoreceptor cells are oriented towards the light source. Below the photoreceptors, a layer of retinal nerve cells transmits signals to the optic ganglion, facilitating visual processing (Young, 1997; Dill-Okubo et al., 2021; Katz & Lyons, 2023).

The optic lobe is widely recognized as a vital region of the brain that is responsible for the processing of visual information received from the retina. It serves a critical role in the interpretation of visual signals, the integration of sensory inputs from other modalities, and the generation of appropriate behavioral responses. The optic lobe receives the visual signals that have been initially processed by the photoreceptor cells and subsequently engages in further processing to generate a coherent and meaningful representation of the visual environment. This intricate process involves complex neural circuits and mechanisms that enable the integration and analysis of visual information within the optic lobe (Saidel, 1982; Young, 1997; Bonadè et al., 2020; Baden & Nilsson, 2022; Napoli et al., 2022).

The optic lobe, a crucial component of the cephalopod brain, exhibits a complex structure comprising various layers and regions. One prominent layer is the medulla, which encompasses a multitude of neurons responsible for processing visual information. The medulla is further divided into distinct zones or regions, each contributing to specific aspects

of visual processing. Photoreceptor cells, situated predominantly in the retina, serve as specialized light-detecting cells that initiate the visual signal. Ganglion cells receive input from the photoreceptors and transmit visual information to other brain regions. Interneurons, or intermediate neurons, reside between the photoreceptors and ganglion cells, playing a role in integrating and modulating visual signals before transmission. Alongside these cell types, additional structures within the optic lobe warrant attention. The lamina, positioned between the photoreceptor layer and the medulla, aids in the organization and processing of visual information. The neuropil, a dense network of nerve fibers and synaptic connections within the medulla, facilitates communication and signal transmission among different neurons. Notably, the connectivity within the optic lobe is exemplified by the optic nerve, formed by the axons of ganglion cells, which acts as a conduit for relaying visual information to higher brain centers. This intricate arrangement and interplay of cellular components and structures in the optic lobe contribute to the remarkable visual capabilities observed in cephalopods (Saidel, 1979; Young, 1997; Liu et al., 2017; Songco-Casey et al., 2022;).

The optic lobe of cephalopods, including octopuses, encompasses specific regions that play crucial roles in the processing and transmission of visual information. These regions include the outer cortex, optic tract region, radial column zone, and central tangential zone. The outer cortex, situated in the outermost layer of the optic lobe, serves as the initial site for the processing and integration of visual signals. It receives input from photoreceptor cells and sends signals to other regions of the optic lobe for further processing and analysis. The optic tract region is where the optic tract, carrying visual information from the retina, enters and terminates. This region is responsible for relaying and distributing visual information to various parts of the optic lobe and other brain regions involved in visual processing. The radial column zone, found within the medulla, exhibits a column-like arrangement of neurons and fibers. In cephalopods, this region is associated with the distribution of photoreceptor cells and the neural

pathways related to their function. It plays a significant role in the processing of visual signals received from the photoreceptors and contributes to the integration and modulation of visual information. The central tangential zone, also located within the medulla, occupies a central position with a tangential orientation. It is typically associated with other types of neurons and pathways involved in visual processing. This region likely contributes to the integration, coordination, and refinement of visual signals before they are transmitted to other brain regions. Together, these distinct regions within the optic lobe of cephalopods work in harmony to process, relay, and integrate visual information, enabling the remarkable visual capabilities observed in these fascinating creatures (Saidel, 1979; Young, 1997; Liu et al., 2017).The research conducted on the octopus visual system has recently been described, specifically in the case of *O. bimaculoides*. While the researchers have identified several subtypes within the major cell populations, they acknowledge that there is likely additional diversity yet to be explored in future studies. Through such investigations, a better understanding of cephalopod visual function can be achieved (Songco-Casey et al., 2022). The molecular mapping presented in this study, which focuses on the optic lobe, provides a roadmap for future research, enabling the exploration of the functional, developmental, and evolutionary aspects of the cephalopod visual system.

In this study, we investigated the expression and localization of photoreceptors, including Retina Rhodopsin, Retinochrome, Melanopsin Rhodopsin, Rhabdomeric, and Rhodopsin kinase, in different zones of the optic lobes of *O. vulgaris*. Our findings reveal distribution patterns and their involvement in visual information coordination. This is the first recorded instance of these photoreceptors being found in the optic lobes of *O. vulgaris*. Cephalopods, in general, possess only four types of opsins (canonical r-opsins, non-canonical r-opsins, xenopsins, and retinochromes), indicating limited diversity of opsins in these organisms. Previous study conducted by Bonadè et al. (2020) has reported the presence of

opsins in tissues other than the eyes and neural tissues. However, our study detected retina rhodopsin, retinochrome, melanopsin rhodopsin, rhabdomeric, and rhodopsin kinase in the optic lobes, confirming their presence in neural tissues. According to the Bonadè et al., 2020 reported the specific duplication of retinochrome and xenopsin in squids. They also identified five sequences of opsins, including rhodopsin (r-opsin1) and retinochrome, expressed in the eyes and skin of common cuttlefish (*S. officinalis*). Furthermore, *O. bimaculoides* genome analysis (Albertin et al., 2015) revealed the presence of rhodopsin, rhabdomeric opsin, peropsin, and retinochrome. Another study conducted by our research group reported the presence of rhodopsin kinase (Ov-GRK1) in the suckers of *O. vulgaris* (Al-Soudy et al., 2021).

The localization of rhodopsin has been reported in the outer cortex and radial column zone of the optic lobe in *O. vulgaris* (Figure 2.2). Rhodopsin (r-opsin1) has been previously identified in the eyes of cephalopods, including octopuses. It is expressed during late embryogenesis in hatchlings (stage 30) of *S. officinalis*, found in all layers of the retina and skin, as well as in the margin of the optic lobes. Similarly, in *O. vulgaris*'s optic lobes, rhodopsin localization confirms its presence and involvement in the vision of cephalopods, including octopuses. This finding provides strong evidence for the presence of rhodopsin in the optic lobe of *O. vulgaris*, consistent with findings in *S. officinalis* (Paternolli et al., 2009; Imarazene et al., 2017; Bonadè et al., 2020). Nevertheless, rhodopsin plays a crucial role in the visual system of cephalopods and is considered the main molecule responsible for light detection (Bonadè et al., 2020), This finding confirms the first reported evidence of rhodopsin in the optic lobes of *O. vulgaris*, in addition to *O. bimaculoides*, *S. officinalis*, and *Doryteuthis pealeii*. It has been previously reported that rhodopsin also plays a vital role in the dynamic changes in skin color for camouflage, indicating the autonomous photosensitivity of the skin, which later facilitates light detection in the eyes (Kingston et al., 2015b; Bonadè et al., 2020). Importantly, the presence of rhodopsin in the cortex of the optic lobes has been emphasized in

the study by Bonadè et al. (2020). Therefore, our findings strongly support and provide the first confirmation of rhodopsin's presence, with clear localization of these genes and cells, ensuring its functions. In invertebrates and vertebrates, rhodopsin presence is similar, with the main difference being that in vertebrates, rhodopsin is hydrolyzed into free retinal and opsin, while in invertebrates, rhodopsin forms a stable pigment called metarhodopsin (Paternolli et al., 2009). Additionally, this report highlights the presence of rhodopsin in the optic lobe of cephalopods (*O. vulgaris*), in addition to the eye, skin, and light organ (photophore) (Kingston et al., 2015b; Bonadè et al., 2020).

The localization and expression of retinochrome mRNA transcripts in the optic lobes of *O. vulgaris* have shown a similar distribution pattern to that of rhodopsin, as found in this study (Figure 2.3). This suggests the possibility of simultaneous expression of both rhodopsin and retinochrome in the optic lobes of *O. vulgaris*. While it has been mentioned that rhodopsin and retinochrome may be expressed differently in adults of *S. officinalis*, co-expression of these two opsins has been reported in embryos by Bonadè et al. (2020). Our findings strongly suggest the co-expression of both opsins (rhodopsin and retinochrome) in adult stages of cephalopods, including *O. vulgaris*. Many studies have reported the presence of retinochrome together with rhodopsin in various species, such as *D. pealeii* and *S. officinalis*, including *O. vulgaris* in this study (Kingston et al., 2015b; Bonadè et al., 2020). Additionally, both rhodopsin and retinochrome have been reported in muscles, hair cells (epithelial primary sensory neurons), arm axial ganglia, and the sucker peduncle of *D. pealeii* (Kingston et al., 2015b). They have also been found in the eyes and skin of *S. officinalis* (Bonadè et al., 2020). This finding suggests the presence of both opsins' genes together in the optic lobes, along with the other selected opsin genes.

In addition to the outer cortex and the radial column zone of the optic lobe, retinochrome transcripts are also expressed in the optic tract region. The significance of

rhodopsin in the eye's rhabdomere cannot be underestimated, as it serves as the primary molecule responsible for vision, as described in the Japanese flying squid (*Todarodes pacificus*). On the other hand, retinochrome demonstrates considerably higher photosensitivity, allowing it to respond to even the slightest traces of light that have passed through both the rhabdomeres and the dense black pigment layer. Given their close proximity, it is plausible that retinochrome may serve as a complementary component in the process of visual excitation (Hara & Hara, 1980). However, it has been reported that photoreceptors located in small parolfactory vesicles along the optic tract of *T. pacificus* contain both rhodopsin and retinochrome, with their known function of electrically responding to light, which is essential for vision in the retina (Kingston et al., 2015b). In contrast, our localization only observed the presence of retinochrome transcripts in the optic tract joining the optic lobe of *O. vulgaris*, which is not similar to the detection of both rhodopsin and retinochrome in *T. pacificus*'s optic tract, as described by (Kingston et al., 2015b). In the eyes, the potential involvement of retinochrome lies in the recycling process of the retinal photopigment, restoring it to its initial state before interacting with r-opsins within the rhabdomes (Bonadè et al., 2020).

Nonetheless, despite previous suggestions that the optic lobe may not contain retinochrome, many studies conducted using optic lobe extracts have confirmed its presence. Furthermore, it has been reported as the first evidence that retinochrome is detected in extraocular, photosensitive tissues (Hara & Hara, 1980). In our study, we have provided visual evidence of the presence of retinochrome in the optic lobe through the use of the WMISH technique, following the pioneering work of Hara & Hara (1980). Both rhodopsin and retinochrome have been traditionally associated with vision. However, our findings further confirm and demonstrate their involvement in photoreception by organs other than the eye (Hara & Hara, 1980). Moreover, it has been reported that retinochrome acts as a photo-isomerase in squid (Provencio et al., 1998).

The expression of mRNA transcripts of rhabdomeric in the optic lobe of *O. vulgaris* shows a high localization in the cortex and the radial column zone of the medulla of the optic lobe. Positive signals of expression are also observed in the optic tract region and the central tangential zone of the optic lobe (Figure 2.4). This finding represents the first evidence of rhabdomeric localization in the optic lobes of cephalopods. Previously, rhabdomeric photoreceptor cells have been reported in the eyes of many protostomian species. In cephalopods, rhabdomeric photoreceptors have been reported in species such as Japanese spineless cuttlefish (*Sepiella japonica*), *S. officinalis*, Hawaiian bobtail squid (*Euprymna scolopes*), and *D. pealeii*, which are representatives of lophotrochozoans, a monophyletic group of protostomes that includes cephalopods, bivalves, and polychaetes (Bonadè et al., 2020; Kingston & Cronin, 2016). Similar to our findings, the presence of rhabdomeric photoreceptors in the optic tract of *T. pacificus* has been reported, which aids in light detection by parolfactory vesicles. Furthermore, the presence of rhabdomeric photoreceptors has been reported in the light organ of *T. pacificus* (Kingston & Cronin, 2016). The role of rhabdomeric photoreceptors is to maintain the chromophore in both the native (dark) and photoactivated (light) states and depolarize in response to light using a Gq-type signal transduction pathway. In addition to cephalopods, rhabdomeric photoreceptors have been reported in several species, including larval eyespots and pigment-cup eyes of polychaetes (*Platynereis dumerilii*), mollusks, echinoderms, and bivalves (Bonadè et al., 2020; Roberts et al., 2022).

The expression of melanopsin has been reported in various brain regions and the skin of many animals. While it is considered of vertebrate origin, it has also been found to have homologs in invertebrate opsins (Provencio et al., 1998; Kingston & Cronin, 2016). Although the presence of many opsins is well-documented, their functions and roles are not well understood in most animals (Kingston & Cronin, 2016). Phylogenetic analyses have shown that melanopsins belong to the group of opsins responsible for visual pigments in cephalopods

and arthropods, suggesting a common ancestor for both vertebrates and invertebrates, as melanopsins are very similar (Drivenes et al., 2003; Matsuyama et al., 2012). It has been found that melanopsin, along with rhodopsin, acts as a non-visual photoreceptor located in vertebrate neurons in the inner retina, absorbing blue light to trigger the "biological clock" or circadian clock of mammals and other animals, including invertebrates. This suggests their involvement in circadian rhythm regulation in clock neurons of the brain, working together with photosensitive receptors such as cryptochromes (Drivenes et al., 2003; Zhgun et al., 2015; Kingston & Cronin, 2016; Bonadè et al., 2020). Interestingly, melanopsins have been found in vertberates to be very similar to invertebrate opsins, such as octopus rhodopsin (Drivenes et al., 2003). Melanopsin was highly expressed in the optic tract region of the optic lobes, with strong positive signals. Additionally, melanopsin expression was predominantly distributed in the radial column zone of the medulla in the optic lobes (Figure 2.5). It has been reported that melanopsin contains an aromatic residue (Tyr-103), which is often associated with light detection (Provencio et al., 1998; Drivenes et al., 2003; Kingston & Cronin, 2016). Considering the above facts, our findings provide the first-time evidence of the localization and expression of melanopsin in the optic lobe of *O. vulgaris*.

The localization of rhodopsin kinase mRNA transcripts in the optic lobes of *O. vulgaris* has revealed its distribution and expression in the cortex, radial column zone of the medulla, and the inner medulla (central tangential zone, T.Z). Additionally, clear expression of rhodopsin kinase was observed in the optic tract region of the optic lobe (Figure 2.6). The higher and intense expression of rhodopsin kinase in the outer layer areas of the optic lobes suggests the responsibility of the cortex for visual information processing, while the medulla serves as the motor command center for dynamic body patterning and is involved in visual learning and memory processes (Liu et al., 2017). Rhodopsin kinase has been found in several tissues as an extraocular or non-visual photoreceptor, such as in the skin and suckers of *O.*

vulgaris (Al-Soudy et al., 2021), and the light organ of bobtail squid (*Euprymna scolopes*)(Kingston et al., 2015b). Rhodopsin kinase is known to interact with other visual phototransduction components, including retinochrome, Gqα, and visual arrestin (Kingston et al., 2015b). Interestingly, the presence of rhodopsin kinase (Ov-GRK1) in the arm suckers of *O. vulgaris* has been associated with the ability to sense diffuse light and contribute to their camouflage ability by matching the background, enabling the rapid adjustment of color patterns without involving the central nervous system (Al-Soudy et al., 2021). The presence of rhodopsin kinase in the optic lobe of *O. vulgaris*, which is considered an extraocular photoreceptor in cephalopods, is an exciting finding. It adds to the understanding of the role and distribution of rhodopsin kinase in different tissues and highlights its involvement in visual processes beyond the traditional visual phototransduction pathways.

Table 2. 3. Localised photoreceptors and their functions

Photoreceptors	**Functions**
Retina Rhodopsin	main molecule responsible for light detection
Retinochrome	responsible for light detection and co expressed with rhodopsin
Melanopsin Rhodopsin	involvement in circadian rhythm regulation in clock neurons of the brain
Rhabdomeric	aids in light detection by parolfactory vesicles and depolarize in response to light
Rhodopsin kinase	responsibility of the visual information processing and act as extraoccular phtoreceptor

In conclusion, the expression and localization of various photoreceptors in the optic lobe of *O. vulgaris* provide valuable insights into the complexity of its visual system. The specific distribution and expression patterns of retina rhodopsin, retinochrome, melanopsin rhodopsin, rhabdomeric, and rhodopsin kinase suggest their distinct roles in light perception and visual processing. Investigating the influence of environmental factors on their expression and localization can shed light on how cephalopods adapt to their visual surroundings and

regulate their behavior. Exploring the molecular mechanisms underlying the expression and localization of these photoreceptors may have implications beyond cephalopod biology. It could potentially contribute to the development of new therapeutic strategies for vision-related disorders or inspire the design of artificial visual systems. Continued research on the expression and localization of photoreceptors in *O. vulgaris* will contribute to a deeper understanding of cephalopod vision and its broader implications in sensory biology.

CHAPTER 3

MOLECULAR EVIDENCE FOR THE PRESENCE OF G PROTEIN-COUPLED RECEPTORS GENES INVOLVED IN ARM SUCKERS OF *O. vulgaris* AND EVIDENCE FOR SMELL-BY-TOUCH BEHAVIOUR

3.1 INTRODUCTION

G protein-coupled receptors (GPCRs) stand as a remarkable and extensive family of integral membrane proteins found in eukaryotes, contributing significantly to cell signaling across biological membranes. Their importance is underscored by their involvement in a wide range of cellular responses, allowing them to mediate signals from both endogenous and exogenous ligands and stimuli (Zucchi et al., 2006; Ritschard et al., 2019; Barreca et al., 2022; Hauser et al., 2022; Kim et al., 2022).

All animals recognize a vast array of odorants they encounter using GPCRs, which possess a characteristic 7-transmembrane domain (7TM) (Zucchi et al., 2006; Ritschard et al., 2019; Barreca et al., 2022; Hauser et al., 2022; Kim et al., 2022). GPCRs are recognized as the largest and most diverse family of integral membrane proteins involved in cell signals across biological membranes in eukaryotes (Ritschard et al., 2019; Barreca et al., 2022; Hauser et al., 2022; Kim et al., 2022). This group is involved in most of the cellular responses and helps to mediate the endogenous and exogenous ligands and stimuli (Barreca et al., 2022). However, the main signaling modality of GPCRs is the engagement and activation of G proteins, which are heterotrimeric proteins (Hauser et al., 2022). GPCRs are classified into six classes based on the sequence homology and similarities of the functions: Class A (rhodopsin-like receptors), Class B (secretin receptors family), Class C (metabotropic glutamate receptors), Class D (fungal mating pheromone receptors), Class E (cAMP receptors), and Class F (frizzled and smoothened receptors) (Barreca et al., 2022).

Notably, GPCRs share a common architectural framework despite their various physiological roles. Their structure includes an extracellular N-terminus and a cytosolic C-terminus, both separated by seven transmembrane α-helices peptides. These helices are interconnected by three intracellular and three extracellular peptide loops, forming a characteristic and well-conserved arrangement in GPCR membranes (Barreca et al. 2022). This structural unity underpins the versatility of GPCRs in transducing signals across a wide spectrum of biological processes.

Within the realm of sensory biology, cephalopods, exemplified by the common octopus (*Octopus vulgaris*), exhibit a captivating adaptation in the domain of olfaction. These remarkable marine creatures navigate their intricate underwater environments, in part, by relying on their advanced olfactory capabilities. At the heart of this sensory prowess lies a complex network of olfactory receptors, which enable them to detect and interpret a wide array of chemical cues present in the surrounding water (Derby & Sorensen, 2008; Polese et al., 2015, 2016; Di Cosmo & Polese, 2017; Barreca et al. 2022; Mollo et al., 2017, 2022).

The molecular evidence for the presence of olfactory receptor genes in the arm suckers of cephalopods offers a deeper understanding of their sensory adaptations. This sensory system allows these cephalopods to decode the chemical language of their aquatic habitat, providing crucial information for their behavior, foraging, and even predator avoidance strategies. This intricate sensory diversity, intertwined with their complex behavior and survival, underscores the extraordinary world of cephalopods, making them a subject of enduring scientific fascination and a testament to the diversity of sensory adaptations in the animal kingdom (Polese et al., 2015, 2016; Di Cosmo & Polese, 2017; Bagheri et al., 2020; Barreca et al. 2022; Buresch et al., 2022).

The sense of smell, known as olfaction, is defined as the ability to detect chemical stimuli or molecules coming from a distant source. In terrestrial environments, this occurs via

volatile molecules, whereas in aquatic environments, it occurs through water-soluble odorant molecules coming from the source. Stimulating molecules play a vital role in olfactory and gustatory perceptions. Therefore, olfactory perceptions can be differentiated from gustatory perceptions, as olfaction (the sense of smell) is considered a distance sense, whereas gustation (the sense of taste) is considered a contact sense. However, these chemical senses (olfaction and gustation) depend on the spatial range and degree of volatility and solubility of chemosensory molecules (Derby & Sorensen, 2008; Mollo et al., 2014, 2017, 2022; Polese et al., 2015). Nevertheless, the solubility of chemosensory molecules changes from hydrophilic to hydrophobic; hence, species need to adapt their olfactory systems accordingly. Discriminations between olfaction and gustation based on the spatial signals range may be confusing without reflecting the molecular interaction of the ligands and receptors (Derby & Sorensen, 2008; Mollo et al., 2017, 2022). Furthermore, it has been reported that aquatic animals have a diverse ability to sense chemical stimuli, and both olfaction and gustation, with their receptors, are conserved in chemosensory organs that can synchronize based on the molecules they recognize. Therefore, synchronization is helpful in the integration of different types of signals in different channels to discriminate, recognize, and receive memory inputs from the surrounding environment (Derby & Sorensen, 2008).

The senses in cephalopods have been proven to contribute to their evolutionary success in different types of habitats. Among these, the octopus is considered the most advanced and successful species, using both distance and contact chemoreception to explore the environment with its various sensory modalities (Maselli et al., 2020a). This demonstrates that octopuses have the most sophisticated sense organs among invertebrates. As advanced marine invertebrates, octopuses may use both distance and contact chemoreception to investigate their surrounding environment (Maselli et al., 2020a; Polese et al., 2016). Although cephalopods use both distance and contact chemoreception, they mostly rely on the visual system as visual

predators, while using other systems to perceive environmental cues under limited light conditions (Polese et al., 2015, 2016; Di Cosmo & Polese, 2017; Bagheri et al., 2020; Buresch et al., 2022).

Octopuses have been observed to use their arms when vision is not possible. They forage blindly in the surrounding environment by using their arms with multimodal sensing through their suckers. It has been reported that octopuses also rely on chemotactile and mechanotactile approaches to identify prey when visual cues are not available, especially during night foraging (Bagheri et al., 2020; Buresch et al., 2022). Additionally, octopuses are able to recognize themselves using their arm suckers and chemosensory cues to avoid self-entanglement (Nesher et al., 2014). A recent study has reported the ability of octopuses to detect water-insoluble compounds through unique chemotactile receptors (van Giesen et al., 2020). Interestingly, specialized sensory receptors were first described in octopuses, specifically in *O. vulgaris*, in 1962, with several other receptors located in the epithelium covering the infundibulum and rim of the sucker, including the morphology of the chemoreceptors (Graziadei, 1962).

Further, most studies have revealed the importance of the arm suckers of octopuses and their multimodal sensing capabilities that play a vital role in their living environments. As some studies have prioritized attention on chemical sensitivity, it has been proposed that chemotactile discrimination also involves olfaction by the arm suckers, which is not considered conventionally. Thus, it allows octopuses to recognize odorants that are insoluble in seawater or have less solubility. Therefore, this peculiar behavior of octopuses has been proposed as "smell by touch" (Di Cosmo & Polese, 2017; Di Cosmo et al., 2018). Moreover, early behavioral studies have revealed that the gustatory system of *O. vulgaris* can distinguish objects based only on their chemical characteristics by direct tactile contact through suckers, termed "taste by touch" (Wells, 1963; Packard, 2004). However, recent behavioral studies with

electrophysiological methods have proven that chemotactile sensory cells in the suckers of *Octopus bimaculoides* have the ability to detect environmentally relevant chemicals. Accordingly, both autonomous arms and whole animals react to these stimuli (Fouke & Rhodes, 2020; van Giesen et al., 2020). Similarly, it is likely that *O. vulgaris*'s gustatory system also has receptors distributed on the suckers, where the aquatic equivalent of taste takes place (Wells, 1963; Graziadei & Gagne, 1976; Lee, 1992; Anraku et al., 2005).

Furthermore, to date, no further studies have been conducted or published, focusing specifically on the presence of GPCRs genes in cephalopods except for the rhodopsin like receptors in the octopus suckers, (Al Sayed et al.,2021) Although GPCRs genes were reported in the genome of *O. bimaculoides* (Albertin et al., 2012, 2015), their potential role in octopus olfaction was not explained or studied in detail. The study found two differentially expressed GPCRs in arm suckers, and these receptors were localized to an area in which chemosensory cells were proposed (Graziadei, 1962; Graziadei & Gagne, 1976). These findings suggest that the presence of these receptors may play a vital role in the diffuse sensory system. Furthermore, we conducted a behavioral experiment to demonstrate that octopuses have the ability to detect insoluble water molecules (limonene) using their suckers. We demonstrated that octopuses have the ability to recognize and respond to insoluble compounds, which typically act as olfactory cues mediating "smell by touch" behavior. Therefore, the main aim of this study is to provide evidence for the "smell by touch" by combining molecular and behavioral analyses.

3.2 MATERIAL AND METHODS

3.2.1 ANIMALS SELECTION AND REARING

The adult wild-caught *O. vulgaris* specimens (n=2, males, body weight ~600g) were collected from the Bay of Naples (Italy) between September and December 2022 for the purpose of conducting behavioral experiments. The animals were then promptly transferred to the cephalopod facility of Professor Anna Di Cosmo at the Department of Biology, University of Naples Federico II, Naples, Italy (UNINA).

The animals were accommodated and acclimatized for 15 days in fiberglass tanks (50 x 50 x 50 cm) filled with seawater using a dedicated circulatory system. The system in the facility maintained the seawater temperature at 18°C and natural photoperiod (12 hrL: 12 hrD). During the acclimatization period, the animals were fed different foods, mainly anchovies, shrimps, and mussels, to avoid repeat exposure during the trial periods. Every day, water quality parameters were checked, and tank cleaning was performed to maintain the animals' health status and monitor stress signs. The animals were also exposed to objects located in the tanks (Polese et al., 2014; Maselli et al., 2020).

In addition, two other animals that were collected following the same procedure as described above in 2019 were used for tissue collection in the molecular experiments. The octopuses were anesthetized with isoflurane insufflation and dissected under sterile conditions (Polese et al., 2014).

The total RNA extracted from the arm suckers of *O. bimaculoides* was obtained from the Marine Biological Laboratory (MBL) in Woods Hole, Massachusetts, United States of America (USA) by Dr Al-Sayed Al-Soudy, a former PhD student from Prof Anna Di Cosmo's lab, during his three-month training with Dr. Joshua Rosenthal in 2019. These samples were used to confirm the presence of GPCRs genes in both species.

The animals' housing, handling, monitoring, and experimental procedures were performed according to the guidelines and instructions provided for cephalopod welfare, in order to protect and use animals in research. All research was approved in accordance with the European Directive 2010/63 EU L276, the Italian DL. 4/03/2014, no. 26, and the ethical principles of Reduction, Refinement, and Replacement. (Project n° 608/2016-PR-17/06/2016; protocol n° DGSAF 0022292-P-03/10/2017).

3.2.3 TISSUE SAMPLES COLLECTION

The expression of TAAR genes was evaluated using octopus arm suckers. Based on their size and location, we chose four different types of arm suckers from the two frontal arms (R1 and L1), namely: distal (DIS), middle (MID), proximal large (PROX-L), and proximal big (PROX-B) (Figure 3.1). The olfactory mucosa (OM) and olfactory lobe (OLF) were used as positive controls, while the hepatopancreas (HEP) was used as a negative control. All tissue samples were preserved in RNA Later and kept in a -80°C freezer until total RNA extraction.

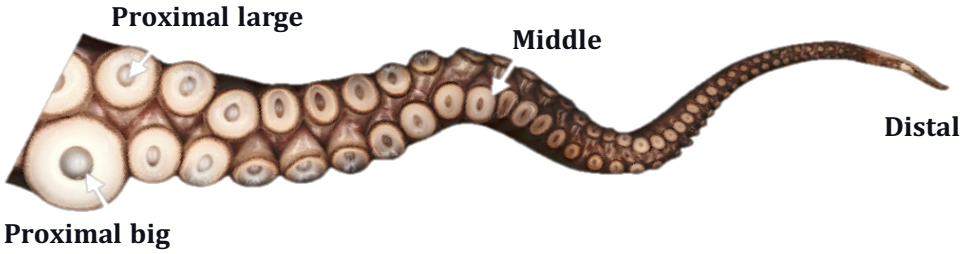

Figure 3. 1. Different types of suckers in Octopus arm suckers.

The middle suckers from L1 (N=2) and R1 (N=2) were additionally collected without causing any damage to the suckers. The collected suckers were fixed overnight with 4% PFA (4% Para-formaldehyde in PBS (phosphate buffered saline), pH 7.4) at 4°C. The fixed tissues were dehydrated using a graded methanol (MeOH) series (25% MeOH; 50% MeOH; 75% MeOH

and 100% MeOH) with 1X PBST (phosphate buffered saline with 0.1% Tween-20) for 15 minutes each. The tissues were then stored in 100% methanol PBST at -20°C until further use.

3.2.3 RNA EXTRACTION AND ANALYSIS OF GPCRs GENES EXPRESSION

The total RNA from the tissues of DIS, MID, PROX-L, PROX-B, OLF, and HEP was extracted using the RNeasy minikit (Qiagen USA) following the manufacturer's instructions. The extracted total RNA was stored in a -80°C freezer until future experiments. Using the RNA extracted from the tissues of both *O. vulgaris* and *O. bimaculoides*, cDNA (complementary DNA) was synthesized by the QuantiTect® Reverse Transcription Kit (Qiagen, USA) according to the manufacturer's instructions. The primers were designed based on the GPCRs (TAAR2 (XM_029783425.1) and TAAR7 (XM_029787211.1)) genes sequence available for both *O. vulgaris* and *O. bimaculoides*, acquired from available genomes (Albertin et al., 2015; Zarrella et al., 2019) from the National Center for Biotechnology Information (NCBI) database using Geneious Prime® 2021.1.1 (https://www.geneious.com). The validation of the primers and GPCRs genes in suckers was performed with synthesized cDNA by Polymerase Chain Reaction (PCR) reactions and sequencing approaches.

Table 3. 1. Designed Primers for gene expression for GPCRs genes in *O. vulgaris*

Primer name	**Primer sequence**
(GPCRs)	**RT-PCR and sequencing in *O. vulgaris***
OV-TAAR2 F	AAGGCAAGACACAATGGCTG
OV-TAAR2 R	ACACTGTGTGGTAAGGCAGA
OV-TAAR7 F	CTGGAGTGTCACCGGAAGAC
OV-TAAR7 R	AGTATGTCGTGCGAGCCAAT
Ubiquitin_ F	TCAAAACCGCCAACTTAACC
Ubiquitin_ R	CCTTCATTTGGTCCTTCGTC

The PCRs were performed using the following mixture in a final volume of 20 µL for both species: 100 ng of synthesized cDNA template, 0.2 µL of Pfu DNA polymerase (Thermo Scientific, Waltham, MA, USA), 4 µL of 4X Tris buffer with MgCl2, 1.6 µL of dNTPs (each dNTP 2.5 µM), and 0.2 µL of 50 µM of each primer (Table 3.1). The PCR cycling parameters included an initial denaturing step of 98°C for 3 min, 35 cycles of 10 s at 98°C, 30 s at 55-63°C, and 1 min at 72°C, and a final extension step of 5 min at 72°C. The PCR products were used to isolate the fragments related to the size of base pairs chosen for the TAAR primers. The isolated gel fragments were purified using a QIAquick Gel Extraction Kit (Qiagen, USA) and sequenced. The sequenced samples were assembled using the software Geneious Prime® 2021.1.1 (https://www.geneious.com), and the identity of the fragments was confirmed with GenBank BLASTn and BLASTx (BLAST, basic local alignment search tool). All the sequenced data generated in this study were deposited in GenBank (MZ441094-7).

Real-time PCR (quantitative PCR, qPCR) analysis was only performed to understand the gene expression of GPCRs genes in the *O. vulgaris* samples, using the QuantiTect SYBR Green PCR Kit (Qiagen, USA) according to the manufacturer's instructions. The qPCR was performed in a final volume of 25µL, with 50 ng of cDNA and a mixture of 1 mM of each primer, and 12.5µL of QuantiFast SYBR Green PCR Master Mix (2×). The conditions for the Real-time PCR cycling profile were set for a cycle at 95 °C for 5 min, followed by 40 three-step cycles at 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 20 s. All the reactions for qPCR were conducted in a Rotor-Gene Q cycler (Qiagen, USA). The ubiquitin gene was used to normalize the relative expression and to check the cDNA integrity as the expression of the housekeeping gene, which is constitutively and stably expressed in most cells and tissues (Table 3.1). All the samples were tested and run in duplicate during the analyses.

3.2.4 SYNTHESIS OF *IN-SITU* HYBRIDIZATION PROBES FOR WMISH

The digoxigenin (DIG)-labeled single-stranded RNA probes were synthesized for WMISH using the standard PCR method (Al-Soudy et al., 2021) with a specific primer set (Table 3.2) that contained an inserted T7 RNA polymerase promoter sequence. The PCR products were used to synthesize DIG-labeled single-stranded RNA probes with the DIG RNA Labelling Kit (Roche:11175025910). Nitro Blue Tetrazolium chloride/5-Bromo-4-Chloro-3-Indolyl Phosphate (NBT/BCIP) was used as the alkaline phosphatase substrate to detect single mRNA species.

Table 3. 2. Designed Primers for antisense RNA probes for WMISH to localize GPCRs genes

Primer name	**Primer sequence**
GPCRs	**In-situ hybridization antisense probe in *O. vulgaris***
OV-TAAR2 F	GATACCAACACCTACGCACCC
OV-TAAR2 R+ T7	TAATACGACTCACTATAGGGAGAGAGGTAAGCCTTGCTGCTTC
OV-TAAR7 F	ACTGTGGGTTTCTAGTGGCC
OV-TAAR7 R+ T7	TAATACGACTCACTATAGGGAGAGTATGTCGTGCGAGCCAATAG

The PCRs were performed using the aforementioned conditions with cDNA as the template and primers designed with T7. The resulting PCR products were then isolated by running them on a 1.5% agarose gel and purified to be used as templates for in vitro transcription reactions. To synthesize the DIG-labeled single-stranded RNA probes, the RNA Labelling Kit (SP6/T7) (Roche Applied Sciences, Laval, QC, Canada) was used, following the manufacturer's instructions. After synthesis, the RNA probes were purified using the RNeasy MinElute Cleanup Kit (Qiagen, GmbH Valencia, CA, USA), and the concentration was estimated by visualizing one microliter of the sample on a 1.5% agarose gel (Al-Soudy et al., 2021).

3.2.5 WHOLE-MOUNT *IN-SITU* HYBRIDIZATION(WMISH)

The WMISH approach was performed to visualize and localize the two target GPCRs genes in the arm suckers of *O. vulgaris* that were preserved as described above in 100% MeOH in PBST. The fixed suckers were brought to room temperature and sorted based on the types of suckers according to the tissue collection in multi-well culture plates (Thermo Fisher Scientific Inc., USA). All selected suckers were rehydrated using a descending series of methanol in PBST: 75%, 50%, and 25% MeOH in PBST for 15 minutes each at RT. The completed hydration process was performed twice in 100% PBST for 10 minutes each with gentle shaking using a shaker. Then, the suckers were subjected to the incubation and digestion processes using a 20 µg/ml in PBST Proteinase-K reagent mixture at 37°C for 20 minutes. The post-fixation process was conducted in 4% PFA for 20 minutes at room temperature, and then the suckers were washed three times in PBST for 5 minutes each (Al-Soudy et al., 2021).

After post-fixation, the suckers underwent a pre-hybridization step for 2 hours. The pre-hybridization solution was prepared by combining 50% formamide (Sigma, USA), 5X saline-sodium citrate (SSC), 1X Denhardt's solution (Sigma, USA), 500mg/ml yeast tRNA (#10109495001, Roche, Germany), and 500 mg/ml salmon sperm DNA, as described by (Al-Soudy et al., 2021).

For hybridization, a new hybridization solution was prepared by adding 10 µL of heparin (100 mg/mL, H3149, Sigma, USA) to 20 mL of pre-hybridization solution, with the ratio adjusted depending on the number of suckers used. The RNA probes, synthesized at a final concentration of 1-2 µg/mL, were then added to the hybridization solution. The hybridization solution with RNA probes was heated to 80°C for 10 minutes and immediately placed on ice for 5 minutes to denature the probes. The hybridization solution was then added to the suckers and allowed to hybridize overnight at 62-65 °C. Before performing the

hybridization, the multi-well container and hybridization solution were pre-warmed to the hybridization temperature (Al-Soudy et al., 2021).

Post-hybridization washes were conducted on the arm suckers after the overnight hybridization process. A 2X sodium chloride sodium citrate solution with 0.1% Tween-20 (SSCT) was used twice for washing at room temperature (RT). Then, the sections were washed with 5X SSCT-50% formamide (v/v) at RT for 5 minutes. The suckers were then incubated with 20µg/ml RNAs A (Invitrogen 12091021) for 15 minutes at 37°C, followed by another wash with 2X SSCT for 10 minutes. The arm suckers were washed again for 15 minutes with 2X SSCT-50% formamide (v/v), 2X SSCT, 0.2X SSCT, and 1X PBST, using each solution (Al-Soudy et al., 2021).

After the post-hybridization washes, the suckers were incubated with a 1X blocking buffer solution (Roche Applied Science 11096176001) in PBST for 1 hour at RT. Following this, the suckers were incubated overnight at 4°C on a rocker with Anti-Digoxigenin-AP antibody, which was diluted 1:2500 in the blocking buffer solution (Roche 11093274910) (Al-Soudy et al., 2021).

The coloration was performed after labelling with the Anti-Digoxigenin-AP antibody. The suckers were washed five times in 1X PBST for 25 minutes and equilibrated in alkaline phosphatase buffer (AP) containing 100mM NaCl, 50mM MgCl2, 100mM Tris, pH 9.5, and 0.1% Tween-20 at RT. The colour reaction was performed using NBT/BCIP stain solution (11681451001, Roche, Germany) at a 1:200 dilution in AP buffer. The colour reactions were conducted in a light-resistant environment until sufficient intensity was achieved. Control samples were left in staining solution for the same time interval as those incubated with anti-sense probes, while control experiments were performed for each condition using hybridization buffer without the probes.

Following the coloration reaction, the suckers were dehydrated in ascending ethanol concentrations in 1X PBS to minimize background and enhance the specific signal. Then, they were rehydrated in 1X PBS. The whole-mount sucker tissues with anti-DIG-labeled probes were observed under a Carl Zeiss Stemi 305 stereomicroscope with Axiocam ERc 5s using ZEISS ZEN lite Software at both UNINA and MBL.

3.2.6 BEHAVIORAL EXPERIMENT PLAN AND PROTOCOL

The behavioral experiment approach was designed to investigate the ability of octopuses to respond to water-insoluble odorants through discrimination learning based on specific odor stimuli. This approach aimed to shed light on the stimulus-specific discrimination and olfactory memory in *O. vulgaris*. To achieve this goal, the octopuses were trained using an associated learning approach that involved providing them with food rewards (Wells, 1968).

The new experimental protocol has been developed (Figure 3.2) in accordance with the aim of the study and it consists of three main approaches: preparation of jars and ceramic balls, testing trials, and experiment/control behavior trials using two *O. vulgaris* animals collected for the behavioral experiments. These animals were kept at the cephalopod's facility of Professor Anna Di Cosmo in the Department of Biology, UNINA, Naples, Italy from September to December 2022, as described above. Due to limited access and animal availability during the COVID-19 pandemic situation, we were only able to conduct these approaches on the two available animals.

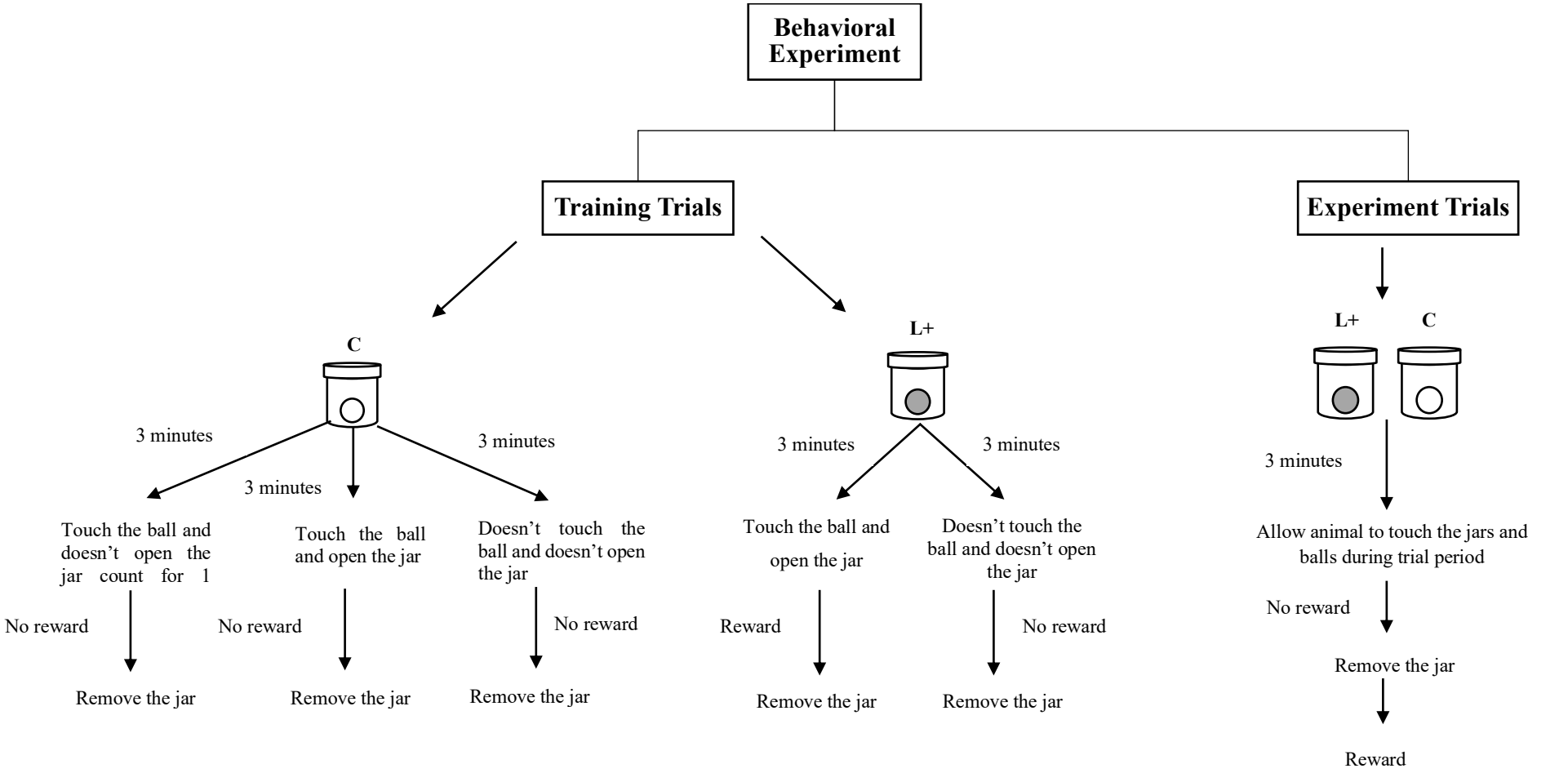

Figure 3. 2. Behavioural Experiment Protocol Plan

The transparent jars (500ml, 7.5 cm x 11.0 cm (diameter x height)) with a hole (2.0 cm diameter) on the top of the lid were used to keep the ~ 8-9 g ceramic balls during the test trials and experiments trials (Figure 3.4). As a stimulant of the purpose of smell by touch, the (R)-(+)-Limonene (Sigma-Aldrich, USA) (Figure 3.3) was used to coat the identical ceramic balls with agarose. The control ceramic ball was coated with a thin smear of 1% agarose using a smooth brush. The positive ceramic ball was coated with 1µL of Limonene mixed with 5 mL of 1% agarose thin smear using the smooth brush. Here we secured and maintained the use identical balls and jars to not make any difference to the vision of the octopus as they are more sensitive to such changes.

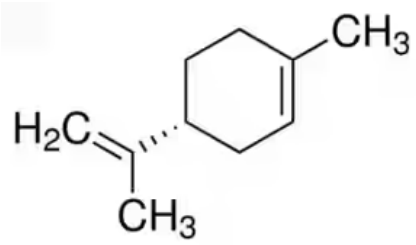

Figure 3. 3. Chemical structure of (R)-(+)-Limonene

Every day during the testing and control trial period, newly coated ceramic balls were prepared before conducting the trials. Testing and control trials were performed for a duration of two to three weeks, excluding Sundays.

Control jar with ceramic ball coated with 1% agarose.
(500ml, 7.5 cm x 11 cm (diameter x height))

Positive jar with ceramic ball coated with (R)-(+)-Limonene 1% agarose.
(500ml, 7.5 cm x 11 cm (diameter x height))

Figure 3. 4. Two types of jars used in behavioral experiments

Testing trials were conducted for both animals, and they were trained for 17-21 days using the following protocol (Figures 3.5 and 3.6). During the testing period, 6 trials were

conducted every day, divided into six trials in the morning and six in the evening, each lasting for three minutes. The jars were introduced according to the sequence described by Fellows in 1967 (Fellows, 1967), assigning number 1 to the ceramic ball coated with limonene + agarose and number 2 to the control ceramic ball coated only with agarose. At the end of each three-minute trial, a three-minute time gap was maintained before starting the next trial.

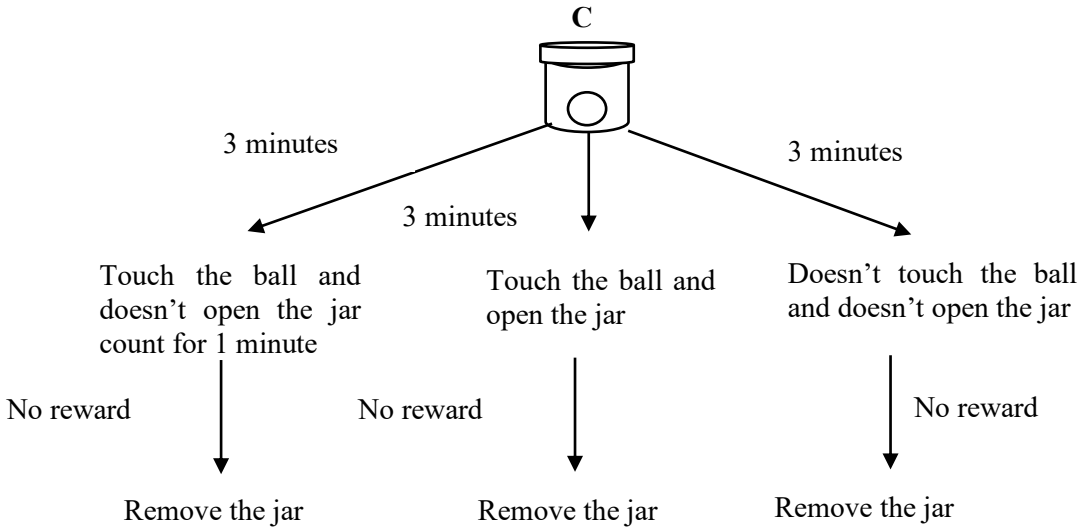

Figure 3. 5. Graphical diagram of protocol used for training trials with control jar

Two people were always present in front of the animal during the trials, and they would switch positions every three trials to avoid any potential bias caused by the octopus having a preference for interacting with a particular person. One person was responsible for introducing the jars, while the other recorded information such as the starting time and the type of jar presented, using a timer.

Before each trial, the animal was gently transferred into an amphora using a hand net. Then, a black divider or screen was placed in front of the amphora to prevent the octopus from seeing the introduction of the jar into the tank. The jar was positioned in the middle of the tank, close to the front glass, based on the size of the tank. The amphora, with the animal inside, was positioned in the middle of the back of the tank. The black divider or screen was removed to

start the three-minute testing trial. The animal was only rewarded if it interacted with the jar that contained the positive ceramic ball. To receive the reward, the octopus had to touch the ceramic ball and then open the jar within the three-minute trial period. To associate the reward with the ceramic ball coated with Limonene, without opening the jar, this practice was conducted for only 2-3 trials at the beginning of the testing period.

If the animal did not come out of the amphora within 30 seconds, a visual lure was used to encourage it to come out and interact with the jars. If the animal did not interact with any of the jars within the three-minute trial period, the jars were removed. The control ceramic ball was positioned in the same way as the positive ceramic ball. If the octopus interacted with the control ceramic ball within the first minute, a jar was removed after one minute. If the octopus did not interact with either ceramic ball or jar within three minutes, the jars were taken out.

All trials were recorded using a GoPro HERO5 Action Camera with 1080 resolution, 24fps, and a narrow screen.

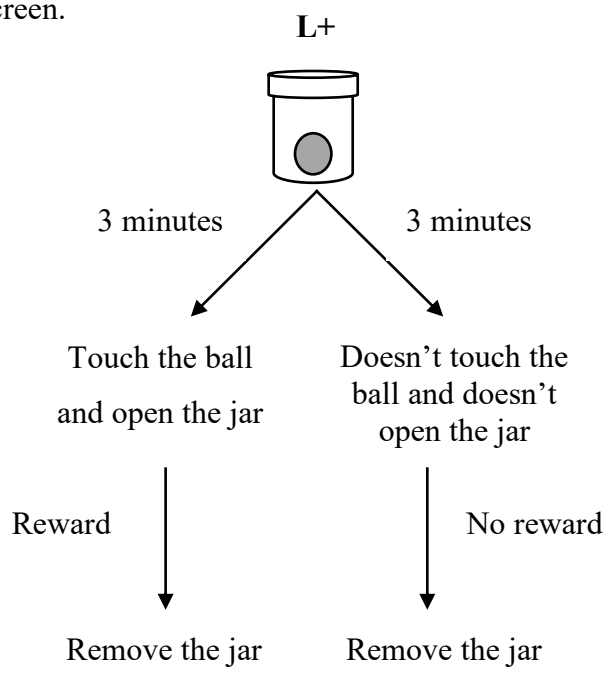

Figure 3. 6. Graphical diagram of protocol used for training trials with positive jar ceramic ball coated with Limonene

After the completion of the testing trial period, a control experiment was conducted for a 3-minute trial period. This was done by introducing both the positive and control ceramic

balls, including jars, together to the octopus (Figure 3.7). The jars were kept at the same distance from the edges of the front glass, with equal distances from each jar. The locations of the jars were noted and changed every day of the trial to avoid side bias to the animal. The animal was rewarded after the end of the 3-minute trial period. All the trials were recorded using the GoPro HERO5 Action Camera Video camera with 1080 resolution, 24fps, with a narrow screen. After finishing all the testing and experiment/control behavior trials, the recorded videos were analyzed using the Behavioral Observation Research Interactive Software (BORIS) (Friard & Gamba, 2016) to extract the ethograms with behavioral types involved during the experiments.

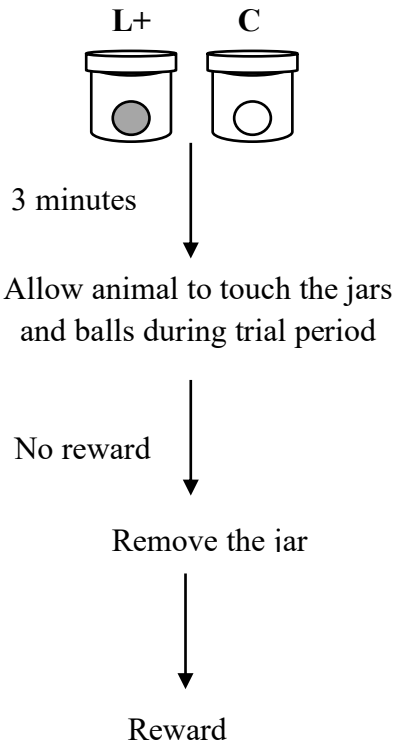

Figure 3. 7. Graphical diagram of protocol used for experiment/control behavior trials

3.3 RESULTS

3.3.1 PCR GEL ELECTROPHORESIS RESULTS OF GPCRs GENES

The confirmation of primers and cDNA was performed by conducting a PCR reaction using cDNA as a template, which was synthesized from RNA extracted from arm suckers of *O. vulgaris* and *O. bimaculoides*. The gel results showed the presence of GPCRs (TAAR2 and TAAR7) genes in all types of arm suckers from *O. vulgaris*, as well as in arm suckers from *O. bimaculoides*. These genes were strongly expressed in control tissue samples of OM and OL, which were used as control samples during the experiment (Figure 3.8).

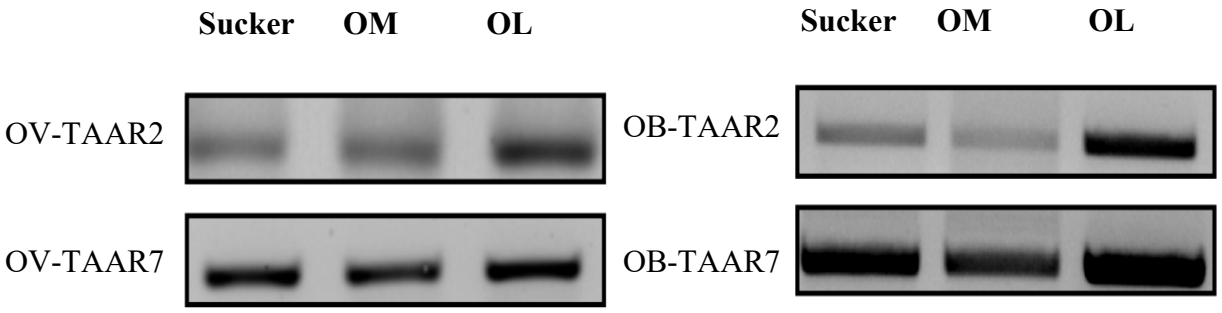

Figure 3. 8. PCR gel electrophoresis results for selected GPCRs genes for cDNA synthesised from *O. vulgaris* and *O. bimaculoides*

3.3.2 GENES EXPRESSIONS OF GPCRs

The gene expressions of selected GPCRs were evaluated in all four types of sucker tissues, including the OM and OL of *O. vulgaris* (Figure 3.9A), using a qPCR approach. Our findings revealed that the selected GPCRs genes were differentially expressed in all four types of sucker tissues (Figure 3.9B). The TAAR2 GPCR gene was expressed at higher levels in PROX-L compared to all other selected tissues, whereas the TAAR7 GPCR gene was upregulated in all selected tissues except for PROX-B. Among those tissues, the highest expression was observed in OLF tissue (~332 times that in OM) and was also highly expressed in DIST (~41 times that in OM). The tissues representing PROX-L and PROX-B exhibited higher expression of

TAAR2 GPCRs, while MID and DIST tissues displayed higher expression of the TAAR7 GPCRs gene.

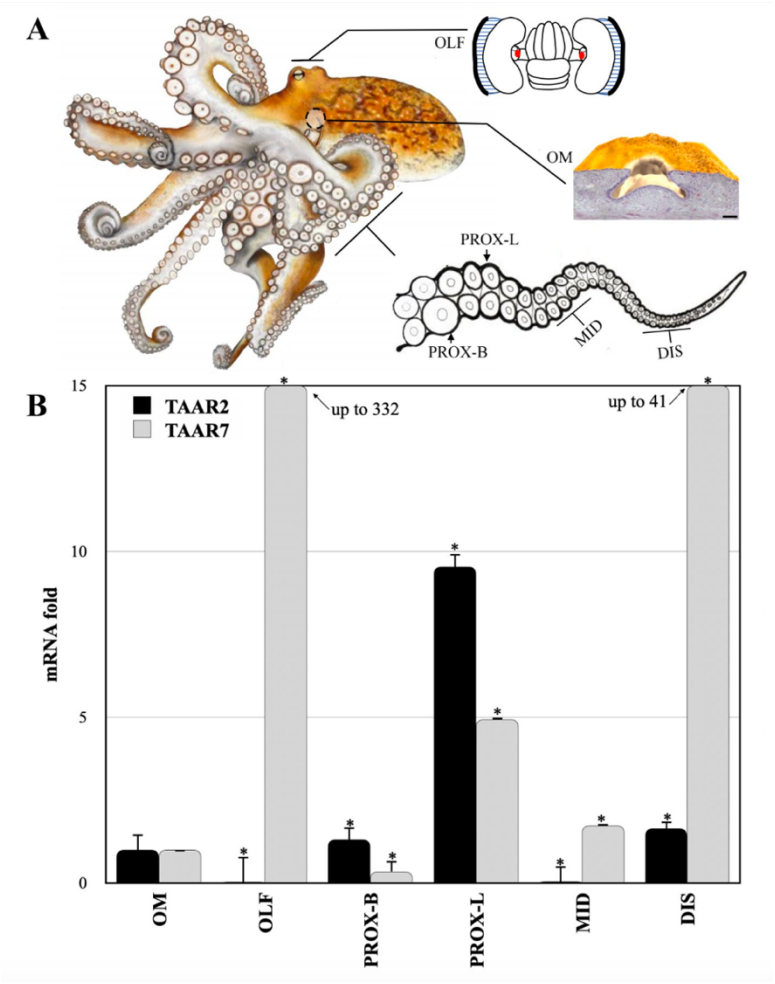

Figure 3. 9. Octopus anatomy and GPCRs gene expression analysis.

A) Schematic of O. vulgaris' anatomy, highlighting the tissues sampled for gene expression analysis: olfactory mucosa (OM), olfactory lobe (OLF), and hepatopancreas (HEP). Four sucker types: proximal big (PROX-B), proximal large (PROX-L), middle (MID), and small distal ones (DIS).

B) Gene expression analysis for mRNA of GPCRs (TAAR2 and TAAR7) of different tissues. Relative mRNA expression levels were measured using real-time analysis and calculated by the 2 (−ΔΔC(T)) method. Each sample (n=2) was tested and run in duplicate. Hepatopancreas was used as negative control (not shown).

3.3.3 LOCALISATIONS OF GPCRs GENES IN ARM SUCKERS

The localization of GPCRs genes transcripts or mRNA expression in the arm suckers of *O. vulgaris* was performed using the WMISH with antisense RNA probes composed of the gene sequences. The results of WMISH showed that GPCR (Figure 3.10A, B) genes were widely and regularly expressed in the outer border of the epithelium rim (RIM). The localization of the GPCR gene was confined to intra-epithelial cells, which were organized as large and globose elements and formed clusters (Fig.9A). Furthermore, the GPCR gene distribution in arm suckers was more diffuse and included globose clusters as well as slender and spindle-shaped cells (Fig.9B). Furthermore, the expression of GPCRs in arm suckers tended to decrease from the infundibulum (IF) towards the acetabulum and was distributed in the internal surface of the suckers. All GPCRs were found in the same region surrounding the epithelium under the toothed cuticle and were more cuboidal under the smooth cuticle. They also appeared on the lateral surfaces of the suckers. The negative controlled suckers did not show any specific coloration in all areas of the arm suckers.

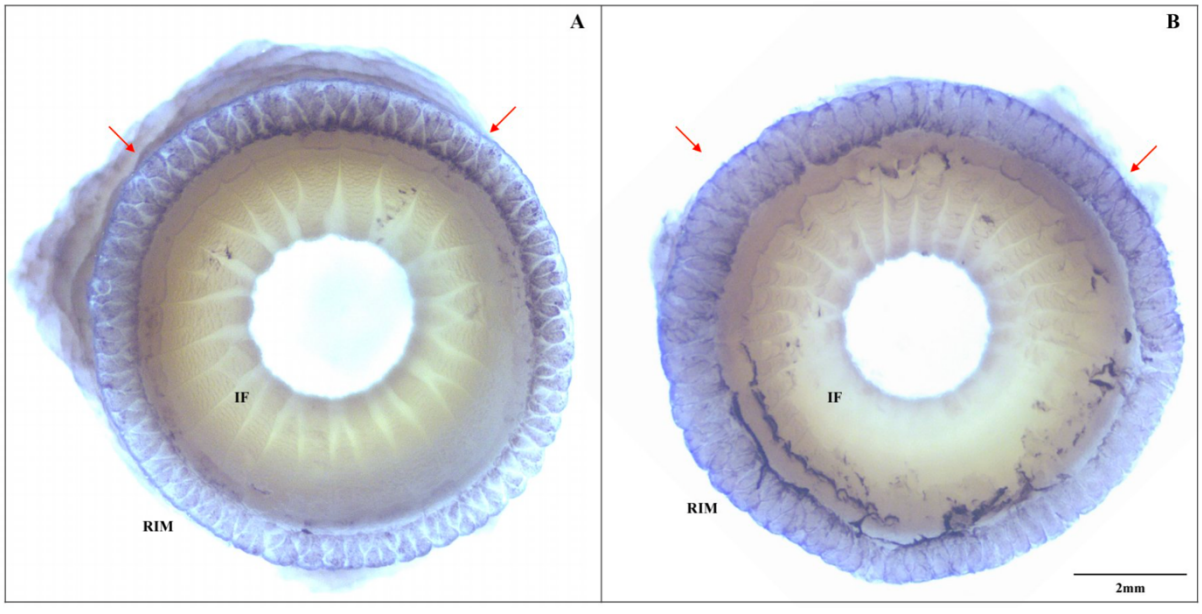

Figure 3. 10. Whole-mount *in-situ* hybridization of the GPCRs genes in the rim of an *O. vulgaris* middle sucker.

The receptor expression of GPCR TAAR2 (A) and GPCR TAAR7 (B) is present in the epithelium of the rim (RIM), but not in the sucker infundibulum (IF). Red arrows indicate positive signals. The dotted lines delimit the clusters of positive cells.

3.3.6 BEHAVIORAL EXPERIMENT

The behavioral assay revealed the ability of the octopus to recognize water-insoluble odorants used in our experiment. Two ceramic balls were used in the assay to stimulate the octopus by placing agarose ceramic balls coated with limonene and uncoated ceramic balls in the tank. These discrimination behavior approaches enhanced olfactory memory in *O. vulgaris* by showing the animal's response to a food reward.

Following the suggested protocol in this chapter, we analyzed each trial using BORIS software. After reviewing the extracted dataset, we trimmed the experiment and control trials to 23 trials each (n=23) to obtain an equal number for both experiments during the analysis. The fewer animal replications were due to limited accessibility to the animal facility and difficulties in finding wild-captured animals during the COVID-19 pandemic period in 2021-2022. The ethograms have taken after the analysis of training (pre) trials and experiment/control (post) trials analyzed for three days have clearly showed octopuses were prefer to spent more time with control jar with agarose-coated ceramic ball with limonene (L+) over the uncoated ceramic ball (C) (Figures 3.11-3.14).

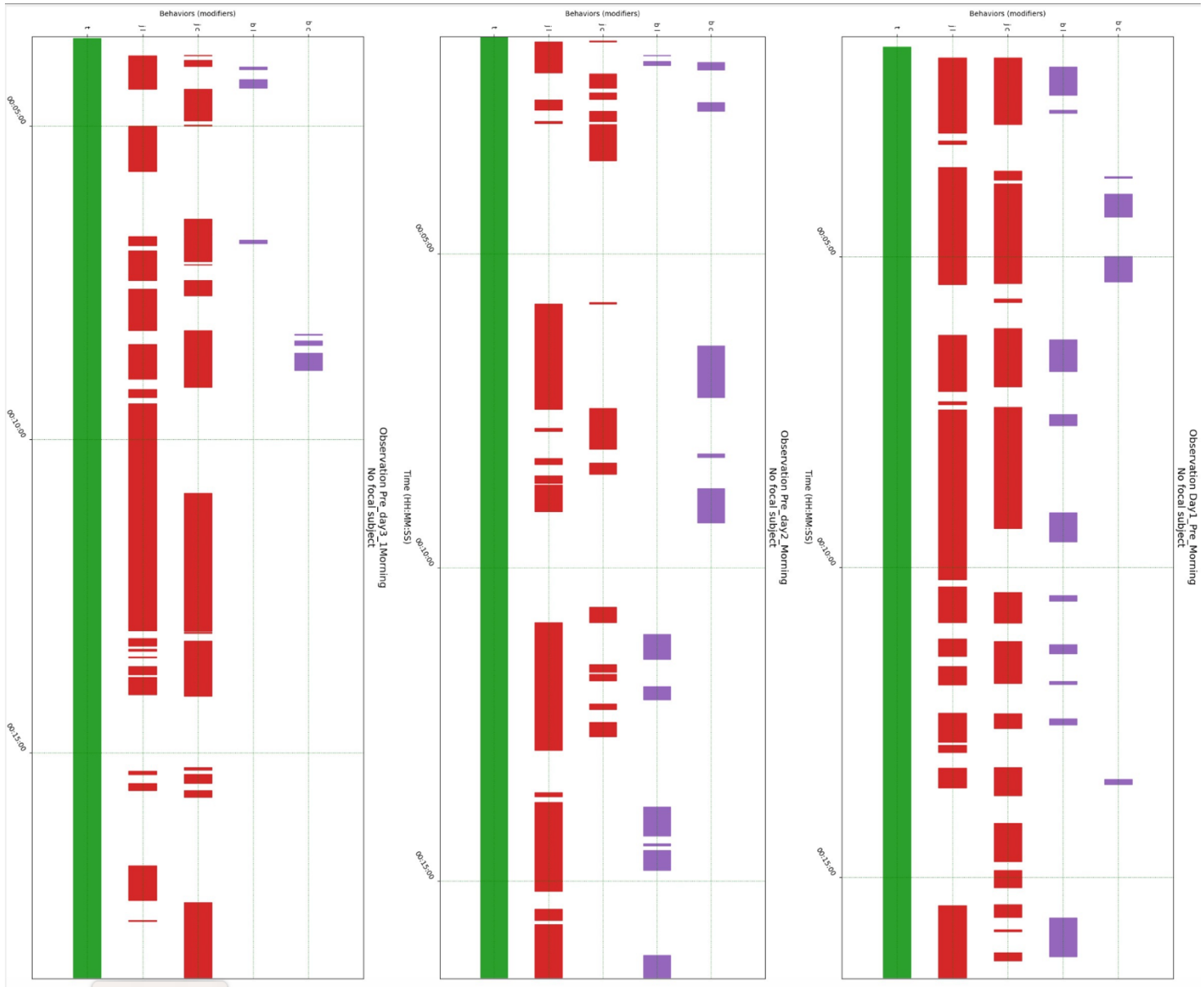

Figure 3. 11. Behavioral response of *O. vulgaris* with jars, agarose-coated ceramic ball with limonene (L+) and uncoated ceramic ball (C) in morning training trials.

(The time spend of Octopuses with jars, agarose-coated ceramic ball with limonene (L+) and uncoated ceramic ball (C) in training (pre) trials.)

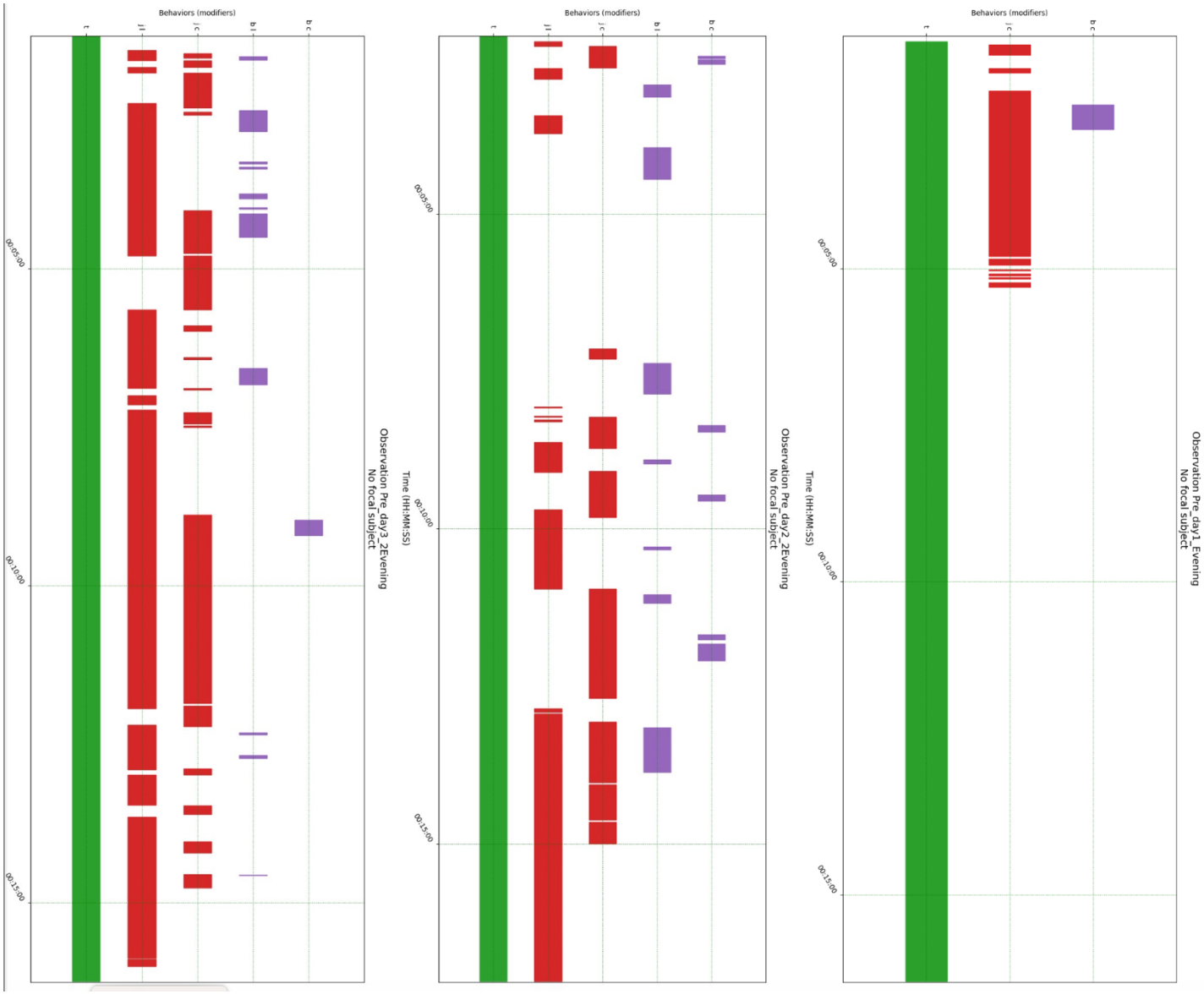

Figure 3. 12.. Behavioral response of *O. vulgaris* with jars, agarose-coated ceramic ball with limonene (L+) and uncoated ceramic ball (C) in evening training trials.

(The time spend of Octopuses with jars, agarose-coated ceramic ball with limonene (L+) and uncoated ceramic ball (C) in training (pre) trials.)

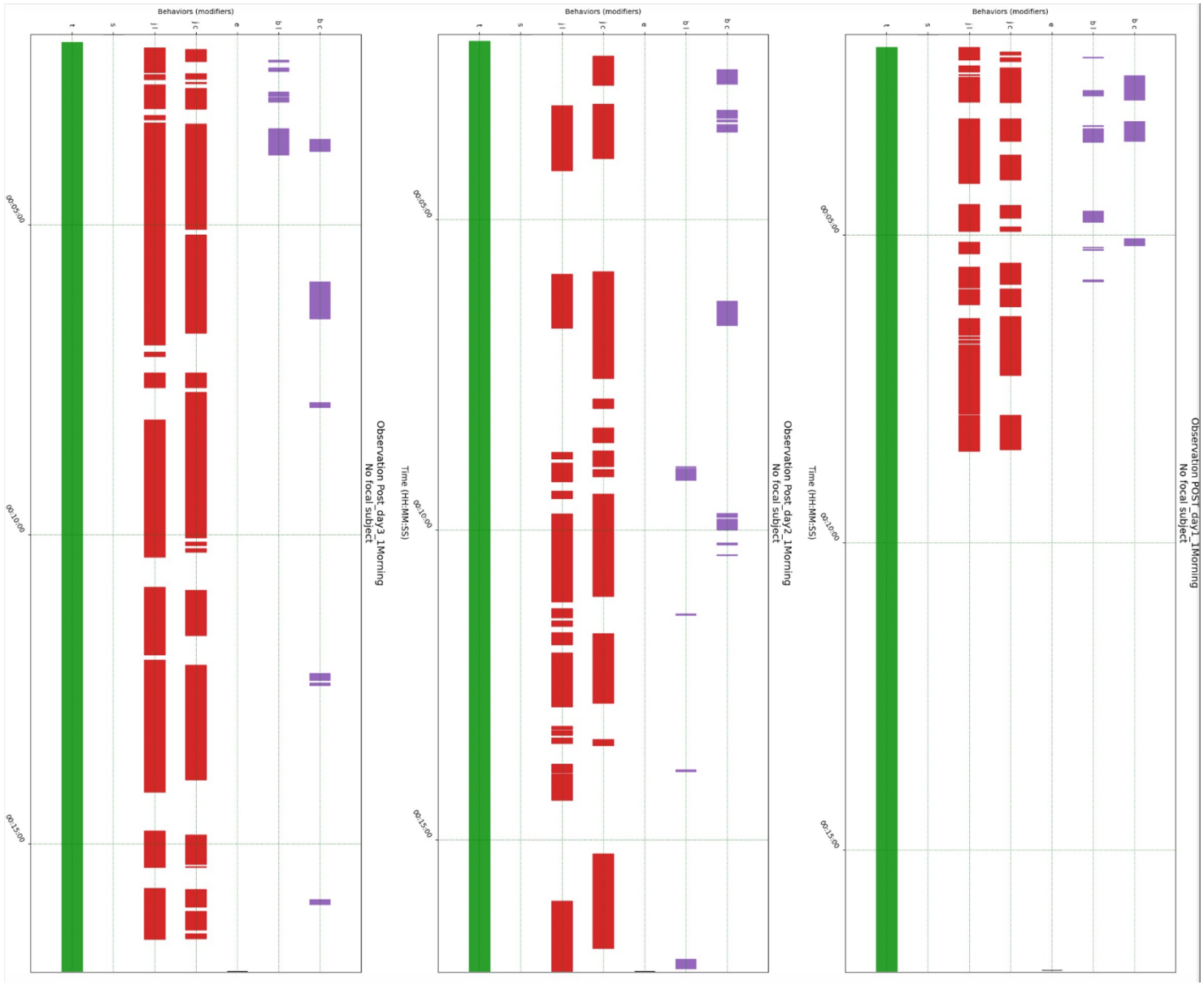

Figure 3. 13. Behavioral response of *O. vulgaris* with jars, agarose-coated ceramic ball with limonene (L+) and uncoated ceramic ball (C) in morning training trials.

(The time spend of Octopuses with jars, agarose-coated ceramic ball with limonene (L+) and uncoated ceramic ball (C) in experiment/control (post) trials. [3:end of 3 minutes, r: reward, l: lure, s: screen removal, t: trial duration, w: try to open jar, b: ball interaction, j: jar interaction, o: opened jar, 1: 1 min from ball touch and f: begging for food])

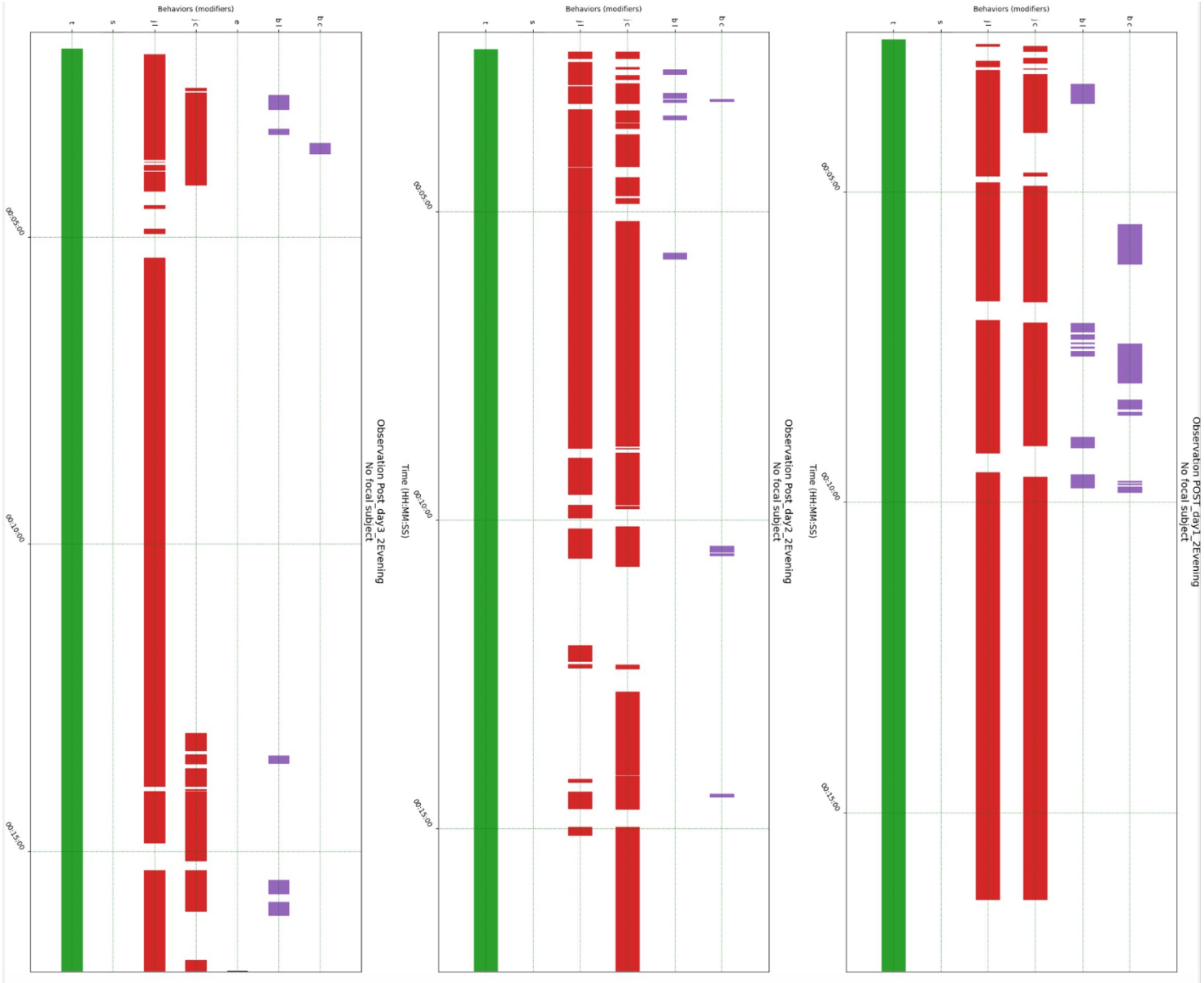

Figure 3. 14.. Behavioral response of *O. vulgaris* with jars, agarose-coated ceramic ball with limonene (L+) and uncoated ceramic ball (C) in evening training trials.
(The time spend of Octopuses with jars, agarose-coated ceramic ball with limonene (L+) and uncoated ceramic ball (C) in experiment/control (post) trials. [3:end of 3 minutes, r: reward, l: lure, s: screen removal, t: trial duration, w: try to open jar, b: ball interaction, j: jar interaction, o: opened jar, 1: 1 min from ball touch and f: begging for food])

The animal choice during the experiments clearly indicated a preference for the agarose-coated ceramic ball with limonene (L+) over the uncoated ceramic ball (C) (Figure 3.10). This preference was further confirmed by the octopus's willingness to spend more time with the L+ jar and ball (Figure 3.10). We observed the same preferences in both trial experiments with both individuals. During both the training and experiment/control trials, the octopuses exhibited stereotypical exploratory behaviors, using sweeping arm motions that allowed their suckers to probe the agarose ball containing the chemical compound (limonene). This clearly indicates the highly learning abilities of octopuses in response to pairing two types of agarose-coated ceramic balls with and without limonene with food rewards.

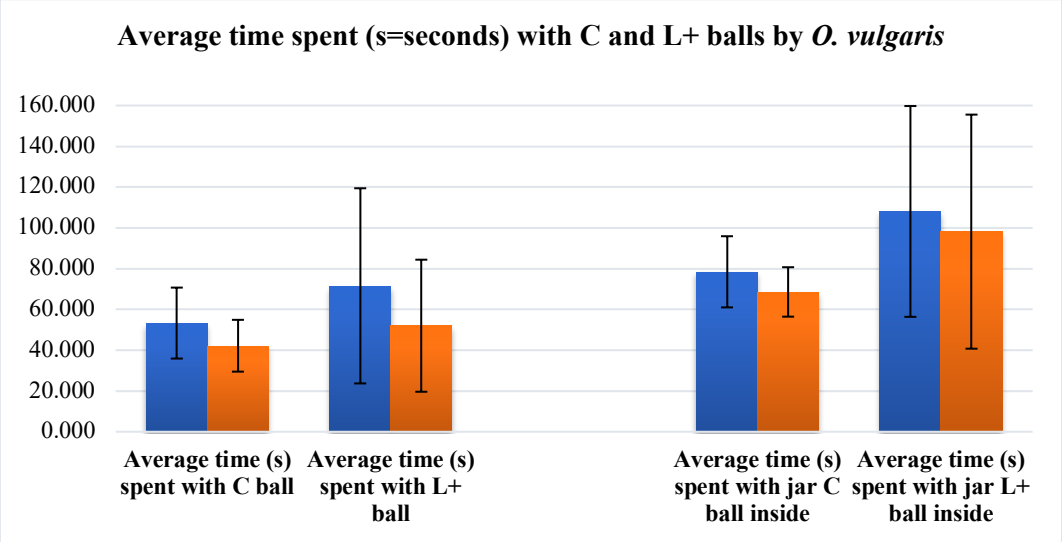

Figure 3 15. Average time (s=seconds) spent by *O. vulgaris* by each jar with agarose coated ceramic ball with Limonene (L+) and without Limonene (C).

3.4 DISCUSSION

Octopuses possess the most advanced and complex nervous system among invertebrates, with a majority of their neurons located in their arm suckers (Mather & Dickel, 2017; Deryckere & Seuntjens, 2018; Mouritsen & Styrbæk, 2021). As a result, they have sophisticated and well-developed sensory organs, including olfactory systems that detect chemosensory stimuli from their external environment (Borrelli & Fiorito, 2008; Polese et al., 2016; Di Cosmo & Polese, 2017; Deryckere & Seuntjens, 2018; Shigeno et al., 2018; Amodio et al., 2020; Maselli et al., 2020a). The olfactory organ of *O. vulgaris* is particularly well-studied for its role in chemical perception, allowing the octopus to sense water-soluble molecules from a distance (Polese et al., 2015). This is critical because a significant proportion of the octopus's neurons are located outside the central nervous system, spread throughout its arms and suckers, and many of its behaviors rely on these organs (Sumbre et al., 2001; Grasso, 2008; Grasso & Basil, 2009; Zullo et al., 2019). Octopus arms are used for a variety of functions, including crawling, walking, disruptive camouflage, signalling, mimicry, conspecific aggression, mating, general manipulation of the environment, and capturing or extracting prey (Wells, 1978; Mather, 1998; Hanlon & Messenger, 2018; Al-Soudy et al., 2021).

However, to date, there have been limited studies conducted on how octopuses recognize and respond to insoluble molecules, including the ability of the arm to "taste" food-related complex odors, which has been previously demonstrated (van Giesen et al., 2020). Furthermore, the chemotactic response of *O. vulgaris* has not been thoroughly examined with a focus on the "smell by touch" approach. Therefore, our study aims to present the first-ever genetic and behavioral evidence for a "smell by touch" system in the suckers of the *O. vulgaris* arm, which is revealed by the functional expression of GPCRs olfactory receptors.

Nevertheless, earlier descriptions of octopus suckers did not relate them to olfactory sensing or function. However, recent studies have proposed that octopuses exhibit "smell by

touch" behavior, and that arm suckers have the ability to recognize water-insoluble odorants (Polese et al., 2015, 2016; Di Cosmo & Polese, 2017). This suggestion prompted us to investigate the chemo-tactile sensation involved in "olfaction".

The involvement of GPCRs, which are olfactory receptors, in octopus arm suckers is a necessary area of study. Because, the relative expression of these two GPCRs in different tissues could be involved in different types of functional purposes.

We observed different expressions of GPCRs in various suckers throughout the arm, denoting differential use of suckers and areas of the arm in sensing and manipulation tasks of the octopus. In addition, our findings showed extremely upregulated GPCR expression in PROX-L suckers, which are sparse, markedly large relative to the surrounding suckers, and present only in males (Wells, 1967). The upregulation specifically in PROX-L suckers suggests a potential role in detecting chemical cues emitted by female octopuses. The WMISH results clearly showed that the GPCR gene is confined to the intra-epithelial cells (Figure 2.9A), organized as large and globose elements, forming clusters that resemble the type 2b cells described by Graziadei and Gagne (Graziadei & Gagne, 1976) as one of the primary sensory cells in the octopuses' sucker, with a potential chemo-sensing function. Additionally, GPCR is expressed in the OLF, and the OM showed downregulated expression compared to the OLF.

The OLF had the highest expression of GPCR, whereas DIS suckers showed the highest expression among the suckers (Figure 3.9 B). It is understandable that the DIS suckers showed high expression, as octopuses use it when interacting with the environment and when exploring their surroundings. Furthermore, when the animal detects and grasps something, they bring it directly to their mouth by creating pseudo joints that point at which the arm bends (Gutfreund et al., 1996, 2006). The MID suckers belong to these arm bend areas, which do not interact with the grasped item. Therefore, it is likely that the GPCR role in the DIS suckers is indeed

searching for chemical cues during explorative behaviors such as foraging (Buresch et al., 2022) or individual recognition (Nesher et al., 2014).

The WMISH results showed a more diffuse distribution of the GPCR genes also in the rim edge of the sucker, with the positivity extending to slender and spindle-shaped cells. These are more likely to resemble the Type 2a cells (Figure 3.9B) described by Graziadei and Gagne (Graziadei & Gagne, 1976). Among the other tissues we examined, OLF had the highest expression of GPCRs, which could be related to its modulatory function in chemoreception and reproduction (Polese et al., 2015).

Our findings strongly suggest that the discovered GPCRs could potentially be involved in octopus arm suckers' detection of water-insoluble molecules, including substances like limonene and other water-insoluble organic compounds. However, further validation of these identified GPCRs requires additional investigation and comparison with other potential GPCRs present in octopus arm suckers.

The associated learning behavioral approach clearly demonstrated the octopus's ability to recognize water-insoluble odorants. This discrimination behavior approach enhanced olfactory memory in *O. vulgaris* by showing the animal's response to a food reward with progressive results. The cognitive abilities of *O. vulgaris* are similar to those of vertebrates with higher-level memory capacity (Bublitz et al., 2021), which supports the recognition of water-insoluble limonene that we used in our experiments. Therefore, our behavioral assay indicates that clearly octopuses can discriminate between two types of ceramic balls through contact chemoreception using their gustatory system. However, sometimes octopuses are unable to perform the task due to lack of interest and depending on the experimental designs. Then, all behavioral performance depends on the protocol and octopus stress, experimental conditions, rearing conditions, and the approach of the protocol, etc. (Bublitz et al., 2021).

The behavioral assay has demonstrated the ability of the octopus to recognize the water-insoluble odorant used in our experiment. Two ceramic balls were used in the assay to stimulate the octopus, one coated with limonene and the other without. This discrimination behavior approach enhanced olfactory memory in *O. vulgaris* by eliciting a response to a food reward. However, during both the training and experimental/control trials, octopuses displayed stereotypical exploratory behavior involving sweeping arm motions that allowed their suckers to probe the water-insoluble odorant, and they showed a higher willingness to stay with the jar and agarose-coated ceramic ball with limonene.

Traditionally, olfaction has been considered a distance sense, allowing animals to detect chemical stimuli from a remote source, while taste is defined as a contact sense that requires physical contact with the chemical source for detection. However, in the marine environment, aquatic olfaction is largely mediated by odorant molecules that are dissolved in water and detected from a distance (Ache & Young, 2005; Brönmark & Hansson, 2012). On land, odorant compounds are generally recognized at a distance by the olfactory system and are transported through the air as small, insoluble molecules smaller than approximately 300 Da (Mori et al., 2006; Touhara & Vosshall, 2009). In contrast, these molecules must be directly touched in order to be sensed as olfactory signals in the water (Giordano et al., 2017). While some cephalopod species may possess genetic underpinnings required for both olfactory systems, octopuses, which have a benthic lifestyle and rely heavily on their arms, are particularly well-suited for studying tactile-olfactory perception.

Using a combination of molecular approaches, our study demonstrates that octopus suckers are capable of detecting odors through tactile contact, indicating that chemical sensing is not limited to the olfactory organ alone (Polese et al., 2015, 2016; Di Cosmo & Polese, 2017). The octopus central sensory organs are known to collect chemical and photic stimuli, while the many suckers on each of its eight arms form a second, diffuse, and multi-modal sensory system.

In summary, this chapter, the focus lies on exploring the remarkable sensory capabilities of octopuses, with particular emphasis on their olfactory system and the intriguing "smell by touch" behavior exhibited through their arm suckers. Through comprehensive investigations, it becomes evident that octopuses possess a highly developed nervous system, with a substantial number of neurons located in their arm suckers, enabling them to detect and respond to chemical stimuli from their surrounding environment. While previous studies have extensively examined the olfactory organ's role in chemical perception, there has been limited research concerning octopuses' ability to recognize water-insoluble molecules. Therefore, this chapter presents ground-breaking genetic and behavioral evidence supporting the presence of GPCRs in the arm suckers of *O. vulgaris*. Furthermore, behavioral assays provide compelling support for the octopus's ability to discriminate between water-insoluble odorants, showcasing their advanced cognitive abilities. Additionally, this chapter sheds light on the fascinating sensory modalities of octopuses (*O. vulgaris*), offering valuable insights into their complex nervous system and sensory processes, thus contributing significantly to the understanding of these enigmatic creatures.

CHAPTER 4

ENHANCING ANIMAL WELFARE THROUGH AN ANESTHESIA PROTOCOL FOR *O. vulgaris*

4.1 INTRODUCTION

In the evolving landscape of animal welfare, the scope of our ethical considerations is expanding. Historically, the welfare of animals has predominantly centered around mammals, often seen through a human-centric lens. However, our understanding of animal sentience has been undergoing a profound transformation. It is becoming increasingly evident that sentience, or the capacity to experience pain and suffering, is not limited to mammals but extends to other animal groups, including decapod crustaceans (such as crabs and lobsters) and cephalopod molluscs (like octopuses) (Passantino et al., 2021; Paolucci et al., 2023). This paradigm shift compels us to broaden our perspective and consider the welfare of all animals, particularly invertebrates, during experimental procedures.

The use of anesthesia in invertebrates is crucial for ensuring their humane treatment, yet this subject is marked by its complexity and the diversity of animals it encompasses (Lewbart et al., 2012). In clinical practice, general anesthetics serve a multifaceted role. They act as muscle relaxants, analgesics (pain relievers), anesthetics to induce unconsciousness, and amnesics to prevent the formation of traumatic memories (Lewbart et al., 2012). Achieving all these objectives with a single substance is a challenging task, often necessitating the use of multiple adjuvants, which results in the administration of complex drug combinations. In clinical settings, patients remain blissfully unaware of surgical procedures, and they retain no memory of the experience, thus allowing them to assert the absence of pain. However, this scenario becomes significantly more intricate when applied to invertebrates. The question of

whether invertebrates, particularly cephalopods, can experience pain has become a subject of intense scientific inquiry (Elwood, 2011; Crook et al., 2013; Sneddon, 2015).

Among Cephalopods, *O. vulgaris* which exhibit behaviors that strongly suggest their capacity to perceive and respond to noxious stimuli. They demonstrate pain avoidance by actively steering clear of locations associated with painful or aversive experiences (Crook, 2021). Similar indicators of pain perception are being recognized in arthropods (Elwood, 2023). This growing body of evidence necessitates a meticulous and compassionate approach when conducting experimental procedures on invertebrates to minimize their stress and potential for pain during physiological experiments.

In the realm of anesthesia for cephalopods, magnesium chloride ($MgCl_2$) has emerged as a candidate for consideration. Although primarily recognized as a muscle relaxant, there have been contentious discussions concerning its ability to ensure pain-free anesthesia (Elwood, 2011; Abbo et al., 2021). The mechanism of action of $MgCl_2$ involves its competition with calcium ions, effectively inhibiting neurotransmitter release in peripheral regions without significant penetration into the central nervous system (Winlow et al., 2018). In 2018, $MgCl_2$ was proposed as a potential adjunct to anesthesia (Winlow et al., 2018), prompting a deeper exploration of this concept. A more comprehensive discussion of the mode of action of magnesium salts as muscle relaxants is available elsewhere (Winlow et al., 2018).

When it comes to volatile anesthetics, isoflurane has gained prominence for cephalopod anesthesia (Winlow et al., 2018; Polese et al., 2014). Isoflurane, a non-flammable halogenated ether compound, is commonly used in both clinical and veterinary settings. When administered via the respiratory system, it offers direct access to both the peripheral and central nervous systems (Winlow et al., 2018).

Recent research has yielded a successful anesthesia protocol for *O. vulgaris* using clinical anesthesia (Polese et al., 2014). However, this protocol is characterized by a relatively

extended induction period, nearly two hours from the commencement of anesthesia to full recovery. This approach involved a gradual increase in isoflurane concentrations in seawater, ranging from 0.5% to 2.0%, with the recognition of a critical transition occurring at 1.0% isoflurane. At this concentration, the chromatophores, specialized pigment cells, began to exhibit uncontrolled flashing—a reliable sign of the loss of central motor control in *O. vulgaris* (Polese et al., 2014). Subsequently, the animals entered a state of relaxation, became unresponsive to tactile stimuli, and achieved full anesthesia. To gauge anesthesia and recovery, physiological markers, such as the respiratory pumping rate, and behavioral tests measuring local withdrawal responses and chromatophore pattern loss were employed (Polese et al., 2014). However, the extended duration of anesthesia raised concerns about the stress experienced by the animals and the quantity of anesthetic agents used.

This study introduces an innovative approach that involves the utilization of $MgCl_2$ as both a pre-anesthetic agent and an adjunct to anesthesia, resulting in significantly reduced induction times for successful anesthesia and facilitating a rapid recovery process. This groundbreaking technique not only minimizes the utilization of anesthetic agents but also reduces the stress experienced by animals during experimentation. The implications of this research extend beyond refining invertebrate anesthesia; they hold the promise of enhancing animal welfare across experimental domains.

4.2 MATERIAL AND METHODS

4.2.1 ANIMALS ACQUISITION

Adult specimens of *O. vulgaris*, nine (9) specimens of *O. vulgaris* (5 males, 4 females, weighing approximately 500-1000g, were collected from the Bay of Naples (Italy) and transported to Professor Anna Di Cosmo's cephalopod facility at the Department of Biology, University of Naples Federico II, Naples, Italy. Upon arrival, animals were placed in fiberglass tanks measuring 50 x 50 x 50 cm, filled with seawater using a dedicated circulatory system. Animal was allowed to acclimate for a period of 15 days. The facility maintained a seawater temperature of 18°C and followed a natural photoperiod of 12 hours of light and 12 hours of darkness. Throughout the acclimatization period, the octopus was provided with a varied diet consisting mainly of anchovies, shrimps, and mussels. Water quality parameters were monitored daily, and regular tank cleaning was conducted to ensure the well-being of the animals and detect any signs of stress.

Following the acclimatization period, the animal was kept in the same tank without any assigned tasks or activities, serving as the control animal which not subjected to any experimental manipulation or intervention (Polese et al., 2014; Maselli et al., 2020a). The animals' housing, handling, monitoring, and experimental procedures were performed according to the guidelines and instructions provided for cephalopod welfare, in order to protect and use animals in research. All research was approved in accordance with the European Directive 2010/63 EU L276, the Italian DL. 4/03/2014, no. 26, and the ethical principles of Reduction, Refinement, and Replacement. (Project n° 608/2016-PR-17/06/2016; protocol n° DGSAF 0022292-P-03/10/2017).

4.2.2 ANAESTHESIA SETUP

Inhalational anesthesia delivery system designed for aquatic animals was used in this experiment. The surrounding seawater was aerated using a power pump and controlled by a

flowmeter (at a rate of 1.8 L/min). This aerated mixture, in combination with isoflurane from a vaporizer, was then channeled into the bath through an air stone (Figure 4. 1). It is important to note that, with the exception of introducing the anesthetic into the aquatic medium, this technique mirrors a standard clinical practice, ensuring that isoflurane is carried to the animal using air as a carrier gas. This method guarantees that the animal maintains proper oxygenation throughout the procedure. All inhalational anesthetics are introduced to an animal's system via an air-water interface, the respiratory epithelium in both vertebrates and octopi, but in this case it was via the aquatic medium. The experimental setup involved a bath enclosed and externally vented, holding 1,600 mL of seawater, consistently maintained at 18°C during the experiment. Each animal was given a minimum of 10 minutes to acclimate to the bath environment before we commenced the delivery of the air and isoflurane gas mixture through the air stone. Past experiments employing gas-liquid chromatography have demonstrated that this method achieves equilibration in about 10 minutes (Polese et al., 2014; Di Cosmo et al.,2023).

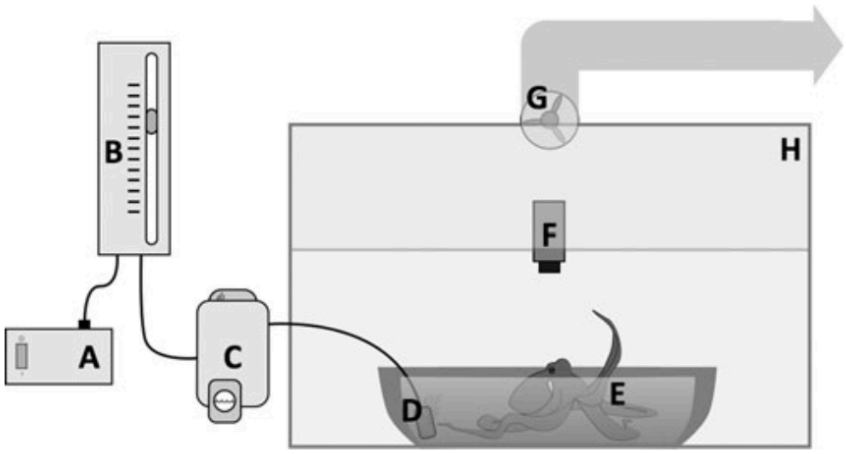

Figure 4. 1. Anaesthetic delivery set up

Electric pump (A), flowmeter (B), and a specific (isoflurane) vaporizer (C) terminating in an air stone (D) in a tank (E) filled with 1.6 L of seawater. The ambient temperature of the water was 18◦C. A recording camera (F) was placed just over the tank to record the movements and

color changes of the animal. The whole system was placed in an enclosure (H) and air was expelled to the outside using an extractor fan (G).

4.2.3 ANAESTHESIA PROTOCOL

After the acclimatisation period, animals were first moved gently into an experimental tank containing 1% $MgCl_2$ in aerated seawater for a period of 10 min. Then the anaesthetic was insufflated in the airflow for 5 min at 1% concentration, after which the animal was maintained in fresh clean seawater until full recovery. To determine the depth of anaesthesia, we used two criteria: physiological, the respiratory rate as judged by the frequency of respiratory pumping, and behavioural, changes of the chromatophore pattern and withdrawal of the arms and siphon. We recorded the responses with a web camera (GoPro Hero10, 4K video at 30 frames per second (fps) in full-screen, internal auto-focus lens system).

The respiratory rate was taken every minute, just before the touch test was performed, to avoid any change due to stimulation. Animals had widely varying pre-control respiratory rates (24–45 cycles per minute) as judged by the pumping movements of the mantle, so for analytical purposes, we normalised the respiratory rate, with 100% being the observed pre-control value (Polese et al., 2014; Di Cosmo et al.,2023). Touch stimuli induced withdrawal of the arms and siphon, and we monitored the changes chromatophore pattern throughout each experiment. The withdrawal responses were elicited by the gentle application of a blunt Plexiglas probe to the arms and siphon.

For analytical purposes, we categorised the responses as strong, medium, weak, or abolished, and these were given numerical values of 6, 4, 2, and 0, respectively. Using imageJ software (Collins, 2007) we measured a spot 10 pixels in size from the interbrachial membrane on frames taken every 5 min throughout the experiments.

The colour intensity of each spot was measured based on the additive red, green, blue (RGB) colour model whereby a zero intensity (value, 0) for each component gives the darkest

colour (no light, considered to be black) and full intensity for each component gives white (value, 255). One-way analysis of variance (ANOVA) was used to assess the significance of differences between the data at time 0 and those at other times.

4.3 RESULTS

4.3.1 ACTIONS OF 1% $MgCl_2$ ON ANIMAL BEHAVIOUR

Upon exposure to 1% $MgCl_2$ in aerated seawater, most animals initially exhibited signs of distress. However, within three minutes of $MgCl_2$ application, a visible relaxation was observed in the animals, indicated by a 4.17% reduction in the median normalized respiratory rate. By the 5-minute mark, all animals had turned pale in color (Figure 4.2) and displayed weakened responses to touch (Figure 4.3). After 10 minutes, the animals were became pale, unresponsive, and immobile, accompanied by a 19.50% reduction in the median % normalized respiratory rate. Consequently, the animals appeared to be in a state of paralysed, yet they continued to maintain a steady respiratory rate (Figure 4.4). Interestingly, none of the animals exhibited chromatophore-related flashing reactions at any stage, likely attributed to the inactivation of transmitter release from chromatophore motor neurons.

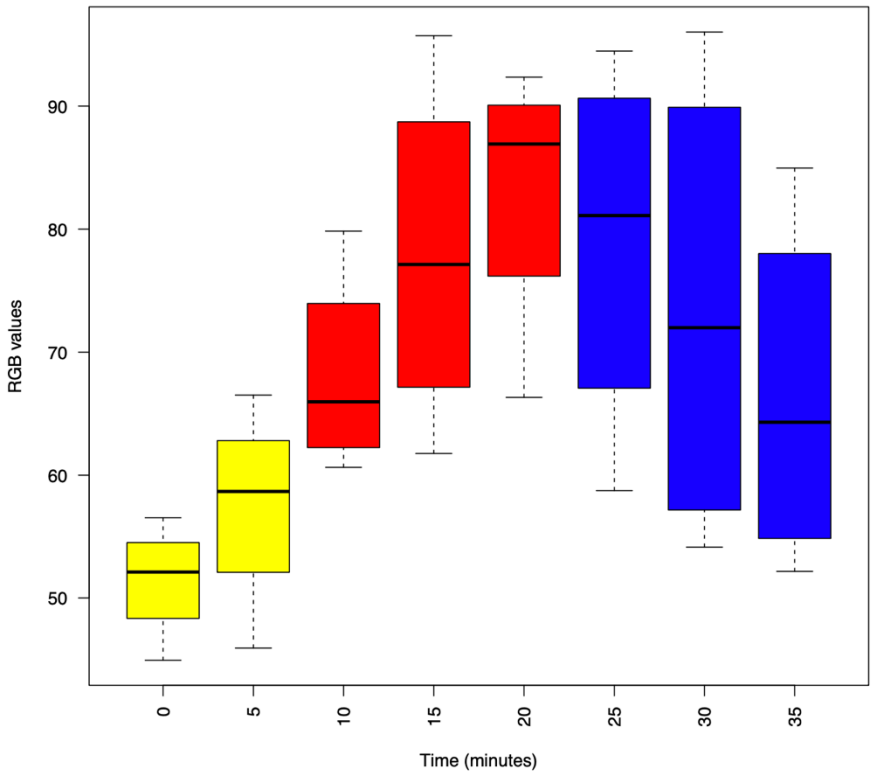

Figure 4. 2. Box plot of the colour intensity of the interbrachial membrane as the concentration of isoflurane was progressively increased and then decreased.

Yellow, 1% $MgCl_2$: Red, 1% $MgCl_2$ and 1% isoflurane; Blue, recovery in fresh aerated seawater. Color intensity was measured using the RGB model whereby a zero intensity (value, 0) for each component gives the darkest color (no light, considered to be black) and full intensity for each component gives white (value, 255). Thus, the higher the recorded value the paler the interbrachial membrane and vice versa.

4.3.2 ADDITION OF I% ISOFLURANE

Following the 10-minute exposure to 1% $MgCl_2$, 1% isoflurane was introduced into the experimental tank by bubbling. While the animals remained unresponsive and continued to grow paler (Figure 4.2), their respiration rate decreased, signifying the onset of isoflurane's anesthetic effect (Figure 4.4). In clinical and veterinary contexts, a reduced respiratory rate serves as a clear indicator that anesthesia is taking effect. After five (5) minutes of anesthesia with isoflurane, the isoflurane supply was turned off, leading to a reduction in the normalized respiratory rate to 54.17% ± 3.59% (mean ± SE) of the normalized baseline. At this point, the pale and unresponsive animals were gently transferred to a different experimental tank that contained fresh aerated seawater to facilitate their recovery.

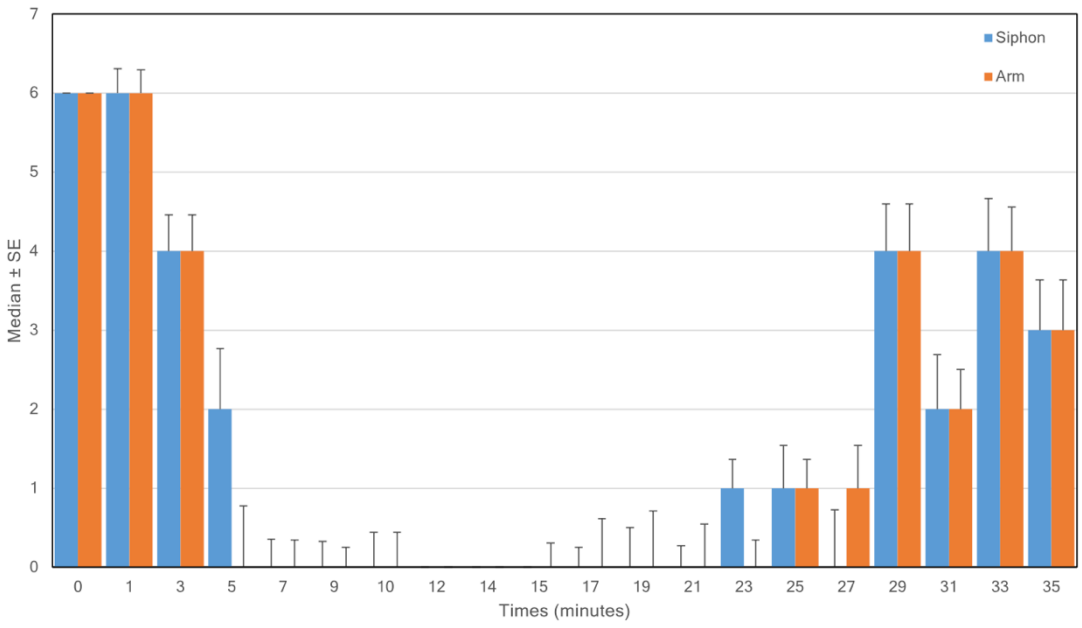

Figure 4. 3. Relative strength of the withdrawal of octopus siphon and arms in response to a touch stimulus versus time (6=strong, 4=medium, 2=low, and 0=none) in 9 animals.

0-10min in 1% $MgCl_2$; 10-15 min in 1% $MgCl_2$ and 1% isoflurane; 15-35 min in fresh aerated seawater.

4.3.3 RECOVERY FROM ANAESTHESIA

In six (6) cases, the animals fully regained their color patterns, respiratory frequencies, and arm and siphon reflex sensitivity within 10-15 minutes. One animal experienced a rapid recovery within seven (7) minutes, while two others took approximately 20 minutes to recover (Figure 4.4).

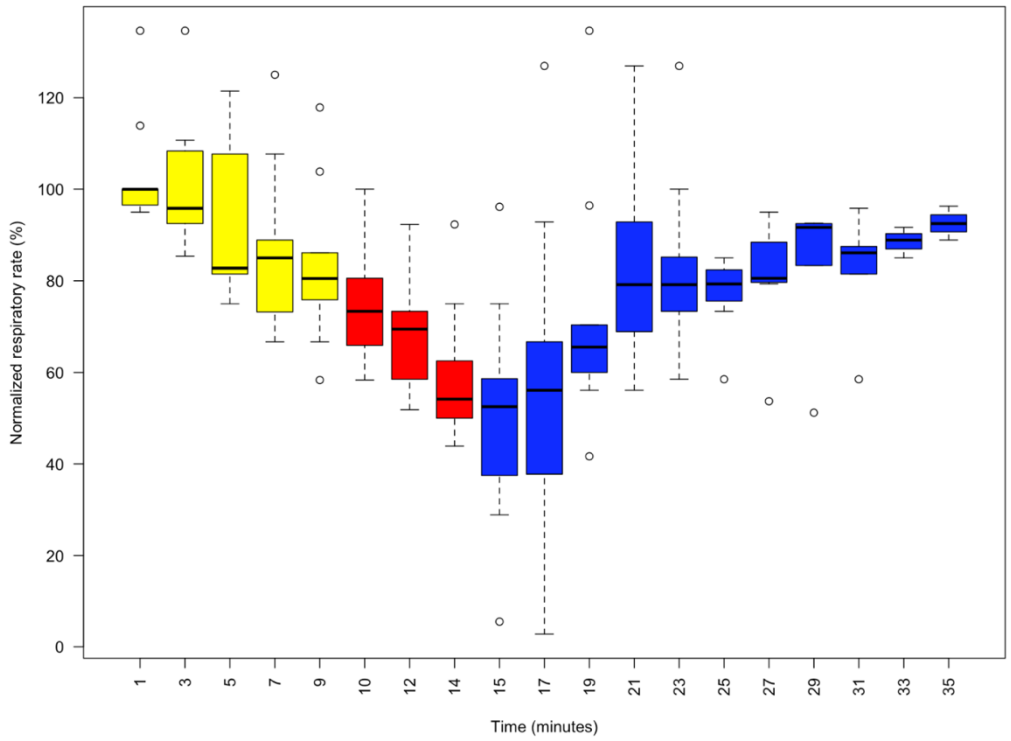

Figure 4. 4. Boxplot of the normalised respiratory rate (%) as determined by the number of mantle contractions per minute in nine (9) animals. Readings were taken at two (2) minutes intervals.

Yellow phase: $MgCl_2$ 1% (10 minutes). Red phase: isoflurane 1% and 1% $MgCl_2$(5 minutes). Blue: recovery phase in fresh aerated seawater. As respiratory rate variability between

animals is substantial, the outlying circles indicate the recorded maximum and minimum normalised respiratory rates. It should be noted that after 10 min in 1% $MgCl_2$, the respiratory rate stabilised at about 80% of its normalised value, followed by a further gradual decline after 1% isoflurane, which was terminated by changing the medium to clean aerated seawater.

4.4 DISCUSSION

Here we illustrated the pre-anesthetization of *O. vulgaris* using $MgCl_2$ for a 10-minute period, followed by full anesthesia with 1% isoflurane for five minutes. The maximum recovery time was approximately 20 minutes. During the initial 10 minutes, we observed a gradual decline in the normalized respiratory rate (%) due to the presence of $MgCl_2$ alone, resulting in a 20% decrease in the normalized value. Subsequently, the introduction of 1% isoflurane led to a significant 44% reduction in the respiratory rate, which persisted into the subsequent phase when the animals were transferred to fresh aerated seawater. However, after an additional two (2) minutes, the respiratory rate began to decline, with notable variations among different animals. Yet, after 20 minutes (approximately 35 minutes into the overall experiment) of replenishing the bathing solution with clean, aerated seawater, all animals made a complete recovery. Although the normalized respiratory rate did not reach 100% in all animals (median ± standard error, 90.69 ± 1.60), however, all the animals were in good health. They were closely monitored for the following 7 days, during which they displayed normal behavior and remained in excellent condition.

This upgraded and developed new protocol is notably superior to our earlier study (Polese et al., 2014) in which we administered increasing doses of isoflurane (ranging from 0.5% to 2.0% in seawater) to 10 *O. vulgaris* specimens over a period of approximately 40 minutes, followed by a recovery period of up to 1 hour. The evident advantage of the new protocol lies in its significantly reduced stress on the animals and a considerably lower isoflurane usage. Moreover, the animals displayed minimal signs of discomfort in the presence of $MgCl_2$, and there were no flashing responses as the animals paled in color, and their touch reflexes decreased as they became immobilized.

Notably, in the presence of 1% $MgCl_2$ in aerated seawater, the respiratory rate experienced a reduction of less than 20% and stabilized after approximately 10 minutes. Upon

the addition of 1% isoflurane, the respiratory rate gradually decreased as expected, as a declining respiratory rate signifies an increasing depth of anesthesia. Additionally, isoflurane is recognized for its suitability in maintaining anesthesia (Pauca et al., 1973), as it also enhances muscle relaxation, while $MgCl_2$ mitigates the early excitatory phase of anesthesia, a concept we previously proposed (Guedel, 1937; Winlow et al., 2018).

While $MgCl_2$ has been employed as an anesthetic for octopods and other cephalopods (Abbo et al., 2021), it is typically applied externally, allowing little or no access to the central nervous system (CNS). In our perspective, using $MgCl_2$ as a standalone anesthetic is entirely inappropriate. $MgCl_2$ primarily functions as a muscle relaxant, although it may possess minimal sedative properties, as previously mentioned (Polese et al., 2014). Therefore, we disagree with the notion that it can serve as a sole anesthetic substance, as suggested in other sources (Abbo et al., 2021), where increasing concentrations of $MgCl_2$, up to 3.75%, were employed to immobilize octopus and cuttlefish specimens over a duration of approximately 20 minutes. These effects were then compared with those of ethyl alcohol, another non-anesthetic substance. It appears that these substances temporarily suppressed evoked activity in the pallial nerve, but no detailed records were provided. To gain a better understanding of nervous activity in these circumstances, it is crucial to provide clear demonstrations of electrical activity, potentially utilizing the recently published techniques by Gutnick et al. 2023.

Properties of general anesthetics, such as clinical anesthetics like isoflurane and related compounds, have well-established systemic and cellular effects that have been observed in mammals and in gastropod mollusks like Lymnaea stagnalis et al., 2019, as well as in *O. vulgaris* (Polese et al., 2014). These effects include interactions with ion channels and voltage-gated channels for sodium, potassium, and calcium, with a tendency to inhibit synaptic transmission (Hao et al., 2020). These properties are general in nature and should not be overlooked by researchers working with invertebrates. Additionally, Keltz and Mashhour 2019

have recently presented compelling evidence that a mechanistic framework for the actions of general anesthetics is emerging from studies spanning a wide range of species, "from *Paramecium* to primates." This framework includes organisms like *Drosophila*, *C. elegans*, gastropod mollusks such as *Lymnaea stagnalis*, and even plants. The appropriate maintenance anesthesia for experiments involving *O. vulgaris* has not yet been determined. Further experiments are necessary to establish the ideal level of anesthesia for fresh animals that have been relaxed with $MgCl_2$. It is likely that a slightly higher concentration of isoflurane will be required if the animal is to undergo surgery.

In summary, 1% magnesium chloride serves as a valuable pre-anesthetic agent for the isoflurane anesthesia of *O. vulgaris*. It reduces the required concentration of isoflurane for achieving complete anesthesia, significantly minimizing stress on the animal and expediting the anesthesia process.

A note regarding the use of vaporizers: The application of volatile general anesthetics does not always necessitate the use of vaporizers, although they do simplify the procedure. Volatile anesthetics can alternatively be dissolved in saline at appropriate concentrations, a method employed by Dickinson et al., 1995. It's important to note that isoflurane is usually considered a controlled substance and may not be freely available, making collaboration with veterinarians the preferred method for its acquisition.

CHAPTER 5

RNA EDITING MECHNAISM OF EGR1 (EARLY GROWTH RESPONSE 1) AND PROTEIN DIVERSIFICATION OF *O. vulgaris*

5.1 INTRODUCTION

The concept of the central dogma has been a fundamental principle in molecular biology since its introduction in the 1950s, stating that DNA is transcribed into mRNA, and subsequently, mRNA is translated into proteins. However, recent studies have unveiled a new layer of complexity in this process, known as RNA editing. RNA editing is a post-transcriptional mechanism that modifies the nucleotide sequence of mRNA without altering the original DNA sequence. This process is mediated by the enzyme ADAR (Adenosine Deaminase Acting on RNA), which converts adenosine (A) to inosine (I), mimicking the behavior of guanine (G). As inosine (I) is recognized as guanosine (G) by the translational machinery, RNA editing can result in "recoding," enabling a static genome to dynamically encode a diverse proteome. While A-to-I mRNA editing is present in other animals, it has been observed to be significantly more prevalent in coleoid cephalopods. A-to-I(G) RNA editing is the most common form of RNA editing identified in metazoans, primarily occurring in noncoding regions of the genome. However, certain animal groups, such as coleoids, exhibit RNA editing within their coding regions, particularly in the nervous system. This revelation has revolutionized our understanding of the role of the genetic code in determining protein synthesis. Extensive research on RNA editing in the nervous system of coleoids, including squids, has demonstrated that a majority of transcripts undergo extensive recoding through A-to-I(G) RNA editing. Studies on the octopus genome have also indicated that RNA editing plays a crucial role in the evolution of cephalopod neural and morphological innovations (Garrett & Rosenthal, 2012b;

Rosenthal & Seeburg, 2012; Alon et al., 2015; Albertin et al., 2015, 2022; Liscovitch-Brauer et al., 2017; Di Cosmo et al., 2018b; Rosenthal & Eisenberg, 2023).

RNA editing has emerged as a crucial regulatory mechanism impacting proteins essential for neural excitability. Notably, A-to-I(G) RNA editing has been demonstrated to modify the activity of key potassium channels, calcium channels, and glutamate receptors, which play critical roles in the proper functioning of the nervous system (Rosenthal & Seeburg, 2012). Furthermore, investigations into the mechanism and regulation of RNA editing in cephalopods have revealed intriguing insights. The findings suggest a trade-off between transcriptome plasticity and genome evolution in cephalopods, highlighting the dynamic interplay between RNA editing and genomic stability in these remarkable organisms (Liscovitch-Brauer et al., 2017).

Studies on octopuses have revealed that A-to-I(G) RNA editing occurs not only in noncoding regions but also in coding regions, resulting in non-synonymous changes that can alter amino acids, a phenomenon known as protein recoding. Notably, higher levels of RNA editing have been detected in conserved sites within coding regions, particularly in non-synonymous positions, indicating the functional significance of this process in octopuses (Garrett & Rosenthal, 2012a; Rosenthal & Seeburg, 2012; Alon et al., 2015; Liscovitch-Brauer et al., 2017; Rangan & Reck-Peterson, 2023; Rosenthal & Eisenberg, 2023). These findings highlight the widespread nature of RNA editing, expanding the diversity of genetic information beyond the constraints of the DNA code. In cephalopods, including octopuses and squids, RNA editing is observed at high levels, suggesting its crucial role in their biology and evolutionary processes. Recent advancements in octopus genome research have provided valuable insights into the mechanisms of RNA editing and its impact on cephalopod biology.

The octopus genome has been discovered to harbor an extensive number of A-to-I(G) RNA editing sites, surpassing 100 million, which are distributed across a majority of its genes.

This level of editing surpasses that observed in other animals, including humans. The abundance of RNA editing in cephalopods, such as octopuses, is believed to contribute to their unique neural and morphological characteristics, such as their intricate nervous system and the remarkable ability to change skin color and texture (Albertin et al., 2015). Interestingly, RNA editing is not confined to coding regions alone but also encompasses non-coding regions, including microRNA precursors. In cephalopods, it has been demonstrated that RNA editing occurs in the nucleus through the involvement of pre-mRNA splicing machinery, leading to the editing of microRNA precursors (Alon et al., 2015). This indicates that RNA editing influences gene regulation and has the potential to impact gene expression levels. Despite its advantages, a trade-off between transcriptome plasticity and genome evolution has been observed in cephalopods. Liscovitch-Brauer et al. (2017) discovered that highly edited genes are more conserved across cephalopod species, suggesting that RNA editing may be constrained by the necessity to maintain gene function. Furthermore, studies have shown that A-to-I(G) RNA editing can influence proteins crucial to neural excitability, potentially contributing to the development of neurological disorders (Rosenthal & Seeburg, 2012).

Cephalopods, such as octopuses, cuttlefish, and squid, display remarkable levels of A-to-I(G) RNA editing, resulting in non-synonymous changes within their proteomes. A specific study focusing on *Octopus bimaculoides* revealed that RNA editing percentages range from 48% to 65% across different tissues, with the highest levels observed in neural tissues (~60%) (Liscovitch-Brauer et al., 2017; Birk et al., 2023; Rangan & Reck-Peterson, 2023). Additionally, other cephalopod species, including *Octopus vulgaris*, cuttlefish (*Sepia officinalis*), Hawaiian bobtail squid (*Euprymna scolopes*), and striped pajama squid (*Sepioloidea lineolata*), also exhibit substantial levels of RNA editing (Rosenthal & Eisenberg, 2023). These findings highlight the prevalence of RNA editing as a prominent mechanism in

cephalopods, underscoring its potential significance in shaping their biology and evolutionary adaptations.

Therefore, RNA editing in cephalopods is believed to play a significant role in the function of the nervous system and brain physiology. Within the neural transcriptome, RNA editing is prominently observed in genes associated with neuronal and cytoskeletal functions. Additionally, higher levels of RNA editing are found in adult cephalopods compared to juveniles, indicating its involvement in development and potentially aging processes (Liscovitch-Brauer et al., 2017). Recent findings have shown that cephalopods exhibit substantially higher levels of RNA editing compared to humans, with recoding accounting for 11-13% of the global RNA editing activity in neural tissues, whereas mammals show less than 1% recoding (Liscovitch-Brauer et al., 2017; Di Cosmo et al., 2018b; Rosenthal & Eisenberg, 2023). This elevated level of RNA editing in cephalopods may have contributed to their distinctive behavioral and cognitive abilities, which have evolved over time. Furthermore, it is suggested that RNA editing serves as an important mechanism for the rapid evolution of neural function and behavior in cephalopods, representing a highly dynamic and adaptable process that can rapidly respond to changing environments and selective pressures (Albertin et al., 2015; Liscovitch-Brauer et al., 2017; Rosenthal & Eisenberg, 2023).

Octopuses and other cephalopods have demonstrated remarkable resilience and adaptability in various environmental conditions, allowing them to thrive worldwide. Numerous studies have indicated that these fascinating creatures possess a distinctive capacity to modify RNA, thereby altering the protein composition encoded by their genes (Liscovitch-Brauer et al., 2017; Di Cosmo et al., 2018b). This inherent ability, combined with their remarkable adaptability, likely contributes to the success of cephalopods in diverse habitats, as previously discussed. Their capability to dynamically adjust their genetic information through

RNA editing enables them to rapidly respond to environmental challenges, ensuring their survival and evolutionary fitness.

Moreover, to date, research conducted on octopuses has identified specific genes that undergo extensive RNA editing, including those encoding potassium channels, glutamate receptors, and serotonin receptors (Albertin et al., 2012, 2015; Liscovitch-Brauer et al., 2017; Rosenthal & Eisenberg, 2023). Additionally, studying RNA editing in octopuses can provide valuable insights into the molecular neuro-epigenetic mechanisms underlying reversible, state-dependent modifications from cellular processes to complex behaviors (Di Cosmo et al., 2018b). Therefore, investigating the RNA editing of specific genes in octopuses holds significant importance due to their unique and captivating characteristics. Octopuses exhibit a wide range of complex behaviors, extraordinary cognitive abilities, and remarkable camouflage techniques that have fascinated scientists for years. By delving into the mechanisms of RNA editing and identifying the genes involved, researchers can gain critical insights into the genetic foundations of these exceptional traits. Understanding the contribution of RNA editing to genetic regulation and modification in octopuses will deepen our understanding of their physiological adaptations and evolutionary history. Furthermore, studying RNA editing in octopus' genes may have broader implications for comparative genomics and find applications in fields such as neuroscience and biotechnology, where knowledge of RNA editing mechanisms can advance gene therapies, drug development, and our understanding of the intricacies of the human genome. Overall, the exploration of RNA editing in specific octopus' genes is not only crucial for unraveling their biological mysteries but also holds potential for broader scientific and practical advancements.

The detection of RNA editing in the Egr1 (Early Growth Response 1) gene is of significant importance due to the crucial role this gene plays in various biological processes. Egr1 is a transcription factor that regulates gene expression in response to extracellular stimuli,

playing a critical role in cellular growth, differentiation, and responses to stress and injury. RNA editing refers to post-transcriptional modifications that can alter the coding sequence or regulatory regions of a gene, thereby affecting protein function and cellular processes. Investigating RNA editing in the Egr1 gene can provide insights into the regulatory mechanisms that fine-tune its activity and modulate downstream signaling pathways. Understanding the extent and functional consequences of RNA editing in Egr1 can illuminate its role in diverse physiological and pathological contexts, such as development, neuroplasticity, and disease progression. Moreover, since dysregulation of Egr1 has been implicated in various disorders, including cancer, cardiovascular diseases, and neurological conditions, studying RNA editing in this gene may unveil novel therapeutic targets or diagnostic markers.

Therefore, detecting and characterizing RNA editing in the Egr1 gene can significantly enhance our understanding of fundamental biological processes, contributing to advancements in both basic and clinical research. Additionally, since the Egr1 gene is associated with the early detection of stress conditions in animals, understanding its behavior under various stress conditions is crucial. This understanding will help us comprehend how RNA editing influences animals' survival under different stress scenarios. Based on this, we used two types of animals, wild and controlled, exposing them to different stress environments to deepen our understanding. Hence, the aim of this chapter is to detect and characterize RNA editing events in the Egr1 gene of *O. vulgaris* under different environmental conditions, with the objective of understanding how these conditions impact RNA editing patterns and their potential implications for gene function and organismal adaptation.

5.2 MATERIAL AND METHODS

5.2.1 ANIMALS ACQUISITION AND TISSUE COLLECTION

Adult specimens of *O. vulgaris*, weighing approximately 650-800g, were collected from the Bay of Naples (Italy) and transported to Professor Anna Di Cosmo's cephalopod facility at the Department of Biology, University of Naples Federico II, Naples, Italy. Upon arrival, one individual was placed in fiberglass tanks measuring 50 x 50 x 50 cm, filled with seawater using a dedicated circulatory system. Animal was allowed to acclimate for a period of 15 days. The facility maintained a seawater temperature of 18°C and followed a natural photoperiod of 12 hours of light and 12 hours of darkness. Throughout the acclimatization period, the octopus was provided with a varied diet consisting mainly of anchovies, shrimps, and mussels. Water quality parameters were monitored daily, and regular tank cleaning was conducted to ensure the well-being of the animals and detect any signs of stress.

Following the acclimatization period, the animal was kept in the same tank without any assigned tasks or activities, serving as the control animal which not subjected to any experimental manipulation or intervention (Polese et al., 2014; Maselli et al., 2020a). Upon arrival, a one animal was subjected to anesthetization using isoflurane insufflation and subsequently dissected under sterile conditions. This animal served as the representative of a wild catch specimen. After one month of being housed in the tanks, the other animal underwent the same anesthetization procedure using isoflurane insufflation. Subsequently, this animal was also dissected under sterile conditions (Polese et al., 2014).

After sacrificing the animals, the optic lobes, supraesophageal and subesophageal masses were carefully collected and immediately preserved in RNA Later, a reagent that helps stabilize RNA and prevent degradation. The preserved tissues were then stored in a -80°C freezer to maintain their integrity until RNA extraction. In addition, arm muscle sections from each animal were dissected and stored in Eppendorf tubes filled with 96% alcohol. These tissue

samples were kept at a temperature of -20°C to preserve the DNA until the DNA extraction process. All research was approved in accordance with European Directive 2010/63 EU L276, the Italian DL. 4/03/2014, no. 26, and the ethical principles of Reduction, Refinement, and Replacement (Project n° 608/2016-PR-17/06/2016; protocol n° DGSAF 0022292-P-03/10/2017).

5.2.2. DNA AND RNA EXTRACTIONS

The extraction of total genomic DNA was performed using the QIAamp DNA Mini Kit from Qiagen, following the manufacturer's instructions. The extracted DNA was carefully stored in a -20°C freezer to maintain its stability and integrity for future experiments.

For the extraction of total RNA, the RNeasy minikit from Qiagen USA was utilized, following the manufacturer's instructions. The chosen tissues were used to isolate the total RNA, which was then stored in a -80°C freezer to ensure its preservation until further experiments. To generate cDNA (complementary DNA), the extracted total RNA was subjected to reverse transcription using the QuantiTect® Reverse Transcription Kit from Qiagen USA, according to the manufacturer's provided instructions. This process facilitated the synthesis of cDNA from the RNA samples, enabling downstream applications in gene expression analysis and other experiments. The primer combinations for amplifying the *O. vulgaris* Egr1 gene were designed using gene sequences obtained from the National Center for Biotechnology Information (NCBI) database. The specific gene sequence used for primer design had the accession number KY986710. The primer design process was performed using SnapGene 5.2.5 software (www.snapgene.com).

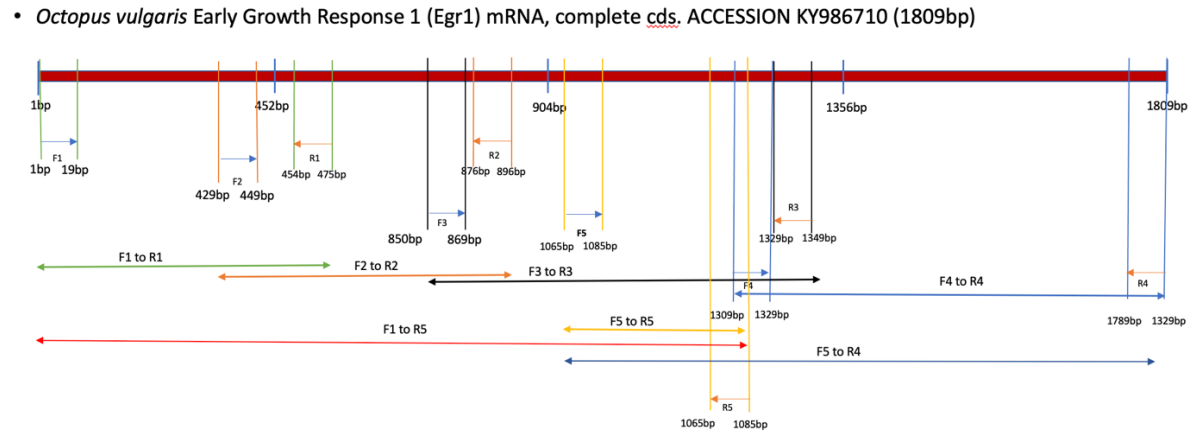

Figure 5. 1. *Octopus vulgaris* Egr1 gene mapping with primers used

To design the primers for the *O. vulgaris* Egr1 gene, the complete gene sequence was divided into several sections using SnapGene 5.2.5 software (www.snapgene.com) (Table 5.1). The Egr1 gene, depicted in Figure 4.1, spans a length of 1809 nucleotides.

To validate the primers and Egr1 gene expression in the tissues, Polymerase Chain Reaction (PCR) reactions were performed using synthesized DNA and cDNA samples. The PCR products were then subjected to sequencing approaches to confirm the accuracy and specificity of the amplified fragments (Alon et al., 2015; Liscovitch-Brauer et al., 2017).

Table 5. 1. Designed primers for selected Egr1 gene in *O. vulgaris*

***Octopus vulgaris* Egr 1 RNA Editing Primers**				
Primer	**Sites**	**Sequence**	**Tm**	**Amplicon**
Egr-1 F1	1 to 19	ATGATTATGGAAGGCCTGG	53°C	475 bp
Egr-1 R1	454 to 475	GCTGTCCCTGTACTTGGGTTGC	61°C	
Egr-1 F2	429 to 449	CAACACAGCCACACCACCTGG	63°C	467 bp
Egr-1 R2	876 to 896	GATGTTGTTTGGAAAGATGTC	52°C	
Egr-1 F3	850 to 869	TTAAGCTGTAGCCAGCAGTC	57°C	499 bp
Egr-1 R3	1329 to 1349	GGTTTAACTGGGTACAACTTC	54°C	
Egr-1 F4	1309 to 1329	GCAGTAAGCACTGGTCAACTG	57°C	500 bp
Egr-1 R4	1789 to 1809	TCACAGTGATGCTGTGGTCAC	59°C	
Egr-1 F5	1065-1085	CCAGGGTATCAAGACAGAGCC	59°C	258bp
Egr-1 R5	1303-1323	ACCAGTGCTTACTGCTGTTGT	60°C	

The PCR was performed using the following mixture in a final volume of 20 µL for both species: 100 ng of synthesized DNA or cDNA template with a mixture of 0.2 µL of Pfu

DNA polymerase (Thermo Scientific, Waltham, MA, USA), 4 µL of 4X Tris buffer with MgCl2, 1.6 µL of dNTPs (each dNTP 2.5 µM), and 0.2 µL of 50 µM of each primer. The PCR cycling parameters included an initial denaturing step of 98°C for 3 min, 35 cycles of 10 s at 98°C, 30 s at 55-63°C, and 1 min at 72°C, and a final extension step of 5 min at 72°C. The PCR products were used to isolate the fragments related to the size of base pairs chosen photoreceptor primers. The isolated gel fragments were purified using a QIAquick Gel Extraction Kit (Qiagen, USA) and sequenced. The sequenced samples were assembled using SnapGene 5.2.5 software (www.snapgene.com) by selecting open reading frames (ORFs), and confirmation was performed with GenBank BLASTn and BLASTx (BLAST, Basic Local Alignment Search Tool) to confirm the identity of the fragments.

5.2.3. DETECTION OF RNA EDITING SITES

To detect RNA editing events in the Egr1 gene, primer combinations were designed to amplify specific regions of the gene using PCR products derived from cDNA samples. Tissues from two different animals, kept under different conditions, were collected for this analysis. The genomic DNA and cDNA sequences obtained from the samples were first confirmed by comparing them with the sequences available in the NCBI database. Subsequently, the chromatogram peak area from the cDNA sequencing was manually analyzed using SnapGene 5.2.5 software (www.snapgene.com) with aligning those using ORFs. This analysis allowed for the identification and characterization of potential RNA editing events occurring in the Egr1 gene.

In cDNA sequencing, the DNA template is initially reverse-transcribed into complementary DNA (cDNA) and then subjected to sequencing. During the sequencing process, the presence of a non-complementary base, such as an adenosine instead of a guanosine, at an RNA editing site can give rise to two distinct peaks in the chromatogram, corresponding to both the edited and unedited bases. By quantifying and comparing the peak

areas of these two peaks, we can determine the frequency or occurrence of the RNA editing event. This approach was employed in our study to specifically identify A-to-G RNA editing, using the formula: % A-to-G editing = (G peak area / (A peak area + G peak area)) x 100 (Liscovitch-Brauer et al., 2017). The resulting calculated value represents the editing percentage (%) for the cDNA samples at the particular site that was analyzed.

Using the same approach and formula, we extended our analysis to detect various other types of modifications, including A-to-C, A-to-T, C-to-A, C-to-G, C-to-T, G-to-A, G-to-C, G-to-T, T-to-A, T-to-C, and T-to-G. Based on the distribution of RNA editing events observed in the samples, we classified the number of occurrences or modifications into three distinct categories: 15-50%, 50-85%, and >85%. However, for the purpose of our study, we made the decision to consider editing events with an occurrence rate greater than 50% as the most likely RNA editing events (Wang & Mohlhenrich, 2019), encompassing all potential types of modifications within the Egr1 gene.

5.2.4. DETECTION OF RECODING PROTEOME WITH A-TO-G EDITING

After aligning the codon triplet in the genomic DNA or the sequence deposited in the NCBI for the Egr1 gene sequence with the codon triplet (nucleotide sequence) in the cDNA sequence, we identified recoding events when the codon triplet in the cDNA sequence differed from the codon triplet in the genomic DNA sequence due to A-to-G RNA editing (Liscovitch-Brauer et al., 2017). To detect recoding events resulting from A-to-I(G) RNA editing, we compared cDNA samples with the genomic DNA or the NCBI deposited sequence for the Egr1 gene. By aligning the nucleotide sequences of the genomic DNA or the NCBI deposited sequence with the nucleotide sequence of the cDNA (Liscovitch-Brauer et al., 2017), we determined if the codon triplet in the cDNA sequence differed from the codon triplet in the genomic DNA sequence due to A-to-G RNA editing. In such cases, we considered it a recoding event.

5.3 RESULTS

5.3.1 DETECTION OF A-TO-G RNA EDITING SITES

The detection of RNA editing events in the selected Egr1 gene has yielded promising results, indicating the presence of editing events. The Egr1 gene, which encompasses 1809 nucleotides (bp) in the coding region or coding sequences (CDS) (GenBank: KY986710.1), has exhibited various levels of A-to-I/A-to-G RNA editing events. The specific numbers of different editing event levels are reported in Table 5.2, and their distribution is illustrated in Figure 5.2, as categorized previously.

Table 5. 2. A-to-G(I) Editing events reported in nervous system

Tissues	Animal Type	A-to-G(I) Editing sites % Value:		
		15-50%	50-85%	>85%
Optic lobe	WILD	1	0	0
	CONTROL	0	70	27
Supraesophageal_mass	WILD	0	0	0
	CONTROL	3	0	0
Subesophageal_mass	WILD	2	0	1
	CONTROL	1	0	1

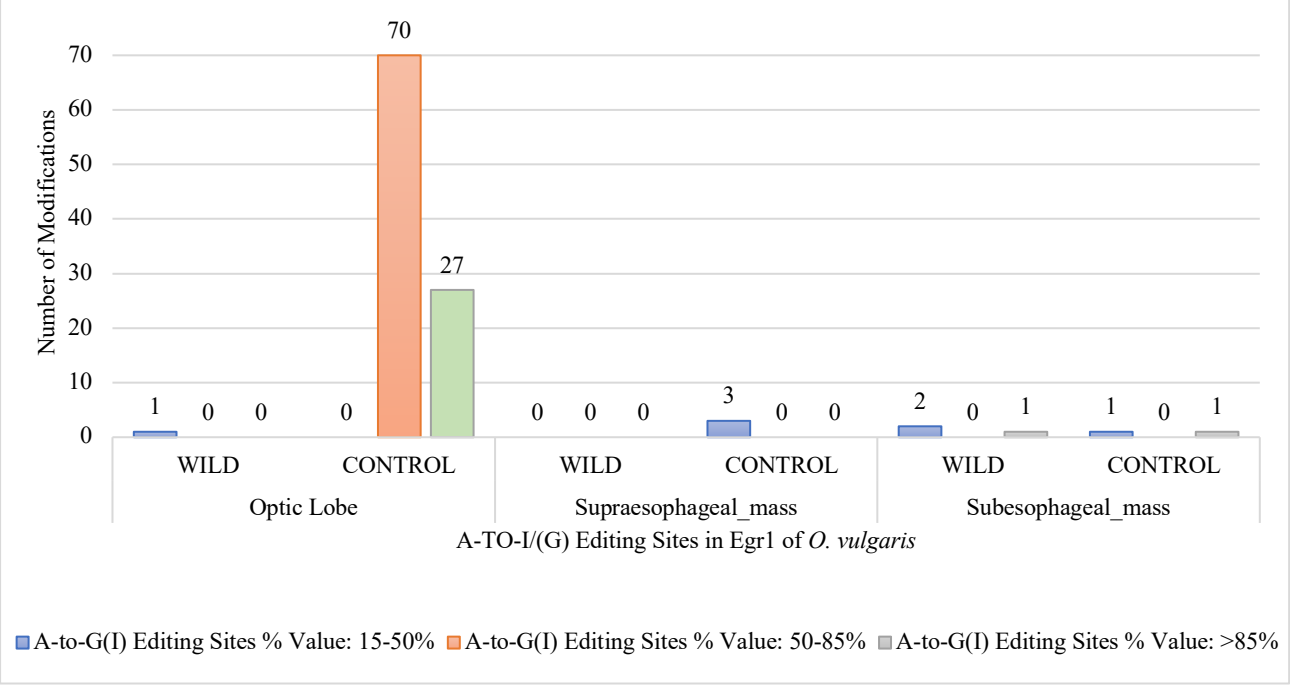

Figure 5. 2. A-to-G(I) Editing sites reported in Egr1 gene of *O. vulgaris*

The analysis revealed that the levels of A-to-G modification were higher in the controlled animal compared to the wild animal. Additionally, when examining other types of possible modifications in the tissues, the optic lobe exhibited the highest number of modifications within the 50-85% range, rather than exceeding 85%. The chromatogram visuals provided further evidence of the possible editing events, as both A and G peaks were visible (Figure 5.3).

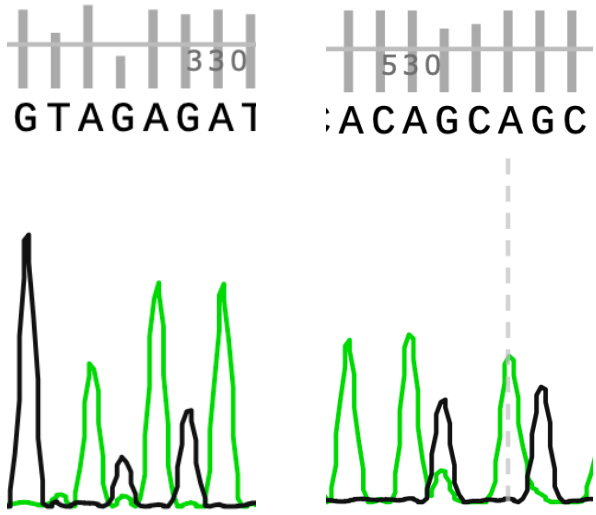

Figure 5. 3. A to G modification chromograms visuals

5.3.2 DETECTION OF OTHER RNA MODIFICATION TYPES SITES

The analysis also included other potential types of modifications, such as A-to-C, A-to-T, C-to-A, C-to-G, C-to-T, G-to-A, G-to-C, G-to-T, T-to-A, T-to-C, and T-to-G, in addition to A-to-G RNA editing (Table 5.3). Similar to A-to-G RNA editing, editing events for these other types of modifications were evaluated based on a threshold of greater than 50% to determine the likelihood of editing events occurring at specific sites in the Egr1 gene, as described earlier (Wang & Mohlhenrich, 2019).

Table 5. 3. Different types of modification types reported in Egr1 gene due to RNA editing.

Tissues	Animal Type	AG	AC	AT	CA	CG	CT	GA	GC	GT	TA	TC	TG
Optic lobe	WILD	1	1	0	0	0	0	0	0	0	0	0	0
	CONTROL	**97**	**69**	**47**	**63**	**31**	**55**	**41**	**5**	**34**	**71**	**43**	**36**
Supraesophageal mass	WILD	0	0	0	0	0	0	0	0	0	0	0	0
	CONTROL	0	0	0	3	0	0	1	0	2	1	0	0
Subesophageal mass	WILD	1	0	0	0	0	0	0	0	0	0	0	0
	CONTROL	1	0	0	0	0	0	0	0	0	1	0	0

The results demonstrated variations in the number of potential modification events among different types of modifications (Figure 5.4). Among these modification types, the highest number of events was observed in A-to-G modification, as indicated in Table 5.3. Furthermore, our findings revealed that A-to-G editing events accounted for 5.4% of all editing events throughout the entire 1809bp region of the Egr1 gene, surpassing the occurrences of other types of modifications.

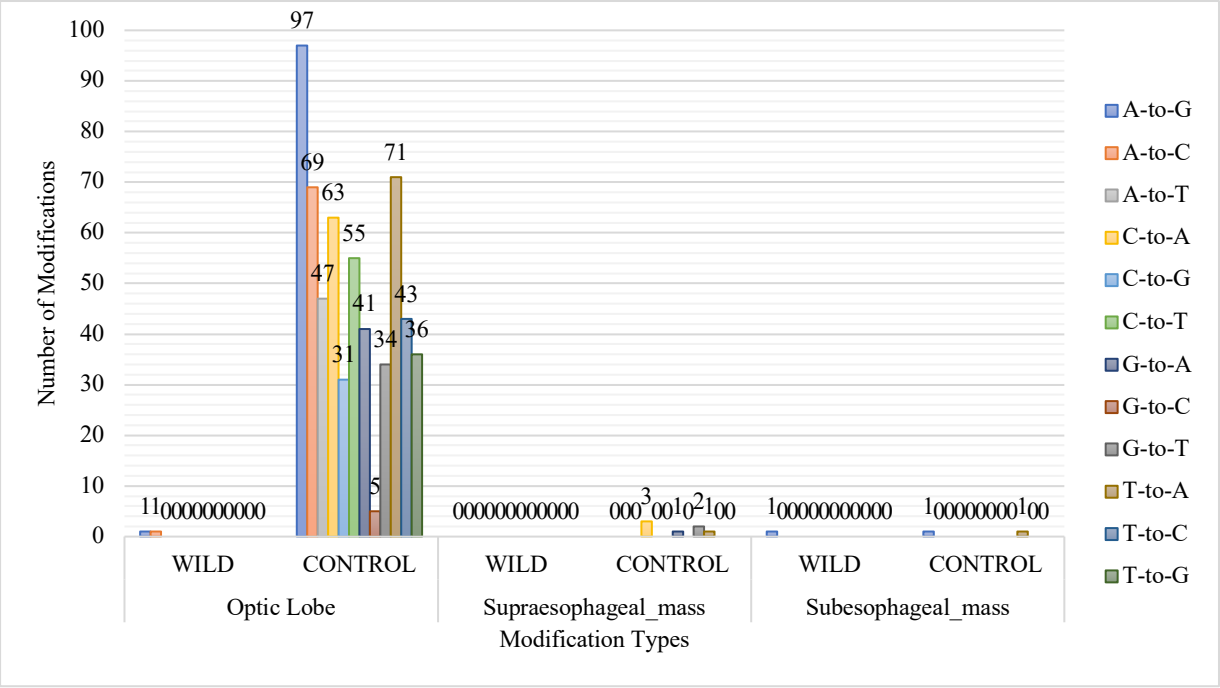

Figure 5. 4. modification types recorded for Egr1 gene in *O. vulgaris*

Furthermore, the analysis revealed that the majority of editing sites detected in the Egr1 gene of *O. vulgaris* were concentrated between positions 400 and 1281 out of the total 1809bp. It is noteworthy that most of the editing events occurred in the middle region of the coding sequence (CDS) of the Egr1 gene. Occurrence of editing events outside this region was found to be rare, except for A-to-C and T-to-G modification types, which appeared at positions 71 and 247, respectively, with a higher occurrence rate of >10%. This finding suggests that the majority of editing events for all types of modifications are predominantly confined to the middle region of the Egr1 gene.

5.3.3 DETECTION OF RECODING PROTEOME WITH A-TO-G EDITING

The proteome was thoroughly examined to assess whether A-to-G RNA editing events at specific positions in RNA transcripts resulted in changes to the codon triplets. However, no nucleotide positions were found to have altered the codon triplets, indicating that all nucleotides remained unchanged. This finding implies that the RNA editing events occurred without recoding the codon triplets, thereby preserving the amino acid sequence. Consequently, no any non-synonymous changes were detected in the Egr1 gene, affirming that the synonymous changes in the coding region did not induce any alterations in protein function. It is worth emphasizing that these results unequivocally demonstrate that synonymous A-to-G RNA editing does not impact proteins and, thus, does not affect protein function. In summary, the observed synonymous A-to-G RNA editing changes did not give rise to modifications in proteins and, consequently, had no impact on protein function.

The editing events for other types of modifications have been meticulously documented and presented both in tabular form (Table 5.4 to Table 5.14) and visually through graphical representations (Figure 5.5 to Figure 5.15). The tables provide a comprehensive overview of the different types of modifications and the corresponding editing events observed at specific sites in the Egr1 gene. The graphical figures offer a visual representation of the distribution

and frequency of these modification events across the gene. Together, these tables and figures provide a comprehensive and detailed analysis of the editing events for various types of modifications in the Egr1 gene.

5.3.4 OTHER TYPES OF MODIFCATIONS

5.3.4.1 A-to-C EDITING EVENTS

Table 5. 4. A-to-C Editing events reported in nervous system

Tissues	Animal Type	A-to-C Editing events		
		15-50%	50-85%	>85%
Optic lobe	WILD	0	1	0
	CONTROL	20	0	69
Supraesophageal mass	WILD	3	0	0
	CONTROL	2	0	0
Subesophageal mass	WILD	0	0	0
	CONTROL	0	0	0

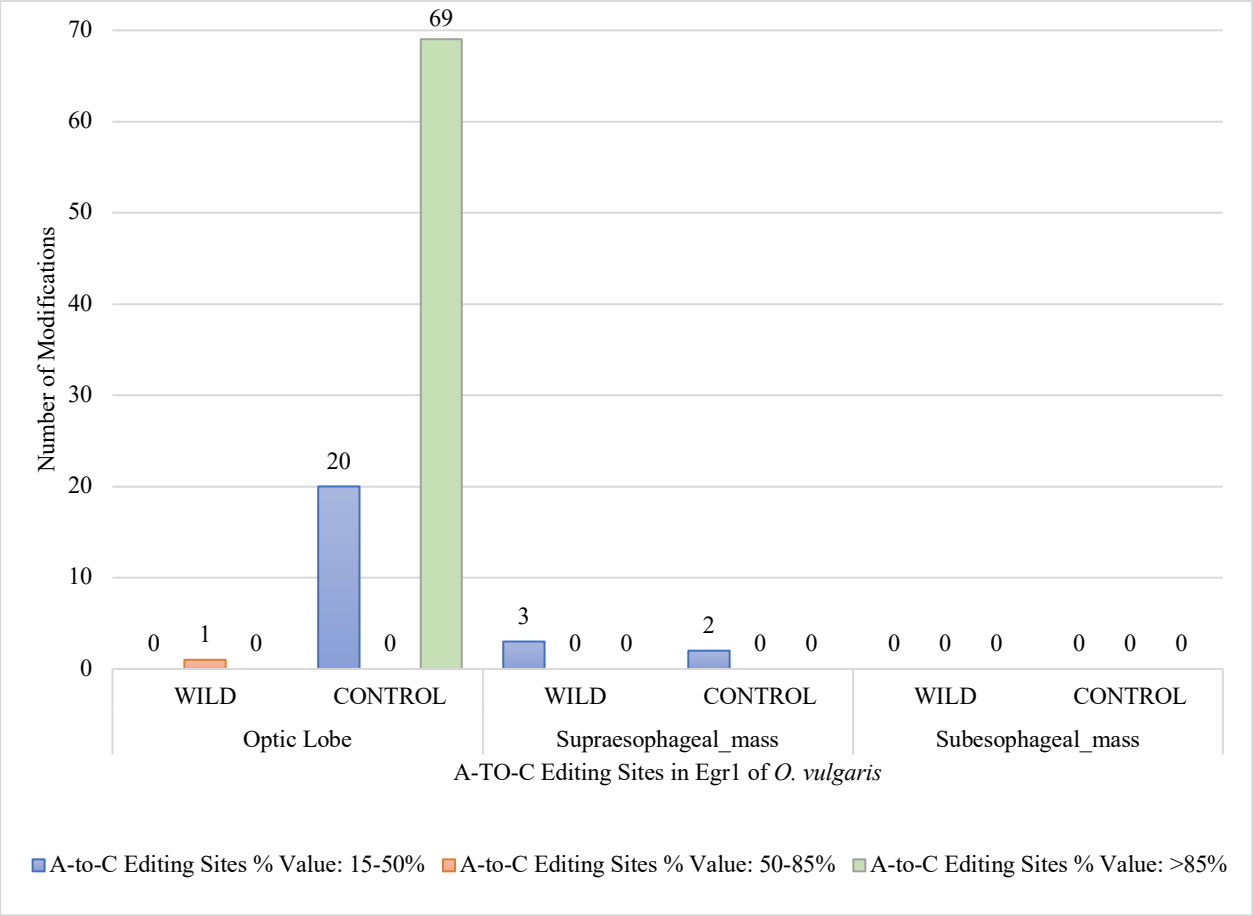

Figure 5. 5. A-to-C Editing sites reported in Egr1 gene of *O. vulgaris*

5.3.4.2 A-to-T EDITING EVENTS

Table 5. 5. A-to-T Editing events reported in nervous system

Tissues	Animal Type	A-to-T Editing events		
		15-50%	50-85%	>85%
Optic lobe	WILD	0	0	0
	CONTROL	34	9	38
Supraesophageal mass	WILD	1	0	0
	CONTROL	1	0	0
Subesophageal mass	WILD	0	0	0
	CONTROL	0	0	0

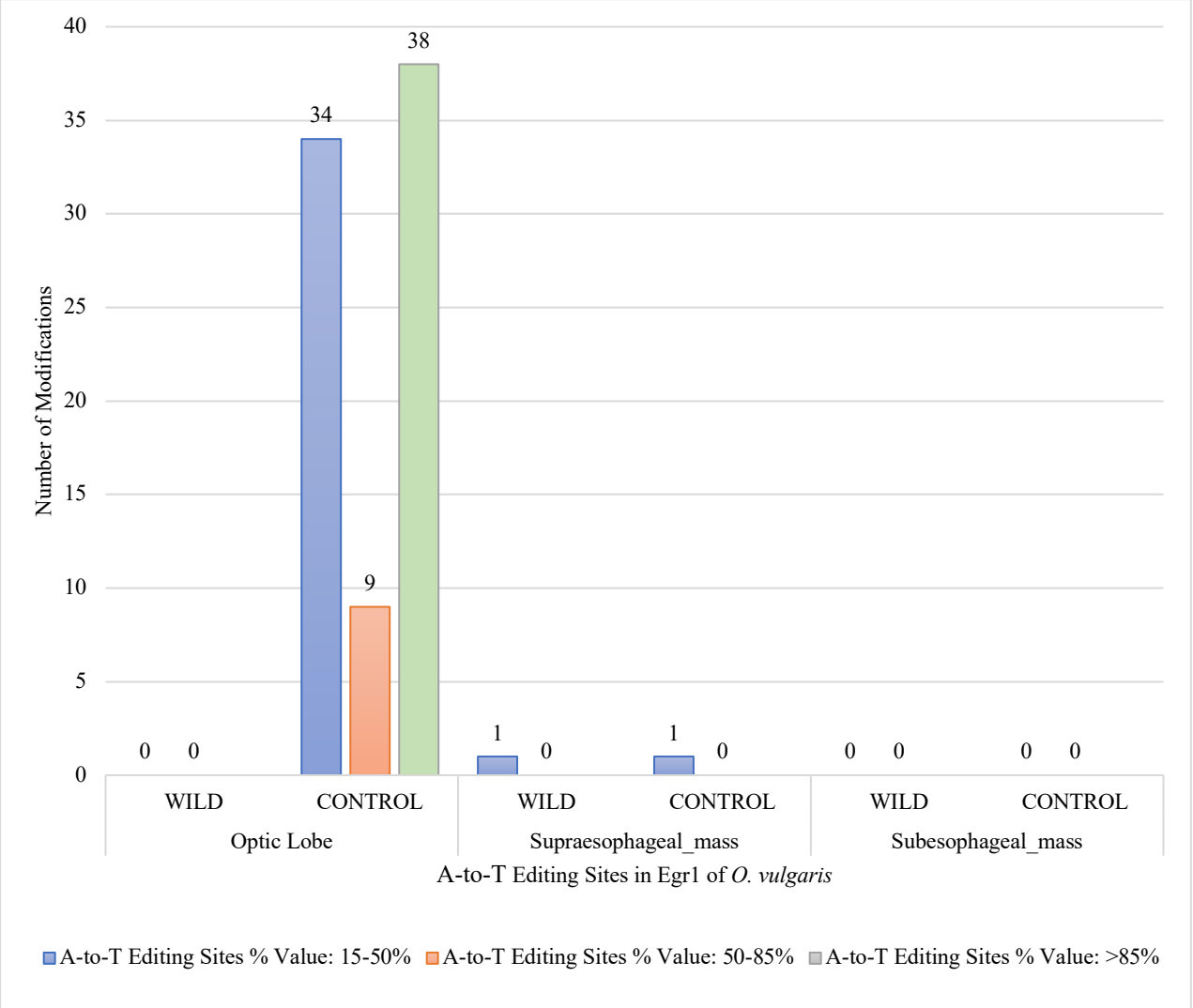

Figure 5. 6. A-to-T Editing sites reported in Egr1 gene of *O. vulgaris*

5.3.4.3 C-to-A EDITING EVENTS

Table 5. 6. C-to-A Editing events reported in nervous system

Tissues	Animal Type	C-to-A Editing events		
		15-50%	50-85%	>85%
Optic lobe	WILD	0	0	0
	CONTROL	31	17	46
Supraesophageal mass	WILD	2	0	
	CONTROL	0	2	1
Subesophageal mass	WILD	0	0	0
	CONTROL	0	0	0

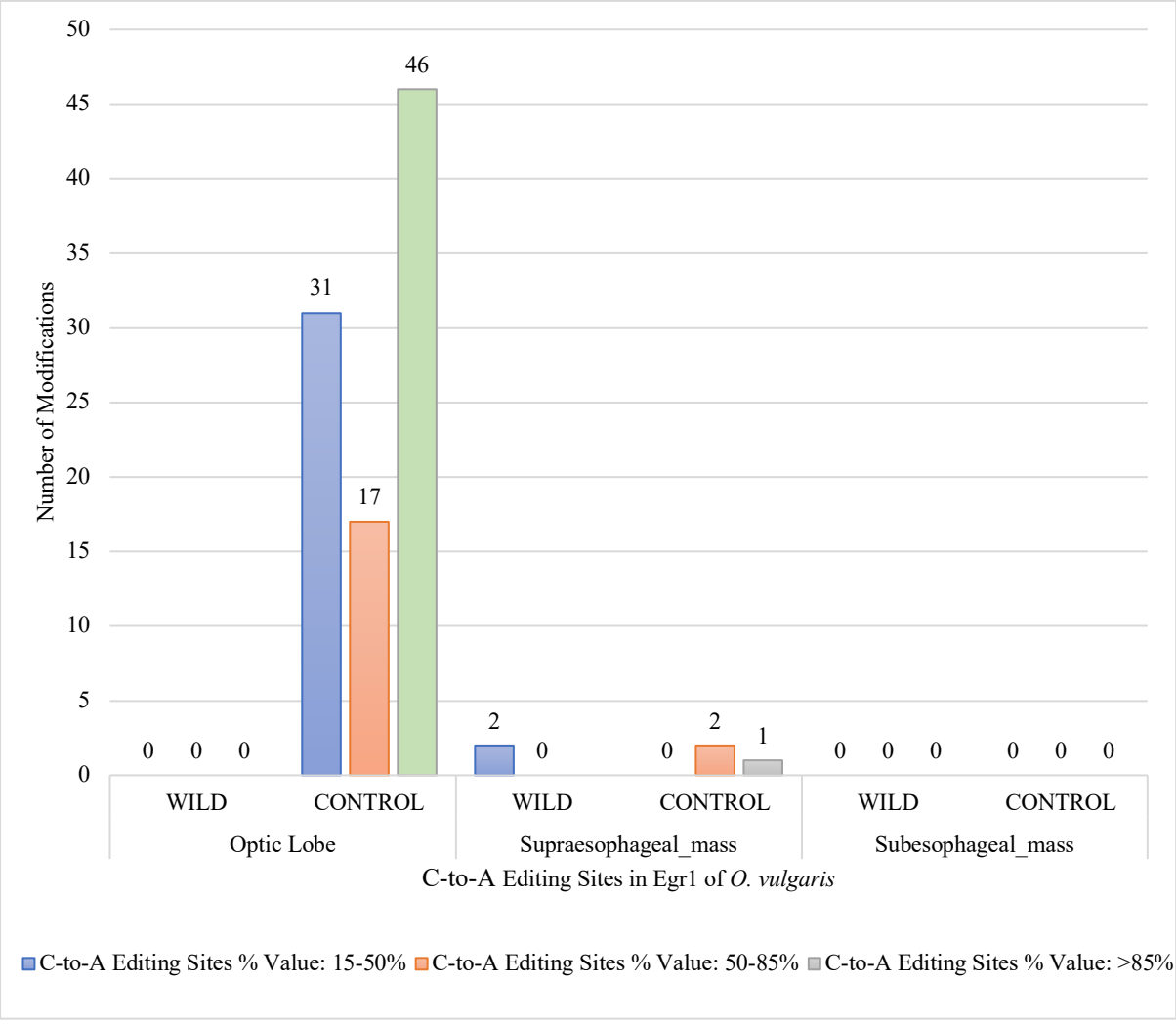

Figure 5. 7. C-to-A Editing sites reported in Egr1 gene of *O. vulgaris*

5.3.4.4 C-to-G EDITING EVENTS

Table 5. 7. C-to-G Editing events reported in nervous system

Tissues	Animal Type	C-to-G Editing events		
		15-50%	50-85%	>85%
Optic lobe	WILD	0	0	0
	CONTROL	55	10	21
Supraesophageal mass	WILD	0	0	0
	CONTROL	3	0	0
Subesophageal mass	WILD	0	0	0
	CONTROL	0	0	0

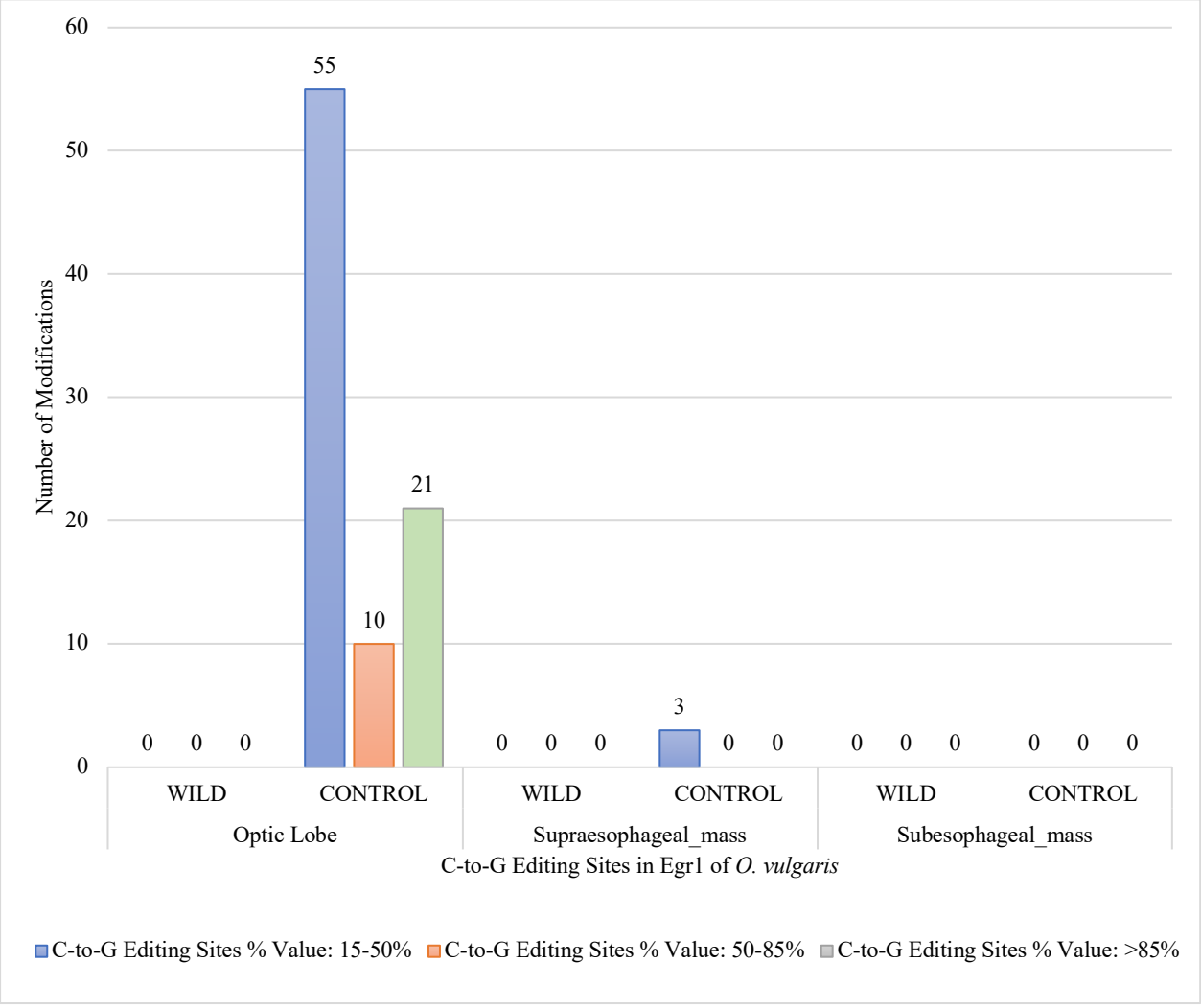

Figure 5. 8. C-to-G Editing sites reported in Egr1 gene of *O. vulgaris*

5.3.4.4 C-to-T EDITING EVENTS

Table 5. 8. C-to-T Editing events reported in nervous system

Tissues	Animal Type	C-to-T Editing events		
		15-50%	50-85%	>85%
Optic lobe	WILD	0	0	0
	CONTROL	31	10	45
Supraesophageal mass	WILD	0	0	0
	CONTROL	0	0	0
Subesophageal_mass	WILD	0	0	0
	CONTROL	0	0	0

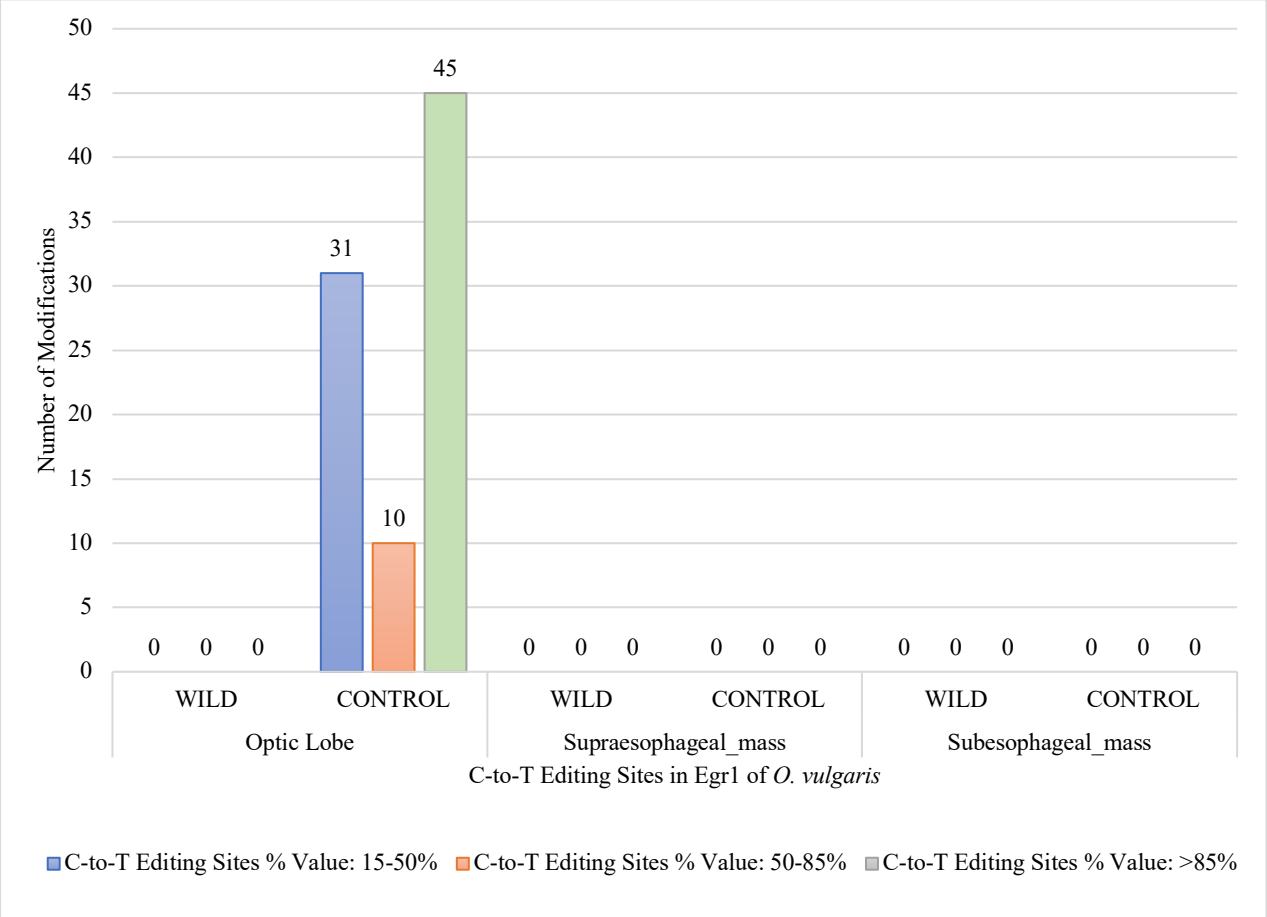

Figure 5. 9. C-to-T Editing sites reported in Egr1 gene of *O. vulgaris*

5.3.4.5 G-to-A EDITING EVENTS

Table 5. 9. G-to-A Editing events reported in nervous system

Tissues	Animal Type	G-to-A Editing events		
		15-50%	50-85%	>85%
Optic lobe	WILD	0	0	0
	CONTROL	15	3	38
Supraesophageal mass	WILD	0	0	0
	CONTROL	1	0	1
Subesophageal mass	WILD	0	0	0
	CONTROL	1	0	0

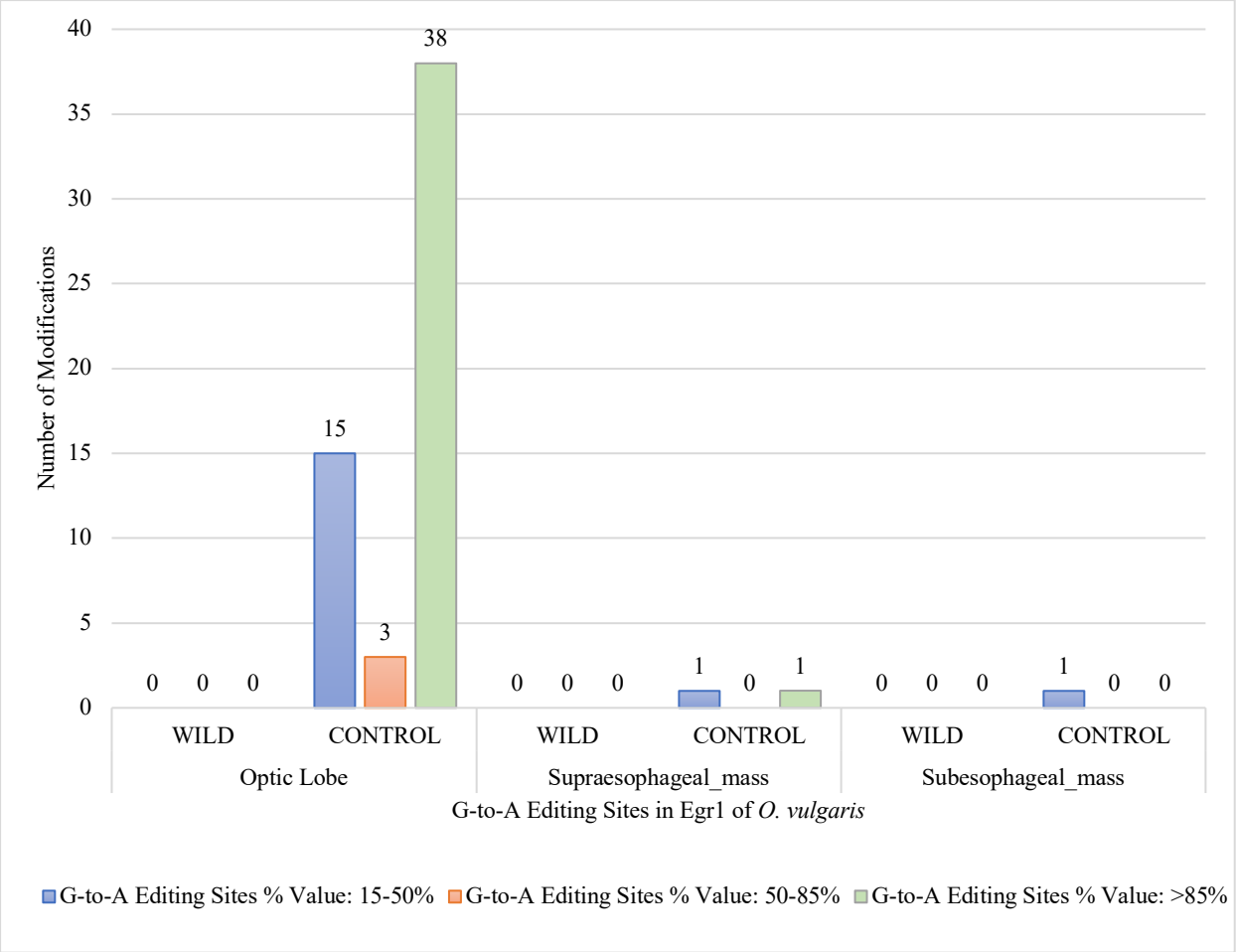

Figure 5. 10. G-to-A Editing sites reported in Egr1 gene of *O. vulgaris*

5.3.4.6 G-to-C EDITING EVENTS

Table 5. 10. G-to-C Editing events reported in nervous system

Tissues	Animal Type	G-to-C Editing events		
		15-50%	50-85%	>85%
Optic lobe	WILD	0	0	0
	CONTROL	31	3	2
Supraesophageal mass	WILD	0	0	0
	CONTROL	0	0	0
Subesophageal mass	WILD	0	0	0
	CONTROL	0	0	0

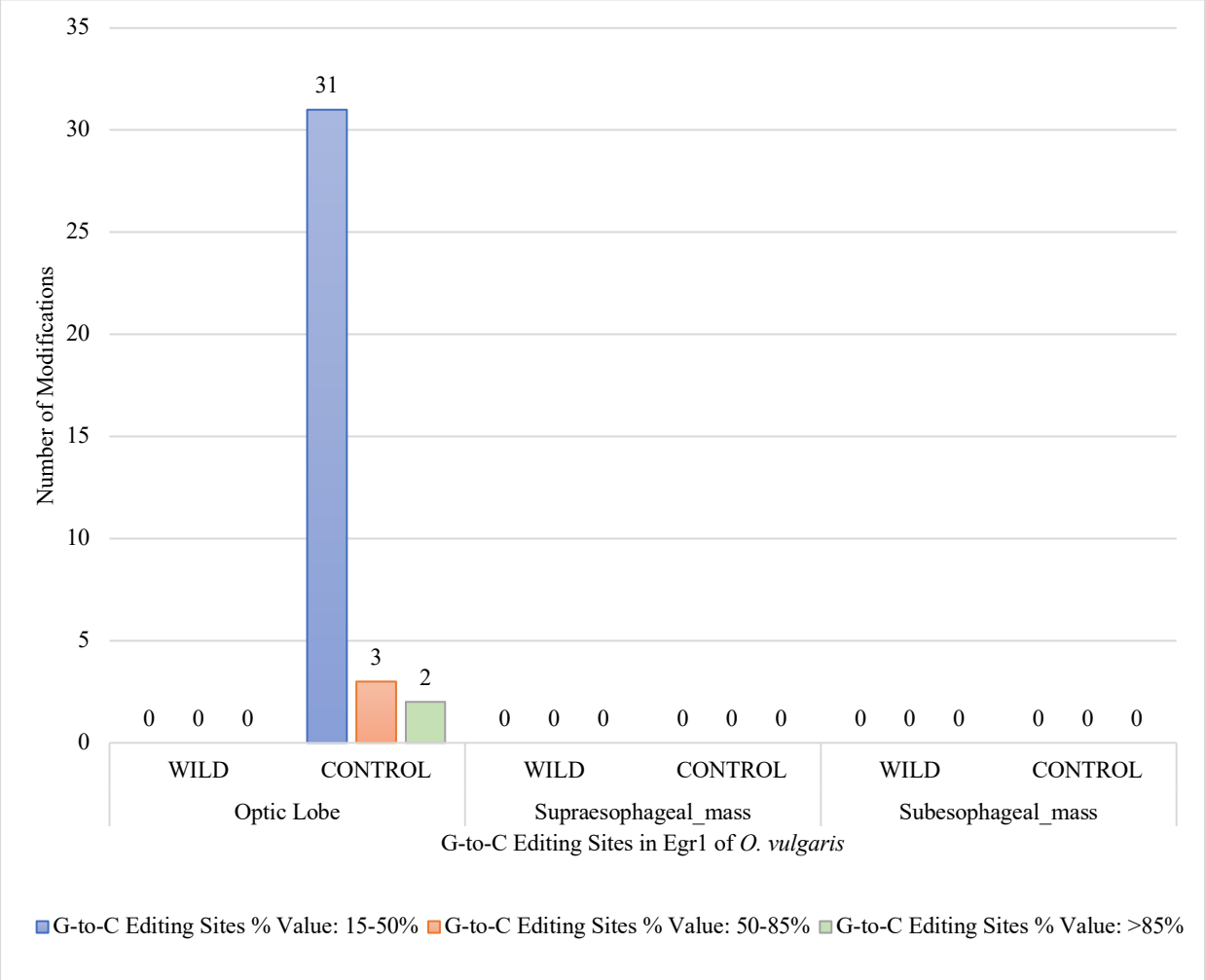

Figure 5. 11. G-to-C Editing sites reported in Egr1 gene of *O. vulgaris*

5.3.4.7 G-to-T EDITING EVENTS

Table 5. 11. G-to-T Editing events reported in nervous system

Tissues	Animal Type	G-to-T Editing events		
		15-50%	50-85%	>85%
Optic lobe	WILD	0	0	0
	CONTROL	28	8	26
Supraesophageal mass	WILD	0	0	0
	CONTROL	0	1	1
Subesophageal mass	WILD	0	0	0
	CONTROL	0	0	0

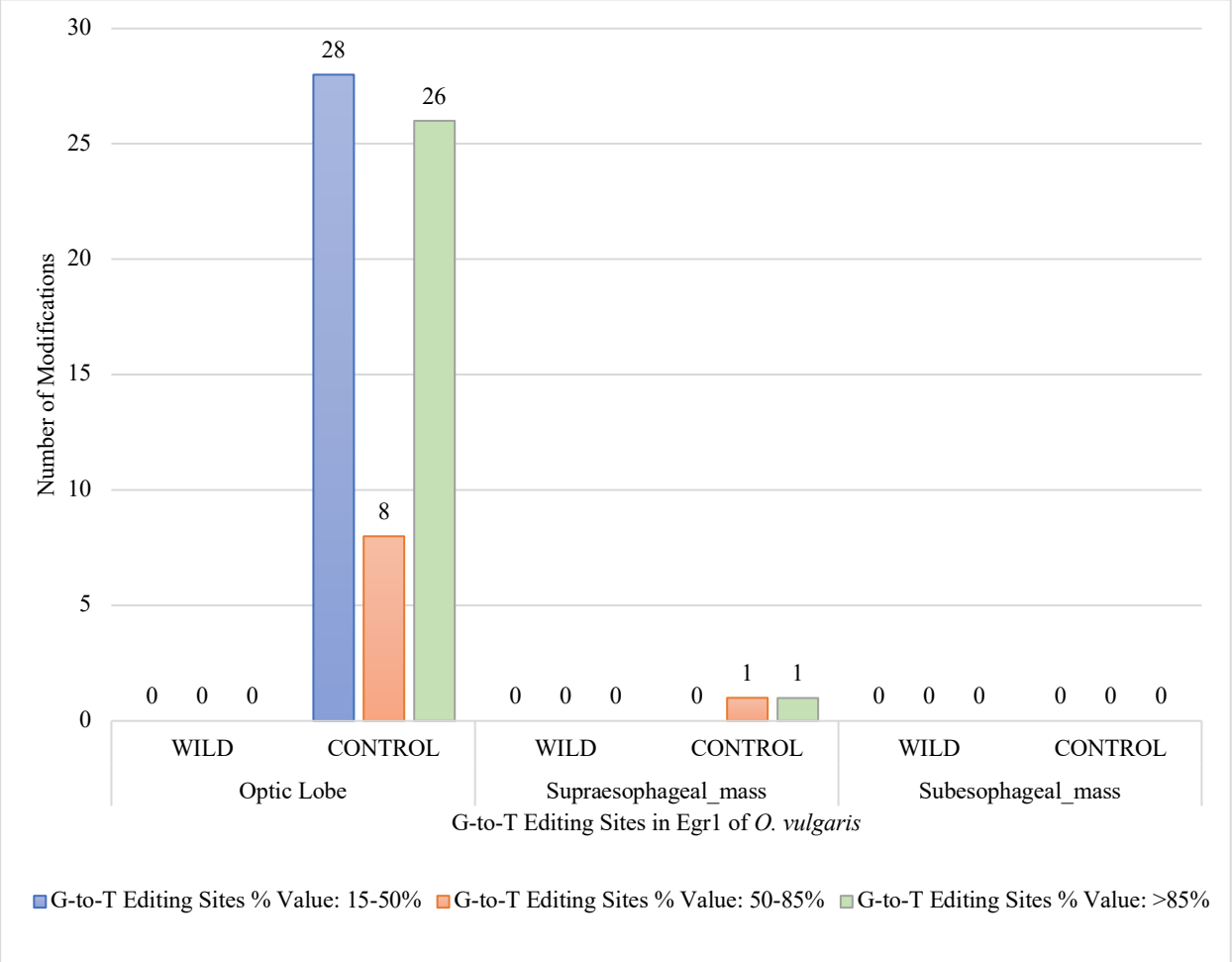

Figure 5. 12. G-to-T Editing sites reported in Egr1 gene of *O. vulgaris*

5.3.4.8 T-to-A EDITING EVENTS

Table 5. 12. T-to-A Editing events reported in nervous system

Tissues	Animal Type	T-to-A Editing events		
		15-50%	50-85%	>85%
Optic lobe	WILD	0	0	0
	CONTROL	19	20	51
Supraesophageal mass	WILD	0	0	0
	CONTROL	0	0	1
Subesophageal mass	WILD	1	0	0
	CONTROL	1	1	0

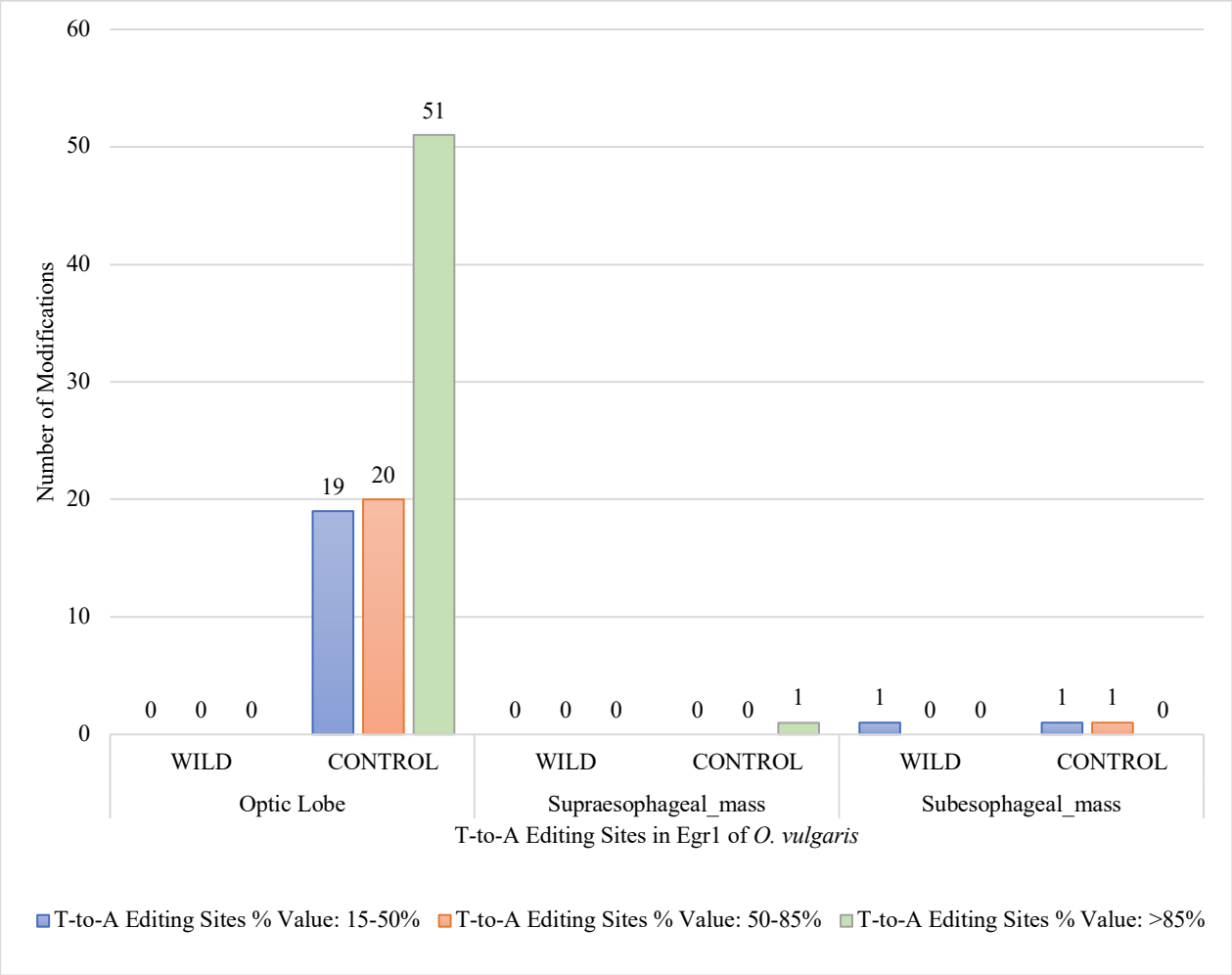

Figure 5. 13. T-to-A Editing sites reported in Egr1 gene of *O. vulgaris*

5.3.4.9 T-to-C EDITING EVENTS

Table 5. 13. T-to-C Editing events reported in nervous system

Tissues	Animal Type	T-to-C Editing events		
		15-50%	50-85%	>85%
Optic lobe	WILD	0	0	0
	CONTROL	28	7	36
Supraesophageal mass	WILD	0	0	0
	CONTROL	1	0	0
Subesophageal mass	WILD	0	0	0
	CONTROL	0	0	0

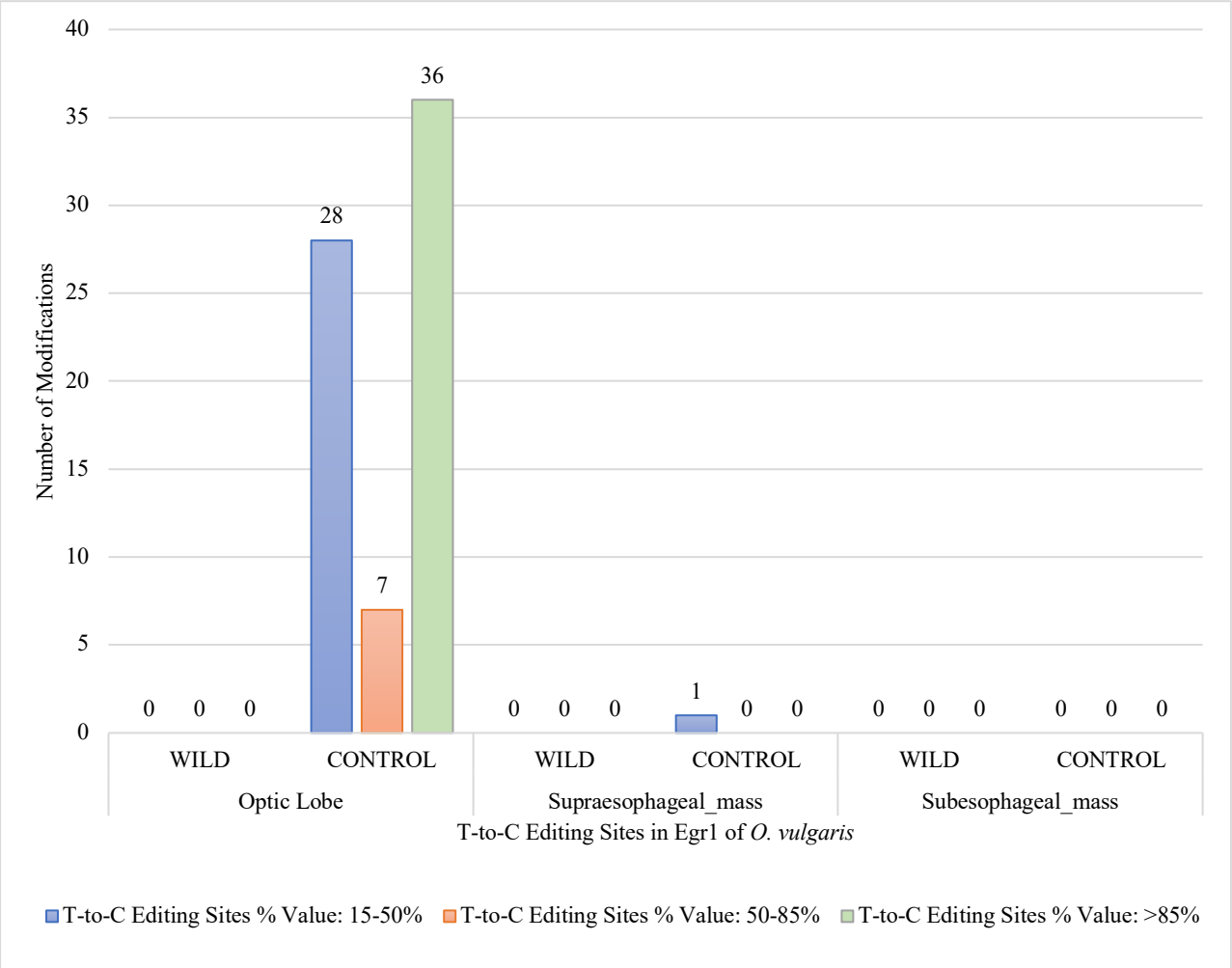

Figure 5. 14. T-to-C Editing sites reported in Egr1 gene of *O. vulgaris*

5.3.4.10 T-to-G EDITING EVENTS

Table 5. 14. T-to-G Editing events reported in nervous system

Tissues	Animal Type	T-to-G Editing events		
		15-50%	50-85%	>85%
Optic lobe	WILD	1	0	0
	CONTROL	50	12	24
Supraesophageal mass	WILD	0	0	0
	CONTROL	0	0	0
Subesophageal mass	WILD	0	0	0
	CONTROL	0	0	0

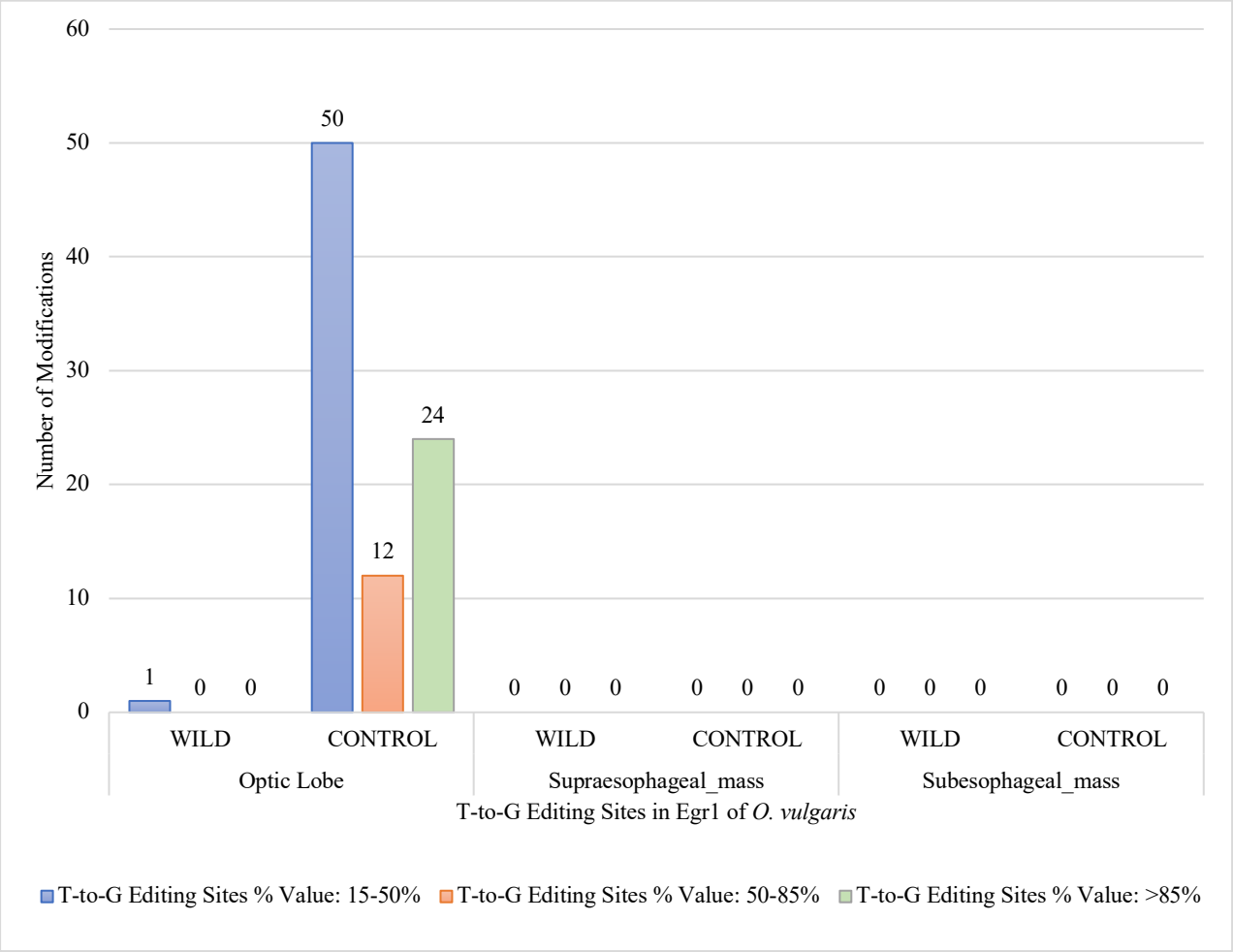

Figure 5. 15. T-to-G Editing sites reported in Egr1 gene of *O. vulgaris*

4.4 DISCUSSION

The phenomenon of A-to-I(G) RNA editing in cephalopods has garnered significant research attention, offering valuable insights into the intricate genetic regulation and post-transcriptional modifications of these captivating marine creatures. Adenosine-to-inosine (A-to-I) conversion, a unique type of RNA editing, plays a crucial role in modifying the genetic information carried by RNA molecules, thus impacting protein diversity and functionality. Cephalopods, including octopuses and squids, exhibit an exceptional and extensive pattern of A-to-I(G) RNA editing, surpassing that observed in most other species. Exploring the mechanisms, functional implications, and evolutionary significance of this process in cephalopods is pivotal to understanding their biology and addressing broader questions regarding adaptation and genomic flexibility. This chapter focuses on the intricate realm of A-to-I(G) RNA editing in cephalopods, with specific emphasis on the Egr1 gene. Our investigation aims to uncover the regulatory mechanisms governing Egr1, identify crucial genetic targets influenced by this editing process, and explore the potential implications of these findings for the biology of these remarkable organisms. By comprehensively examining the role of A-to-I(G) RNA editing, particularly within the context of the Egr1 gene, we aim to provide a comprehensive understanding of how it shapes the genetic landscape of cephalopods.

Overall, coleoid cephalopods, including octopuses, squids, and cuttlefishes, show a high prevalence of RNA editing, primarily observed in the neuron system, accounting for approximately ~60% of reported editing events. However, no editing has been reported in the nautilus (*Nautilus pompilius*) or *Aplysia californica* yet. In contrast to coleoid cephalopods, RNA editing for protein recoding is rare in most organisms. Yet, in humans and mice, millions and thousands of editing events have been reported, respectively. Most of these editing sites occur in double-stranded RNA (dsRNA) structures formed by inverted repetitive elements in non-coding sections. Weak editing sites account for approximately ~3% of recoding in humans,

while mammals exhibit numerous editing events without clear adaptive advantages. In the realm of invertebrates, limited attention has been given to RNA editing. Drosophila melanogaster, a well-studied invertebrate, has reported around ~4% recoding sites among mRNA, with 1,300 recoding sites conserved across *Drosophila* lineages. These editing events have been found to occur under positive selection and play a role in fine-tuning nervous function. Similarly, in cephalopods, highly edited sites tend to be conserved across coleoid taxa, strongly indicating that cephalopod editing is under positive selection and confers phenotypic advantages (Rodriguez et al., 2012; Liscovitch-Brauer et al., 2017; Birk et al., 2023; Rosenthal & Eisenberg, 2023).

Recent studies have focused on the role of A-to-I(G) RNA editing in cephalopods, particularly in relation to temperature changes (Liscovitch-Brauer et al., 2017; Birk et al., 2023; Rosenthal & Eisenberg, 2023). Many of these editing events occur in the nervous system and are involved in the regulation of Ca^{2+} and K^{+} ion levels in nerves (Liscovitch-Brauer et al., 2017; Birk et al., 2023; Koenig, 2023; Rosenthal & Eisenberg, 2023). Specifically, the study found that crucial editing sites in the Synaptotagmin-1 and Kinesin-1 genes play vital roles in adapting to lower temperatures without altering the genomic DNA sequence under cold conditions. Notably, these editing events occur within hours to days in response to temperature changes (Birk et al., 2023; Koenig, 2023). Similarly, it has been reported that RNA recoding occurs in temperature-specific kinesin variants within 24 hours in response to environmental temperature changes (Rangan & Reck-Peterson, 2023). Therefore, such RNA editing mechanisms can assist animals, especially those in marine environments, in rapidly adapting to changing environmental conditions, including temperature fluctuations caused by upwelling events, seasons, or migrations. It is expected that RNA editing may lead to recoding events that result in changes to amino acids and protein synthesis within several days to a week, allowing organisms to reach a new steady state following a temperature change. However, it has been

noted that this mechanism may not be sufficient to handle rapid temperature changes caused by tides or crossing thermoclines in marine environments (Birk et al., 2023).

Besides, there are several noteworthy examples of mRNA editing occurring in the GRIK family of ionotropic glutamate (kainate) receptors, particularly in GRIK2. This editing is conserved in the genomes of both *D. pealeii* (squid) and mammals, resulting in the substitution of Tyr512Cys in squid and Tyr571Cys in humans and mice. Furthermore, other genes, such as dynein (DYNC1H1), the ATPase Na^+/K^+ Transporting Subunit Beta 1 (ATP1B1), and the squid CELF2 gene (known to associate with RNA editing enzymes in mammals), have also been reported to undergo RNA editing (Albertin et al., 2022), further other genes subjected to RNA editing of octopuses have been reported by Albertin et al., 2012. Therefore, it is imperative to conduct comprehensive functional studies on these genes in cephalopods, particularly with a neural-specific focus, in order to unravel the true secrets behind these intriguing mechanisms (Albertin et al., 2022).

Based on this concept, our study focused on the immediate early gene and transcription factor, Egr1. The Egr1 gene is an intriguing gene that acts as a mediator and regulator of synaptic plasticity and neuronal activity in organisms, both under physiological and pathological conditions (Duclot & Kabbaj, 2017). Remarkably, there have been no previous reports on the study of the Egr1 gene in octopuses, making our research the first to investigate the RNA editing mechanisms of the Egr1 gene in *O. vulgaris*. Additionally, it has been reported that Egr1 is involved in a broad range of activities in the nervous system, playing specific roles such as participating in extracellular stimulation and transcriptional events that contribute to long-term functional changes in neurons. It also plays a role in the regulation of visual cortical development, protein emergence within cells, learning new tasks (specifically related to visual discrimination), response to emotional stress and reward, as well as the control of cognition, emotional response, social behavior, and sensitivity to reward in regions such as the medial

prefrontal cortex, striatum, hippocampus, and amygdala. Notably, when aligning amino acids, differences were found between Egr1 proteins within species rather than between species, indicating clear specificities in protein-protein interactions that influence regulation, reactivity, transcriptional control, and ultimately neuronal function. Furthermore, it has been reported that Egr1 expression induces neuronal activities that stimulate neurons, making it a valuable tool for studying the functions of neuronal ensembles involved in higher-order brain functions (Illing, 2002; Duclot & Kabbaj, 2017; Fuss & Schluessel, 2018). Therefore, given the advanced and complex nature of cephalopod brains, utilizing the Egr1 gene to understand A-to-I(G) RNA editing using the nervous system of *O. vulgaris* can provide insights into their behaviors and functional significance.

Therefore, our analysis of the discovery of A-to-I(G) RNA editing has revealed that a higher number of editing sites occurred in the optic lobe of the control animal, with the majority being moderate editing sites (70 editing sites) compared to highly edited sites (27 editing sites) observed in the wild animal. Conversely, the presence of editing sites in the supra and suboesophageal masses were very low and negligible, recording only 1-3 editing sites. It is intriguing to note the impact of the Egr1 gene in two different animals under different conditions: the wild conditions and the controlled conditions of captivity. These two different environmental settings likely triggered distinct responses in the animals, such as stress, predator pressure, food availability, water conditions, and other biotic and abiotic factors. These factors may directly influence a wide variety of stimuli, activating general intracellular signaling pathways that enable *O. vulgaris* to adjust and adapt their neuronal activity and physiological responses to environmental stimuli (Duclot & Kabbaj, 2017). In the wild conditions, the animals may experience predatory pressure and other stressful situations. As we acquired the animals from fishermen, they typically kept the animals in fishing boats under their captivity or inside fishing gears for approximately 18 to 24 hours. This extended period

of captivity and potential stress may hinder the animals' ability to adapt and modify neuronal activities, especially in the context of Egr1 gene modifications. Recent studies have reported that RNA editing can occur within a few hours to days in response to different conditions, such as temperature changes, demonstrating significant levels of RNA editing in octopuses and squids as they adapt to these changes (Liscovitch-Brauer et al., 2017; Birk et al., 2023; Koenig, 2023; Rosenthal & Eisenberg, 2023). Similarly, studies conducted on cephalopods have revealed that RNA editing can vary between different cephalopod species and even among different individuals of the same species, highlighting RNA editing as a highly dynamic and adaptable process in response to rapid environmental changes and selective pressures (Albertin et al., 2015). Furthermore, it has been established that the Egr1 gene can be utilized to investigate neuronal ensembles underlying anxiety, stress response, and stress-related disorders in animals (Duclot & Kabbaj, 2017). In contrast, this may explain why wild *O. vulgaris* exhibited very low levels of editing sites in the Egr1 gene compared to *O. vulgaris* kept under controlled conditions.

The *O. vulgaris* kept under controlled conditions had already undergone a 15-day acclimatization period and were further maintained in optimized conditions for one month, including water quality and food availability. This extended period allowed the animals sufficient time to adapt and survive without experiencing any stress-inducing stimuli or triggers within the facility. As a result, the animals were able to adjust their neurological and physiological conditions to ensure survival. Interestingly, this may have facilitated the animals in organizing their mRNAs to undergo different types of RNA editing, particularly A-to-G RNA modifications. This strongly supports the role of the Egr1 gene, as it has been reported to act as a master regulator of neuronal activities and to coordinate multiple levels of synaptic and neuronal plasticity. Additionally, studies have shown that the regulation of Egr1 is dependent on the nature and duration of stress exposures. Acute physical stressors such as immobilization,

forced swim, and restraint have been found to upregulate Egr1 mRNA expression levels (Hawkins et al., 2006; Duclot & Kabbaj, 2017; Fuss & Schluessel, 2018). Considering these factors, it is evident that the *O. vulgaris* kept under controlled conditions significantly influenced the results of different editing levels and percentages that we have reported here, specifically in relation to the various stages of A-to-G RNA editing, which were observed to be between 50-80% and >80% in the Egr1 gene.

The very recent findings have explained this RNA editing process can be turned on or off at specific editing sites, allowing organisms to respond to changes in their surroundings. For example, if the temperature becomes colder, organisms can activate the editing process at specific RNA sites to modify the RNA sequence in a way that helps them cope with the cold. Similarly, if oxygen levels are low, the editing process can be triggered at certain sites to adjust RNA sequences and enhance oxygen utilization. This flexibility enables organisms to fine-tune their genetic instructions in response to environmental challenges. Not only are physical environmental factors involved in this RNA editing process, but the social environment can also have an impact. Interactions with other organisms, such as mates, rivals, or social groups, can influence the editing process. The social environment may trigger specific RNA editing events that modify RNA sequences to adapt to social cues or dynamics. In overall, RNA editing allows organisms to dynamically respond to changing environmental conditions. By selectively activating or deactivating editing sites in RNA molecules, organisms can acclimate to factors like temperature, oxygen availability, light, or pH (Rosenthal & Eisenberg, 2023).

However, upon examining the mRNA sequences of the Egr1 gene and its recoding under A-to-G RNA editing, we observed that all the modifications occurred at the synonymous level, meaning they did not result in changes to the amino acid sequence of the mRNAs, unlike non-synonymous RNA editing. This finding can be attributed to the fact that the animals were kept under controlled conditions for an extended period, specifically one month following a

15-day acclimatization period. As a result, the amino acid sequence modifications in the Egr1 gene were not observed due to the prolonged duration of the study. To gain a deeper understanding of when the animals are more likely to utilize A-to-G RNA editing modifications, it is crucial to investigate and analyze these modifications in animals kept for different periods of time, ranging from hours to days, with frequent sampling intervals. This approach is similar to studies conducted on temperature differences and their impact, which have been examined in *O. bimaculoides* and *D. pealeii*, focusing on proteins such as kinesin-1, Synaptotagmin-1, and microtubule motor proteins kinesin and dynein. These exposures were limited to hours with various intervals extending up to a few days (3-4days) (Birk et al., 2023; Koenig, 2023; Rangan & Reck-Peterson, 2023). Furthermore, studies have reported on the expression of the Egr1 gene in newts (*Cynops pyrrhogaster*) under different exposure times to light, ranging from minutes to days. It has been shown that the expression of the Egr1 gene can be triggered within short periods of time (Itaru & Sakae, 2005). Considering those above-mentioned experiments, it is essential to investigate the same time frame for the Egr1 gene of *O. vulgaris*, as we have kept the animals under controlled conditions for prolonged periods. Therefore, this could be one of the reasons why the *O. vulgaris* kept under controlled conditions did not exhibit any recoding events.

Similarly, we observed the same results for the wild *O. vulgaris*, where no recoding events were detected. This could be attributed to the stress levels experienced by the animals during handling by fishermen on the vessels, as we do not have precise information about the duration of their captivity until reaching the selling point. To ensure accurate comparisons, it is crucial to collect and preserve the animals as soon as they are caught from the wild, without subjecting them to post-harvest stress caused by fishermen handling on the vessels. This condition has been reported for the Egr1 gene, as it is known to be regulated by a wide range of environmental events. Furthermore, special attention should be given to the Egr1 gene, as it

exhibits high dynamics and can modify the expression of genes involved in various aspects of synaptic plasticity, including vesicular transport and neurotransmitter release, synaptic architecture, endocytosis, and protein degradation. It has also been reported that Egr1 gene expression is triggered by extracellular stimulation, leading to transcriptional events that contribute to long-term functional changes in neurons (Illing, 2002; Hawkins et al., 2006; Duclot & Kabbaj, 2017).

In addition to the A-to-G RNA editing modifications, we also investigated other types of possible modifications (A-to-C, A-to-T, C-to-A, C-to-G, C-to-T, G-to-A, G-to-C, G-to-T, T-to-A, T-to-C, and T-to-G) for the Egr1 gene. These modifications followed a similar pattern to the A-to-G RNA editing, with higher levels of editing observed in the optic lobe of the control animal compared to the wild animal. The same trend was observed for the supra and suboesophageal masses, with only 1-2 editing sites detected for both types of modifications in both wild and controlled animals. This is an intriguing finding, consistent with previous studies conducted on other types of modifications in cephalopods (Alon et al., 2015; Liscovitch-Brauer et al., 2017), where the highest RNA editing levels were also observed for A-to-G modifications. These results align with the patterns reported by Albertin et al. in 2015. It is important to note that the number of editing sites are lower in our study, as we focused solely on the Egr1 gene, while previous studies analyzed all RNA sequenced transcripts.

The recoding of the Egr1 gene transcript cDNA was also examined in our study. Alignment of genomic DNA with the cDNA sequences of the Egr1 gene clearly showed no changes in the cDNA sequences. Even though the peak areas were altered due to the occurrence of editing sites, the changes were synonymous and did not result in any modifications to the nucleotide sequence of the Egr1 gene transcripts. It is worth noting that most studies conducted using RNA sequencing and transcriptomics analyses have primarily focused on non-synonymous RNA editing, which involves a higher number of editing sites and a greater

percentage of modifications (Albertin et al., 2012, 2015, Rodriguez et al., 2012; 2022; Rosenthal & Seeburg, 2012; Garrett & Rosenthal, 2012a; Alon et al., 2015; Koenig et al., 2016; Liscovitch-Brauer et al., 2017; Birk et al., 2023; Rosenthal & Eisenberg, 2023).

However, a study conducted by Wang & Mohlhenrich in 2019, that calculated diversity score metrics to validate RNA editing and amino acid recoding in all cephalopods, using available databases until 2019, reported that all genes showed RNA editing. Notably, the study found that the Egr1 gene exhibited a very low level of RNA editing score (score for Egr1 = 0.01124) compared to other genes and was classified as a gene with very low-level editing. The authors listed the top 100 highly edited genes along with their diversity score metrics, and they compared their calculations with previous studies to validate their findings (Wang & Mohlhenrich, 2019). Based on this information, it can be concluded that the Egr1 gene in *O. vulgaris* undergoes synonymous RNA editing without recoding events in our experiment, resulting in no changes to the amino acid sequence of the Egr1 gene. These findings represent the first study to report synonymous RNA editing in the Egr1 gene of *O. vulgaris*.

Changing of amino acids sequence codons triplet always not change during the A-to-G RNA editing. It also determines and changes occur depending on where the editing occurs in the mRNA molecule. In some cases, editing may occur in non-coding regions or in regions that do not affect the amino acid sequence, leading to no change in the resulting protein. In other cases, editing may occur in a region that does affect the amino acid sequence, leading to a change in the protein. Therefore, the detection of RNA editing events in cDNA samples cannot rely solely on changes in codon triplets, and other methods such as RNA sequencing or PCR-based methods may need to be employed to detect editing events. When considering the RNA editing mechanisms in cephalopods, including octopuses, the enzymes known as ADARs play a crucial role in this post-transcriptional process. These enzymes are responsible for facilitating a powerful and highly specific alternative mechanism during RNA editing and modifications.

Specifically, ADARs are involved in the conversion of adenosine to inosine, effectively mimicking guanosine during this post-translational process and participating in various biological processes (Birk et al., 2023).

The recoding process may occur at a low frequency and could potentially be adaptive, but it is more likely a byproduct of ADAR activity near targeted sites. A study conducted by Albertin et al. in 2022 reported that 70% of edits in neural tissues are recoding events, while the remaining 30% are synonymous edits. Their findings also suggested that the impact of recoding on protein function can be subtle and specific to each recoded protein, as observed in specific K^+ channels. Moreover, the presence of recoding sites in cephalopods, particularly in conserved amino acid residues that remain uncharacterized, presents an intriguing opportunity. These recoding sites could potentially serve as a valuable tool for identifying functional residues or targets of modulation in proteins found in non-cephalopod organisms. By exploring these recoding sites, we can adopt a distinct approach, diverging from traditional screening methods. The substitutions observed at these sites may represent natural variations that animals utilize to augment or fine-tune protein function in different environments and circumstances (Rangan & Reck-Peterson, 2023).

In cephalopods, two types of ADARs have been reported: ADAR1 and ADAR2. However, it has been observed that ADAR2 catalytic activities are not exceptionally high compared to human and *Drosophila* ADAR2, and they are not heavily involved in high-level recoding in cephalopods. In coleiid cephalopods, it has been reported that there is expression of single genes for both ADARs, each possessing some unique structural features. Further investigation into the involvement of ADAR1 in RNA editing is recommended for a better understanding of its role (Jiang & Zhang, 2019; Rangan & Reck-Peterson, 2023; Rosenthal & Eisenberg, 2023). In our experiments, we also examined the expression of the ADAR1 gene using gel electrophoresis in the same tissues used to analyze the RNA editing of Egr1. The

expression of ADAR1 was clearly detected. To gain a better understanding, it is recommended to study the involvement of ADAR1 in different tissues from the nervous system of *O. vulgaris* using qPCR to determine its expression levels. Moreover, this confirms the involvement of ADAR enzymes in the regulation of the Egr1 gene, as previously analyzed in other animals (Sedmík, 2020).

In the case of our experiment on the Egr1 gene of *O. vulgaris*, the absence of recoding could be attributed to the regulation of the recoding process. The specific RNA sequence and structural motifs surrounding the recoding sites in the Egr1 gene may not be favorable for recoding to occur. These elements act like a hardwired code that determines whether recoding takes place at a particular site. If the sequence and motifs do not provide the necessary conditions for recoding, it is less likely to happen as described by (Eisenberg, 2018). Furthermore, the expression levels of ADARs, which are responsible for catalyzing the recoding process, play a significant role. If the abundance or activity levels of ADAR proteins are insufficient or not properly regulated, recoding may be limited or absent (Eisenberg, 2018). This could be the case for the Egr1 gene in *O. vulgaris* in our experiment.

By understanding the regulatory mechanisms involved in recoding, it can gain insights into the factors that influence its occurrence. Further exploration of the specific RNA sequence, structural motifs, and the expression levels of ADARs in the context of the Egr1 gene could provide valuable information about why recoding did not occur in our experiment. It is through these investigations that we can uncover the conditions necessary for recoding and potentially manipulate them to achieve specific outcomes in protein function.

In conclusion, RNA editing in cephalopods is more prevalent compared to other species, with a high percentage of editing events occurring in the neuron system. RNA editing is under positive selection and may confer phenotypic advantages to cephalopods. Recent studies have explored the role of A-to-I RNA editing in cephalopods in adapting to temperature

changes and its involvement in regulating ion levels in nerves. The Egr1 gene, a transcription factor involved in synaptic plasticity and neuronal activity, has been investigated in our experiment for RNA editing in cephalopods. The study compared A-to-I RNA editing in the Egr1 gene between wild and controlled *O. vulgaris* and found variations in editing levels, likely due to different environmental conditions and stressors. The modifications in the Egr1 gene were observed at the synonymous level and did not result in changes to the amino acid sequence. Therefore, further research is needed to understand the timing and duration of RNA editing in cephalopods and investigate the impact of stress levels on editing events in *O. vulagris*.

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ABBREVIATIONS

2D	Secondary Structures
7TM	7-transmembrane Domain
A	Adenosine
ADAR	Adenosine Deaminase Acting on RNA
ADB	Asian Development Bank
Am	*Alligator mississippiensis*
Bg	*Biomphalaria glabrata*
BLAST	Basic Local Alignment Search Tool
BORIS	Behavioral Observation Research Interactive Software
C	Ceramic Ball
cDNA	Complementary DNA
CDS	Coding region or coding sequences
Cn	*Coilia nasus*
DIG	Digoxigenin
DIS	Distal
Dr	*Danio rerio*
dsRNA	Double-stranded RNA
EC	Extracellular Loop
Egr1	Early Growth Response 1
G	Guanine
GPCRs	G protein-coupled Receptors
HEP	Hepatopancreas
Hs	*Homo sapiens*
I	Inosine

IC	Intercellular Loop
IF	Infundibulum
L+	Limonene
MBL	Marine Biological Laboratory
$MgCl_2$	Magnesium Chloride
MeOH	Methanol
MID	Middle
ML	Maximum Likelihood
Mm	*Mus musculus*
NBT/BCIP	Nitro Blue Tetrazolium chloride/5-Bromo-4-Chloro-3-Indolyl Phosphate
NCBI	National Center for Biotechnology Information
NJ	Neighbour Joining
O.C	Outer Cortex
OB	*Octopus bimaculoides*
Ol	*Oryzias latipes*
OLF	Olfactory Lobe
OM	Olfactory Mucosa
OR	Olfactory Receptor
ORFs	Open reading frames
OT	Optic Tract Region
OV	*Octopus vulgaris*
PBS	Phosphate Buffer Saline
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with 0.1% Tween-20
Pc	*Pomacea canaliculate*

PCR	Polymerase Chain Reaction
PCR	Polymerase Chain Reaction
PFA	Para-formaldehyde
PROX-B	Proximal Big
PROX-L	Proximal Large
r-opsin1	Rhodopsin
R.Z	Radial Column Zone
RIM	Epithelium Rim
RT	Room Temperature
SE	Standard Error
s	Seconds
SSC	Saline-Sodium Citrate
STHRDP	Science and Technology Human Resources Development Project
T.Z	Tangential Zone
TAARs	Trace Amine-Associated Receptors
UNINA	University of Naples Federico II
USA	United States of America
WMISH	Whole-mount *In-situ* Hybridization

PUBLICATIONS

Indexed-peer reviewed Articles:

1. Di Cosmo, A., Maselli, V., Cirillo, E., Norcia, M., **de Zoysa, H.K.S**.; Polese, G., Winlow, W. (2023). The Use of Isoflurane and Adjunctive Magnesium Chloride Provides Fast, Effective Anaesthetization of *Octopus vulgaris*. Animals, 13(22), 1-8pp. https://doi.org/10.3390/ani13223579
2. Valeria Maselli; Alsayed Alsoudy; Joshua J. C. Rosenthal; **H. K. S. de Zoysa**; Tamar Gutnick; Michael J. Kuba; Domenico Fulgione; Maria Buglione; Gianluca Polese; Anna Di Cosmo (2023). Not only the nose knows – TAAR gene expression in the octopus sucker, iScience (Under review).
3. Localize and mapping for photoreceptors molecules in the optic lobe of O. vulgaris (under preparation).

Abstracts: National/Internationals

1. Maselli, V., Norcia, M., Cirillo, E., **de Zoysa, H.K.S.,** Pinto, H. M., Al-Soudy, Al-Sayed, Di Cosmo, A. (2023). *Octopus vulgaris* vision using multiple distinct opsins in the optic lobe. 82° Congresso, Unione Zoologica Italiana (UZI)- 2023. 19-22 September, 2022, Palermo, Italy.
2. Maselli, V., **de Zoysa, H.K.S.,** Pinto, B., Polese, G., Kuba, M. J., Gutnick, T., Norcia, M., Di Cosmo, A. (2022). Sensory System, Behaviour and Differential Gene Expression: New Insights From Common Octopus (Octopus vulgaris). 81° Congresso, Unione Zoologica Italiana (UZI)- 2022. 20-23 September, 2022, Trieste, Italy, 29pp.
3. **de Zoysa, H.K.S.**, Al-Soudy, Al-Sayed, Polese, G., Maselli, V., Di Cosmo, A. (2022). Octopus sucker as the extra-ocular visual system. CIAC- 2022. 2-8 April, 2022, Sesimbra, Portugal.

4. Polese, G., Al-Soudy, Al-Sayed, Maselli, V., Russo T., **de Zoysa, H.K.S.**, Di Cosmo, A. (2021). Octopus' Suckers a Multitasking Sensor. International Bioscience Conference. 25 – 26 November, Serbia.

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1. Cirillo, E., Norcia, M., **De Zoysa, H. K. S.**, Winlow, W., Maselli, V., Polese, G., Di Cosmo, A. (2023). Sviluppo Di Test Comportamentali Per L'accertamento Dello Stato Di Anestesia Profonda in *Octopus vulgaris* a Supporto Dell'animal Welfare. 82° Congresso, Unione Zoologica Italiana (UZI)- 2023. 19-22 September, 2022, Palermo, Italy.
2. Maselli, V., Alsoudy, A., Rosenthal, Joshua J. C., **de Zoysa, H.K.S.**, Gutnic, T., Kuba, Michael J., Fulgione, D., Buglione, M., Norcia, M., Polese, G., Di Cosmo, A. (2023). Not only the nose knows. *Society for Molecular Biology and Evolution (SMBE)* - 23 – 27 July 2023, Ferrara, Italy, 256-257 pp.
3. **De Zoysa, H. K. S.**, Maselli, V., Pinto, B., Polese, G., Kuba, M. J., Gutnick, T., Di Cosmo, A. (2022). Smell by Touch in Common Octopus (*Octopus vulgaris*). 81° Congresso, Unione Zoologica Italiana (UZI)- 2022. 20-23 September, 2022, Trieste, Italy, 101pp.
4. Pinto, B., Maselli, V., Consoli, P., Riveccio, E., Minichino, R., **De Zoysa, H. K. S.**, Polese, G., Di Cosmo, A. (2022). Piano Di Gestione Della Pesca Del Polpo (*Octopus vulgaris*) Nelle Aree Marine Protette Della Regione Campania. 81° Congresso, Unione Zoologica Italiana (UZI)- 2022. 20-23 September, 2022, Trieste, Italy, 80pp.