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Octopus Senses: From Genes To Behavior

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ABSTRACT

Octopuses are intelligent, soft-bodied animals, have complex nervous systems with remarkable cognitive abilities and keen senses that perform reliably in a variety of visual and chemo-tactile learning tasks for exploring and sensing the environment. They have the largest nervous system of any invertebrate, with 500 million neurons distributed centrally and peripherally throughout the body. The nervous system of common octopus (*Octopus vulgaris*), is comprised of central lobes surrounding the esophagus and a pair of optic lobes that together contain approximately a third of the neurons, with the remaining two-thirds distributed within the arms (e.g. in the large axial nerve cords that extends along the center of each of their eight arms). The most obvious characteristic feature of an octopus is its eight long and flexible arms, but these pose a great challenge for achieving the level of motor and sensory information processing necessary for their behaviors. In addition, octopuses have a significant number of lobes of the nervous system dedicated to visual, tactile, and chemosensory perception. In this study, I aimed to provide a comprehensive view on the genetic bases for the tactile form of olfaction, extraocular photoreception in the sucker, localization of photoreceptors molecules in the optic lobe of O*. vulgaris*, as well as to identify the major genes are involved in the adult neurogenesis and then the cognitive system in *O. vulgaris*. I have applied a developed whole-mount *in situ* hybridization, real-time qPCR, and bioinformatic methods, supported by behavioral evidences to provide a comprehensive view on these processes in *O. vulgaris*, highlight how genomic innovation translates into organismal organization novelties. Results achieved contributed to some extent, and promoted interest in this field.

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Prologue and plan of this Ph.D. Project

Octopus is a cephalopod, well known as an intelligent soft-bodied animal, possessing a complex nervous system with an advanced sensory system, and exhibits remarkable cognitive abilities (Hanlon and Messenger, 1996; Hochner et al., 2006). They have evolved an incredible diversity of their sensory systems, including vision and olfaction to extract information from the environment. For cephalopods, particularly octopus, olfaction and vision are most likely the dominant sensory modality. Octopuses possess rich visual and chemo-tactile perception. The nervous system of octopus has significant number of lobes are dedicated to visual, tactile, and chemosensory perception (Shigeno et al., 2018; Grasso and Basil, 2009). These developed sensory systems allow them to achieve sophisticated behaviors to detect food, avoid predators and communicate with conspecifics.

Furthermore, octopuses evolved many unique organs that allow them to sense and explore diverse environments such as arm suckers that function as specialized tactile and chemosensory organs, as well as an elaborate chromatophore system under direct neural control that enables rapid changes in appearance (Hanlon and Messenger, 1996; Kro¨ger et al., 2011). It has been demonstrated that octopuses widely use their suckers for sensing and exploring the environment (Kier and Smith, 1990). Octopus has chemoreceptors on their suckers (Graziadei, 1958 Graziadei and Gagne 1976), which are thought to facilitate a taste-by touch ability (Wells, 1963; Wells et al., 1965). In addition, the octopus sucker epithelium has been shown to contain a variety of specialized sensory receptors, giving them unique features to perform a remarkable variety of sensory functions (Guerin, 1908; Martoja and May, 1956; Rossi and Graziadei, 1958 Graziadei and Gagne 1976; Packard 1988). However, studies related to identifying the function of these sensory receptors at the molecular levels are still scarce.

Last but not least, besides their sensory systems, my attention was focused on the cognitive system that in octopuses is considered as integrated, adaptive system able to perform a myriad of cognitive functions in the brain achieving sophisticated vertebrate-like plasticity and neural control (Shomrat et al. 2008; Edelman and Seth 2009, Young 1991). Octopus show an extraordinary learning ability, cognitive function, and adaptability are linked to the increments of the adult neurogenesis in the neuro-genic zones of the octopus brain (Bertapelle et al. 2017). Altogether these distinctive features of the highly developed sensory

systems (olfaction and vision) of octopus together with the extraordinary adaptive/plasticity of their physiological innovations and behavior are undoubtedly acquired during evolution, and greatly contributed for the animals' survival. These unique behavioral traits, genomic innovation, and neural novelties have garnered significant attention for molecular studies of their neuroethology, which could provide fundamental insights into how octopuses perceive and explore their environment.

To investigate the molecular bases for these intriguing processes including the tactile form of olfaction, extraocular photoreception and adult neurogenesis in *Octopus vulgaris*, I combined the behavioral and biomolecular approaches in order to achieve this goal. In this study, I have applied a developed whole-mount *in situ* hybridization, real-time qPCR, and bioinformatic methods, supported by behavioral analysis to provide a comprehensive view on these processes in the common octopus, *O. vulgaris*.

The overall goals of this PhD project are:

1- To investigate the priority given to chemical *vs*. visual perception to establish the sensorial hierarchy in food choice by *O. vulgaris*.

2- To contribute to the knowledge on the presence of olfactory receptor genes involved the tactile form of olfaction in the arm suckers of *O. vulgaris* and *O. bimaculoides*, that is usually non-olfactory organ, as well as localized their expression at the sensory area of octopus sucker, demonstrating the peripherally distributed octopus nervous system is a key site for processing of olfactory sensory information.

3- To examine and localize the expression of light sensing molecules (like, Ov-GRK1) in the octopus sucker, suggesting that the sucker of *O. vulgaris* has an extra-ocular photoreceptive system for light sensitivity, mediating the phototransduction cascade process.

4- Localize and mapping for photoreceptors molecules in the optic lobe of *O. vulgaris*.

5- To identify the major genes involved in adult neurogenesis processes in *O. vulgaris*.

Despite my experience and previous work were focused on mammalian and large animals including farm animals, my Ph.D. studies carried out under the supervision of Prof. Anna Di Cosmo, gave me a great opportunity to expand the field by integrating

neuroethological and molecular studies to the best of my knowledge. During these years, I had the possibility to experience an environment highly sensitive to the inclusion of cephalopods, as sole representatives among invertebrates, in accordance with the principles and procedures that were approved by the Institutional Animal Care of the University of Napoli Federico II and the Ministry of Health, according to the Italian and European law (European Directive 2010/63 EU L276; Italian DL. 4 March 2014, no. 26) and the ethical principles of Reduction, Refinement and Replacement.

Furthermore, during my Ph.D research activities, I was trained at the Marine Biological Laboratory (**MBL**) in Woods Hole, Massachusetts, the USA with Dr. Joshua Rosenthal, to specifically work in the area of the post-transcriptional processes underlying the generation of RNA editing events in olfactory receptor neurons in the suckers of octopus, as well as to know-how RNA editing is influenced by environmental factors (chemical cues), and how editing helps shape the evolution of this sophisticated animal. I also performed the experiment to localize the expression of OB-TAARs in the arm suckers of *O. bimaculoides* using the whole-mount *in situ* hybridization technique.

I regularly participated in seminars and international conferences. During my Ph.D research activities, I received a "Young Researchers" Award from the Italian Zoological Union (*UZI*), 80th Congress of the Italian Zoological Union (**UZI**) Rome, 23-26 September 2019.

My PhD had the ambitious aim to provide a comprehensive view on the genetic bases for the tactile form of olfaction, extraocular photoreception, mapping for photoreceptors molecules in the optic lobe of *O. vulgaris*, as well as define the major genes are involved in the adult neurogenesis and then the cognitive system in *O. vulgaris*, highlight how genomic innovation translates into organismal organization novelties. I believe to have contributed to some extent, and I think I have also promoted interest in the study. I hope that future studies may contribute in the above lines and that my work, will assist future students.

CHAPTER 1

INTRODUCTION

1- The physiology, behavior, and sensory processing capabilities of cephalopods.

In order to survive, all animals have evolved an incredible diversity of sensory systems to extract information from the environment. The sensory systems of animals are crucial to detect environmental stimuli, and they are then processed through the nervous system to generate appropriate behaviors (Williamson, 2009). The developed sensory system allows animals to achieve sophisticated behaviors such as; finding food, avoiding predators, identifying conspecifics, locating suitable habitat, and attracting mates (Dangles et al, 2009; Jordan and Ryan, 2015; Hauser and Chang, 2017).

Coleoid cephalopods (cuttlefishes, squids, and octopuses) are considered as an ideal model organism for this endeavor. They have highly sophisticated sensory systems (Nixon and Young 2003; Borrelli 2007) that displays outstanding behaviors and high cognitive capacities to cope with diverse environmental conditions in their niches (Hochner et al., 2006; Kuba et al., 2006; Hanlon and Messenger 1998; Hochner et al. 2003, 2006; Fiorito 2008; Hochner 2008, 2010;2012; Huffard 2013). They are unique amongst invertebrates, having evolved large, highly differentiated brains, and a well-developed set of sensory organs (Packard, 1972; Messenger, 1977; Young, 1977, 1989; Budelmann, 1995, 1996; Hanlon and Messenger, 1998; Anderson et al., 2010) provide exciting model systems to investigate how organismal novelties of these animals evolved and adapted to all marine environments.

The evolution of the nervous system is one of the key features of functional adaption of cephalopods to their environment: peripheral and central nervous systems associated with sensorial structures constitute the network of perception and integration of internal and environmental factors (reviewed in Albertin and Simakov, 2019). Additionally, their adaptation to a wide range of marine environments is associated with a suite of morphological novelties that have evolved across the subclass of their sensory organs such as light and adhesive organs, sucker, accessory nidamental glands, toxin-producing salivary glands, among others. They also have a distributed nervous system in the brain, arms and skin therefore, cephalopod's behavior is controlled in three somewhat separate domains - brain decision

making, arms manipulation, and chromatophore skin appearance (Mather and Dickel, 2017). Thus, the nervous system of cephalopods represents a striking example of embodied organization show the highest degree of centralization (Packard, 1972), show remarkable and unique features, in which the central brain acts as a decision-making unit that integrates multimodal sensory information and coordinates the motor commands executed by the periphery (Shigeno et al., 2018).

2- Octopuses

Octopuses are intelligent, soft-bodied animals, have complex nervous systems with remarkable cognitive abilities and keen senses that perform reliably in a variety of visual and chemo-tactile learning tasks (Sanders, 1975; Wells, 1978; Mather, 1995; Boal, 1996; Anderson and Mather, 2010; Hochner, 2008; Mather and Kuba, 2013; Hanlon and Messenger, 2018). They have the largest nervous system of any invertebrate, with 500 million neurons distributed centrally and peripherally throughout the body (Hochner 2008).

The common octopus (*Octopus vulgaris*) is the most studied of all octopus species (Figure1). The nervous system of *O. vulgaris*, is comprised of central lobes surrounding the esophagus and a pair of optic lobes that together contain approximately a third of the neurons, with the remaining two-thirds distributed within the arms (e.g. in the large axial nerve cords that extends along the center of each of their eight arms) (Young, 1971).

The most obvious characteristic feature of an octopus is its eight long and flexible arms, but these pose a great challenge for achieving the level of motor and sensory information processing necessary for their behaviors (Shigeno et al., 2018; Zullo et al. 2009; Packard 1972). The octopus's arms are lined with hundreds of tactile and chemosensory structures, known as suckers that interact with and provide information from the environment (Graziadei and Gagne 1976; Nixon and Young, 2003). Octopus has a significant number of lobes of the nervous system dedicated to visual, tactile, and chemosensory perception (Shigeno et al., 2018; Grasso and Basil, 2009). They have also the ability to integrate both information received visually and chemicals cues perceived by chemosensory organs to engage in complex cognitive tasks that mediate many keys aspects of their living environment. Moreover, the neuromuscular system of the octopus arm combines extreme flexibility with the ability to make precise goal-directed movements and carry out highly sophisticated tasks (Gutnick et al, 2011; Huffard et al 2005; Mather, 1998).

These distinctive features of the distributed nervous system of octopus together with the extraordinary plasticity of their physiological innovations and behavior (unique morphological and neural novelties, such as arms with tactile and chemosensory suckers and a large complex nervous system) are undoubtedly acquired during evolution (Packard, 1972), and have contributed greatly to their success (Hochner, 2012). They compete successfully with vertebrates in their ecological niche using a rich behavioral repertoire more typical of intelligent behavior (Budelmann, 1995; Budelmann et al., 1997; Hochner and Glanzman, 2016; Villanueva et al., 2017).

Through the evolutionary process, a challenging task considering the more than 500 million years of independent evolution (Packard, 1972; Kröger et al., 2011;Roth, 2013; 2015), the structure and function of the central and peripheral nervous system has become more complex. In addition to these unique morphological and neural organization in octopuses, the distinct genetic novelties at the genomic level make them fascinating organisms to study (Albertin et al., 2015; Liscovitch-Brauer et al., 2017; O'Brien et al., 2018). The availability of genomic sequencing for the four octopus species including *Octopus bimaculoides* (Albertin et al., 2015), *O. vulgaris* (Zarrella et al., 2019), *Octopus minor* (Kim et al., 2018), and *Octopus Sinensis* (Li et al., 2020), provide a remarkable opportunity for investigating the genetic basis underlying organismal novelties as well as open up a new era of cephalopod genomics on various aspects of cephalopod biology, ranging from neuroethology to evolutionary genomic.

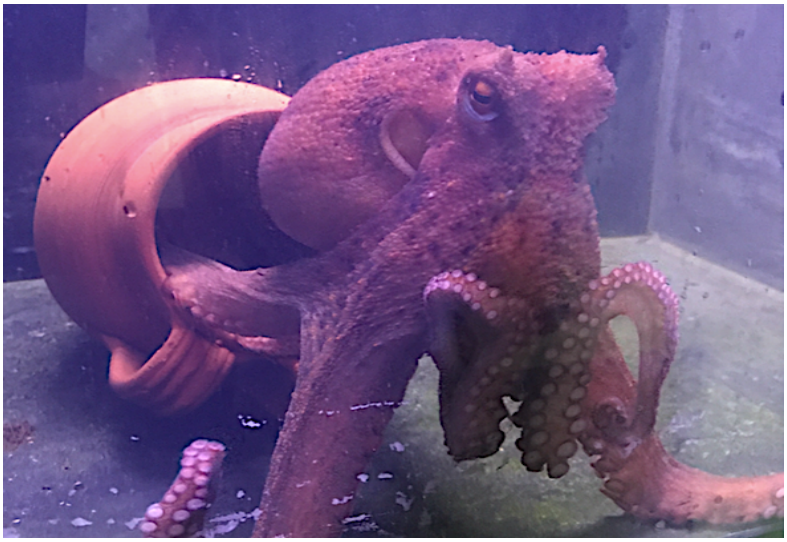

Figure 1. A typical specimen of *O. vulgaris (taken from Di Cosmo's Cephalopod Facilities)*

3- Genetic features underlying organismal novelties in octopus.

The molecular basis of cephalopod innovations first arose with the sequencing of the *O. bimaculoides* genome (Albertin et al., 2015). The published genome and multiple transcriptomes of the *O. bimaculoides* have provided valuable information on genomic traits and various levels of genome organization (e.g., the emergence of novel genes, gene family expansion, dramatic genome rearrangements, RNA editing, and transposable element activity) related to the evolution of neural complexity and morphological innovations. The remarkable innovations in the morphological and functional organization of its nervous system (Figure 2) (Edelman and Seth 2009; Shigeno et al. 2018; Styfhals et al. 2019) are linked to the evolution of an unprecedented complexity coded both at cellular and molecular levels (Shigeno et al. 2018; Shigeno and Ragsdale 2015; Gray and Young 1964; Wang and Ragsdale 2017; Styfhals et al. 2019).

As more octopus genomes are sequenced including *O. vulgaris* (Zarrella et al., 2019) *O. minor* (Kim et al., 2018) and *O. sinensis* (Li et al., 2020), these genome-level studies revealed that massive expansions occurred in several major gene families that involved in neuronal patterning, signaling and cell communication such as; protocadherins (**PCDHs**) and C2H2 zinc fingers (**C2H2-ZNFs)**, additionally the G protein-coupled receptors (**GPCRs**). The independently expanded PCDHs and C2H2 zinc fingers gene families are almost exclusively expressed in nervous tissues in both vertebrates and cephalopods and seem to be involved in both nervous system development and functioning (Albertin et al., 2015; Styfhals et al., 2019; Wang and Ragsdale. 2017; Peek et al., 2017).

The PCDHs, one of the most striking expansions found in octopus genomes: *O. bimaculoides* has 168 protocadherins (Albertin et al., 2015) and the drastic expansions were also observed in the genome of *O. minor*, like 303 (Kim et al., 2018), other sequenced cephalopods seem to have a similarly large complement (Styfhals et al., 2019). Strikingly, differential gene expression analysis revealed that octopus PCDHs are predominantly expressed in the nervous tissues, like vertebrate PCDHs, where they are essential for nervous system functioning (Albertin et al., 2015; Styfhals et al., 2019). Some octopus PCDHs are broadly elevated throughout the nervous system, while others show restricted expression to specific neural tissues. In particular, the optic lobes and the axial nerve cord present an impressive

enrichment in PCDHs (Albertin et al., 2015). These structures will likely be central to the future study of cephalopod PCDHs function.

The C2H2-ZNFs is the largest family of transcription factors in animal genomes, another gene superfamily that is massively expanded in cephalopod genomes. Interestingly, approximately 200~400 C2H2-ZNFs typically have been identified in invertebrates, but in cephalopod, lineages appeared to have had a dramatic increase in the size of this family, with nearly 1,800 and 2,289 members in *O. bimaculoides* and *O. minor*, respectively (Albertin et al., 2015; Kim et al., 2018).

The GPCRs comprise a large superfamily of proteins that possess a characteristic 7-transmembrane domain (**7TM**), mediating signal transduction pathways by sensing a variety of external and internal stimuli (Hanlon and Andrew, 2015; Kobilka, 2007). They play a crucial role in how organisms react to their environments, as well as in homeostatic regulation via endocrine and neuronal functions. Sensory receptors that detect and respond to light, taste, and smell primarily belong to the GPCR superfamily. Larger repertoires of the GPCRs can increase the amount of available sensory information associated with specific functions related to smell, taste, and light perception (Hill et al, 2002; Niimura and Nei, 2007; Thomas and Robertson, 2008), facilitating environmental adaptation (Strotmann et al., 2010). In the octopus genome, such octopus-specific expansions have been observed for GPCRs repertoire, suggesting enabling the evolution of sensory functions relevant to their ecological contexts. The expression profiles of octopus GPCRs repertoire were found to be across various tissues, but most are mainly expressed in tissues outside of the brain, devoted to the evolution and neuronal functionality in the arms and distal structures {GPCRs in suckers, skin, and the axial nerve cord (ANC)} (Ritschard et al., 2019), such as arms with tactile and chemosensory suckers (Nixon and Young, 2003; Young, 1971; Giesen et al., 2020). However, relatively little is known about GPCR diversity and functionality in octopus as well as their exact physiological function remains unclear.

Furthermore, these genome-level novelties in octopus are also accompanied by other sophisticated innovations such as extensive RNA editing, particularly in the nervous system cells, which generates transcript and protein diversity in genes involved in neural excitability, as well as in genes participating in a broad range of other cellular functions (Garrett and Rosenthal, 2012a,b; Liscovitch-Brauer et al., 2017). Lastly, the genome sequencing of several

octopuses showed that repeat elements, in particular transposable elements (TL), with major roles in genome rearrangements and evolution, are abundant, indeed, the genome of *O. bimaculoides* revealed that over 45% of the genome is comprised of repetitive elements (Albertin et al., 2015). The *O. minor* genome is composed of 44% repetitive sequences (Kim et al., 2018), while, 42.26% of repetitive sequences were annotated in *O. sinensis* (Li et al., 2020). The significant proportion of multiple mapping reads suggests that, similar to the *O. bimaculoides* genome, *O. vulgaris* genome has a large number (at least 50%) of repetitive elements (Zarrella et al., 2019). More recently, the identification of long interspersed element (LINE) and full-length transpositionally competent TEs found a similar expansion also in the genome of *O. vulgaris*, suggesting that they might be active in the brain. Transcription and translation measured for one of these elements resulted in specific signals in neurons belonging to areas associated with behavioral plasticity (Petrosino et al., 2021).

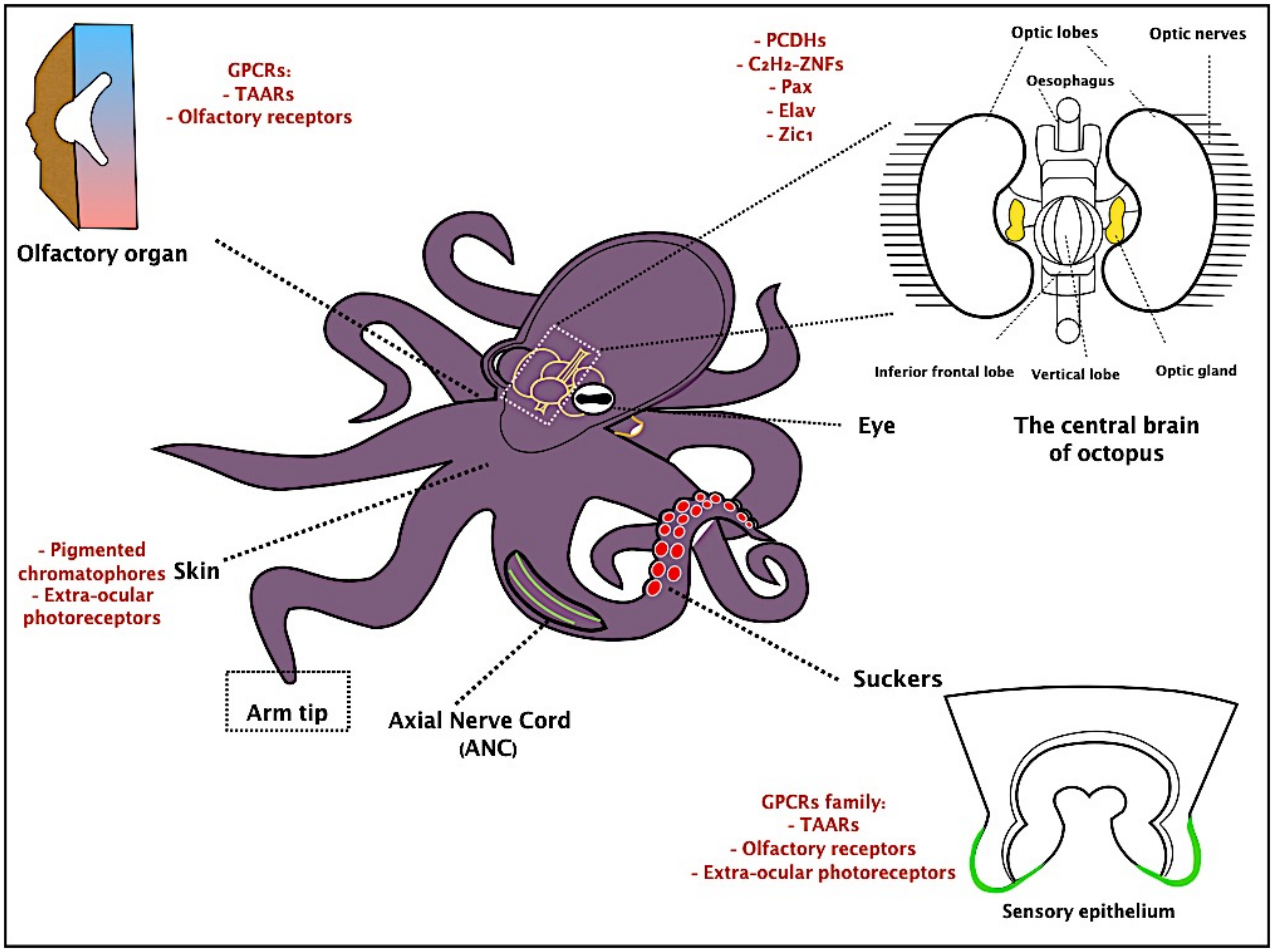

Figure 2. Diagram of sensory and nervous system in octopus from whole animals to sensory organs and brain, including the central brain mass (supra- and sub-esophageal mass), optic lobes, and eight axial nerve cords.

4- Intelligence and cognitive system of the octopus

Intelligence and the cognitive system has known as an integrated, adaptive system able to perform a myriad of cognitive functions in the brain, such as sensory perception, learning and memory, problem-solving tasks, and decision making.

Among invertebrates, *O. vulgaris* is considered as an interesting model organism for studying the comparative cognition in invertebrates (Edelman, 2011; Edelman and Seth, 2009; Mather, 1995; Young, 1991). They display a wide repertoire of outstanding behavior and complex cognitive capabilities mediated by a highly sophisticated nervous system with a high degree of brain plasticity (Hanlon and Messenger 1998; Hochner et al. 2003, 2006; Hochner 2008, 2010, 2012). In many behavioral studies, octopuses show excellent learning and memory recall (Marini et al., 2017; Hochner et al., 2006; Robertson et al., 1994; Young et al.,1995; 1991; Young, 1983; Sanders, 1975; Wells,1978) and sophisticated cognition (Mather and Dickel, 2017; Huffard, 2013; Gutnick et al., 2011; Alves et al., 2009; Edelman and Seth, 2009; Hvorecny et al., 2007; Kuba et al., 2006; Mather,1995).

They also show considerable skills in problem-solving tasks. Critically, octopuses exhibit flexibility in solving problems not only in their natural environment but also when faced with artificial tasks (Richter et al., 2016; Amodio and Fiorito 2013; Anderson and Mather, 2010; Kuba et al., 2010;2003; Mather and Anderson,1999; Fiorito et al., 1990; Schiller,1949; Wells, 1964). Octopuses have extraordinary learning ability and behavioral flexibility by quickly adapting to a change in their living environment. Crucially, the performances of the octopuses in these cognitive abilities are often greatly attributed to generated newborn neurons to improve their brain function and to cope with the challenging environmental conditions (Lindsey and Tropepe, 2006; Bertapelle et al., 2017). Recently, it has been reported that the cognition and learning abilities are also linked to adult neurogenesis in *O. vulgaris* (Bertapelle et al. 2017).

Intriguingly, these results revealed an increment of adult neurogenesis in the specific neurogenic zones of the adult *O. vulgaris*, when challenged with problem-solving tasks (Bertapelle et al., 2017). These results show how the enriched environment affects cell proliferation and synaptogenesis. The Proliferating Cell Nuclear Antigen (PCNA) was used as a marker of cell proliferation and a cytoplasmic isoform of poli (ADP-ribose) polymerase 1 (PARP1) as a marker of neuronal plasticity (Bertapelle et al. 2017, De Lisa et al., 2012).

Moreover, bivariant analysis of flow cytometry using BrdU incorporation allowed assessment of the magnitude of adult neurogenesis in those neuro-genic zones of the adult *O. vulgaris*, in particular, the brain areas involved in learning and memory, and sensory stimuli integration (Di Cosmo et al., 2018). Thus, we highlighted the importance of adult neurogenesis to specifically improve the performance of cognitive and learning abilities of octopus in an enriched challenging environment. However, studies related to identifying the major genes that are involved in this intriguing process are still scarce.

Interestingly, the previous study on the genome of *O. bimacualodies* has discovered a massive expansion of protocadherin (PCDHs) gene family, which regulates the development of neurons and synapses (Albertin et al., 2015). In octopus PCDHs, one large exon encodes all cadherin domains, while editing is reported to be enriched in PCDHs, suggesting RNA editing, in conjunction with gene duplication, as an alternative mechanism to RNA splicing for the generation of diverse protocadherin molecules in the nervous system (Wang and Ragsdale. 2017). Similarly, in chordates, PCDHs are also diversified using extensive alternative splicing to generate unique molecules on cell surfaces for self-recognition, a key component for the development of complex brains (Wang and Ragsdale. 2017). These genomic characteristics may have facilitated the involvement of octopus PCDHs gens in the adult neurogenesis process in *O. vulgaris'* brain and related to their cognitive abilities.

To assess this possibility, in this study, we analyzed the differential gene expression of three protocadherin genes (Oct-PCDHs), three Pax genes, Elav gene and Zic1 gene in specific brain areas (the central part of the supraesophageal mass, the subesophageal mass, and the optic lobes, including the olfactory and peduncle lobes (OOP) on the optic tracts) in adult octopuses kept under the tree different cognitive stimulations: tested (enriched environment), wild (naturally enriched environment), and control conditions (unenriched environment) (Maselli et al., 2020). Our data shows that Oct-PCDHs genes are upregulated in the learning and lower motor centers in the brain of both tested and wild animals (higher in the latter).

5- The optic lobe, visual and extra-ocular photoreceptive systems in octopus

The octopus has always been described as a predominately "visual" animal with a complex visual system characterized by the presence of highly developed eyes, reflecting the important role that vision plays in these marine predators for various aspect of its life, including, social communication, hunting, and adaptive coloration (Hanlon and Messenger, 2018). Octopuses possess sophisticated eyes, keen visual acuity, and complex visual processing in the optic lobes (Hanlon and Messenger, 1996; Grable et al., 2002; Zylinski et al., 2009; Yoshida et al., 2015). The eyes of octopus are well adapted to the habitat and lifestyle of the species. Even with lacking multiple photoreceptor types, they can achieve color discrimination with only a single photoreceptor, which has a monochromatic view of the world (Stubbs and Stubbs, 2016) within a wide range of light conditions (Yoshida et al., 2015; Gagnon et al., 2016). Unlike the vertebrate camera eye, the octopus eyes have an un-inverted retina, a cornea, an iris, a spherical lens, and photoreceptor cells that translate light from the light-sensitive retina into nerve signals which travel along the optic nerve to the brain, where the visual information is processed (Figure 3) (Young, 1960, 1971; Wells, 1966a; Maddock and Young, 1987).

At the neuronal level, the octopus optic lobes, a pair of the large nervous structures (larger than other regions of the brain) located outside the cartilaginous capsule of the brain and connected to the retinae of the eyes (Figure 2), reflecting the importance of visual information to behavior of this animal and essential for the computation of visual input (Young et al 1995; Young, 1960, 1971; Wells, 1966a; Maddock and Young, 1987).

Moreover, octopuses are known as visually oriented animals, learning to use visual landmarks (spatial information) to navigate within their home ranges, memorize a den, and guide their returns to it (Mather et al., 1991). Indeed, *O. vulgaris* can quickly learn for visual object discrimination (Boycott and Young, 1955; Messenger and Sanders, 1972); and recognize familiar conspecifics (Mather et al., 1991; Boal. 2006). They also learn to associate a visual or tactile stimulus with a negative or positive reward by observation learning abilities (Fiorito and Scotto. 1992; Amodio and Fiorito. 2013), learn to use vision to make the precise goal-directed movement by the arms (Gutnick et al, 2011). Octopus can communicate intra-specifically using polarized light signals (Moody and Parriss, 1960, 1961). The photoreceptors in octopus eyes are arranged in a manner to enable this animal the capacity to detect and

process the polarized light through the visual system to enhance the detection and recognition of prey.

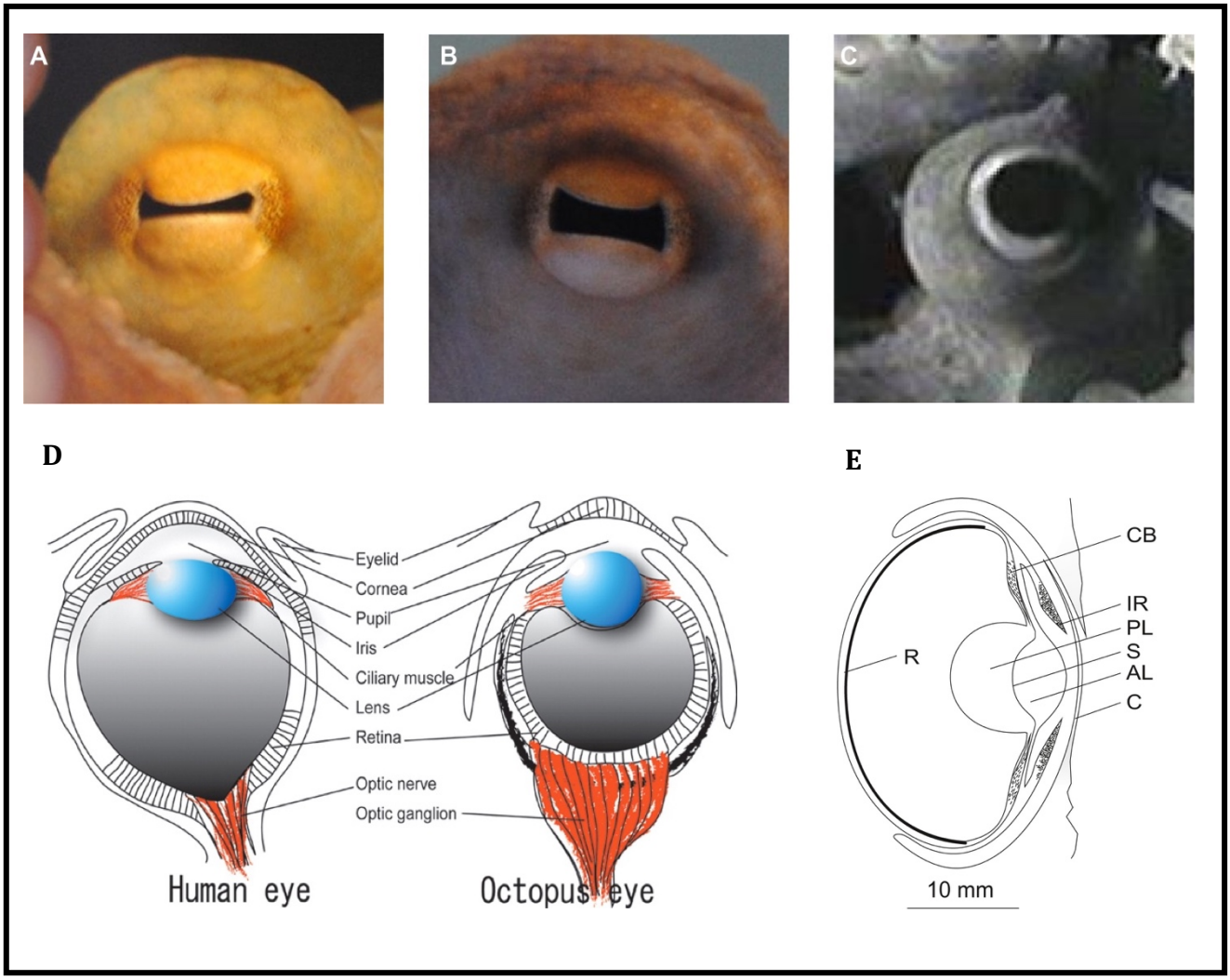

Figure3.Pupil of *Octopus vulgaris*. (**A**) Constricted horizontal slit pupil in bright light. (**B**) intermediate pupil size, and (**C**) fully dilated pupil in dim light conditions. (**D**) Structural comparison between human and octopus eyes (Figure from Ogura et al., 2019). (**E**) Schematic of the eye of *Octopus vulgaris* (longitudinal vertical section). Light falling on the eye of octopus first hits the cornea (C). Beyond the cornea, the light passes the anterior chamber and the pigmented mobile iris (IR) before it is refracted by the spherical lens. The lens, composed of an anterior (AL) and a posterior part (PL) separated by a septum (S), is suspended by the ciliary body (CB). Finally, the light hits the everted retina (R) in the back of the eye (Figure from Budelmann, 1994).

Besides the retinal photoreceptors in their eyes that are devoted strictly to vision, they have photoreceptive structures harboring other photoreceptor types know as extra-ocular photoreceptors typically mediate the phototransduction cascade to perform nonvisual tasks that occur in a wide array of cephalopod species (Messenger, 1967; Hara and Hara, 1976, 1980,

Mauro, 1977; Tong et al., 2009, Mäthger et al., 2010, Kingston et al., 2015a). These types of photoreceptors contribute to visual perception by activating varying G-protein-mediated cascades, that occur upon light stimulation, switching on different cellular responses, and ultimately producing different organismal events (Porter 2016). In cephalopods, especially octopuses, are well known for their remarkable ability to change their appearance by altering their dermal coloration and patterning for camouflage and communication (Young 1973; Hanlon and Messenger, 2018; Messenger. 2001; Borrelli et al., 2006; Norman, et al. 2001). It has been demonstrated that this ability is not only made by detecting visual cues using the eyes but also several extraocular photoreceptors that are expressed throughout dermal tissues have contributed to light detection (dermal light sense) and possibly to signaling and camouflage (Tong et al., 2009; Kingston et al., 2015; Ramirez and Oakley, 2015).

Although extraocular photoreceptors are expressed outside of the eyes and do not involve in image analysis, some of them that have been described are thought to detect light using rhodopsin, identical to those located in the eye (Mathger et al., 2010). Some are also known to use other visual phototransduction components, including retinochrome, Gqα, visual arrestin, and rhodopsin kinase (Gartner, 2000; Tong et al., 2009; Kingston et al., 2015; Kingston and Cronin 2016). The extra-ocular photoreceptive system of octopus plays an important role in most of its lives correlated with a visual distributed sensing. This functional flexibility provides this animal an additional perception for the external environment using the various aspect of non-visual signaling mechanisms related to the benthic lifestyle of adult octopus which can even inhabit shallow water. Additionally, animals can move and orient to the light stimulus, and the regularity in the light-dark periods are signals for circadian rhythms. However, we have little understanding of the nature of these receptors, their genetic and molecular components, and their biological functions are often not well understood. Here, we present the molecular evidence for the expression of four types of photoreceptors in the octopus sucker and further localized their expression at the epithelium of the rim of the sucker of *O. vulgaris*, suggesting that the sucker has an extra-ocular photoreceptive system for light sensitivity. These results suggested that the sucker of *O. vulgaris* is light-sensitive organ mediating the phototransduction cascade process. The octopus sucker has the expressing of phototransduction receptors that are homologous to those used in well-characterized visual systems. Thus, the sucker of *O. vulgaris* seems to function as extraocular

photoreception. These findings help us to understand the adaptations of the octopus visual system to lifestyle and habitat and implications for cephalopod visual system novelties. Future studies will allow the completion of a picture of vision in *O. vulgaris*. The detailed insight will thus be obtained regarding the world of a fascinating invertebrate which otherwise spends its life in a habitat that is still not easily accessible to humans.

6- Smell and chemo-tactile senses of octopus

Chemical senses are undoubtedly the most ancient senses that have evolved, resulting in the widespread sensory modality in all animals. Most animals use a broad range of chemical cues in circumstances that are critical for survival, mediating behaviors and physiological responses related to identifying food, predators, and mates, etc.. (Sorensen and Caprio, 1998; Bone and Moore, 2008). Chemoreception, a process by which organisms respond to chemical stimuli in their environments that depends primarily on the senses of taste and smell.

Among cephalopods, although the octopus is mostly relied on its visual system to perceive environmental cues, chemical perception is a crucial sense in the ecology of octopus particularly under limited light conditions (Polese et al., 2016; Di Cosmo and Polese, 2017; Nilsson et al., 2012; Polese et al., 2015). Octopuses are widely using both distance and contact chemoreception allow them to the sensing of a broad spectrum of chemical and mechanical signals in their environment through multiple chemosensory organs including olfactory organs (Woodhams and Messenger, 1974; Gilly and Lucero, 1992; Budelmann, 1996; Mobley et al., 2008; Polese et al., 2016), the buccal lips and mouth (Emery, 1975), isolated sensory neurons (Baratte and Bonnaud, 2009; Buresi et al., 2014), and chemoreceptors arm suckers (Wells et al., 1965; Graziadei,1962; Graziadei, 1964; Graziadei and Gagne,1976). Thus, they may explore their environment by touch and taste (Wells et al., 1965; Giesen et al., 2020), while their olfactory organs can perceive widespread water-borne molecules arising from a distance (Polese et al., 2016; Di Cosmo and Polese, 2017; Alves et al., 2007; Boal and Golden, 1999; Boyle, 1983;1986; Chase and Wells, 1986; Lee, 1992). Moreover, the full complement of the octopus GPCR superfamily revealed that the *O. bimaculoides* genome contains a large family of chemosensory-like GPCRs; 74 GPCRs are similar to the Aplysia chemosensory GPCRs 57 and 11 GPCRs are similar to vertebrate olfactory receptors (Albertine et al., 2015). However, chemosensory processing remains one of the least understood areas of octopus nervous system organization (Young, 1971).

Regarding the chemo-tactile sense in octopus, when vision is unavailable, octopus foraging behavior is dependent on arm chemo-tactile sensation, reaching into dark crevices and probing other parts of their surroundings (Budelmann, 1996; Walderon et al., 2011; Mather and Kuba, 2013). Additionally, *O. vulgaris* was shown to use chemosensory cues at its suckers to avoid self-entanglement, which plays an important role in a self-recognition mechanism (Nesher et al., 2014). Behavioral and electrophysiological study on *O. bimaculoides* has been Recently confirmed that chemosensory receptor cells in the arms and suckers are able to detect environmentally relevant chemicals and utilizing local neural signaling to integrate sensory cues and carry out arm-autonomous behaviors (Fouke and Rhodes, 2020). These are similar to the early behavioral studies that have also shown that octopuses are able to discriminate among objects based only on their chemical characteristics by their suckers of the arms, evoking a behavior defined as "taste by touch" (Wells, 1963). More recently, Giesen et al., (2020) discovered the molecular basis of chemo-tactile sensation in *O. bimaculoides*, confirming that the arm suckers have a kind of taste-touch ability mediated by unique chemo-tactile receptors (CR). These studies reflect the importance of the chemo-tactile sense in the octopus arms sucker that plays a critical role in many aspects of the chemosensory of ecological events.

In view of the great chemical sensitivity of the octopus suckers, it has been recently hypothesized that the chemo-tactile discriminations in octopuses are also involved in olfaction by their suckers that were traditionally not considered olfactive, allowing them to recognize odorant molecules that are insoluble or have very low solubility, exhibiting a peculiar behavior described as "smell by touch" (Di Cosmo et al., 2018; Di Cosmo and Polese 2017).

In chemosensory research, it has been proposed that the evolutionary transition from aquatic to terrestrial life necessitates the detection of hydrophobic airborne ligands instead of water-soluble hydrophilic molecules (Mollo et al., 2014). In the aquatic environment, marine animals including crustaceans and fishes can use their gustatory systems to recognize distant water-soluble compounds without physical contact with their source (Figure 4). Nonetheless, crustacean and fish are also produce and respond to a variety of hydrophobic compounds (not readily diffuse in water) (Giordano et al., 2017); in particular, little shrimp (*Palemon elegans*) and also fish (*Danio rerio*) use their chemosensory mouthparts to perceive typical odiferous compounds usually smelled by humans which are also widespread in terrestrial organisms.

In the same way, these molecules could be smelled by any other aquatic animals, including octopus, when they are touched through tactile forms of olfaction. Interestingly, this empirical evidence challenges the notion of aquatic olfaction.

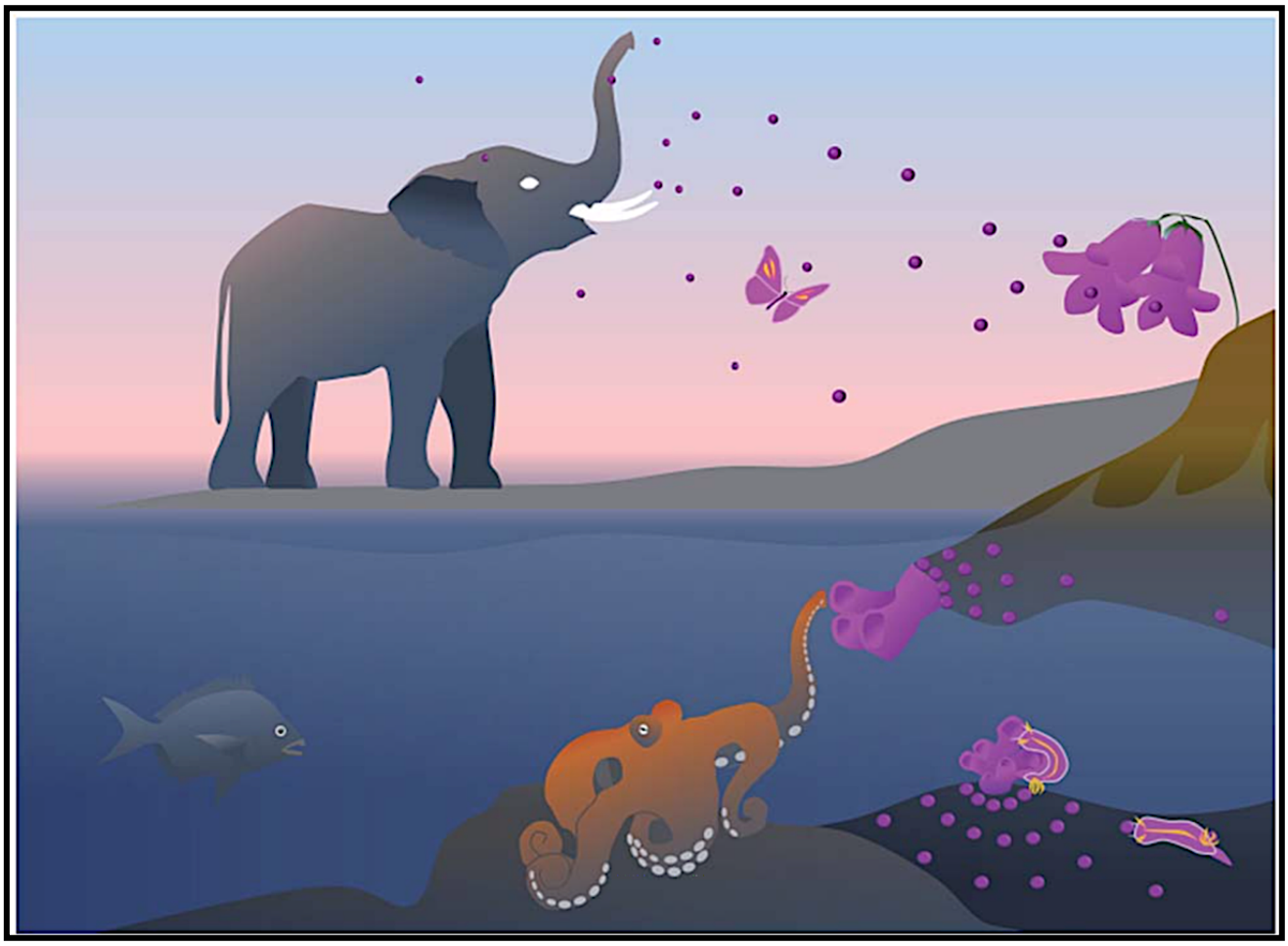

Figure 4. Chemosensory spatial range of an ideal natural molecule (represented by fuchsia spots) that is insoluble in water but volatile. The compound is sensed over a long distance by the highly discerning nose of the elephant, and allows flowers to attract fly pollinators within a three-dimensional olfactory space (top). In the marine environment, the same molecule allows both an octopus to avoid eating a toxic sponge, and the sponge itself to avoid predators, while specialist nudibranchs can use the same signal to find food and mates (bottom). Underwater, however, "tactile" olfactory perceptions occur within a two-dimensional space (Figure from Mollo et al., 2017).

In terrestrial organisms, the distinction has often been made between taste (contact chemo-sensation, where stimuli are often solid or liquid) and olfaction (distance chemo-sensation, where stimuli are often volatile compounds) (Caprio, 1977). But, in the aquatic environment, it is not yet clear how this distinction might be applied to marine animals

including octopus where the same receptors may serve to detect chemicals with both local and distant sources. Thus, the discrimination between olfaction and taste based on the spatial signal range would certainly be confusing without considering specific molecular interactions of the ligands with chemosensory receptors. Therefore, the accurate studies to define the type of chemoreceptors and their ligands based on molecular and genetic data should be certainly desirable and persuasive for a better understanding of all forms of tactile-chemoreception, as well as shed more light on the synchronous of different signals using different channels by the chemosensory organs and integrates them into the central nervous system. In our study, combining molecular and behavioral analyses, considering the type of receptors and octopus behavior in exploring the tactile forms of olfaction, we demonstrated that *O. vulgaris* are able to recognize and respond to the insoluble compounds which typically act as olfactory cues on land by their suckers mediating smell by touch behavior. The results present expression of trace amine-associated receptors (TAARs) genes on the octopus' "gustatory systems", coding for olfactory receptors cells support that the sense of the smell is not restricted to the olfactory organ, but it is diffuse due to the presence of olfactory receptors on the arm suckers.

7- Octopus' arm sucker: Multi-Sensory (multi-functional) integration organ

The octopus sucker represents a fascinating natural system with unique features performing a remarkable variety of tasks to discover how much information coming from contacts with its environment (Packard 1988). Octopuses use their suckers for a myriad of functions such as anchor the body to the substrate or to grasp, manipulate objects and adhesion (Kuba et al., 2006; Grasso, 2008; Grasso and Setlur 2007) and chemo- and mechano-sensing (Graziadei 1962; Graziadei and Gagne 1976a,b; Wells, 1978; Maselli et al., 2020). The octopus arm sucker contains the most effective mechanical and sensory systems. The arms and suckers constitute most of the body mass and account for most of the neurons and muscles; in fact that the vast majority of their behaviors depend upon their arms and suckers, which are functioning as "natural biosensors' (Sumbre et al., 2001; Grasso, 2008; Zullo et al., 2019).

Interestingly, the suite morphological and mechanical features of the sucker (Figure 5) gives the octopus the ability to perform many tasks for sensing and exploring the environment (Smith, 1990). The meridional, circular, and radial muscles (Tramacer et al., 2013; Kier and Smith,1990) controlled by the sucker ganglia achieve a wide array of motor and proprioceptive functions. An octopus sucker consists of two regions, rich in sensorial cells connected to nervous fibers: the infundibulum (AR) and acetabulum (IF) (Figure 5 a). The infundibulum is the exposed, pliable, denticles face of the sucker that is circumscribed on its rim by a ridge (Kier and Smith, 2002; Tramacere et al., 2014). The acetabulum is the more rigid, ellipsoidal cavity of the sucker, consisting of a domed roof featuring a fibrillar surface and smooth surrounding walls (Kier and Smith, 2002; Tramacere et al., 2013; 2014). The epithelium lining the infundibulum consists of tall columnar cells resting on a basal lamina and the inner connective tissue capsule (Graziadei and Gagne, 1976). The attachment process of octopus suckers begins with the infundibulum pressing and conforming to the surface, and the rim sealing the sucker and preventing water leakage (Tramacere et al., 2013; 2014; 2015).

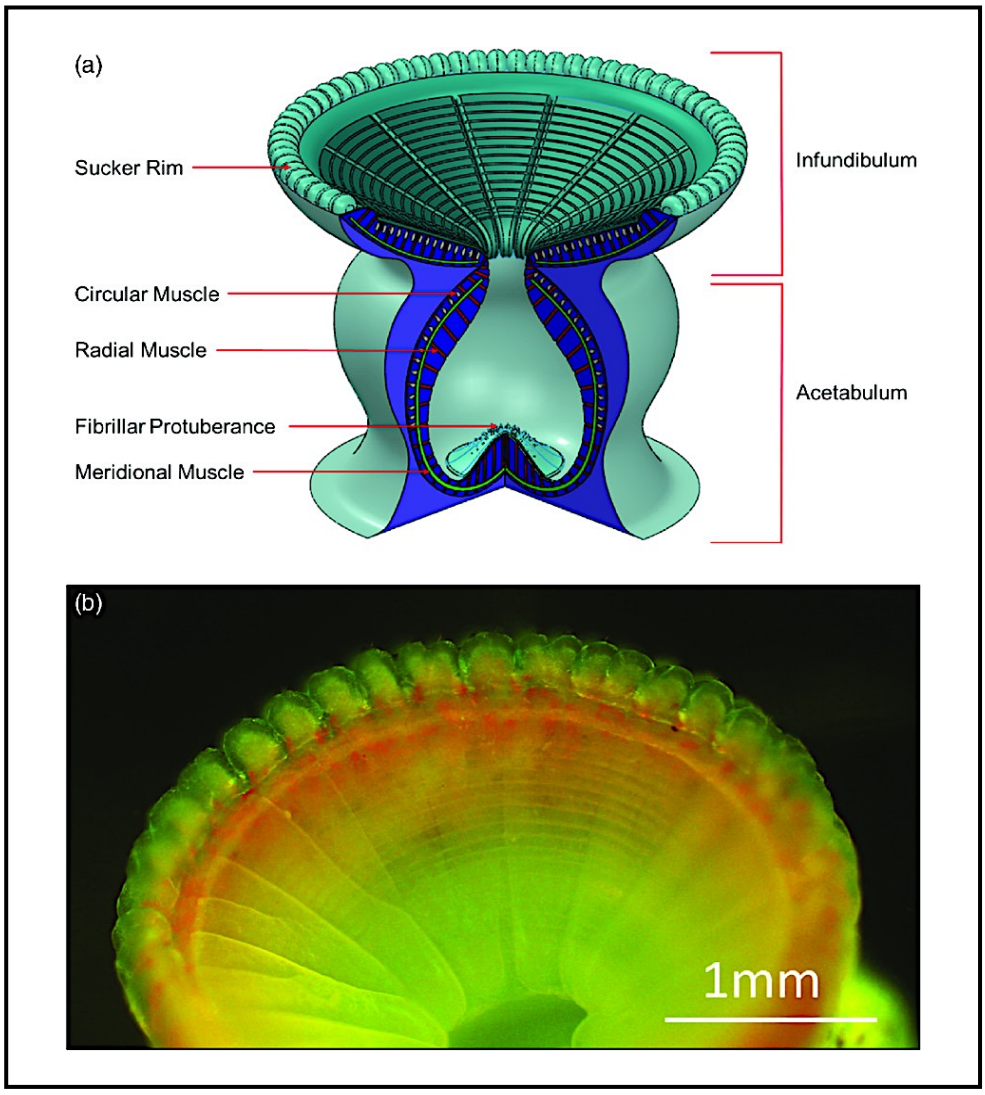

Figure 5. Octopus sucker: a) 3D sucker model with major regions and muscles labeled; b) 4× zoomed image of a sucker, highlighting the infundibulum (Figure from Bagheri et al., 2020).

There are thus numerous sensory cells of various types in the epithelium which covers the exterior border of the sucker. The number is greatest around the rim of the suckers and decreases passing down the stalk of the infundibulum, and there are few in the skin of the surface of the arm itself. In that region, the intra-epithelial receptors consist only of a few cells sending their processes directly to the central nerve cord of the arm (Graziadei; 1962). The arms contain a central axial nerve cord with brachial ganglia located at each sucker (Gutfreund et al., 2006; Young, 1971). Arm ganglia process motor and sensory information, enabling local signal processing that allows the arm, and even individual suckers, to perform autonomous behaviors (Grasso, 2008; Sumbre et al., 2001). In the face that octopuses are largely tactile animals (Wells 1978), many previous studies have focused on the chemo-tactile ability of the sucker (Giesen et al., 2020; Fouke and Rhodes, 2020; Wells 1978), on the sensory

receptors in the suckers (Graziadei 1962; Graziadei and Gagne 1976 a;b) suggesting the sucker serves as the sensory organ for the chemotactic sense. It also uses the sucker as a tactile organ, reacting not only to simple pressure but also making discriminations between certain specific three- dimensional patterns (Wells, 1964). This unique octopus chemo-tactile sense is mediated by suction cups (suckers) along the arms that sense and manipulate prey by utilizing local neural signaling to integrate sensory cues and carry out arm-autonomous behaviors (Fouke and Rhodes, 2020; Hochner, 2012; Sumbre et al., 2001; Wells et al., 1965; Young, 1971).

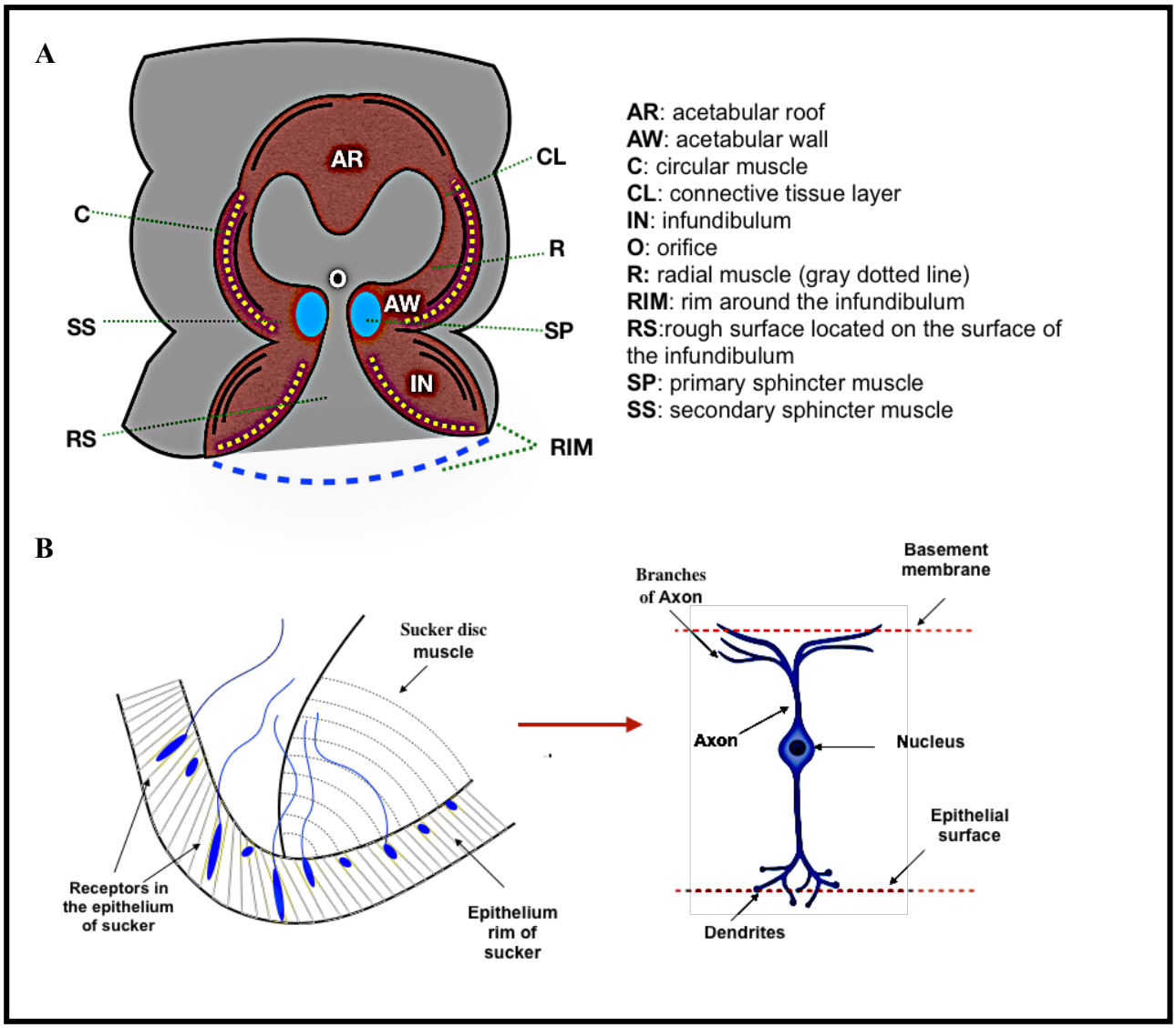

Figure 6. (**A**) Schematic of the anatomy of the suckers and the epithelial rim of sucker. (**B**) Detail of the infundibulum of epithelium rime which cover the exterior border of the sucker and distribution of primary receptors in the epithelium rim *has been given by* Graziadei 1964; Wells et al., 1965; Graziadei & Gagne, 1976).

In this context, early studies have confirmed a huge number of primary receptor cells localized in the sucker rim epithelium are morphologically similar to receptor cells in other animals, which plays a fundamental role in the chemo- and mechano-sensing in the suckers (Graziadei 1962). However, the epithelium of the suckers of the octopus has been shown to contain a variety of sensory receptor cells with unusual cytological and morphological characteristics (Graziadei and Gagne 1976). Many details of the function of these cells are unresolved yet. In our study, we try to better understand the molecular characterization and function of these types of receptors at molecular and cellular levels. A growing body of literature describes unexpected functions for sensory receptors in other organs. In this study, we present current knowledge about the function of sensory receptors found outside their classical sensory organs in octopus. In our study, we present the molecular evidence for the expression of four types of photoreceptors in the octopus sucker and further localized their expression at the epithelium of the rim of the sucker, suggesting the sucker has an extra-ocular photoreceptive system for light sensitivity. Finally, our study demonstrated that suckers have the extraordinary ability to detect a wide range of sensory information in several sensory modalities necessary for their appropriate behaviors considering the diverse environments and stimuli, mediating a variety of functions and - in certain cases–sense light.

CHAPTER (2)

Sensorial Hierarchy in *Octopus vulgaris*'s Food Choice: Chemical *vs.* Visual

5. Introduction

The sensory systems of animals are crucial to detect environmental cues, and they are then processed through the nervous system to generate appropriate behaviors (Williamson, 2009), such as finding food, avoiding predators, identifying conspecifics, locating suitable habitat, and attracting mates (Dangles et al., 2009; Jordan and Ryan, 2015; Hauser and Chang, 2017). In aquatic systems, as on land, chemical cues affect not only individual behavior and population dynamics, but also community organization and ecosystem function.

Use of sensory modalities may be related to the ecology of the species, as prey or predator. Animals use different sensory modalities to search for food such as chemical, vibrational, tactile, sound, heat, and visual senses (Atema et al., 1988). Among them, while vision enables marine animals to swim directly to food items when they see it, chemoreception is essential to detect and locate food items, especially for animals active at night or in the deep ocean (Prosser, 1973; Weissburg et al., 1993; Derby and Steullet, 2001). Several studies suggest that aquatic species rely more strongly on chemical perception rather than vision one when discriminating between harmless and dangerous hetero-specifics (Gerlai, 1993; Kiesecker et al., 1996; Mathis and Vincent, 2000; Ferrari et al., 2010). On the other hand, aquatic environments are particularly prone to the variability of the visual and chemical conditions. For example, turbidity could reduce the efficacy of visual cues even if octopus is capable of polarized vision (Moody and Parriss, 1961; Talbot and Marshall, 2011; Stubbs and Stubbs, 2016), whereas currents may disrupt chemical information.

Accordingly, the sensory system's capabilities in cephalopods have been inextricably associated with their evolutionary success, allowing them to occupy many ecological niches of the sea from shallow waters to the deep sea. Coleoids are endowed with a highly sophisticated nervous system (Nixon and Young, 2003; Young, 1971; 1972) and an exceptionally large brain that includes more than 30 differentiated lobes (Young, 1971; Hanlon and Messenger, 2018). Among them, *O. vulgaris* (hereafter octopus), well known as an intelligent soft-bodied animal, has a significant number of lobes of the nervous system dedicated to visual, tactile, and chemosensory perception (Shigeno et al., 2018; Grasso and Basil, 2009). Its

nervous system has a high degree of cross-connectivity (Young, 1971; Shigeno et al., 2018; Budelmann,1996) able to integrate sensory inputs coming from the environment through its well-developed sensory organs (Young, 1971; Polese et al., 2016; Well,1963; Wells et al., 1965).

Indeed, octopus has a rich repertoire of complex behaviors (Figure 1) that includes problem-solving, visual, and chemo-tactile. In particular, the abilities of coleoids to perceive environmental cues have been mainly attributed to its visual systems. Although, under limited light conditions, the chemical signals are the primary important source as sensory inputs (Polese et al., 2016; Di Cosmo and Polese, 2017; Nilsson et al., 2012; Poles et al., 2015). Thus, coleoids have remarkable abilities to recognize chemical cues through the buccal lips and mouth(Emery, 1975), isolated sensory neurons (Baratte and Bonnaud, 2009; Buresi et al., 2014), and arm suckers (Wells et al., 1965; Graziadei,1962; Graziadei, 1964; Graziadei and Gagne,1976). Thus, they may explore their environment by touch and taste, while their olfactory organs are able to perceive at distance (Polese et al., 2016; Di Cosmo and Polese, 2017; Alves et al., 2007; Boal and Golden, 1999; Boyle, 1983;1986; Chase and Wells, 1986; Lee, 1992), sensing a broad spectrum of chemical signals (Budelmann, 1996).

It has been reported that chemosensory cues are important in decision-making in octopuses (Budelmann,1996; Boal and Golden, 1999; Boyle, 1983;1986; Chase and Wells, 1986; Lee, 1992; Hanlon and Shashar, 2003; Walderon et al., 2011; Wood et al., 2008). Training experiments for testing chemical discrimination have been done in octopus to demonstrate its ability to distinguish between objects based on their chemical differences using their arm suckers and described this ability as taste by touch (Wells, 1963; Wells et al., 1965), while odor discrimination was tested to assess perceptions of water-born chemical stimuli at distance (Walderon et al., 2011). Furthermore, it has been highlighted that the octopus's olfactory organ is able to change shape, from relaxed to erect to perceive water-soluble compounds such as salts, sugars, amino acids, amines, peptides, proteins, and functionalized hydrocarbons, which allows the animal to orient itself to detect the spatial gradient of these chemical cues, helping in navigation and triggering spatial memories (Di Cosmo et al., 2018; Polese et al., 2016; 2015; Huffard, 2013). Octopuses also possess a self-recognition mechanism, which consists of the attachment reflex inhibition of their own suckers, due to chemical signals in the skin (Nesher et al., 2014).

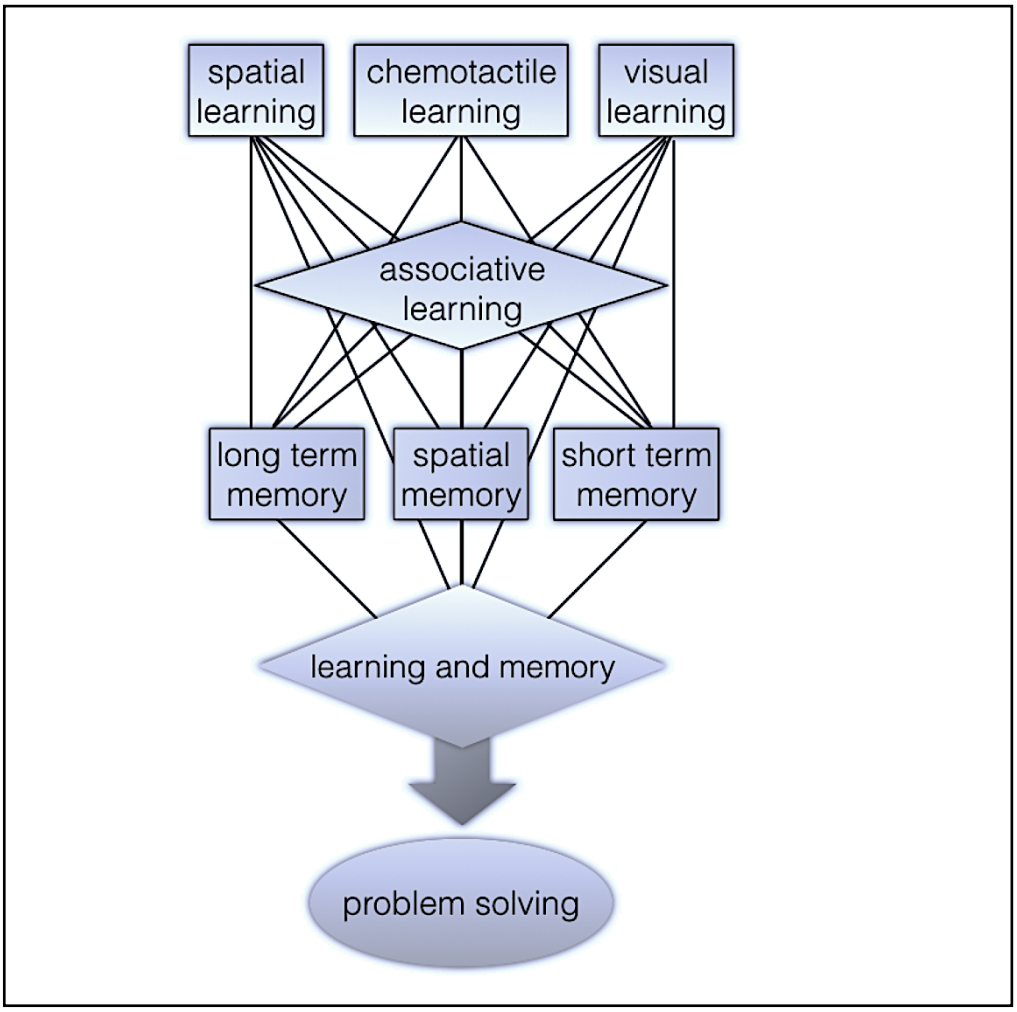

Figure 1. Octopus's problem-solving abilities (Anderson and Mather, 2007; Finn et al., 2009; Kuba et al., 2014; Maldonado, 1963; 1965; Moriyama and Gunji, 1997; Richter et al., 2016) **through learning and memory abilities** (Hanlon and Messenger, 2018; Godfrey-Smith, 2013; Hochner and Brown, 2003: Hochner et al., 2006; Mather and Dickel, 2017; Mather and Kuba, 2013; Sanders, 1975; Shomrat et al., 2015; 2008; Wells, 1978; Young, 1964) **[associative learning** (Amodio and Fiorito, 2013; Fiorito and Scotto, 1992; Tokuda et al., 2016), **spatial memory** (Boal et al, 2000; Gutnick et al., 2011; Mather, 1991), **visual learning** (Amodio and Fiorito, 2013; Boycott and Young, 1956; Fiorito and Chichery, 1995; Messenger and Sanders, 1972), **chemo-tactile learning** (Wells and Wells, 1956), **long-term and short-term memory** (Hochner and Brown, 2003; Sanders, 1975; Shomrat et al., 2015; Marini et al., 2017; Wells,1967)**].**

Recently, it has been hypothesized that olfaction in octopus is not restricted to the olfactory organ, but it is also extended to other structures such as the suckers, that were traditionally not considered olfactive. In particular, octopus exhibits a peculiar performance that can be defined "smell by touch", useful to detect odorant molecules that in water are insoluble or have a very low solubility (Di Cosmo et al., 2018; Di Cosmo and Polese, 2017; Mollo et al., 2014; 2017).

However, the octopus has always been described as a predominately "visual" animal with a complex visual system characterized by the presence of highly developed eyes

(Budelmann,1996; Grable et al., 2002; Hanlon and Messenger, 1996; Yoshida et al., 2015; Zylinski et al., 2009). Analogously to vertebrate, octopus eyes are equipped with an un-inverted retina, a cornea, an iris, and a lens. Even if they have just one type of receptor cell and only rhodopsin as pigment, octopuses have the ability to recognize the plane of polarized light based on rhabdomeres dichroism. Moreover, it has been proposed that they are able to discriminate colors (Stubbs and Stubbs, 2016a) within a wide range of light conditions (Yoshida et al., 2015; Grable et al., 2002; Hanlon and Messenger, 1996), even if this mechanism is largely discussed due to the turbid aquatic environment and it should be confirmed by behavioral experiments (Stubbs and Stubbs, 2016b; Gagnon et al., 2016).

Besides their eyes, octopus can detect light to trigger the animal's color changes using other visual senses. In fact, they can even perceive light through the skins (Ramirez and Oakley, 2015), and they can camouflage with the high-fidelity color to natural and artificial backgrounds (Akkaynak et al., 2013; Buresch et al., 2015; Chiao et al., 2011; Hanlon et al., 2013 Kühn, 1950; Mäthger et al., 2010). Experiments for testing visual discrimination have been established in octopus (Young, 1961). For example, they can quickly learn to visually discriminate between a series of objects (Boycott and Young, 1956; Messenger and Sanders, 1972), learn to use vision to direct an arm to a target (Gutnick et al., 2011), and recognize familiar conspecifics using vision (Mather, 1991; Boal, 2006; Mather,1991). Octopuses could be visually oriented as well, learning to use visual cues to choose and memorize a den, and take the correct route to return to it (Mather, 1991). When both chemical and visual information is available, octopuses combine information from all sensory inputs that they perceive and then the animals can camouflage themselves, escape a predator, or chase prey in the wild, or open jars for food in captivity (Richter et al., 2016; Anderson and Mather, 2010; Bertapelle et al., 2017). This integration of several sensory inputs may occur at central and/or peripheral levels (Di Cosmo et al., 2018; Di Cosmo and Polese, 2016), but the relative contribution of each sense remains poorly understood. Our study investigated the priority given to chemical vs. visual perception to establish the sensorial hierarchy in food choice by *O. vulgaris.*

2. Materials and Methods

2.1. Animals

Specimens of *O. vulgaris* (n = 4, bodyweight 600 ± 50 g, mean ± SD) were collected from the Bay of Naples (Italy) between June 2018 and October 2018. The animals were transferred to the Di Cosmo's cephalopod facility at the Department of Biology, University of Naples Federico II, Italy and kept individually in large fiberglass tanks (50 × 50 × 50 cm) filled with seawater (Di Cosmo et al., 2015; Polese et al., 2015;). Water temperature was kept at 18 ± 1 °C (mean ± SD), and illumination was maintained with natural photoperiod using LED tubes as a light source. All tanks were enriched by adding an amphora (as a den) and rocks (two rocks, about 6 cm^3). An acclimation period of 15 days was initiated before any experiments were performed. During this time, octopuses were fed ad libitum with crabs (*Carcinus sp.*), a different type of food than was used during trials to reduce the effects of repeated exposure on food choice.

The experiments in the present study were conducted in accordance with the principles and procedures that were approved by the Institutional Animal Care of the University of Napoli Federico II and the Ministry of Health (Project n° 608/2016-PR-17 June 2016; protocol n. DGSAF 0022292-P-3 October 2017), and according to the Italian and European law (European Directive 2010/63 EU L276; Italian DL. 4 March 2014, no. 26; the ethical principles of Reduction, Refinement and Replacement).

2.2. Experimental Design

To establish the priority given to chemical *vs.* visual cues in food choice, we defined a behavioral experimental design (Figure 2). Firstly, we tested the octopus food preference (**FP**), giving them three different food types (anchovy, *Engraulis encrasicolus*; clam, *Ruditapes philippinarum*; mussel, *Mytilus edulis*) for 7 days. All foods were placed within octopus's visual field at the same distance and simultaneously. In the **FP** test, we evaluated the first food eaten among three provided that should correspond to the favorite one. Then, to investigate an octopus's ability to identify the jar containing their favorite food, we subjected the octopuses to five problem-solving tasks (T1, T2, T3, T4, T5, Figure 2, Figure S1) as following :

- **T1** (positive control)–food provided in transparent screw-jars with pierced lid.
- **T2**–food provided in sealed (not pierced) and transparent screw-jars.
- **T3**–food provided in no-transparent (blind) screw-jars with pierced lid.
- **T4** (confusion task)–food provided in the blind screw-jars with pierced lid supplied outside with a realistic picture of the food that results different from what is inside.
- **T5** (negative control)–food provided in completely blind and sealed screw-jars.

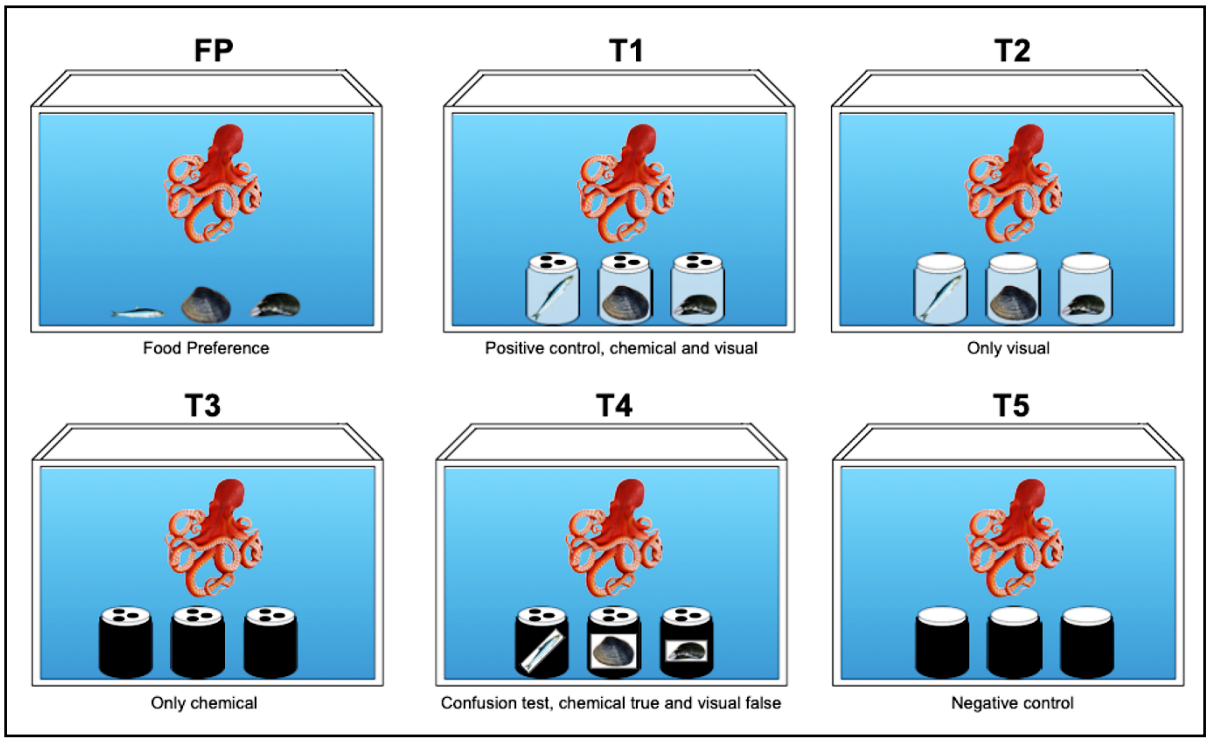

Figure 2. Experimental design to establish the sensorial hierarchy in food choice in *Octopus vulgaris* between chemical and visual cues. Food preference test (**FP**); food types provided in the transparent screw-jars with pierced lids (**T1**), positive control, both chemical and visual cues; food types in transparent screw-jars with no-pierced lids (**T2**), only visual cues; food types in blind screw-jars with pierced lids (**T3**), only chemical cues; food types in screw-jars with pierced lids with outside a photo of food (anchovies, clam, mussel) that is different from the food inside (**T4**), chemical true and visual false cues; food types inside blind screw-jars with no-pierced lids as control (**T5**), negative control, both chemical and visual cues are absent.

Each octopus was exposed to 5 trial days for each task. All experiments (FP, T1-T5) were conducted once per day and recorded for at least 1 hr. with a digital camera (GoPro Hero 5) positioned on the front of the aquarium (20 cm), to analyze octopus's choice and behavioral responses, such as exploring, selecting the jar, and eating. We performed FP followed by T1 as first, then animals were tested with the tasks from T2 to T5 randomly.

In the FP tests, we considered the food that was eaten, while in the tasks we considered four behaviors (1, jar touched; 2, jar opened; 3, food touched; 4, food eaten) and the time that animals spend to choose the jar to open, from the very first touch to the grab and wrap of the jar starting to open it (Δt).

2.3. Statistical Analysis

We examined videos using a high-resolution media player (QuickTime 7, Apple Inc., Cupertino, CA, USA) for behavioral analysis and we recorded data into an Excel data sheet (Microsoft Excel 15.32). Data are expressed in percentages and to analyze the data, we used GraphPad Prism 8 software, SPSS (IBM Corp., Armonk, NY, USA) and R cran (Team, 2018), performing the Friedman and Wilcoxon matched-pairs tests on ranks within and between experimental conditions.

3. Results

3.1. Food Preference in *O. vulgaris*

During acclimatization, animals readily recovered their normal behavioral repertoire and did not show any sign of distress. During the food preference test (FP), octopuses approached and explored the different food items presented (Table 1). Animals readily grabbed and fed based on their individual choice. All octopuses touched the three kinds of food provided, exhibiting no significant differences in the first touch for the proposed foods (**Table 1, Figure S2, Table S1**).

Table 1. First touch during the food preference test (%).

Food	Me	IQR (Q1; Q3	Friedman Test (Chi-Square; *p*-value)
Anchovies	33.33	25.00; 55.00	1.524; 0.467
Clams	33.33	29.16; 45.00	
Mussels	33.33	12.50; 40.00	

Median (Me), Interquartile Range (IQR): Q1–first quartile, Q3–third quartile, Friedman test.

Although, evaluating the first food eaten, octopuses showed a significative preference for anchovies as a first choice (high-preference), followed by clams (moderate-preference), and mussels (low-preference; **Tables 2 and 3, Figure S2, Table S2**).

Table 2. Food preference test (%).

Food	Me	IQR (Q1; Q3	Friedman Test (Chi-Square; p-value)
Anchovies	66.67	66.67; 90.00	11.120; **0.004**
Clams	20.00	0.00; 33.33	
Mussels	0.00	0.00; 0.00	

Median (Me), Interquartile Range (IQR): Q1–first quartile, Q3–third quartile, Friedman test, *p*-values < 0.05 are marked in bold.

Table 3. Food preference test. Wilcoxon matched-pairs test significance *p*-value, *p*-values < 0.05 are marked in bold.

Food	Anchovies	Clams
Clams	**0.027**	
Mussels	**0.018**	0.216

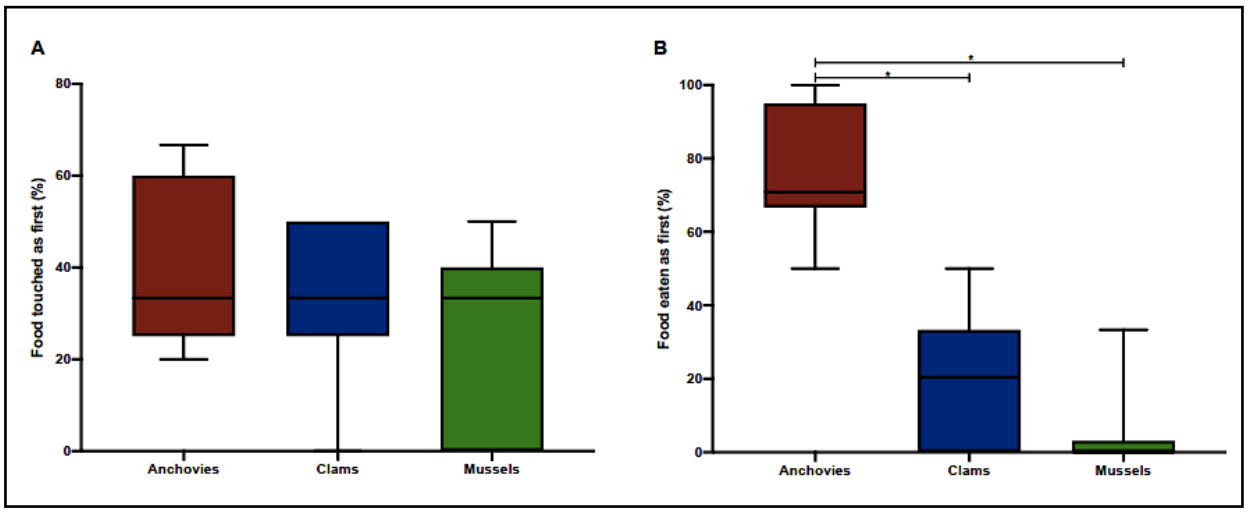

Figure S2. First touch and food preference in *O. vulgaris*. (**A**) Boxplot of the food that was touched first (Friedman test, $p>0.05$); (**B**) Boxplot of food that was eaten first (Friedman test, $p<0.05$). Wilcoxon matched pairs test significance is denoted with asterisks * for $p<0.05$.

3.2. Food Choice under Different Problem-Solving Tasks

In five of discrimination tasks (T1-T5), jars containing different foods were placed into the bottom of the tank, and all animals performed behaviors such as touching, exploring, and opening the jars.

The results revealed that octopuses did not show significant differences in jar-touching behaviors among any of the tests (Friedman test T1-T5, Chi-square = 38.460, p = 0.000). Conversely, they generally showed high significant variance in food recognition ability associated with the jar-opening task during all discrimination experiments (**Tables 4 and 5, Figure S3, Table S3**). In T1 (both chemical and visual discriminations are available), octopuses revealed the most significant ability to distinguish the food inside the jars and subsequently open the jar that contained the preferred food (anchovies), then the mussel, whereas the clam was ignored. In T2 (only visual discrimination), the octopuses had a significant decrease in recognition of the jar that contained the preferred food. In T3 (only chemical discrimination),

the octopuses showed a highly significant difference in selecting the jar containing the preferred food. In the confusion task T4 (real chemical and false visual cues), the octopuses had to overcome this confusion effect by following one of the two cues to find the right jar containing the preferred food. In T4, octopuses exhibited high significant ability in food recognition guided by chemoreception in the opening selected jar containing anchovies. In the control group (T5), without any possibility to distinguish the food inside the jars neither by vision nor by chemosensory cues, all octopuses showed no difference in choosing the jar.

Table 4. Food choice under different tasks in *O. vulgaris* (%)

Task	Food	Me	IQR (Q_1; Q_3)	Friedman Test (Chi-Square; p-Value)
T1 Combined Chemical and Visual Cues	Anchovies	100.00	75.00; 100.00	8.400; 0.015
	Clams	0.00	0.00; 0.00	
	Mussels	0.00	0.00; 25.00	
T2 Only Visual Cues	Anchovies	50.00	0.00; 100.00	3.500; 0.174
	Clams	0.00	0.00; 0.00	
	Mussels	0.00	0.00; 75.00	
T3 Only Chemical Cues	Anchovies	100.00	58.33; 100.00	7.625; 0.022
	Clams	0.00	0.00; 16.67	
	Mussels	0.00	0.00; 25.00	
T4 Real Chemical and False Visual Cues	Anchovies	100.00	75.00; 100.00	8.400; 0.015
	Clams	0.00	0.00; 0.00	
	Mussels	0.00	0.00; 25.00	
T5 Negative Control	Anchovies	0.00	0.00; 100.00	0.400; 0.819
	Clams	0.00	0.00; 50.00	
	Mussels	0.00	0.00; 100.00	

Median (Me), Interquartile Range (IQR): Q_1–first quartile, Q_3–third quartile, Friedman test, *p*-values < 0.05 are marked in bold.

Table 5. Food choice under different tasks in *O. vulgaris*. Wilcoxon matched-pairs test significance p-value, *p*-values < 0.05 are marked in bold.

T1 Combined Chemical and Visual Cues	**Anchovies**	**Clams**
Clams	**0.034**	0.317
Mussels	**0.046**	
T3 Only Chemical Cues	**Anchovies**	**Clams**
Clams	**0.039**	0.655
Mussels	**0.049**	
T4 Real Chemical and False Visual Cues	**Anchovies**	**Clams**
Clams	**0.034**	0.317
Mussels	**0.046**	

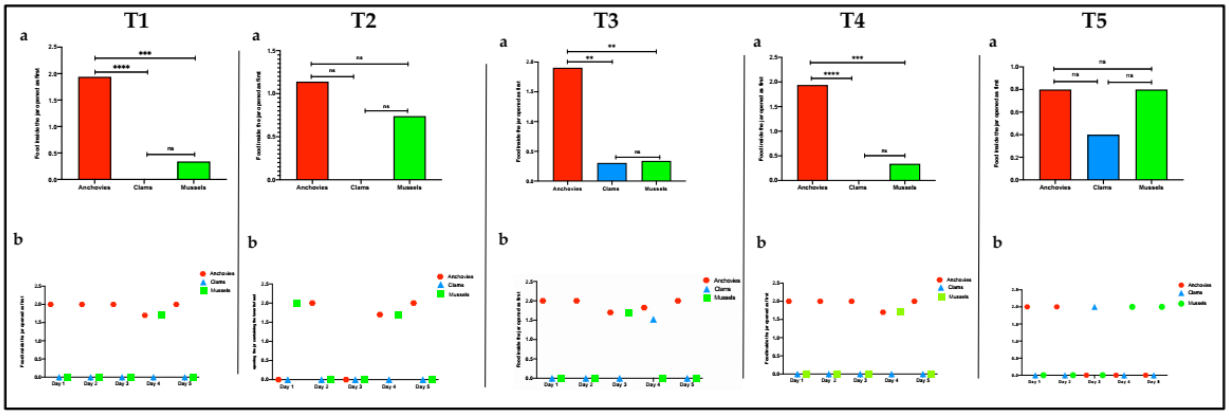

Figure S3. Food choice under different tasks in *O. vulgaris*. **T1** combined chemical and visual cues; **T2** only visual cues; **T3** only chemical cues; **T4** real chemical and false visual cues; **T5** negative control. (a) five day trend preferences; (b) single day preferences. Friedman test T1-T5, Chi-square = 38.460, p<0.05. Data were log transformed, Wilcoxon matched pairs test significance is denoted with asterisks * for p <0.05.

3.3. Food Preference Compared to Jar Choice under Different Problem-Solving Tasks.

We analyzed the octopus's food preference considering only food eaten, after jar opening (Tables 6 and 7, Table S4).

Octopuses exhibited no significant difference in the first eaten food comparing FP to each discrimination task, in fact, the first choice was always anchovies, the second clams, and then mussels. In T1, in which octopuses can use both chemical and visual cues, the first food eaten resulted in 100% anchovies, ignoring clams and mussels. Similarly, in T2, in which animals can use just visual cues, the first food eaten resulted in 100% anchovies as above. In T3, where animals perceived just chemical cues, the first food eaten resulted in anchovies, followed by clams, and ignoring mussels. Later, in T4, in which octopuses perceived the right chemical cues while the visual one was false, the first food eaten resulted in 100% anchovies, avoiding clams and mussels. In the control task, where octopuses were not allowed to perceive either visual or chemical cues, the choices among the three food were randomly directed.

Table 6. Maintained food preference during problem-solving tasks in *O. vulgaris* (%).

Task	Food	Me	IQR (Q1; Q3)	Friedman Test (Chi-Square; *p-Value*)
T1 Combined Chemical and Visual Cues	Anchovies	100.00	100.00; 100.00	10.000; **0.007**
	Clams	0.00	0.00; 0.00	
	Mussels	0.00	0.00; 0.00	
T2 Only Visual Cues	Anchovies	100.00	100.00; 100.00	8.000; **0.018**
	Clams	0.00	0.00; 0.00	
	Mussels	0.00	0.00; 0.00	
T3 Only Chemical Cues	Anchovies	100.00	83.33; 100.00	9.500; **0.009**
	Clams	0.00	0.00; 16.67	
	Mussels	0.00	0.00; 0.00	
T4 Real Chemical and False Visual Cues	Anchovies	100.00	100.00; 100.00	10.000; **0.007**
	Clams	0.00	0.00; 0.00	
	Mussels	0.00	0.00; 0.00	
T5 Negative Control	Anchovies	0.00	0.00; 50.00	0.000; 1.000
	Clams	0.00	0.00; 50.00	
	Mussels	0.00	0.00; 50.00	

Median (Me), Interquartile Range (IQR): Q1–first quartile, Q3–third quartile, Friedman test, p-values < 0.05 are marked in bold.

Table 7. Maintained food preference during problem-solving tasks in *O. vulgaris*. Wilcoxon matched-pairs test significance *p*-value.

T1 Combined Chemical and Visual Cues	**Anchovies**	**Clams**
Clams	0.025	
Mussels	0.025	1.000
T2 Only Visual Cues	**Anchovies**	**Clams**
Clams	0.046	
Mussels	0.046	1.000
T3 Only Chemical Cues	**Anchovies**	**Clams**
Clams	0.034	
Mussels	0.034	0.317
T4 Real Chemical and False Visual Cues	**Anchovies**	**Clams**
Clams	0.025	
Mussels	0.025	1.000

3.4. Time Spent to Choose Which Jar Opening under Different Problem-Solving Tasks.

To elucidate which sense was most important in food choice, we compared the time spent to recognize the preferred food inside the jar using visual and combined or separately (Figure 3, Tables 8 and 9, Table S5). We considered the time spent by an octopus from the very first touch and choice of the jar to open (Δt, Figure 3). All octopuses opened the jars to reach the food independently from the task proposed, and showed relatively differences in time needed to make the choice (Figure 3). Octopuses spent a few seconds to recognize the preferred food when chemical and visual cues were available for T1 (Δt (s) = 31.0, Me), while the time increased considerably when one of the two or both senses were limited; in fact, for T2 (visual cues only), Δt (s) = 394.50 s (Me); in T3 (chemical cues only), Δt (s) = 25.00 s (Me); in T4 (true chemical and false visual cues), Δt (s) = 22.50 s (Me); and finally, in T5 (negative control), Δt (s) = 1932.00 (Me).

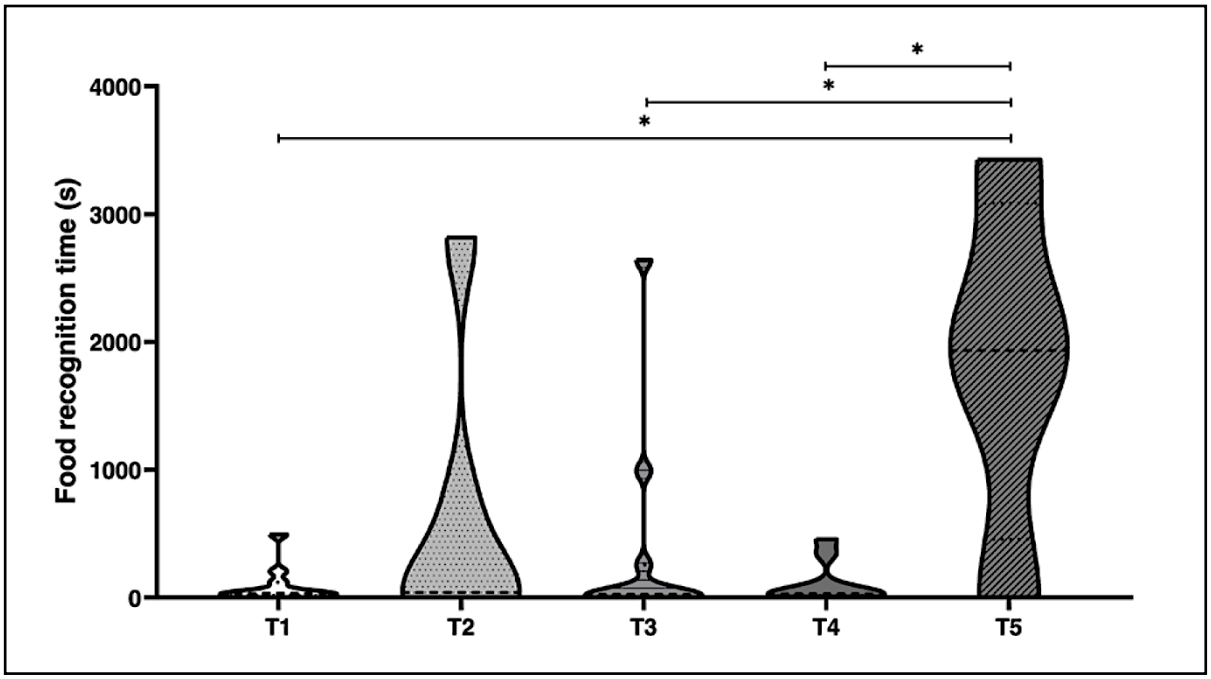

Figure 3. Violin plot of the time spent (Δt) by *O. vulgaris* in problem-solving. **T1** combined chemical and visual discriminations; **T2** only visual discrimination; **T3** only chemical discrimination; **T4** real chemical and false visual discriminations; **T5** negative control. Δt (s): time spent to choose the jar to open, from the very first touch to the grab and wrap of the jar starting to open it. Wilcoxon matched-pairs test vs T1: significance is denoted with asterisks * for $p < 0.05$.

Table 8. Time spent (Δt) by *O. vulgaris* in problem-solving (second).

Task	Me	IQR (Q1; Q3)	Friedman Test (Chi-Square; *p*-Value)
T1 Combined Chemical and Visual Cues	31.00	18.50; 47.25	9.055; 0.059
T2 Only Visual Cues	394.50	35.00; 1263.50	
T3 Only Chemical Cues	25.00	13.00; 30.00	
T4 Real Chemical and False Visual Cues	22.50	15.25; 147.50	
T5 Negative Control	1932.00	1356.00; 2401.00	

Median (Me), Interquartile Range (IQR): Q1–first quartile, Q3–third quartile, Friedman test.

Table 9. Time spent (Δt) by *O. vulgaris* in problem-solving. Wilcoxon matched-pairs test significance p-value, *p*-values < 0.05 are marked in bold.

	T1 Combined Chemical and Visual Cues	**T2** Only Visual Cues	**T3** Only Chemical Cues	**T4** Real Chemical and False Visual Cues
T2 Only Visual Cues	0.117			
T3 Only Chemical Cues	0.944	0.108		
T4 Real Chemical and False Visual Cues	0.346	0.442	0.780	
T5 Negative Control	**0.009**	0.407	**0.009**	**0.029**

4. Discussion

In the wild, octopuses are generalist and opportunistic predators that prey on a great variety of species (Ambrose, 1984; Mather,1991). There were strong differences in prey preference among individual octopuses and the prey choice could be varied according to several factors such as predation risks, interspecific competition, or local prey abundance (Anderson et al., 2008). In captivity, octopuses have shown preferences for selected preys (Scheel et al., 2007; Vincent et al.,1998; Mather and Anderson, 1993), making their food choice by using their sophisticated sense organs. Among these, much attention has been given to vision (Budelmann,1996; Grable et al., 2002; Zylinski et al., 2009). They also possess sensitive olfactory organs that they use to detect chemicals in the water (Polese et al., 2016; 2015; Gilly and Lucero,1992; Woodhams, P.L.; Messenger, 1974). However, they also possess suckers that have excellent tactile and chemical sensitivity to perceive chemicals by touch (Di Cosmo and Polese, 2017; Chase and Wells, 1986; Graziadei, 1964). Both chemical and visual information is elaborated and stored in specific brain lobes, located in the supra esophageal mass and optic

lobes (Young,1971;1961). Nevertheless, no previous study has addressed the question on which sense has the priority in the food search and choice, although most of the behavioral studies, performed prior to this one, were focused on just their visual capabilities (Gutnick et al., 2011; Messenger and Sanders, 1972; Alves et al., 2007a,b; Byrne et al., 2002; Kuba et al., 2006; Mather, 1985; Boal, 1996).

Chemical perception is undoubtedly the first sense that evolved, resulting in widespread sensory modality in all animals. The biological system generated an enormous number of receptors genes to detect and recognize chemicals, but chemoperception resulted largely underappreciated by scientists, even in light of sensory drive evolution theory (Yohe and Brand, 2018; Endler, 1992). Here, we establish the priority given to chemical versus visual perception in octopus's food choice just using the ethological approach. Our experiments are performed on a small animal group made by four samples that allow us to investigate the octopus's behavior, in according with the 3Rs rules (reduction, refinement, replacement) as allowed by the Italian law (European Directive 2010/63 EU L276; the Italian DL. 3 April 2014, no. 26). On the basis of the octopus's exploratory behaviors observed during food preference test (FP), animals tested show a clear preference for the anchovies. However, the first food touch was not always consistent with the food preference (Figure 3), exhibiting a peculiar exploratory behavior when they approach a new environment (Mazzolai et al., 2018; Hanlon and Messenger, 2018;1996; Anderson and Mather, 2010; Mather, 2006; Kuba et al., 2003; Mather and Anderson, 1993; Allen et al., 1986; Messenger,1983). Although the fact that octopuses did not touch the preferred food immediately is a clear sign that they cannot rely just on visual perception when they approach a prey, evidently, they need to acquire more information about what they see, using other senses like chemical and tactile, to understand the nature of what they are going to eat. To this end, octopuses are equipped with arms containing a widespread chemotactic sensory system concentrated in the hundreds of suckers (Graziadei and Gagne,1976; 1973; Wells,1967; Wells, 1963; Graziadei,1962; Wells and Wells, 1953). Thus, food choice in octopuses is driven by multiple sensory cues; nevertheless, a hierarchy in sensory perceptions could be hypothesized.

In our experiments, it was clear that they are mainly attracted by the physical presence of it, without recognizing the preferred food by vision at distance. Subsequently, after a random first touch (Figure 3), octopuses start an evaluation of the food using tactile and

chemical senses. This allowed us to recognize a temporal hierarchy, where the octopus uses first visual, tactile, and chemical senses, in this order. Our observations are in agreement with previous studies in which it has been reported that octopuses are visually oriented towards a new given object, and then explored it with their arms (Mather and Anderson, **1993;** Kuba et al., 2006a,b). In this case, we are not able to define which sensory cues are dominant by which they arrive at a decision on food choice, because this behavior represents the result of the integration of different sensory information coming from visual, tactile, and/or chemical systems sequentially to perform a suitable behavior.

To understand whether there is a sensorial hierarchy between visual and chemical, to establish which is dominant in decision-making, we tested octopuses with five discrimination tasks. Problem-solving and flexible tool-use are considered hallmarks of cognitive abilities and intelligence (Byrne and Bates, 2007; Gibson, 1986). In the wild, octopuses exhibit behavioral flexibilities in solving many kinds of problem. For instance, the giant octopus, while attached to a rock can use one of the arm tips to attract a seagull, then when the seagull gets close the sea surface and within the range of the animal's arm, it grabs and draws the bird into the water (https://youtu.be/LNwegprmtx8). In captivity, octopus also exhibits cognitive abilities in solving problems, when challenged with artificial tasks. Octopuses could retrieve L-shaped food containers from crevices, with or without visual access and independently from the spatial orientation of containers (Richter et al., 2016) or learn how to unscrew a jar to reach the food (Bertapelle et al., 2017). The data here discussed clearly show the ability of octopuses to open jars during all five discrimination tasks successfully.

Our findings indicate that octopuses recognize the jar containing the anchovy, that resulted to be the preferred food in FP test (Tables 2 and 3), in all discrimination tasks ($T1^{-}$ T4), with the exception of the negative control (T5) (Tables 4 and 5). However, the task in which it is evident that the dominant sense is the chemical one, it is the confusion task (T4), where, despite the fact that octopuses were cheated with a false picture of the food inside, they picked up anchovy in the 100% of the cases. This evidence is corroborated when we excluded the chemical cues, focusing exclusively on the visual sense (T2), in which the jar containing anchovy was selected in only 50% of the cases. The negative control experiment (T5) reinforces our claim, in fact, that the chemical and visual information was not used by octopuses in solving this task, so the choice was randomly made. These findings are consistent with Mather

and collegues (Mather and Anderson, 2018) recently reporting that octopuses did not open the jar to get a small crayfish inside, because chemical cues from herring were smeared on its surface. Our experimental design (Figure 2) allowed us to demonstrate that in *O. vulgaris* both chemical and visual perceptions are essential in food choice; nevertheless, the chemical signals are the most important inputs.

On the other hand, when we compared food eaten to jar choice under all different problem-solving tasks, we discovered that the preference to anchovies was maintained, even when the first jar chosen was not containing the anchovy (Tables 4 and 5). In fact, in T2, where octopuses could just see the food inside, in 33.3% of cases, they opened first the jar containing a mussel, but because octopuses do not eat it, they resultantly were forced to look for another "chance to win" the preferred food. Merging the data coming from all tasks (Tables 4 and 5), we observed that the percentage of successful decisions to open as first the jar contained the preferred food, based on chemical cues, were significantly higher than visual one (88.9% vs. 11.1% respectively, Wilcoxon matched-pairs test $p < 0.05$). Furthermore, when we considered Δt, octopuses spent more time to visually discriminate the preferred food than either by combined visual and chemical discrimination or by chemical discrimination only (Figure 4). Despite the fact that the differences encountered in Δt are affected by inter-individual variability, that is well known in this animal, and the limited number of specimens used, these results indicate that octopuses are able to decrease Δt to correctly solve operant tasks based on chemical information (Figure 4), this might be of importance in predation strategy in the wild where the prey is not closed in a jar, but hidden and ready to escape. However, while we posit that octopus, privileges its chemical stimuli over visual ones, we should appreciate that the two combined increase the probability of success in prey (Di Cosmo et al., 2018; Bertapelle et al., 2017; Di Cosmo and Polese, 2016; 2014).

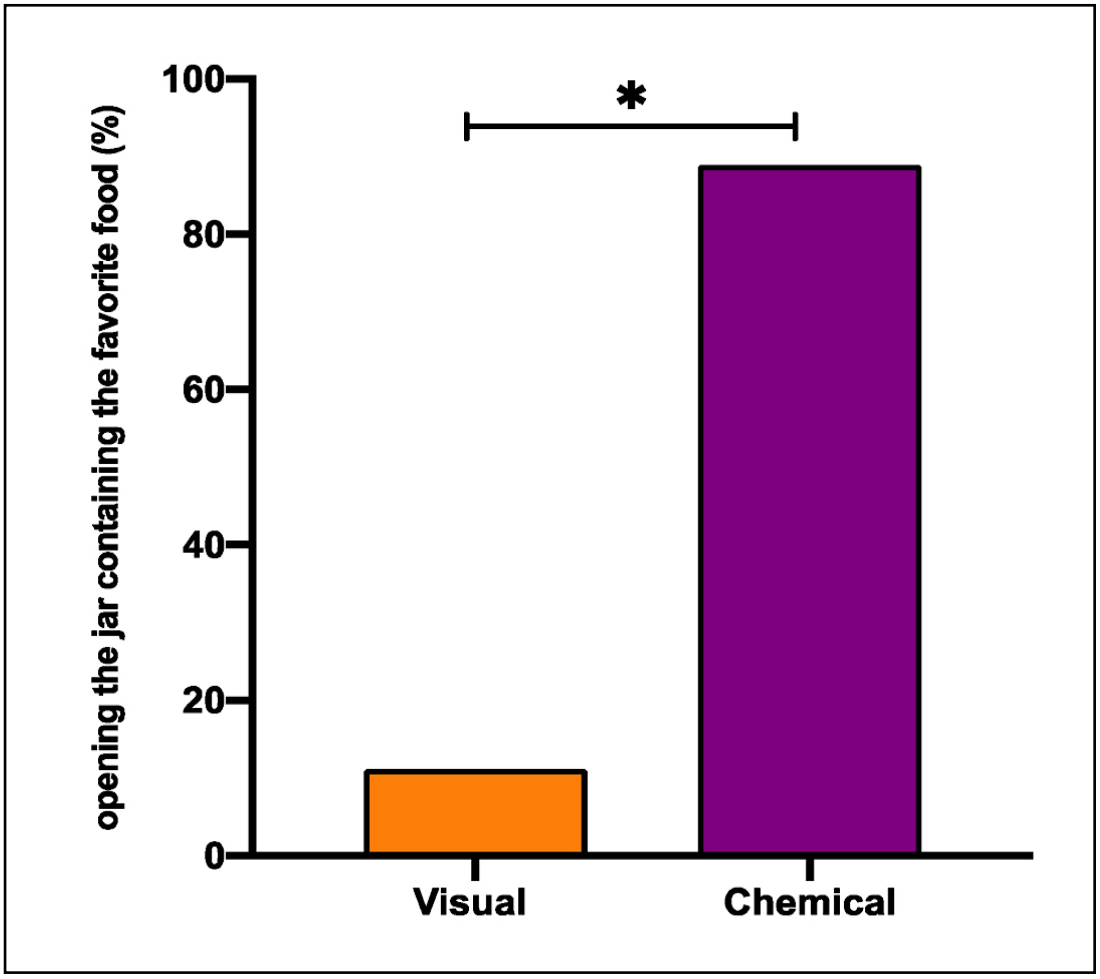

Figure 4. Visual *vs.* chemical perception in *O. vulgaris*. Percentage of the successful decisions to open as first the jar that contained the preferred food, based on chemical (purple) or visual (orange) cues. Wilcoxon matched-pairs test vs. T significance is denoted with asterisks * for $p < 0.05$.

Our results demonstrate involvement of chemosensory sense in octopus food choice behavior, and allows a reassessment of the importance of chemical perception in the ecology of octopus. In fact, octopus is able to detect chemical cues with different spatial ranges through either contact or distant chemoreception. Its capability is based on the presence of the olfactory organ, structure mainly dedicated for hydro-soluble molecules, and the numerous chemoreceptors bared on its sucker rims that are essential to perceive and explore bi-dimensional traces consisting of insoluble molecules released on the seafloor in turn by preys, predators, or conspecifics (Di Cosmo et al., 2018; Huffard, 2013). These considerations will open new perspectives to study the behavior of such an intriguing animal that is the octopus.

Chapter (3)
Smell by Touch in *Octopus vulgaris*: Molecular and Behavioral Evidence

1. Introduction

Olfaction (the sense of smell) is traditionally considered as a distance sense, by which all animals can detect chemical stimuli coming from a distance source, whereas the sense of taste is defined as a contact sense (like touch) that must physically contact with the chemical source for detection. Consequently, in the marine environment, aquatic olfaction is generally thought to be mediated almost extensively by odorants molecules dissolved in water and perceived from a distance (Ache and Young, 2005; Eisthen and Polese, 2006; Smith, 2008; Shi and Zhang, 2009; Brönmark and Hansson, 2012; Krång et al., 2012; Tuchina et al., 2014). However, many insoluble molecules smaller than~300Da known as odorant compounds on the land, which can be transported through air and are generally recognized at distance by the olfactory system (Mollo et al., 2017; Mori et al., 2006; Touhara and Vosshall, 2009). Conversely, these molecules have to be sensed by direct touch in aquatic systems and act as olfactory signals (Mollo et al., 2014; Giordano et al., 2017). For instance, the marine natural product chemists have isolated such insoluble compounds from benthic invertebrates that use them as defensive toxic weapons and as olfactory signals (Mollo et al., 2014; 2017; Giordano et al., 2017).

It has been widely reported that aquatic animals, including crustaceans and fish, can use their gustatory systems to recognize water-soluble compounds without physical contact with its source other than the compounds themselves. These "gustatory" systems can respond to a very low concentration of those chemical cues and evoke behaviors (Caprio and Derby, 2008; Schmidt and Mellon, 2011). However, recent research showed empirical evidence challenges the notion of the aquatic olfaction, indicating that both crustacean and fish are also able to detect hydrophobic compounds (almost insoluble in water) through tactile forms of olfaction (Giordano et al., 2017); in particular, little shrimp (*Palemon elegans*) and also fish (*Danio rerio*) use their chemosensory mouthparts to perceive typical odiferous compounds usually smelled by humans. In the same way, these molecules could be smelled by any other aquatic animals when they are touched. In these cases, both taste and olfaction and their receptors' range are evidently conserved in the chemosensory organs and synchronous them

based on the kind of molecules that can perceive. Thus, the discrimination between olfaction and taste based on the signal spatial range would certainly be confusing without considering specific molecular interactions of the ligands with chemosensory receptors.

Therefore, the accurate studies to define the type of chemoreceptors and their ligands based on molecular and genetic data should be certainly desirable and persuasive for a better understanding the all forms of tactile-chemoreception, as well as shed more light on the synchronous of different signals using different channels by the chemosensory organs and integrates them into the central nervous system. In particular, taste and olfaction are a part of a multimodal system of information transfer. The synchronous use and integration of different signals using different channels have the advantage of improving recognition, discrimination, and memory of inputs by the environment. For example, in *O. vulgaris*, the reproductive behavior is highly regulated and controlled by a complex set of signal molecules such as sex steroids, neuropeptides, and neurotransmitters that guide the behavior from the level of individuals in evaluating mates to stimulating or deterring copulation (Di Cosmo and Polese 2014, 2017) and to sperm-egg chemical signaling that promotes fertilization (De Lisa et al., 2013).

In cephalopods, octopuses, are considered advanced marine invertebrates that frequently utilize both distance and contact chemoreception to explore the environment (Budelmann et al., 1997; Maselli et al., 2020). Unquestionably, they possess a capacity for detecting widespread water-borne molecules arising from a distance source by their olfactory organs (Chase and Wells, 1986; Lee, 1992; Boal and Golden, 1999; Alves et al., 2007; Walderon et al., 2011). Moreover, early behavioral studies showed that the gustatory system of *O. vulgaris*, that is located on the arm suckers, can distinguish objects based only on their chemical characteristics by direct tactile contact, evoking a behavior defined as "taste by touch" (Wells, 1963). Recently, the behavioral and electrophysiological study on *O. bimaculoides* has also confirmed that chemosensory receptor cells in the arms and suckers are able to detect environmentally relevant chemicals and utilizing local neural signaling to integrate sensory cues and carry out arm-autonomous behaviors (Fouke and Rhodes, 2020). It seems that the *O. vulgaris* gustatory system consists of receptors distributed on the suckers, where the aquatic equivalent to taste takes place (Wells, 1963; Graziadei and Gagne, 1973; Lee, 1992; Anraku et al., 2005; Grasso and Basil, 2009). The octopus arm suckers are generally

considered as specialized chemo-tactile organs with high sensitivity, being equipped with millions of distributed sensory receptors allowing the octopus to process in parallel the massive amount of mechanical and chemical information available from its densely innervated suckers (Yekutieli et al, 2005 a,b; Graziadei, 1965, 1971; Young, 1971). Interestingly, in *O. vulgaris* the chemoreceptors have been described on the sucker of the arms (Graziadei, 1962) showed to contain several types of specialized sensory receptor cells localized in the epithelium covering the infundibulum and rim of sucker. In particular, the morphology of these cells suggests the presence of chemoreceptors, as well as tactile receptors (Rossi and Graziadei, 1958; Graziadei, 1964, 1965, 1971; Graziadei and Gagne 1976). More recently, Bellono and his colleagues (Giesen et al., 2020) discovered the molecular basis of chemo-tactile sensation in *O. bimaculoides*. These findings confirmed that the arm suckers have a kind of taste-touch ability mediated by unique chemo-tactile receptors (CR). The results reflect the importance of the chemo-tactile sense in the octopus arms sucker that plays a critical role in many aspects of the chemosensory of ecological events. In view of the great chemical sensitivity of the octopus suckers, it has been recently proposed that the chemo-tactile discriminations in octopuses are also involved in olfaction by their suckers that were traditionally not considered olfactive, allowing them to recognize odorant molecules that are insoluble or have a very low solubility, exhibiting a peculiar behavior described as "smell by touch" (Di Cosmo et al., 2018; Di Cosmo and Polese, 2017). This hypothesis reflects the importance of the sense of touch and raised the fundamental questions about their nature in this species. Therefore, the presence of the olfactory genes on the octopus' "gustatory systems", coding for olfactory receptors (ORs) will provide us molecular evidence supporting the hypothesis that in aquatic environments octopuses are able to recognize by their suckers the insoluble compounds which typically act as olfactory cues on land .

Generally, all animals recognize the vast array of odorants they encounter using G protein-coupled receptors (GPCRs), seven-transmembrane domain proteins that activate G protein-based signaling cascades when triggered by their ligands. Trace amine-associated receptors (TAARs) are a class of chemoreceptors belonging to GPCR superfamily (Bunzow et al. 2001; Borowsky et al. 2001; Lindemann et al. 2005). They are highly expressed in olfactory sensory neurons (OSNs), located in the olfactory epithelium, functioning as a distinct family of olfactory receptors (Liberles and Buck, 2006; Johnson et al., 2012; Pacifico et al., 2012). The

number of functional TAARs varies among species with 6 in human, 15 in mouse, and 17 in the rat (Hashiguchi and Nishida, 2007), unexpectedly they are largely expanded in teleosts including 112 in zebrafish (Hussain et al., 2009), suggesting an important role of TAARs in aquatic olfaction. Despite the TAAR genes are also found to be expressed in the *O. bimaculoides* genome (Albertin et al., 2015), but little progress has been made to reveal their potentiality in the octopus diffuse olfaction. Therefore, we assume that TAARs may have involved in diffuse olfaction by octopus suckers. Here for the first time we identified and localized three TAARs in *O. vulgaris* and *O. bimaculoides* suckers, where they may serve a diffused olfactory chemical receptors. We present evidence of TAARs expression, not only in the olfactory mucosa but also in the suckers of *O. vulgaris*. In addition, we analyzed the evolution of the three TAARs in octopus, correlating to those of invertebrates and vertebrates and predicting the key ligand binding site residues, that are complementary to that of ORs.

On the other hand, olfaction is commonly involved in odor discrimination tasks, where the odor is the marker for important biological events, informs the central nervous system of animals about the chemical composition of the external environment, allowing them to anticipate and rapidly adapt their behavior when they are searching for food or when engaging in social or sexual behaviors (Julliard et al 2017). Although the suckers on the arms of the octopus are demonstrated to be the sensory structures responsible for the observed behaviors (Chase and Wells,1986). Octopuses can also be trained to discriminate between two touched objects which differ only in their chemical characteristics (Wells 1963; Wells et al. 1965; Giesen et al., 2020). However, there is little evidence to show that octopus possesses a capacity for tactile forms of olfaction. In this study, we also conducted a behavioral experiment, in which octopuses exhibited the ability to detect insoluble odor molecules using their sucker. In our study, combining molecular and behavioral analyses, we demonstrated that *O. vulgaris* show olfactory learning and memory through "smell by touch" behavior, suggesting the sense of the smell is not restricted to the olfactory organ, but it is diffuse due to the presence of olfactory receptors on the arm suckers.

2. Material and Methods

2.1 Animals

Adult specimens of *O. vulgaris* (n = 4, body weight 600g±50g, mean ± SD) were collected from the Bay of Naples (Italy) between February 2019 and July 2019, and transferred to the Di Cosmo's cephalopod facility at the Department of Biology, the University of Naples Federico II, Italy. Animals were housed individually in fiberglass tanks (50x 50x 50 cm) with natural circulating seawater (temperature: 18 ± 1 °C, mean ± SD) (Di Cosmo et al., 2015; Maselli et al., 2020; Polese et al., 2014) (Di Cosmo et al., 2015; Polese et al., 2014). The animal house maintained with natural photoperiod cycles (12L:12D cycle). Illumination was provided by led tubes as artificial light sources. All tanks were environmental enriched by adding an amphora (as a den) and rocks (two rocks, about 6 cm3). Before starting the behavioral experiments, in order to reduce the potential stress related to the change of environment, octopuses were acclimatized for 15 days (Maselli et al., 2020). During this time, octopuses were fed ad libitum with crabs (*Carcinus sp.*), a different type of food than was used during trials to reduce the effects of repeated exposure on food choice. Octopus behavior and health status were monitored daily for signs of stress in accordance with the guidelines of animal welfare (Di Cosmo et al., 2015; Fiorito et al., 2014).

Our research is approved to 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). All handling, housing and experimental procedures were carried out in accordance with relevant guidelines and regulations to the protection and use of animals in research. Novel animals were used for molecular experiments, octopuses (N=2) were anaesthetized by isoflurane insufflation and dissected in sterile conditions (Polese et al., 2014).

For the evaluation of the expression of octopus-TAARs we dissected suckers from distal (DIS), middle (MID), and proximal (PROX) part of the octopus' arms L1 and R1. The olfactory mucosa (OM) was used as positive control, meanwhile optic lob (OL) and hepatopancreas (HP) as negative controls. Samples were immediately preserved in RNA-later, cut and upon freezing ice. In the laboratory, they were immediately processed for RNA extraction. Total RNA was extracted from tissues using the RNeasy minikit (Qiagen USA) and stored at −80 °C for further experiments.

For whole-mount *in situ* hybridization preparations, middle octopus suckers (MID) from L1 (N=2) and R1 (N=2) arms were collected and fixed with 4% PFA (4% Para-formaldehyde in PBS, pH 7.4) overnight at 4°C. Fixed specimens were dehydrated in a graded methanol series (25% MeOH; 50% MeOH; 75% MeOH and 100% MeOH) with 1X PBST (phosphate buffered saline with 0.1% Tween-20) for 15 min each and stored in 100% methanol at - 20°C until use.

2.2 Expression of TAAR genes in the arm suckers of octopuses

The total RNA was extracted from octopus distal (DIST), middle (MID), and proximal (PROX) part of the octopus' arm, the olfactory mucosa (OM), olfactory lobe using TRIzol® reagent (Gibco BRL, Grand Island, NY, USA) following manufacturer's instruction. For cDNA synthesis, first strand cDNA was obtained using the QuantiTect® Reverse Transcription Kit (Qiagen, USA) primed with random hexamers according to the manufacturer's instructions. We designed three primers pairs (Table 1) based on TAAR genes sequences of *O. vulgaris* (OV-TAAR1, OV-TAAR2 and OV-TAAR7) (Zarrella et al., 2019) and *O. bimaculoides* (OB-TAAR1, OB-TAAR2 and OB-TAAR7) (Albertin et al., 2015) obtained from National Center for Biotechnology Information (NCBI) database.

In order to validate the expression of the TAAR in the octopus's arm suckers, reverse transcription PCR reactions (RT-PCR) were performed. Additionally, the expression of the house keeping β-actin was determined to check the cDNA integrity and used as control for its constitutive and stable expression in most cells and tissues. Gene fragments were amplified by the touch down PCR in a final volume of 20 µL, with 0.2 µL of Pfu DNA polymerase (Thermo Scientific, Waltham, MA, USA), 4 µL of 4X Tris buffer with $MgCl_2$, 1.6 µL of dNTPs (each dNTP 2.5 µM), 0.2 µL of 50 µM of each primer and 100 ng of cDNA template under the following conditions: 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 amplification products from *O. vulgaris* and *O. bimaculoides* were gel excised and cleaned up using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), and sequenced to confirm identity using alignment program of the NCBI BLAST (basic local alignment search tool). Chromatograms were assembled and analyzed using software Geneious version 9.1. PCR products were analyzed with GenBank BLASTn and BLASTx (BLAST, basic local alignment search tool). The analysis of the sequencing confirmed the identity of the fragments. All sequence data generated in this study were deposited in GenBank (Table1).

Table (1): Primers used in this study.

Primer name	bp
	RT- PCR primer sequences for *O. vulgaris*
Ov-TAAR1	287
Ov-TAAR2	306
Ov-TAAR7	269
Ubiquitin	113
	Primer sequences for in-situ hybridization antisense probe
Ov-TAAR1 F	
Ov-TAAR1 R+ T7	875
Ov-TAAR2 F	
Ov-TAAR2 R+ T7	813
Ov-TAAR7 F	
Ov-TAAR7 R+ T7	763
	RT- PCR primer sequences for *O. bimaculoides*
OB- TAAR1	632
OB-TAAR2	552
OBTAAR7	508
β-Actin	121
	Primer sequences for in-situ hybridization antisense probes
OB-TAAR1 F	
OB-TAAR1 R+ T7	1004
OB-TAAR2 F	
OB-TAAR2 R+ T7	1030
OB-TAAR7 F	
OB-TAAR7 R+ T7	1004

Additionally, we performed a real-time PCR using the QuantiTect SYBR Green PCR Kit (Qiagen, USA). PCR was performed in a final volume of 25µL, with 50 ng of cDNA, 1 mM of each primer, and 12.5µL of QuantiFast SYBR Green PCR Master Mix (2×). The PCR cycling profile consisted of a cycle at 95 °C for 5 min, 40 three-step cycles at 95 °C for 15 s, at 60 °C for 20 s, and at 72 °C for 20 s. Quantitative RT-PCR analysis was conducted by using the 2-(ΔΔCt) method (Livak and Schmittgen, 2001). RT-PCR was performed in a Rotor-Gene Q cycler (Qiagen, USA). The ubiquitin gene was used for normalization of the relative expression (Table 1). At the end of each test, a melting curve analysis was done (plate read every 0.5 °C from 55 to 95

°C) to determine the formation of the specific products. Each sample was tested and run in duplicate. We compared and analyzed results using a Wilcoxon two group test and data with *p*-values < 0.05 were considered statistically significant.

2.3 Probe synthesis and whole-mount *in situ* hybridization

For *in situ* hybridizations, digoxigenin (DIG)-labeled single-stranded RNA probes were synthesized using the PCR standard method (Hua et al., 2018) using DIG RNA Labelling Kit (Roche:11175025910) with Nitro Blue Tetrazolium chloride/5-Bromo-4-Chloro-3-Indolyl Phosphate (NBT/BCIP) as the alkaline phosphatase substrate for detection of single mRNA species. To construct the TAAR Digoxigenin (DIG)-labeled single-stranded RNA probes, we performed PCR standard method using specific primer set (Table 1).The PCR fragments were used as templates for in vitro transcription reaction using the T7 RNA polymerase promoter sequence corresponding to forward and reverse primers for the sense and antisense probes, respectively. PCR cDNA fragments were isolated by 1.2% agarose gel and used as templates for in vitro transcription reaction. RNA transcription reaction was performed using the DIG-RNA Labelling Kit (SP6/T7) (Roche Applied Sciences, Laval, QC, Canada) following the manufacturer's instructions. Final probes were cleaned up using RNeasy MinElute Cleanup Kit (Qiagen USA), and one microliter was visualized on a 1.5% agarose gel to estimate concentration.

For whole-mount *in situ* hybridization, fixed suckers were rehydrated by descending methanol series in 75%, 50%, and 25% MeOH in PBST for 15 min each at RT. Completed rehydration was performed twice in 100% PBST for 10 min each with gentle rocking. Tissues were incubated and digested in the detergent mix (20 µg/ml in PBST Proteinase-K) at 37°C for 20 min, post-fixed in 4% PFA for 20 minutes at room temperature, then washed three times in PBST for 5 min each. Tissues were pre-hybridized for 2hr and hybridized overnight at 62°C in hybridization solution (50% formamide, 5X saline-sodium citrate (SSC), 1X Denhardt's solution, 500mg/ml yeast tRNA and 500 mg/ml salmon sperm DNA) . After hybridization, the tissues were incubated with 20µg/ml RNAs A (Invitrogen 12091021) for 15 min at 37°C, then subjected to a series of post-hybridization washes in decreasing concentrations of SSC with 0.1% Tween 20. Tissues were blocked in 1X blocking solution (Roche Applied Science 11096176001) in PBST for 1 hr. at room temperature under gentle rocking, followed by incubation in 1:2500 Anti-

Digoxigenin-AP antibody (Roche 11093274910) in blocking solution overnight at 4°C on a rocker. Tissues washed in PBST five times for 25 min each, equilibrated in alkaline phosphatase buffer (AP) (100mM NaCl, 50mM $MgCl_2$, 100mM Tris, pH 9.5, 0.1% Tween-20) at room temperature. The color reaction was performed in NBT/BCIP stain solution (Roche 11681451001) in AP buffer under the light-resistant environment until the colors reached satisfactory intensity. Control specimens were left in staining solution for the same time interval as those incubated with anti-sense probes. In order to test for nonspecific labeling, negative control experiments were performed for each condition using hybridization buffer only without probe.

After coloration reaction, all tissues were passed through ascending concentrations of ethanol in PBS to remove background and darken the specific signal, re-hydrated in PBS. Whole-mount sucker tissues with DIG-labeled probes were observed colourimetrically under a Carl Zeiss Stemi 305 stereomicroscope with Axiocam ERc 5s .

2.4 Sequence analysis and Molecular phylogenetic reconstruction of the octopus TAAR genes

The phylogenetic relationships were created by aligning the amino acid sequences of the TAAR from *O. vulgaris*, and *O. bimaculoides* with corresponding sets of the known TAAR amino acid sequences from 15 other animals, including vertebrate and invertebrates (Hashiguchi and Nishida, 2007; Lindemann et al., 2005). For more sensitive research we used the identified TAAR genes from some aquatic animals and mollusks. In addition to TAAR sequences, we used human rhodopsin receptor and several biogenic amine receptors from zebrafish, human and chicken were used as out-groups following a previous study (Tessarolo et al., 2014). Six olfactory receptors (OR) sequences are also included in our analysis as additional outgroups. The phylogenetic trees were constructed using the Neighbour Joining (NJ) and Maximum likelihood (ML) methods with confidence in the resulting tree branch topology measured by bootstrapping through 500 iterations. Unrooted trees were constructed by a neighbor-joining method with Poisson correction of distances, as implemented in MEGA7 software. All alignments and phylogenetic analyses were carried out using MEGA7 (Kumar et al., 2016). Amino acid alignments were carried out using the MUSCLE algorithm (Edgar, 2004).

To confirm the functionality of given Ov-TAAR and Ob-TAAR genes, we constructed a WebLogo (Crooks et al., 2004), highlighting conserved amino acid residues from the alignment of the 6 full-length Ob-TAAR genes. Additionally, because the TAARs are members of the GPCR class of proteins to predict their secondary structures (2D), including the transmembrane (TM) protein topology, the N-terminal, intercellular loop (IC), extracellular loop (EC) and C-terminal regions, TMHMM 2.0 (Krogh et al., 2001) and Protter (Omasits et al., 2014) were used.

2.5 Behavioral experiment (Olfactory stimulation)

In this study, we developed behavioral experiment to study how octopus response to insoluble odors molecules which are working as olfactory cues in aquatic environment. The behavioral experiment design was performed using an odor-associative learning task, targeted the sensory epithelium area in octopus's arm suckers. The octopuses were trained to associate olfactory stimulus to food reward.

2.5.1 Preparation of olfactory odor stimulus

In our behavioral experiments, two odor stimuli were used for olfactory discrimination task. Behavioral tests was divided into two experiment: The first experiment with the natural product (extract homogenized fish, anchovy, *Engraulis encrasicolus*) infused in agarose gel as agarose ball shape. 200mL of 1.5% agarose infused with the indicated compound was then added to the empty plastic ball, resulting in a uniform depth of agarose ball (+F). The odorless ball used as control one, in which agarose was independently prepared and only contained seawater (-F).

The second experiment with artificial odor molecules (Limonene) infused in agarose gel (+L ball with odorous stimulus) and the odorless ball used as control one (-L agarose ball without odorous stimulus. The Limonene ((R)-(+)-Limonene; 97.8% purity; 136.24 M.W.; C14634100; Dr. Enrenstorfer GmbH- Bgm; Augsburg, Germany), shows a characteristic odors when exposed to air. The limonene concentration value was assigned according to previous studies (Budelmann et al., 1997; Chase and Wells, 1986; Lee, 1992). The odor stimulus used during our olfactory stimulation experiment has a very low solubility. Notably, there were no differences in the surface texture for the two balls or any other differences shape. The odor solution was emulsified in filtered seawater and fresh odor solution was prepared every day for each trial with the same concentration (2*109ppm).

2.5.2. Behavioral test

In these experiments, we placed the agarose balls (+F and -F; for the first experiment) or (+L and -L; for the second experiment) into two different plastic screw-jars (20 × 15 cm, height 10 cm) with a small hole for reaching, allowing animals only one opportunity to perceive the odor stimuli through insert their arm in the hole, touching and inspecting the ball inside the jar.

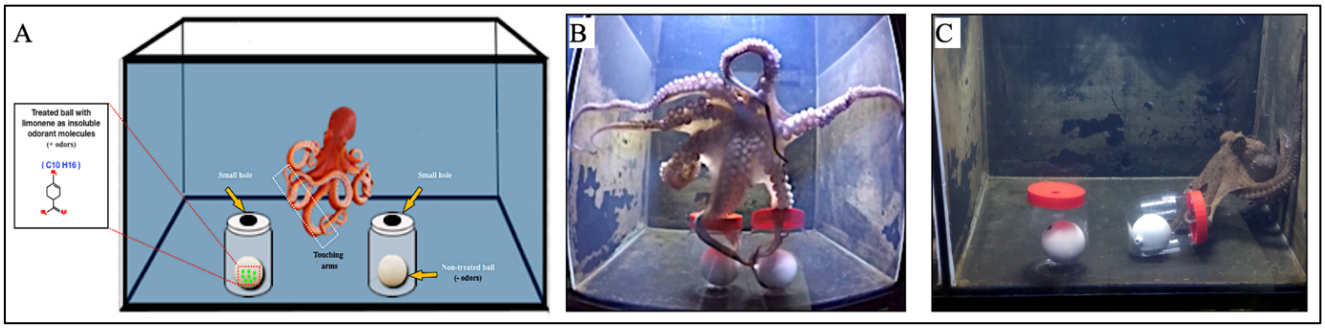

Figure 1. Diagram of behavioral experimental set-up to test the *O. vulgaris* ability to discriminate to insoluble odors molecules (lemon like, +L) or extract homogenized fish (*E. encrasicolus,* +F) relative to odorless balls used as negative controls (-L or -F). Octopus respond to olfactory stimuli by extending an arm inside the plastic screw-jars through the pierced lid. (A) Octopus respond to olfactory stimuli by extending their arm sucker inside the plastic screw-jars through the pierced lid and touch the balls. (B) manipulate: actively explores the jar (C) select the jar to open.

After acclimatization period, octopuses were challenged with a task consisting in two screw-jars (with a small hole for reaching) with (+F and -F) or (+L and -L) agarose balls, that were gently presented into the experimental tank at equidistance and simultaneously to the octopus, (Figure 1A). The octopus approached the screw-jars, extending its arm into the jars, touching the balls inside, making a choice by attacking one of two jars (Figure 1 B,C). Food reward (anchovy, *Engraulis encrasicolus*) was delivered within 5 ± 1 s (mean ± SD) when the octopus made a choice by attacking only the jar contain + agarose balls . This behavioral design allowed animal to perform an olfactory-associative learning task, in which they required to learn that +F or L+ (ball with odor) was the stimulus that allow them to gain a food reward) (Figure 1B).While the other screw-jar with (odorless) was not rewarded with food (Figure 1C). Animals that during a trial attacked only jar (odorless agarose ball; -F or -L) was feed after 6 hours from trial with a mussel (*Mytilus edulis*).

We randomized the spatial distribution and the position of jars in each trial, to exclude octopus habituating to the food reward created by the jar's position. Octopuses were trained one time per day for 5 days with each experiment. The first three days were considered as training and the remaining seven days were considered test. Data were expressed as mean percentage of correct response (+F or +L choice) for each octopus. Number of touches on both agarose ball. Data were averaged as discrimination score and duration of agarose balls touches for each animals overall trials. All experiments were conducted once per day and recorded for at least one hour with a digital camera (GoPro Hero 5) positioned on the front of the tank (20 cm), to analyze octopus's behavioral responses. We examined videos using a high-resolution media player (QuickTime 7, Apple Inc., Cupertino, CA, USA) for behavioral analysis and we recorded data into an Excel data sheet (Microsoft Excel 15.32).

3. Results

3.1 Expression of TAAR genes in the arm suckers of octopus

To identify the expression of TAAR genes in the arm suckers of *O. vulgaris* and *O. bimaculoides*, we investigated the expression of three octopus TAARs in *O. vulgaris* (OV-TAAR1, OV-TAAR2 and OV-TAAR7) and *O. bimaculoides* (OB-TAAR1, OB-TAAR2 and OB-TAAR7) using reverse transcription PCR reactions (RT-PCR), and verified by showing their express in the olfactory tissues including the whole olfactory mucosa (WOOM) and olfactory lobe (Figure 2). These results showed that the three octopus TAARs are strongly expressed in the octopus's arm suckers for both species, reflecting their potential role in the interaction between the detection of insoluble odor molecules from the environment and regulation the tactile forms of olfaction in octopus. The present of the TAARs expression in the octopus's arm suckers of *O. vulgaris* and *O. bimaculoide* providing an explanation for how octopuses can detect the insoluble odors molecules in their environment and exhibit an precatively behavior known as smell by touch.

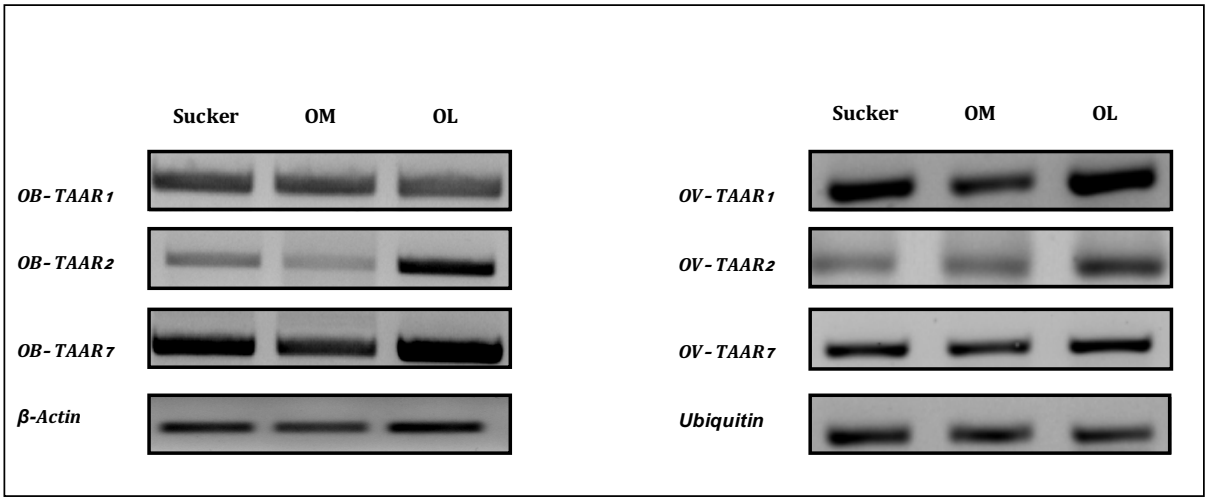

Figure 2:RT-PCR analyses showing the expression of octopus TAAR genes in *O. bimaculoides* (OB-TAAR1, OB-TAAR2 and OB-TAAR7) and *O. vulgaris* (OV-TAAR1, OV-TAAR2 and OV-TAAR7). Expression of octopus TAAR genes in different tissues including sucker, olfactory mucosa (OM) and olfactory lobe. Expression of the house- keeping genes (β-Actin and ubiquitin) are also shown. All PCR products were subjected to sequencing.

In our study, further evaluation for the differential expression of octopus TAARs along the arm sucker of O. vulgaris was performed by using quantitative RT-PCR (qRT-PCR).The expression of OV-TAARs was detected in all six tissues analyzed including olfactory mucosa (OM), optic lobe (OL), proximal big suckers (PROX-B) proximal large suckers (PROX-L), middle

suckers (MID), and distal suckers (DIS). The three OV- TAARs (OV-TAAR1, OV-TAAR2 and OV-TAAR7) showed widely different levels of expression in the six tissues while OV-TAAR7 revealed the highest expression in the optic lobe with approximately 332 times. In addition, the real time qPCR experiments analysis showed a high significative expression of OV-TAAR7 in the middle suckers, and distal suckers respect to OV-TAAR1 and OV-TAAR2 ($p < 0.05$). In the proximal large suckers, the expression of OV-TAAR2 was high significant compared to OV-TAAR1 and OV-TAAR7. On the contrary, there are no significative difference in the gene expression theses in the olfactory lobe. The hepatopancreas tissue did not show any expression, and was used as a negative control.

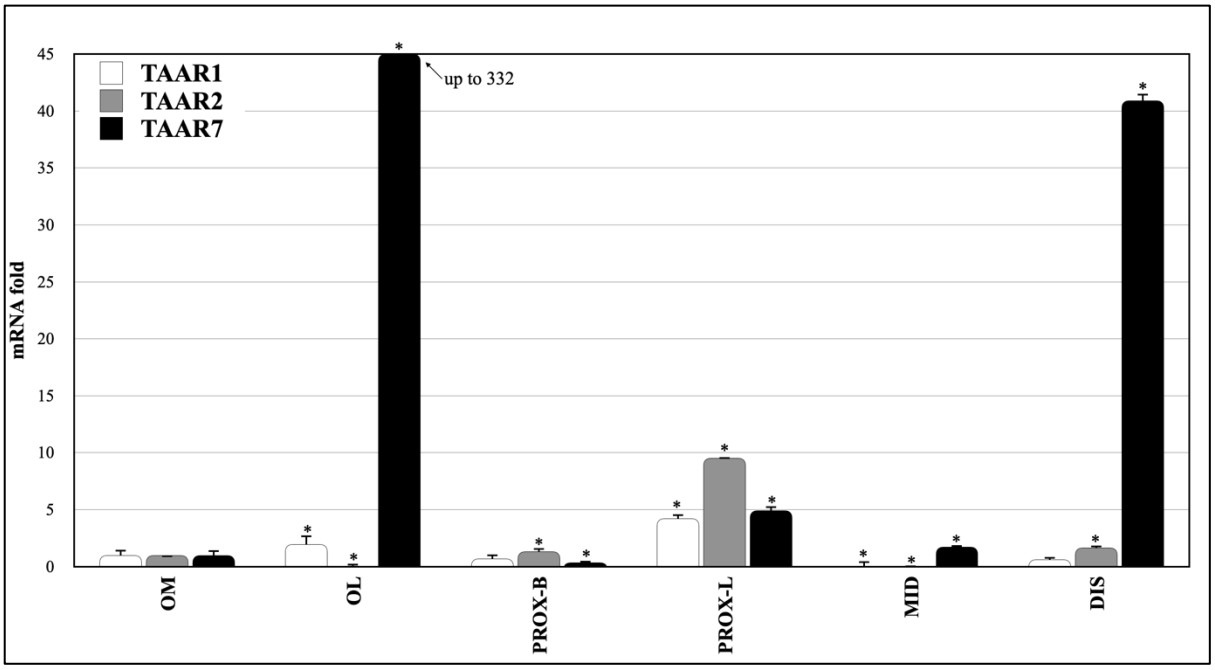

Figure 3. Relative mRNA expression levels of TAAR genes in *O. vulgaris* tissues: olfactory mucosa (OM), optic lobe (OL), proximal big suckers (PROX-B) proximal large suckers (PROX-L), middle suckers (MID), and distal suckers (DIS). *asterisk indicates that the difference vs olfactory mucosa (OM) expression is statistically significant (Wilcoxon two group test, $p<0.05$). Error bars represent the SEM.

3.2 Localization of octopus-TAARs in the sucker using whole-mount *in situ* hybridization.

To localize the expression of octopus-TAAR gene in the arm suckers (gustatory system), we performed whole mount *in situ* hybridization using RNA probes, showing their spatial expression on the sucker of octopus. Full coding regions of these genes were characterized and analyzed their expression and localization in the arm sucker from *O. vulgaris* and *O. bimiceuladoes*.

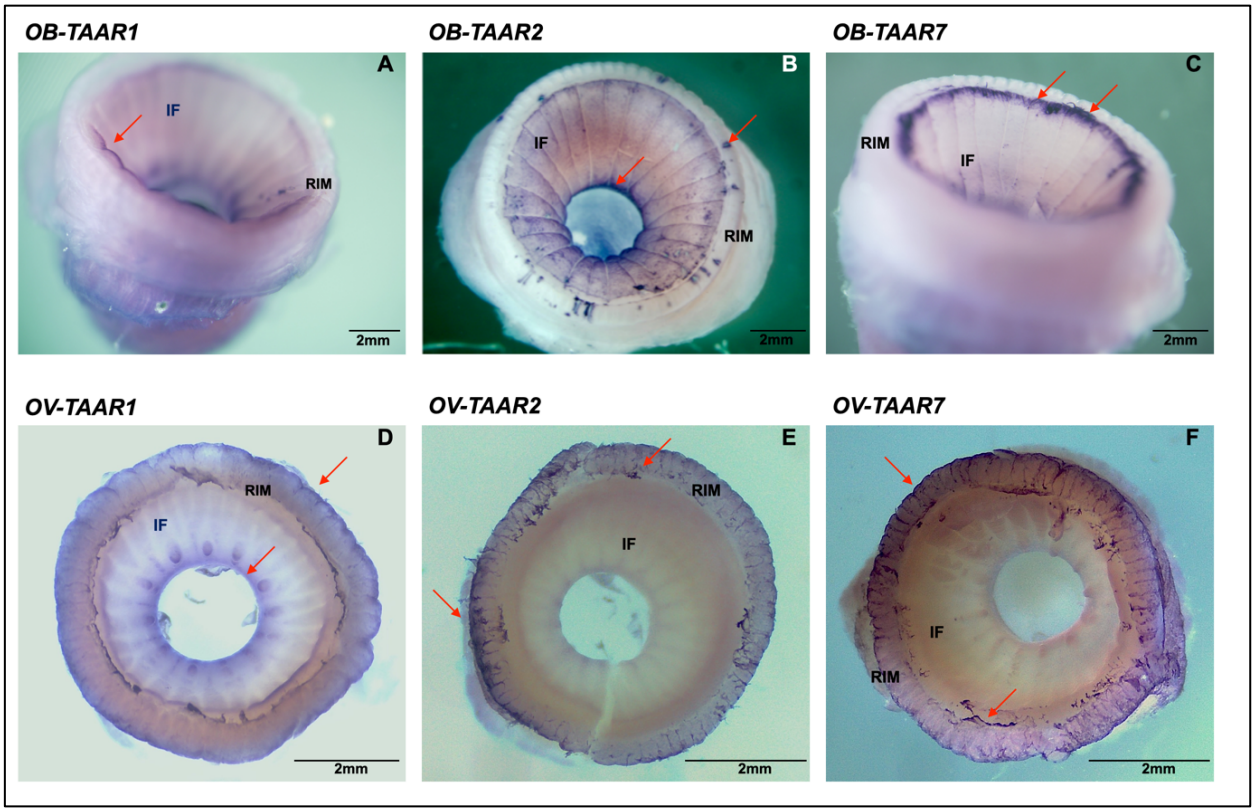

Figure 4. Localization of TAAR mRNA in the sucker of *O. bimaculoides* (A-C) and *O. vulgaris* (D-F) using whole-mount in situ hybridization. Expression of octopus TAAR receptors are distributed in the epithelium rime (RIM) which covers the exterior border of the sucker and the side of the infundibulum (IF). The red arrows indicate positive signals.

The whole mount in situ hybridization experiments on *O. bimiceuladoes* were performed by the Marine Biological Laboratory (MBL) at University of Chicago, Woods hole, USA. Our results showed that the three all TAAR genes are found to be preferentially localized in the epithelial cell covering of the surface of the infundibulum part and the epithelium rim of arm suckers of *O. vulgaris* and *O. bimiceuladoes* (Figure 4) indicting this area is particularly specialized to receive the olfactory cues from environment that evoke smell by touch

responses. it is widely recognized that the infundibulum part is first in contacting a substrate, suggesting that the insoluble odors may be detected by olfactory receptor neurons, which are located within specialized structures distributed on the epithelial surface of the infundibulum. These findings are in agreement with the previous studies that reported that the epithelium of the suckers of the arms of the octopus has been shown to contain a variety of specialized sensory receptor cells (Guerin, 1908; Martoja and May, 1956; Rossi and Graziadei, 1958 ; Graziadei and Gagne, 1967). It is noteworthy that the expression of TAARs tends to decrease from the infundibulum towards the acetabulum, which is the internal surface of the sucker. This suggests the importance of these genes at the level of infundibulum and the rim, the anatomical parts in close contact with the surrounding environment. All TAARs genes are also detected in a similar region surrounding the epithelium under the toothed cuticle and more cuboidal under the smooth cuticle and on the lateral surfaces of the sucker. The cell bodies of primary receptor cells, are scattered among the epithelial cells in the infundibulum part and rim of sucker. Negative control using a sense DIG-labelled probe showed no specific staining throughout all regions of the sensory suckers.

3.3 Sequence analysis and Molecular phylogenetic reconstruction of octopus TAAR genes

To examine the evolutionary relationships of the TAAR genes in *O. bimaculoides* and *O. vulgaris,* together with the 15 other vertebrate and invertebrate species, the neighbor-joining (NJ) and maximum likelihood (ML) methods were used to construct a phylogenetic tree with other 230 OR functional genes. According to our phylogenetic tree, we found that the octopus TAARs could be separated into two major clades (Figure 5). Moreover, octopus TAARs were most closely related to those of invertebrates such as some mollusks, considered the TAAR of Lottia gigantea as the closest ortholog and forming an independent cluster at the branching of other cholinesterases. This position was supported by high bootstrap values (99/95). Interestingly, octopus TAARs showed also a closer relationship with TAARs belonged to Class III in teleost fish as identified by Hussain et al. (Hussain et al., 2009), which seemed to have gained a novel set of ligands under unusually strong positive Darwinian selection and evolved eventually into a new olfactory receptor gene family. Octopus TAARs have clearly distinct from their close relatives, the aminergic neurotransmitter receptors including all major aminergic receptor subtypes (cholinergic, dopaminergic, histaminergic, noradrenergic, and

serotinergic receptors). Octopus TAARs also segregates with maximal bootstrap values from the ORs, which are less closely related, but belong to the same major family of GPCRs, the rhodopsin type GPCRs (Fredriksson et al., 2003). Therefore, octopus TAARs as olfactory receptor may have evolved independently and directly from ancestral GPCRs multiple times across many animals lineage, which may have had a prior olfactory chemosensory function. These observation of clustered TAARs family included in this study is consistent with those found in other previous studies (Hussain et al., 2009; Jiang and Zhang, 2019; Tessarolo et al., 2014; Zhu et al., 2017). We emphasize that the appropriate choice of out-groups is especially relevant for the proper delineation of the TAAR gene family.

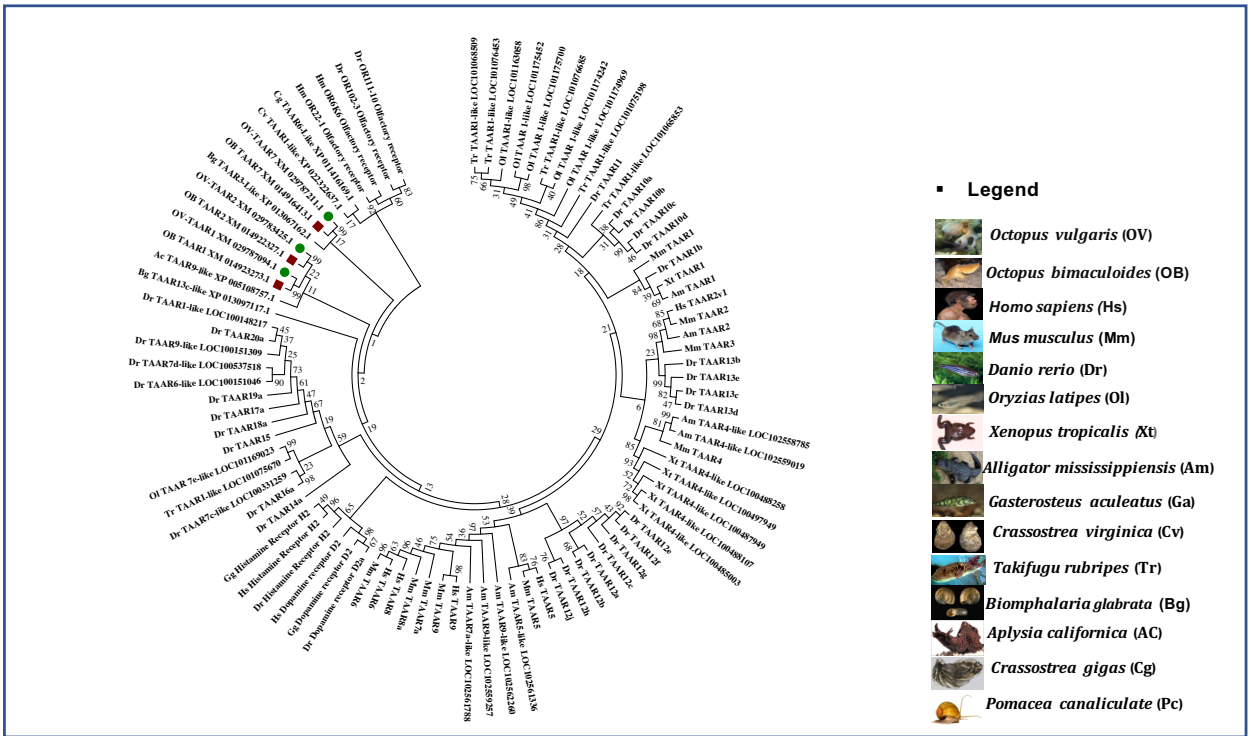

Figure 5. Evolutionary analysis and phylogenetic analysis of the octopus TAAR genes with other species include vertebrate and invertebrate. The neighbor-joining (NJ) and maximum likelihood (ML) methods were used to construct phylogenetic tree for TAAR protein sequence.

To highlight conserved amino acid residues we constructed a WebLogo (Crooks et al. 2004) from the alignment of 6 full-length TAAR genes from *O. vulgaris* and *O. bimoleculdes*. Conservation of amino acid sequence for these gene is displayed as a sequence logo (figure 5A). Because the TAARs are members of the GPCR class of proteins, we used TMHMM2.0 (Krogh et al. 2001) and Protter (Omasits et al. 2014) to predict their secondary structures (2D).

Each amino acid sequence was predicted to contain seven transmembrane (TM) domains with the C-terminal domain being located in the cytosol and the N-terminal domain located extracellularly (Figure 5B).

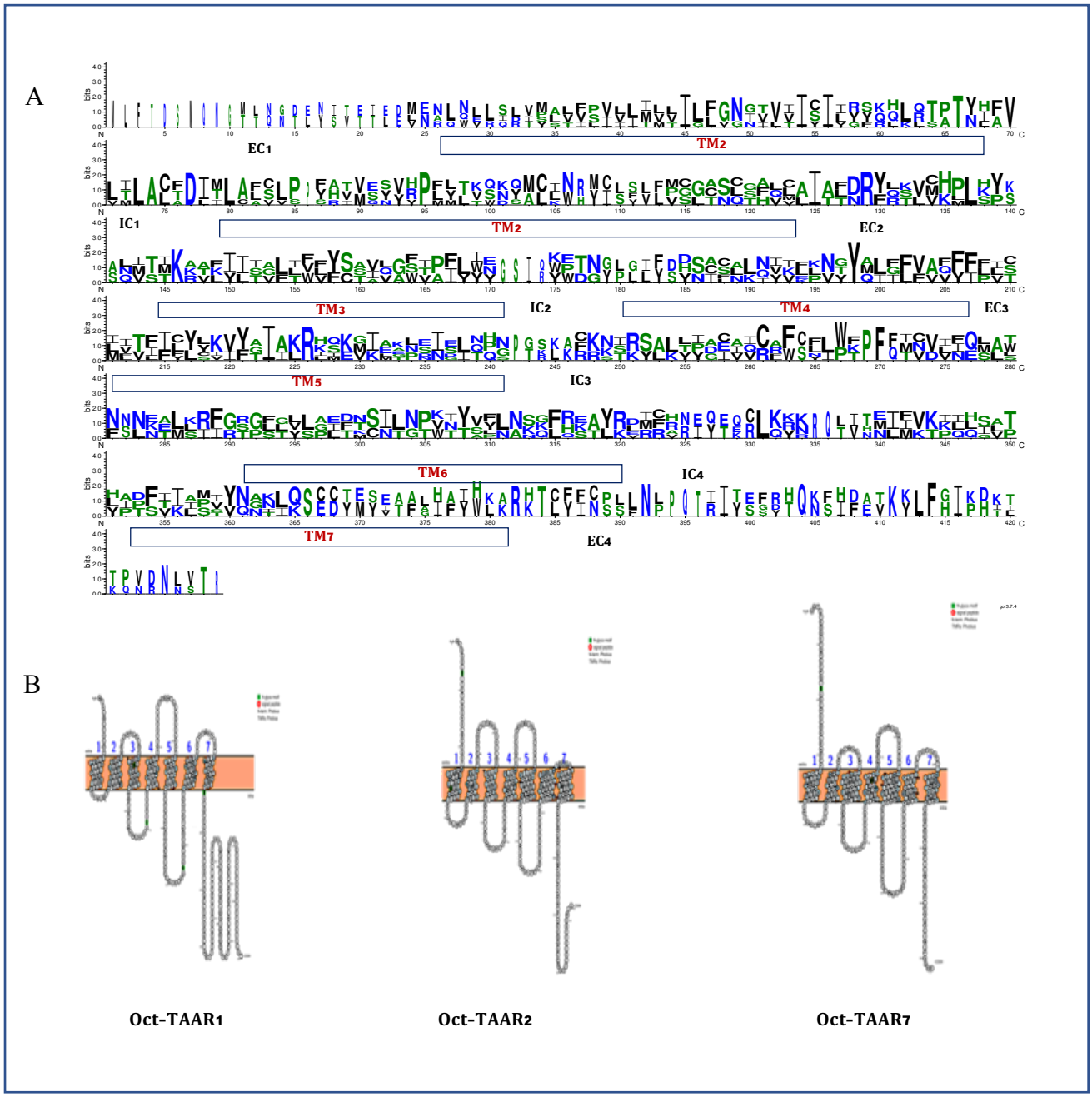

Figure 6. Amino acid sequence conservation in 6 full-length TAAR genes from *O. vulgaris* and *O. bimoleculdes*. (**A**) The open reading frames of the aligned gene set (n=6) was used to build a sequence logo. Putative locations corresponding to the transmembrane regions, (TM1-7), intra-cellular loops (IC1-3), and extra-cellular loops (EC1-3). The height of the 1- letter amino acid code in logo reflected the degree of conservation. (**B**) 2D transmembrane (TM) protein topology of the three octopus TAAR protein sequences.

3.4 Behavioral assay

The behavioral experiment procedures were performed for olfactory discrimination task using an odor-associative learning strategy, targeting the sensory area of epithelia in octopus's arm suckers for touching and perceive the olfactory stimuli. Octopuses were subjected to discriminate between two agarose balls (+ & -) and associate olfactory stimulus with the + ball (chemical odor-containing) with the food reward.

Figure7. **Behavioral response of *O. vulgaris* to insoluble odors molecules**. Octopus respond to olfactory stimuli: (A) touching and interacting behavior of *O. vulgaris* to agarose balls with fish extract (+F) or odorless (-F); (B) touching and interacting behavior of *O. vulgaris* to agarose balls with limonene (+L) or odorless (-L).

The behavioral assay in which octopuses reached arms inside jars through a small hole to explore the +agarose balls (fish extract or lemon compound) or the control balls (agarose ball with only seawater). Octopuses exhibited stereotypical exploratory behaviors involving sweeping arm motions in which suckers probed the agarose ball containing chemical compounds) (Figure1 B,C). Octopuses spent significantly more time touching the + agarose ball (chemical odor-containing) than the control ball, n = 5 trials, $p < 0.01$, two-tailed Student's t test (Figure). Preliminary experiments indicated that octopuses are able to discriminate between the two agarose balls (+&-) through contact chemoreception using their gustatory system. In addition, octopuses exhibited highly learning abilities in response to pairing (+F and +L) with food reward. Histogram with overlaid density plot showing the distribution of duration of touches for both balls (Figure 7A,B).

4. Discussion

The chemosensory world presents a common challenge to all animals. All animals must recognize and respond to chemosensory information in their environment. To achieve the perception of the external world and complex chemosensory stimuli of cephalopods large repertoires of olfactory and gustatory receptors are employed in well-developed sensory organs considered the most sophisticated of all those of invertebrates (Packard, 1972; Messenger, 1977; Young, 1977, 1989; Budelmann, 1995, 1996; Anderson et al., 2010). The octopus nervous system is among the most complex of invertebrates with the majority of neurons dedicated to semi-autonomous execution of arm and sucker behaviors during exploratory foraging and prey capture in their benthic environment (Hanlon and Messenger, 1996; Hochner, 2012; Young, 1971). Although the chemical perception through the sense of smell to perceive water-soluble molecules from distance by the olfactory organ of *O. vulgaris* has been well studied (Polese et al., 2015). our knowledge of how octopuses recognize and respond to insoluble molecules (very low solubility smaller than ~300 Da) by the chemotactic form is still poorly understood. In particular, the molecular basis and mechanisms of chemo tactile behavior and how octopuses distinguish and bind insoluble molecules in the surrounding environment still unclear. Our results demonstrate that octopuses can recognize and respond to several insoluble compounds which typically act as olfactory cues on land and elicit distinct

chemotactile behaviors, thus establishing a molecular basis for this aquatic tactile form of olfaction or smell by touch.

Aquatic chemosensation is poorly understood compared with its terrestrial counterpart. Aquatic chemical sensing has been associated with waterborne hydrophilic molecules; however, poorly soluble terpenoids associated with terrestrial olfaction have been demonstrated to elicit contact-dependent behavioral responses in aquatic organisms (Giordano et al., 2017; Long and Hay, 2006). Thus, It has also been believed that the chemosensory world of marine animals is limited to water-soluble molecules, and does not include volatile, chemical stimuli (Ache and Young. 2005; Caprio and Derby. 2008). recent findings give evidence indicating that chemoreception of volatile/odorant lipophilic compounds, almost insoluble in water and act as olfactory cues, can occur in aquatic environments by the way of smell. This preconceived evidence has recently contradicted by showing that both crustacean and fish can detect hydrophobic compounds act as olfactory signals by a tactile form of olfaction (Giordano et al., 2017), in particular, they show that little shrimp (Palemon elegans) and also fish (Danio rerio) have a sense volatile biomolecules using their chemosensory mouthparts to perceive typical odiferous compounds usually smelled by humans. In the same way, this strongly suggests that other benthic animals, such as octopus, could recognize chemical stimuli adherent to the substrate by their arm suckers (gustatory systems) allowing them to shape their sophisticated behavior defined as smell by touch.

Interestingly, the gustatory systems of *O. vulgaris* are consists of sensory receptors distributed on the suckers, considered the aquatic equivalent to taste (Wells, 1963; Graziadei and Gagne, 1973; Grasso and Basil, 2009). They are equipped also with many chemosensory neurons located in their suckers, exhibit a peculiar behavior that can be provocatively described as ‘taste by touch (Wells, 1963). Moreover, the molecular basis of chemo-tactile sensation, which is thought to facilitate a taste-by-touch ability in octopus has been recently discovered (Giesen et l., 2020). These findings confirmed that the arm suckers have a kind of “taste by touch ability” mediated by unique chemotactic receptors (CR). Given the distinction between olfaction and gustation based upon spatial criteria is prevalent in the literature on aquatic chemical communication (Giordano et al., 2017). Thus, the debate about the terms taste and olfaction concerning the chemosensory systems and chemotactic sensation of octopus is not yet clear and requires further elaboration. Given the great chemical sensitivity of the

octopus suckers, we recently proposed that the chemo-tactile sensation in octopuses are also involved in olfaction by their suckers that were traditionally not considered olfactive, allowing them to recognize odorant molecules that are insoluble or have a very low solubility when they are touched and exhibiting a peculiar behavior described as "smell by touch" (Di Cosmo et al., 2018; Di Cosmo and Polese 2017). Thus, It will be necessary to the presence of olfactory receptor genes in octopus's suckers to meet this task, which is involved in the chemoreception, considered the tactile form of olfaction in the octopus suckers. Despite the ubiquity of this hypothesis, little is known about the sensory mechanisms that drive octopus to interact with these types of molecules.

In the current work, we present the first genetic evidence for the chemo-tactile capability of octopus, which is mediated by the functional expression of olfactory receptors in the arm suckers of *O. vulgaris* and *O. bimaculoides* to detect insoluble odor molecules from the environment.

TAARs, as olfactory receptors, are expressed in the olfactory epithelium of mice indicated that both trace amines and their receptors have roles in olfaction, can recognize trace amine substances and related compounds (Liberles and Buck 2006; Hussain et al. 2009; Liberles and Buck 2009). The finding by Liberles and Buck (2009). TAARs are found in all vertebrate genomes examined thus far (Hashiguchi and Nishida, 2007). Three of TAARs genes have been identified in the *O. bimaculoides* genome (Albertin et al., 2015) and *O. vulgaris* genome (Zarrella et al., 2019). Therefore, we hypothesize that TAARs may have an important role in octopus olfaction.

In this study, our results showed that octopus-TAARs are strongly expressed in the olfactory tissue and the octopus's arm suckers (Figure1). These results are consistent with the function of the octopus-TAARs as olfactory receptor genes reflecting that these receptors could interact with insoluble odor molecules in the octopus chemical environment, which may be related to the tactile form of olfaction by the octopus' arm suckers that are usually non-olfactory organ (smell by touch behavior in octopus).

Additionally, gene expression analysis revealed that the expression of OV-TAARs was detected in all six tissues analyzed including olfactory mucosa (OM), optic lobe (OL), proximal big suckers (PROX-B) proximal large suckers (PROX-L), middle suckers (MID), and distal suckers (DIS). The three OV- TAARs (OV-TAAR1, OV-TAAR2 and OV-TAAR7) showed widely

different levels of expression in the six tissues while OV-TAAR7 revealed the highest expression in the optic lobe with approximately 332 times. In addition, the real time qPCR experiments analysis showed a high significative expression of OV-TAAR7 in the middle suckers, and distal suckers respect to OV-TAAR1 and OV-TAAR2 ($p < 0.05$). In the proximal large suckers, the expression of OV-TAAR2 was high significant compared to OV-TAAR1 and OV-TAAR7. The presence of the TAARs expression in the octopus's arm suckers of *O. vulgaris* and *O. bimaculoides* providing an explanation for how octopuses can detect the insoluble odors molecules in their environment and exhibit an precatively behavior known as smell by touch. The results also showed the three octopus-TAARs in O. vulgaris are expressed at a higher level in the olfactory mucosa, suggesting that these genes also function as potential chemosensory receptors in the olfactory organ are involving in distance perception process for detecting the water-soluble chemical cues from the distance. Early behavioral studies have already shown that octopuses and cuttlefish are capable of distance chemoreception (Lee 1992; Boal and Golden 1999).

To better understand the specialized roles of octopus-TAARs in mediating this behavior requires characterization of the projections of these genes the mechanisms controlling TAARs expression, we characterized the octopus-TAAR genes expressed in the sucker and olfactory organs of *O. vulgaris* (OV-TAAR1, OV-TAAR2, and OV-TAAR7) and *O. bimaculoides* (OB-TAAR1, OB-TAAR2, and OB-TAAR7). In our phylogenetic tree, we found that the octopus TAARs could be separated into two major clades. Moreover, octopus-TAARs were most closely related to those of TAAR mollusks, considered the TAAR of *Aplysia californica* (Ac) as the closest one to Octopus-TAAR1 and TAAR2 in both *O. vulgaris* and *O. bimaculoides*. While, the TAAR of *Biomphalaria glabrata* (Bg) is most closely to octopus-TAAR7 in both species. Interestingly, they showed also a closer relationship with TAAR genes belonged to class III in teleost fish which have been identified by Hussain et al. (2009), which seemed to have gained a novel set of ligands under unusually strong positive Darwinian selection and evolved eventually into a new olfactory receptor gene family. Also, octopus-TAAR genes are segregated with maximal bootstrap values from the ORs, which are less closely related, but belong to the same major family of GPCRs, the rhodopsin type GPCRs (Fredriksson et al. 2003). This result suggests that octopus-TAARs as olfactory receptors may have evolved independently and directly from ancestral GPCRs multiple times across many animal lineages, which may have had a prior

olfactory chemosensory function. This observation of clustered TAAR gene family included in this study is consistent with those found in other previous studies (Hussain et al., 2009; Jiang and Zhang, 2019; Tessarolo et al., 2014; Zhu et al., 2017). These findings could also support the functionality of given octopus-TAARs to be associated with the detection of olfactory cues in their environment using the suckers of an octopus.

It has been previously described in detail the description of the anatomy of the sucker and of the general arrangement of the epithelial lining of the organ where the sensory receptors are located (Graziadei, 1964). A diagram (Figure 6 in chapter 1) shows the three regions of the sucker (epithelium lining the acetabulum (AC); epithelium lining the infundibulum (IF) and the epithelium lining the rim (RIM) where these sensory cells occur. The sensory receptor cells are particularly frequent on the rim of the sucker (RIM) and in the epithelium of the infundibulum (IF). In the epithelium of the rim which covers the exterior border and the side of the infundibulum, there are various types and forms of specialized sensory receptor cells, the structure of these cells presumably suggests the presence of chemoreceptors as well as tactile receptors in the sucker (Guerin,1908; Martoja and May 1956; Rossi and Graziadei, 1958 and Graziadei and Gagne, 1973). These findings are reported that these cells are sensory neurons but the molecular cell types and their localization in the sucker are not yet certainly known. In the current study, we localized the expression of mRNA TAARs in the arm sucker of *O. vulgaris* and *O. bimaculoides* using whole-mount *in situ* hybridization experiment. Localization of TAAR mRNA in the sucker of *O. bimaculoides* (A-C) and *O. vulgaris* (D-F) using whole-mount in situ hybridization. Expression of octopus TAAR receptors are distributed in the epithelium rime (RIM) which covers the exterior border of the sucker and the side of the infundibulum (IF).

The results exhibited similarities in localization of expression among the 6 distinct octopus-TAARs (Figure 4, *O. bimaculoides* (A-C) and *O. vulgaris* (D-F)). Expression of octopus TAAR receptors are distributed in the epithelium rime (RIM) which covers the exterior border of the sucker and the side of the infundibulum (IF). The most striking expression pattern of octopus-TAAR genes was found in the sensory epithelium of the infundibulum (IF), where the cuticle is present with small openings correspond to the distal end of the sensory cells allowing them to come in free contact with the surroundings and reach the surface of a substrate (Graziadei,1964). These results confirm the suggestion proposed based on the

morphological feature of a cell type that they are possibly chemosensitive rather than mechanoreceptors since they have access to the external environment through pores in the cuticle and they seem to be similar to some sensory cells of the oral lip of these animals (Graziadei, 1960). Here, we assume that the sensory cell in the epithelium lining of the infundibulum (IF), maybe a specialized region that participates in olfactory processing using octopus's suckers. In this work, the widespread of octopus-TAAR receptors must play an important role in the animal's responses to insoluble odor molecules in the environment. In the behavioral experiment, we tested how octopuses respond to insoluble odor compounds which are act as olfactory signals in the aquatic system when they touch them.

The behavioral experiment procedures were performed using an odor-associative learning strategy, targeted the sensory area of epithelia in octopus's arm suckers for touching and perceive insoluble odors molecules. Octopuses spent significantly more time touching the+ agarose ball (chemical odor-containing) than the control ball. The results indicated that octopuses can discriminate between two agarose balls (+&-) through contact chemoreception using their gustatory system. Also, octopuses exhibited highly learning abilities in response to pairing (+) with a food reward.

Taken together, our results point toward supporting our hypothesis, the expression of octopus-TAARs in the octopus's sucker provides direct evidence for the function of the olfactory receptor through the contact chemoreception. Also, behavioral evidence for the response to the insoluble odor molecules mediating touch- smell arm behavior.

CHAPTER (4)

See through the arms: extra-ocular photoreceptive system in the sucker of *Octopus vulgaris*

1- Introduction

Cephalopods are known for their ability in camouflaging quickly, consisting of change their appearance through the alteration of the skin color pattern. They achieve this by detecting the surrounding environment using their complex eyes. Their eyes are specialized light-sensitive structures, involved in image forming vision. Once acquired the visual input, their brain sends output signals to chromatophores, iridophores, leucophores, and papillae, resulting in color pattern and/or shape change (Cloney and Brocco 1983, Hanlon and Messenger 1988, Allen et al., 2009, Chiao et al., 2010). Cephalopod's embryo becomes able to answer a light stimulation when eyes' rhabdomeres express the retinal (Romagny et al., 2012). Even though cephalopods lack multiple photoreceptor types in the retina and are equipped with just a single photoreceptor class, they are able to determine the spectral composition of objects because their photoreceptor cells contain a variety of light-sensing and other molecules that interact with each other to ensure the transduction of signals (Yau 2009, Chaves et al., 2011, Yoshida et al., 2015, Bonadè et al., 2020). Furthermore, Cephalopods possess well-studied extra-ocular photoreceptors (Messenger 1967, Mauro and Sten-Knudsen 1972, Messenger and Sanders 1972, Young 1972, Hara and Hara 1976, Hara and Hara 1980, Mathger et al. 2008, Tong, et al. 2009, Mäthger et al. 2010, Kingston et al. 2015). Mäthger et al. (2010), for instance, identified the presence of rhodopsin transcripts in fin and mantle tissue of the cuttlefish, *Sepia officinalis*, which suggests that cephalopods may have dermal photoreceptors that function using the same phototransduction pathway as those in the retina. Light-activated chromatophore expansion was described in *Octopus bimaculoides* where it has been evaluated the expression of r-opsin in the skin (Ramirez and Oakley 2015).

Kingston and colleagues (Kingston et al. 2015a;b), instead identified the presence of mRNA transcripts and proteins (rhodopsin, retinochrome, and Gqa) involved in phototransduction in cephalopods' derma, in particular in a component of chromatophore organs and fin muscle in squid and cuttlefish. Surprisingly in hatchings, *Dorotheutis pealei* small hair cells, known as mechanoreceptors, co-express rhodopsin and retinochrome

suggesting their multimodal sensory function working simultaneously as mechanical and light sensitive structures (Kingston et al., 2015b). The same authors found rhodopsin and retinochrome in the arm ganglia and the sucker peduncle nerves too, suggesting that also these tissues work in a multimodal way detecting tactile and photic information. Very recently it has been found that the octopus arm tip reacts in response to illumination folding it in a reflex-like manner (Katz et al., 2021).

Other extra ocular light-sensitive structures are represented by the dorsal vesicles (Young et al., 1979), parolfactory vesicles (Messenger 1967), and epistellar bodies (Young 1973). For instance in the squid Abraliopsis the dorsal vesicles have a role in regulating bioluminescence emitted from light organs (Young. 1979), while in the deep-sea squid, Todarodes pacificus, the parolfactory vesicles respond electrically to light and express rhodopsin and retinochrome (Young 1962, Messenger 1967, Hara and Hara 1976, Hara and Hara 1980, Cloney and Brocco 1983, Allen, Michels et al. 1986). The bobtail squid, *Euprymna scolopes*, instead, possesses extra-ocular photoreceptors located in its bioluminescent light organ, expressing rhodopsin, arrestin, and rhodopsin kinase proteins (Tong, Rozas et al. 2009), all involved in the regulation of the luminance emitted from the organ (Jones and Nishiguchi 2004). Furthermore, the epistellar body of the *Eledone cirrhosa* has photoreceptors that respond to light with a rhodopsin-like spectral sensitivity (Cobb and Williamson 1998, Kingston et al., 2015).

In the octopus, the arm suckers have unique features to perform a remarkable variety of functions (Packard 1988), such as anchoring the body to the substrate, grasping, manipulating, and investigating objects (Kuba, Byrne et al. 2006, Kuba, Byrne et al. 2006). The octopus arm sucker contains an extremely effective mechanical and sensory system. Earlier studies focused on the sensing properties of the octopus suckers, demonstrating that a wide range of primary receptor cells is present in the suckers (Graziadei 1962, Graziadei and Gagne 1976, Wells 1978). Most of these cells are morphological modifications of the ciliated bipolar cells, that represent the common neuroreceptor archetype throughout the animal kingdom (Graziadei and Gagne 1976), and 4 types of primary receptor cells were described in the epithelium of the octopus suckers rim (Graziadei 1962). Based on their morphology, Graziadei & Gagne (1976) hypothesized their role in mechano- chemo- and photo-reception. Recently van Giesen and colleagues (2020) described the molecular basis of chemotactile system located

in the arm suckers. However, the molecular characterization and function of photoreceptor types in the arm suckers remain unclear to date.

The opsin family is a multigenic family of G protein coupled receptors (GPCR) (Feuda et al. 2012, Porter et al. 2012, Ramirez et al. 2016) and in eumetazoans there are at least 9 opsins paralogs (Ramirez et al. 2016) 6 of which have been identified in mollusks, and only 4 have been detected in cephalopods, in the genome of *Octopus bimaculoides*: rhodopsin, rhabdomeric opsin, peropsin, and retinochrome (Albertin, Simakov et al. 2015).

Rodopsin's role in vision is well known; upon absorption of light, it activates a G-protein cascade that generates an electrical response at the surface membrane of the retinal rod cells. This response encodes the absorption of single photons, and upon transfer through the visual pathway it ultimately elicits visual sensations (Alfinito and Reggiani 2015). It consists of an apoprotein opsin and 11-cis-retinal chromophore bounded by Schiff-base linkage (Murakami and Kouyama 2008, Shukolyukov 2012).

In the current study, we sequenced the *O. vulgaris* GRK1 gene defining a phylogenetic tree and performing a 3D structure model prediction. Then f*or the first time, besides to state the presence of O. vulgaris* GRK1 gene expression in eyes and skin, we show its expression in the suckers rim epithelium. Furthermore, we also quantify the relative mRNA in different sucker types at several arm levels. Taking together our data extend the touch/chemo sensations of these structures (Maselli et al. 2020, van Giesen et al. 2020) to the light-sensing ability suggesting the sucker of *O. vulgaris* as extra-ocular photoreceptive system.

2. MATERIAL AND METHODS

2.1 Tissue collection and fixation

Adult specimens of *O. vulgaris* (body weight 800g±50g, mean ± SD) were collected from the Bay of Naples (Italy) and transferred to the Di Cosmo's cephalopod facility at the Department of Biology, the University of Naples Federico II (Italy). Adult specimens of *O. vulgaris were* anesthetized by isoflurane insufflation (Polese, Winlow et al. 2014) and tissues were dissected under sterile conditions following institutional guidelines. For gene analysis on RNA, we dissected four sucker types (Proximal big, proximal large, middle, distal; Figure 1), skin, eye (retina), and heart (as negative control), then sampled were snap frozen, put in Trizol. Dissected samples were stored at −80 °C for further experiments.

For whole-mount *in situ* hybridization arm suckers from L1 were isolated and a fixative 4% PFA (4% Para-formaldehyde in PBS, pH 7.4) was added directly at 4 °C overnight. Fixed tissues were dehydrated in a graded methanol series (25% MeOH; 50% MeOH; 75% MeOH and 100% MeOH) with 1X PBST (Phosphate-buffered saline with 0.1% Tween-20) for 15 min each and stored in 100% methanol at -20°C until use.

Our research is approved to 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).

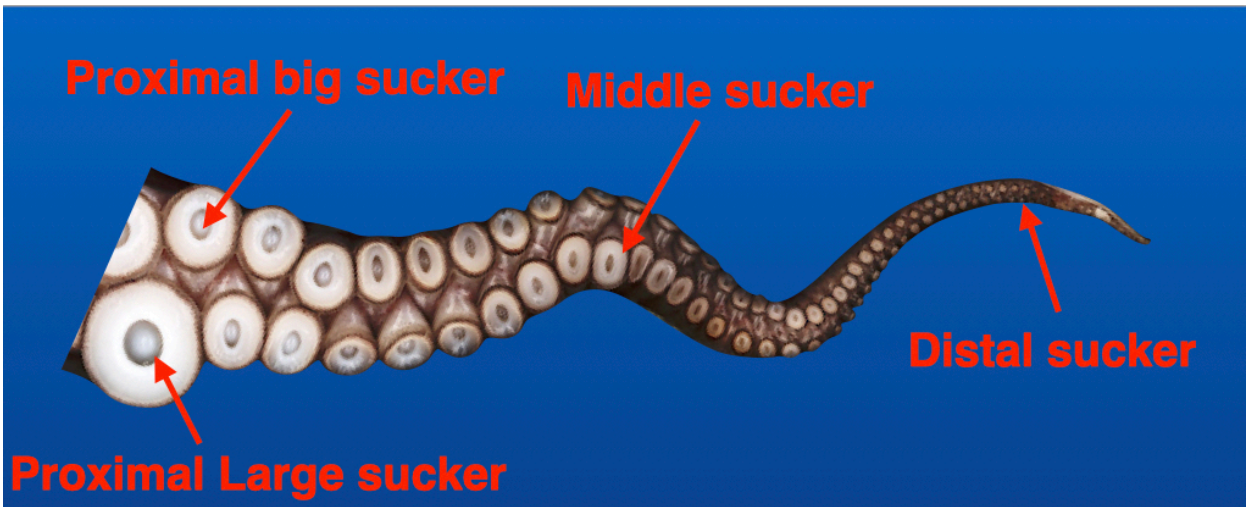

Figure 1 - Image description of four sucker types: proximal big, proximal large, middle, distal.

2.2 Expression analysis of Rhodopsin kinase (Ov-GRK1) in different tissues and sequencing

Total RNA was extracted using the RNeasy minikit (Qiagen, USA), following the manufacturer's protocol. The quality and amount of purified RNA were analyzed spectrophotometrically with Qubit 3.0 (Thermo Scientific Inc., Waltham, MA, USA). RNA of 1000ng was reverse transcribed with the QuantiTect® Reverse Transcription Kit (Qiagen, USA). Specific PCR primers were designed with the software Geneious 9.1 (Biomatters, Auckland, New Zealand, available from http://www.geneious.com), using the coding sequence for Rhodopsin_kinase (GRK1) gene from the genome of *O. bimaculoides* (Albertin, Simakov et al. 2015) (Table 1).

PCRs were performed in a final volume of 20 µL, with 0.2 µL of *Pfu* DNA polymerase (Thermo Scientific), 4 µL of 4× Tris buffer with $MgCl_2$, 1.6 µL of dNTPs (each dNTP 2.5 µM), 0.2 µL of 50 µM of each primer, and 100 ng of cDNA template under the following conditions: An initial denaturing step of 98 °C for 3 min; 35 cycles of 10 s at 98 °C; 30 s at 60 °C and 1 min at 72°C; and a final extension step of 5 min at 72 °C. PCR products were purified from unincorporated primers using Exonuclease I and Fast Alkaline Phosphatase (Thermo Scientific). The sequencing reaction was performed using the BigDyeTM Terminator Cycle Sequencing chemistry (Applied Biosystems, Foster City, CA, USA). Sequences were purified using DyeEx 2.0 Spin Kit (Qiagen, USA) and analyzed by an ABI 3100 automated sequencing instrument (Perkin-Elmer, Genetic Analyzer, Foster City, CA, USA). Chromatograms were assembled and analyzed using software Geneious version 9.1. PCR products were analyzed with GenBank BLASTn and BLASTx (BLAST, basic local alignment search tool). Additionally, we performed a real-time PCR on four sucker types (proximal big, proximal large, middle, and distal), skin, eye (retina), and heart (as negative control), using the QuantiTect SYBR Green PCR Kit (Qiagen, USA). PCR was performed in a final volume of 25µL, with 50 ng of cDNA, 1 mM of each primer, and 12.5µL of QuantiFast SYBR Green PCR Master Mix (2×). The PCR cycling profile consisted of a cycle at 95 °C for 5 min, 40 three-step cycles at 95 °C for 15 s, at 60 °C for 20 s, and at 72 °C for 20 s. Quantitative RT-PCR analysis was conducted by using the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen 2001). RT-PCR was performed in a Rotor-Gene Q cycler (Qiagen, USA). The ubiquitin gene was used for normalization of the relative expression (Table 1). At the end of each test, a melting curve analysis was done (plate read every 0.5 °C from 55

to 95 °C) to determine the formation of the specific products. Each sample was tested and run in duplicate.

We compared and analyzed real-time PCR results using a Wilcoxon two group test and data with p-values < 0.05 were considered statistically significant.

Table 1 - Primers used in this study

Primer pairs used in RT-PCR	**Primer sequences (5′→3′)**
Ov-GRK1 F	CCGCCTCTCATTCCTCCAAG
Ov-GRK1 R	AGATCTCTCCTTCCACAATCACA
Ubiquitin_ F	TCAAAACCGCCAACTTAACC
Ubiquitin_ R	CCTTCATTTGGTCCTTCGTC
For WM-ISH probe	
Ov-GRK1 F	CCGCCTCTCATTCCTCCAAG
Ov-GRK1 R + T7	*TAATACGACTCACTATAGGGGAGA*AGATCTCTCCTTCCACAATCACA

2.3. Whole-mount *in situ* hybridization

To generate the Ov-GRK1 Digoxigenin (DIG)-labeled single-stranded RNA probe, we performed PCR standard method using specific primer set (Table 1).The PCR fragments were used as templates for *in vitro* transcription reaction using the T7 RNA polymerase promoter sequence corresponding to forward and reverse primers for the sense and antisense probes, respectively. PCR cDNA fragments were isolated by 1.2% agarose gel and used as templates for in vitro transcription reaction. RNA transcription reaction was performed using the DIG-RNA Labelling Kit (SP6/T7) (Roche Applied Sciences, Laval, QC, Canada) following the manufacturer's instructions. Final probes were cleaned up using RNeasy MinElute Cleanup Kit (Qiagen USA), and one microliter was visualized on a 1.5% agarose gel to estimate concentration.

For whole-mount *in situ* hybridization, fixed suckers were rehydrated by descending methanol series in 75%, 50%, and 25% MeOH in PBST for 15 min each at RT. Completed rehydration was performed twice in 100% PBST for 10 min each with gentle rocking. Tissues were incubated and digested in the detergent mix (20 µg/ml in PBST Proteinase-K) at 37°C for 20 min, post-fixed in 4% PFA for 20 minutes at room temperature, then washed three times in PBST for 5 min each. Tissues were pre-hybridized for 2hr and hybridized overnight at 62°C in hybridization solution (50% formamide, 5X saline-sodium citrate (SSC), 1X Denhardt's solution, 500mg/ml yeast tRNA and 500 mg/ml salmon sperm DNA) . After hybridization, the tissues were incubated with 20µg/ml RNAs A (Invitrogen 12091021) for 15 min at 37°C, then subjected to a series of post-hybridization washes in decreasing concentrations of SSC with 0.1% Tween

20. Tissues were blocked in 1X blocking solution (Roche Applied Science 11096176001) in PBST for 1 hr. at room temperature under gentle rocking, followed by incubation in 1:2500 Anti-Digoxigenin-AP antibody (Roche 11093274910) in blocking solution overnight at 4°C on a rocker. Tissues washed in PBST five times for 25 min each, equilibrated in alkaline phosphatase buffer (AP) (100mM NaCl, 50mM $MgCl_2$, 100mM Tris, pH 9.5, 0.1% Tween-20) at room temperature. The color reaction was performed in NBT/BCIP stain solution (Roche 11681451001) in AP buffer under the light-resistant environment until the colors reached satisfactory intensity. Control specimens were left in staining solution for the same time interval as those incubated with anti-sense probes. In order to test for nonspecific labeling, negative control experiments were performed for each condition using hybridization buffer only without probe.

After coloration reaction, all tissues were passed through ascending concentrations of ethanol in PBS to remove background and darken the specific signal, re-hydrated in PBS. Whole-mount sucker tissues with DIG-labeled probes were observed colourimetrically under a Carl Zeiss Stemi 305 stereomicroscope with Axiocam ERc 5s .

2.4 Molecular phylogenetic analysis

To construct the evolutionary relationships of the Rhodopsin kinase receptor we aligned the sequence of *O. vulgaris* kinase (Ov-GRK1, XM_029795790.1) with those of other amino sequences of vertebrate Rhodopsin kinase (RK) and Bilaterian GPCR Kinases, together with 6 previously identified cephalopod RK sequences, including *Enteroctopus dofleini*, *Loligo forbesii, Doryteuthis pealeii, Euprymna scolopes* and *O. bimaculoides*.

Protein sequences were aligned with the MUSCLE algorithm (Edgar 2004), included in the software package MEGAX (Kumar, Stecher et al. 2018) with default parameters. ProtTest v3.4.2 was used to establish the best evolutionary model (Darriba, Taboada et al. 2011). Bayesian tree was constructed using MrBayes v3.2.7 (Ronquist, Teslenko et al. 2012) and Bayesian inference phylogenies were run for 1,000,000 generations. Markov chain Monte Carlo (MCMC) was used to approximate the posterior probability of the Bayesian trees. Bayesian analyses included four independent MCMC runs, each using four parallel chains composed of three heated and one cold chain. Ten per cent of initial trees were discarded as burn-in.

Phylogenetic trees were rendered using FigTree (http://tree.bio.ed.ac.uk/software/figtree (Stöver and Müller 2010).

2.5 Prediction of 3D structure model

To estimate sequence similarity of OV-GRK1 protein and the GRK1 protein sequence of *O. bimaculoides* (XP_014774259) was aligned with rhodopsin kinase sequences of the light organ (ACB05677) and the eye (ACB05676) of *E. scolopes*. The alignment was performed using CLUSTALW (Larkin, Blackshields et al. 2007) and colored according to the CLUSTALX scheme using JALVIEW (Waterhouse, Procter et al. 2009).

Homology modeling of the Ov-GRK1 protein structure was constructed using the SWISS-MODEL Web server (http://swissmodel.expasy.org). The three-dimensional (3D) structure of Ov-GRK1 protein was built based on the target-template alignment using ProMod3 (Guex, Peitsch et al. 2009). The human G-protein coupled receptor kinase 2 (Thal, Homan et al. 2012) (PDBe ID:3v5w) was selected as the template (sequence similarity: 66.32 % and The QMEAN Z-score : 1.35). The target sequence was searched with BLAST and HHBlits (lightning-fast iterative protein sequence searching by HMM-HMM alignment) against the primary amino acid sequence contained in the SWISS-MODEL template library (SMTL) (Camacho et al. 2009, Mirdita et al. 2017, Steinegger et al. 2019). The global and per-residue model quality has been assessed using the global model quality estimation (GMQE) scoring function (Studer et al. 2020).

To visualize the predicted model, the graphical representations of the protein structures for the Ov-GRK1 structure was created using PyMOL (version 1.3) (DeLanoScientific, San Carlos, CA). Additionally, the physiochemical characteristics such as half-life, number of amino acid, theoretical *pI*, and extinction coefficient for the Ov-GRK1 protein were predicted by the ProtParam tool (https://web.expasy.org/protparam).

3. RESULTS

3.1 Sequencing and Expression analysis of Ov-GRK1 gene

The analysis of the sequencing confirmed the identity of the fragments. All sequence data generated in this study were deposited in GenBank (accession numbers MW483824).

Here, we present molecular evidence of the Ov-GRK1 gene expression in the epithelium rim of different type of *O. vulgaris* suckers (Figure 2;3). RT-PCR amplification revealed that Ov-GRK1 gene is expressed in the suckers and in the skin and retina of *O. vulgaris* (Figure 3). There is a significative difference in the gene expression among different type of suckers: up-regulated in distal big and middle suckers than distal large ones, meanwhile there are no significant differences in the gene expression among skin, suckers distal big and proximal one. Ov-GRK1gene expression in the retina tissue results about 9 fold than in distal big suckers. The heart tissue did not show any expression, and was used as a negative control.

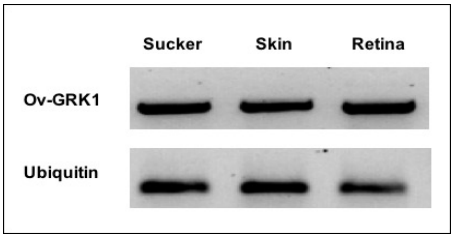

Figure2: RT-PCR analyses showing the tissue distribution of Ov-GRK1 transcripts in *O. vulgaris*. Expression of the house- keeping gene ubiquitin is also shown. All PCR products were subjected to sequencing.

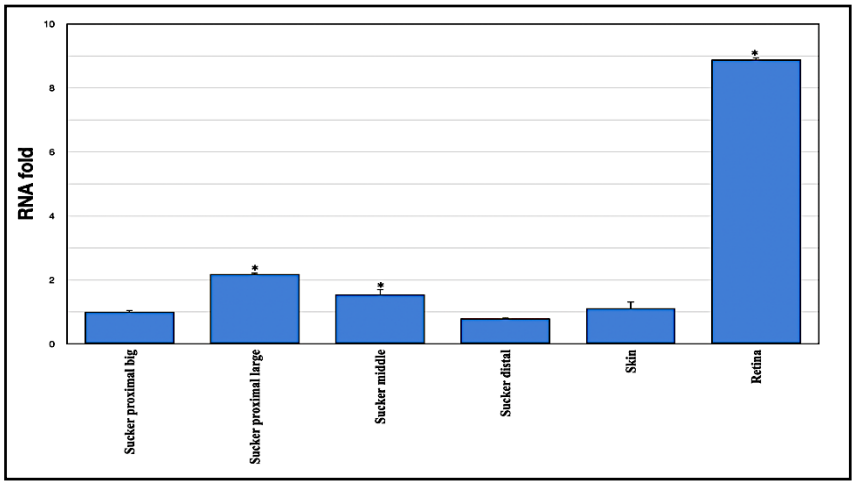

Figure 3: **Figure 2. Gene expression analysis for mRNA of Rhodopsin kinase receptor gene (Ov-GRK1) of different tissues.** *Relative mRNA expression levels were measured using real-time analysis and calculated by the 2(−ΔΔC(T)) method. Each sample was tested and run in duplicate. The heart tissue was used as negative control. The ubiquitin gene was amplified as an internal control. No-template controls were included. Relative mRNA fold change in gene expression was compared to the proximal big sucker (set y = 1). * asterisk indicates that the difference vs. sucker proximal big is statistically significant (Wilcoxon-test, p<0.05). Error bars represent the SEM*

3.2 Localization of Ov-GRK1 transcript by whole-mount in situ hybridization in the sucker of *O. vulgaris*

In order to localize Ov-GRK1 transcript in the sucker of *O. vulgaris* we performed the whole-mount *in situ* hybridizations with probes constructed on the Ov-GRK1 gene.

Ov-GRK1 mRNA was expressed in the epithe m of the arm sucker rim (Fig. 4A, B, C). Ov-GRK1 gene expression was exclusively located around the outer border of the epithelium rim (RIM), but no expression was detected in the epithelium lining of infundibulum (IF) part of the arm sucker (Fig. 4B). This receptor expression is widely and regularly distributed around the epithelium of the rim (Fig. 4B, C). We observe an unusual branched shape (Fig. 3C): a high expression is present in the external portion of the rim, which spread in numerous finger-like and laminar projections in the inner part of the rim (Fig. 4C).

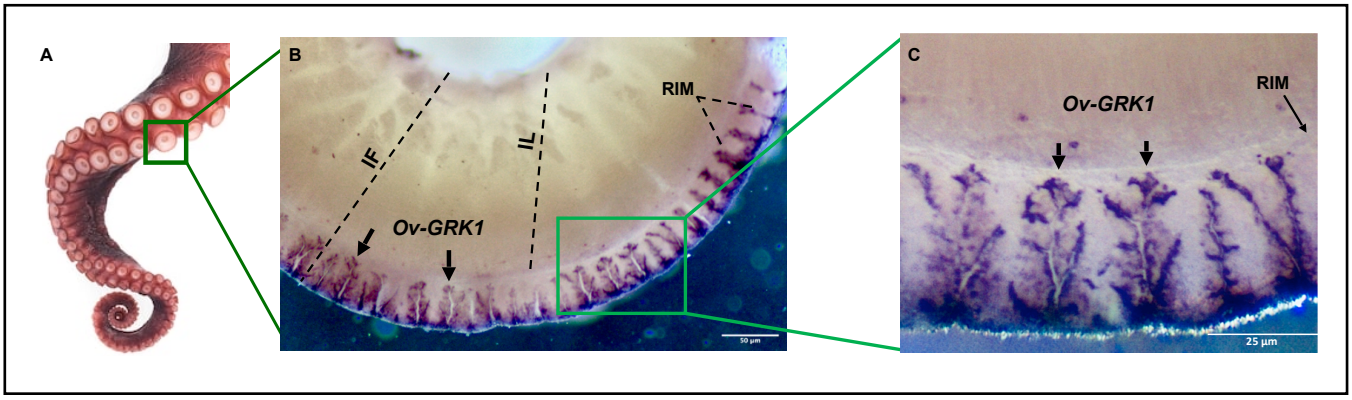

Figure 4:**Expression and localization of *Ov-GRK1* mRNA by whole month *in situ* hybridization in the rim of the sucker and optic lobe of *O. vulgaris***. **A**) Octopus arm with array of the suckers. **B**) The receptor expression is present in the epithelium of the rim (arrows). **C**) higher magnification of the portion of the epithelium rim inside the green rectangle showing *Ov-GRK1* expression. Epithelium of the rim (RIM), the infundibulum part of the sucker (IF), infundibulum lumen (IL). Arrows indicate positive signals.

3.3 Molecular phylogenetic construction

The sequence analysis showed that Rhodopsin kinases from *O. vulgaris* and *O. bimaculoides* were almost identical (99.80% sequence identity). Moreover, *E. scolopes* Rhodopsin kinase identified in eyes (ACB05676.1) and light organ (ACB05677.1) also showed a high similarity with rhodopsin kinase of *O. vulgaris* (92.33%, and 92.04% of sequence identity, respectively), revealing a high similarity in genes among species (Figure 5).

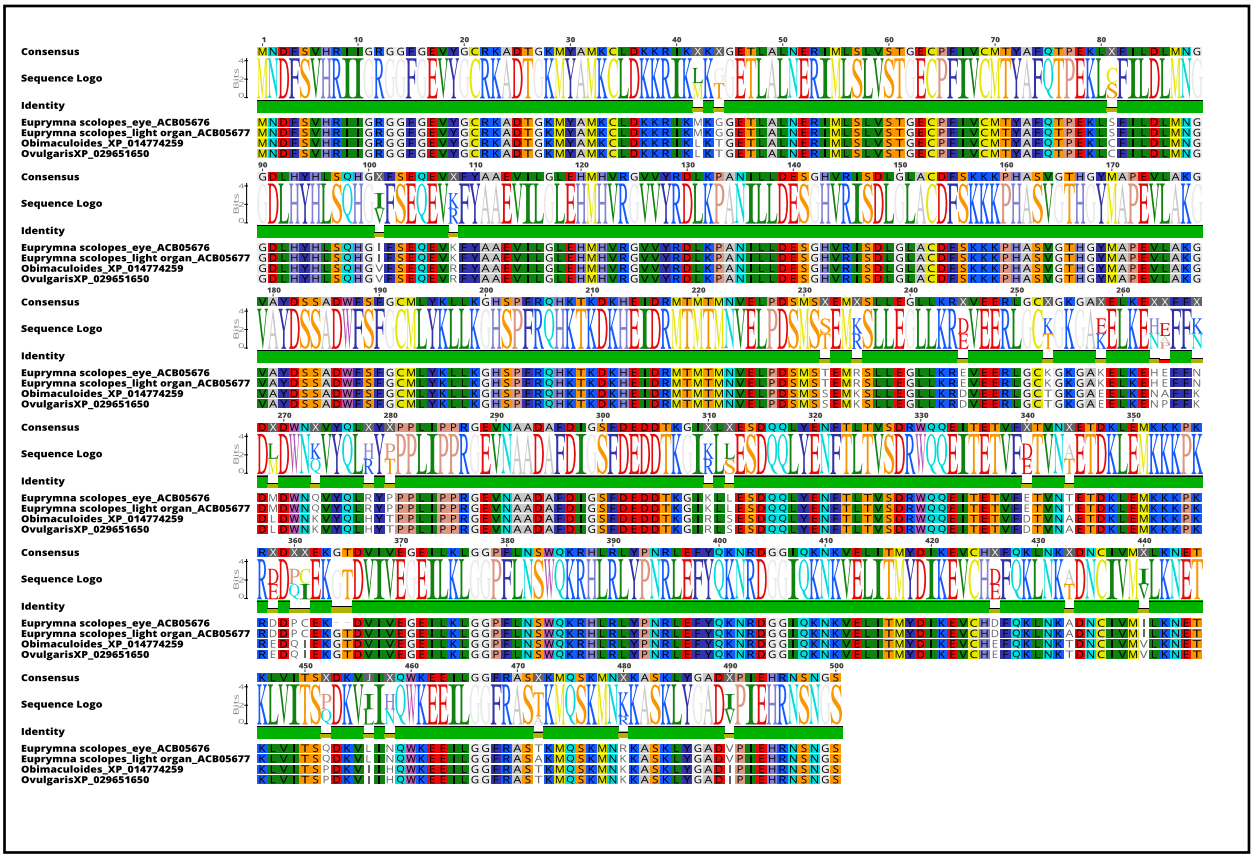

Figure 5: Alignment of Ov-GRK1 amino acid sequences to an *O. bimaculoides* rhodopsin kinase amino acid sequence from GenBank (accession number XP014774259) and to those of *E. scolopes* Rhodopsin kinase extracted from through eyes (ACB05676) and light organ (ACB05677). White line boxes highlight potential amino acids differences among samples. Complete open reading frames are shown

The phylogenetic trees of the Ov-GRK1 genes were reconstructed using maximum likelihood (ML) and Bayesian methods. Given their similar topologies. The Bayesian tree was displayed in Figure 5 because of its higher support values. The analysis of the sequencing confirmed the identity of the fragments. All sequence data generated in this study were deposited in GenBank. Interestingly, the phylogenetic tree shows that Ov-GRK1 is closely related to other GRK1 identified in other cephalopods, including *O. bimaculoides* and *E. dofleini*

(Figure 6). In particular, Ov-GRK1 gene in cephalopod is clearly evolutionary distinct from ecdysozoans and vertebrates GRK1 family .

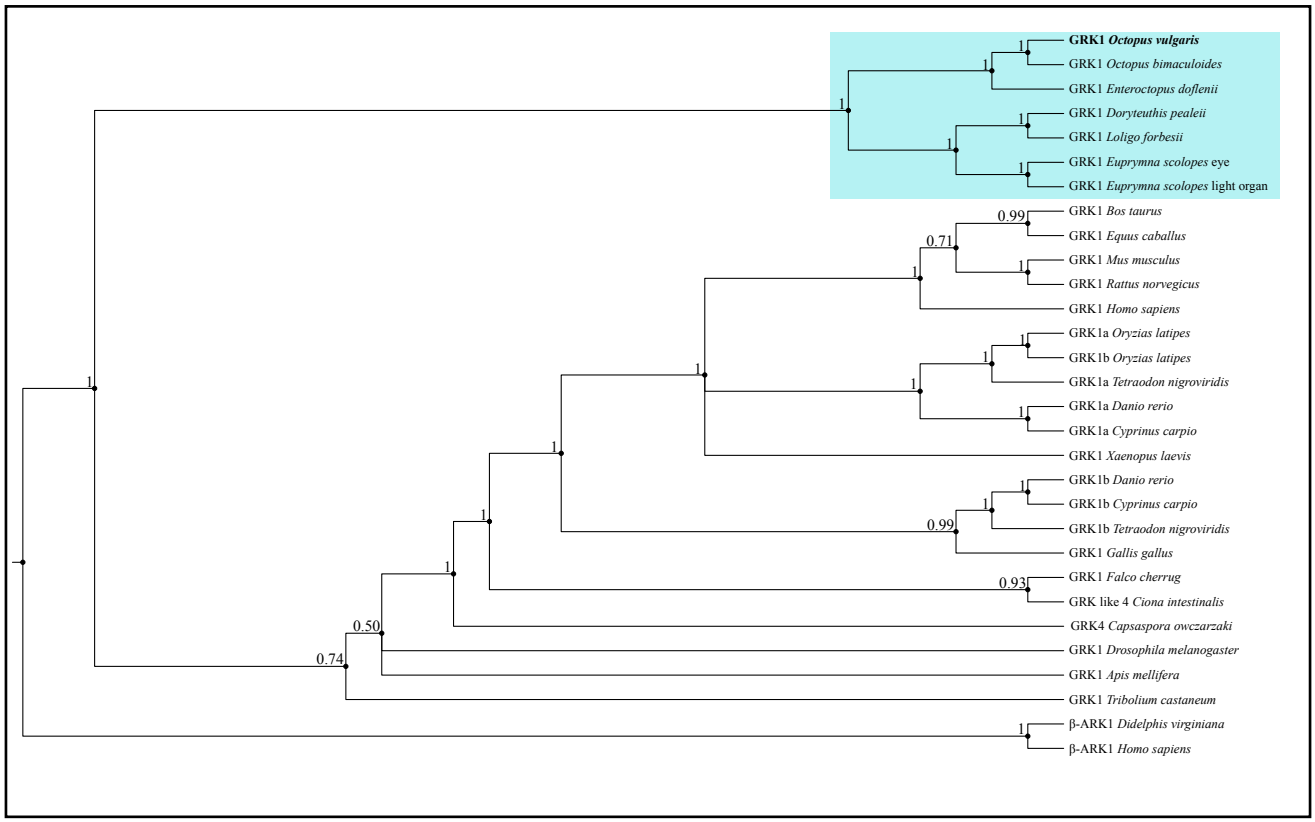

Figure 6: **Bayesian tree of GRK protein sequences**. Bayesian phylogenetic tree performed with LG+G model, constructed with protein sequence from Ov-GRK1 and Rhodopsin kinase receptors of 18 species including other cephalopod, invertebrate and vertebrate. All protein sequences were obtained from GenBank and the accession numbers are presented in Table S1. Bayesian posterior probabilities are represented over nodes; blue box highlights the cephalopod clade and the Ov-GRK1 protein sequences is in bold.

3.4 Prediction of 3D structure model for Ov-GRK1

Due to the lack of availability of a three-dimensional model (3D) structure for octopus GRK1 in the protein database (PDB), the present study predicts 3D structure for Ov-GRK1 protein (Fig. 7B). The predicted model was constructed based on the crystal structure G-protein coupled receptor kinase 1 (GRK1) (6c2y.1.A) showed the maximum sequence identity (66.32 %) to the query protein Ov-GRK1 (Fig. 7A). GRK2 is an enzyme that in humans is encoded by the ADRBK1 gene and it was initially called Beta-adrenergic receptor kinase (βARK or βARK1). Our results showed that the Beta-adrenergic receptor kinase (β-ARK1; modern name GRK2) was the best template obtained through with BLAST and HHBlits (lightning-fast iterative protein sequence searching by HMM-HMM alignment) against the primary amino

acid sequence contained in the SWISS-MODEL template library (SMTL). Ov-GRK1 has been modeled with high accuracy (coverage: 0.98; the global model quality estimation (GMQE) score: 0.77) using a single highest scoring template.

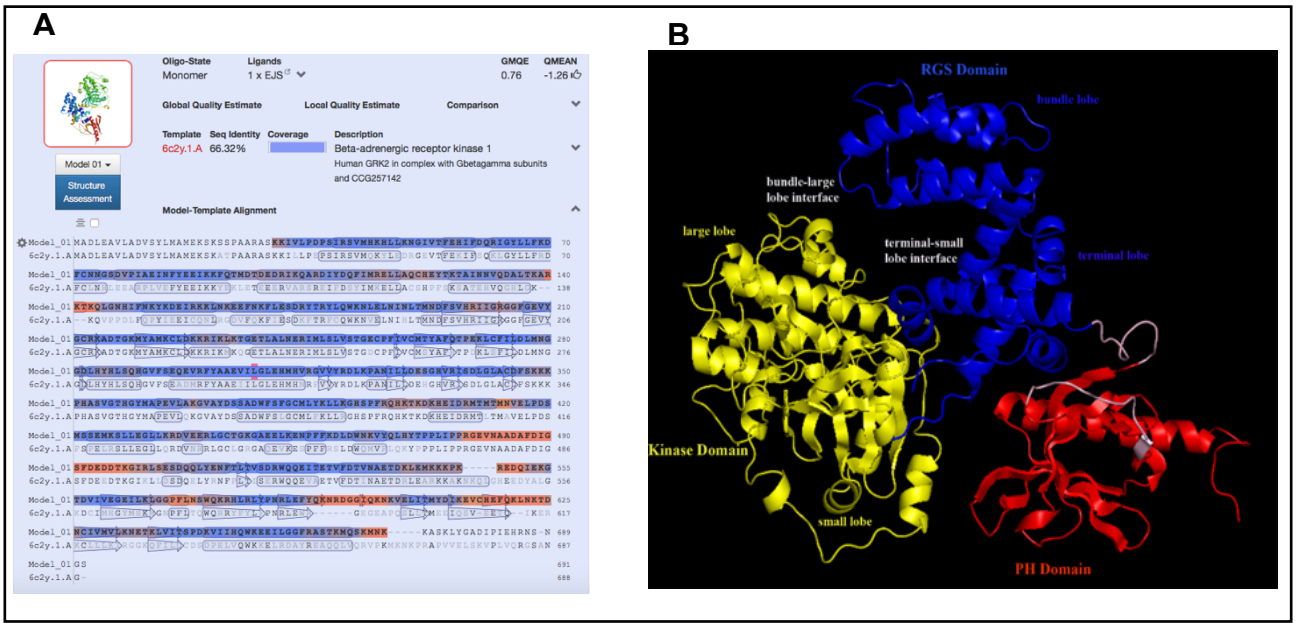

Figure 7: Homology modeling of the Ov-GRK1: (A) Alignment with a potential Model-Template (6c2y.1.A). **(B)** 3D structural view for the prediction of ligand binding sites in Ov-GRK1 generated by 3DLigandSite based on the PDB.

The obtained model is characterized by the presence of three main domains: the RGS domain (Fig. 6B, colored in blue), which comprises two subdomains referred as the bundle lobe and the terminal lobe; the kinase domain (Fig. 7B, colored in yellow), which comprises two subdomains called the small and large lobe; the PH domain (Fig. 7B, colored in red). There are two distinct interfaces between the regulator of G-protein signaling (RGS) and kinase domains; the larger contact interface is between the terminal lobe of RGS and the small lobe of the kinase domain, with a sequence identity and conservation between β-ARK1 and Ov-GRK1 extremely high; the smaller interface is between the bundle lobe of RGS and the large lobe of the kinase, also this contact area is highly conserved. The terminal lobe of RGS also forms an extensive contact interface with the PH domain, which is also highly conserved. The C terminal domain of the protein, located in the domain PH is not structured as expected, because it is likely to become ordered only upon interaction with G β γ subunits. The kinase domain is highly conserved while the RGS and PH domains present the main sequence

differences, this is in agreement with previous finding that the RGS and PH domains of β-ARK1 move as a single domain with respect to the kinase domain between the active and inactive structures (Lodowski, Barnhill et al. 2005). The highest sequence differences characterized by deletions and insertions of amino acids are located in the PH domain in a region of high conformational variability (Figure 3B, colored in pale pink). These differences are not contiguous in the sequence but constitute a surface, which is likely to be peculiar too.

Furthermore, the predicted molecular weight of the Ov-GRK1 was estimated as 79.6 kDa by the ProtParam tool (https://web.expasy.org/protparam), which is similar to that of β-ARK (80 kDa) (Benovic et al. 1987). The physicochemical characteristics such as half-life, number of amino acids, theoretical *pI* (isoelectric point), and extinction coefficient for Ov-GRK1 protein were predicted.

4. DISCUCSION

Classically, the function of sensory receptors foresees their confinement to the sensory organs where they were initially identified. For instance, when we talk about photoreceptors immediately, we link them to the eyes. However, in most animals, the ability to detect and use light for different biological purposes is mediated by visual pigment molecules that act as light sensors for visual and non-visual functions (Shichida and Imai 1998, Oakley and Speiser 2015). In cephalopods, various sensory photoreceptor molecules have been found outside their classical sensory organs, where they respond to different stimuli, initiating signaling cascades in these extraocular systems. There are several well-studied extraocular photoreceptors including rhodopsin in the light organ of cephalopods, parolfactory vesicles of squids, and visual opsins (rhodopsin, retinochrome, Gq-coupled opsin) in their skin that are believed to intrinsically contribute to light detection and likely to dermal patterning suggesting the presence of an extraocular vision in these animals (Hara and Hara 1980, Ban et al. 2005, Kasai and Oshima 2006, Tong et al. 2009, Chen, et al. 2013 a,b, Kingston et al. 2015, Kingston et al. 2015, Ramirez and Oakley 2015).

Thus, the visual system in cephalopods appear not confined to the canonical visual organ but includes extraocular systems that contribute to their complex behavioral interactions, reflecting the important role that light/picture perception plays in their life, in term of social communication, hunting, and adaptive coloration (Hanlon and Messenger 2018).

The recent availability of genomic sequencing for the four octopus species including *O. bimaculoides* (Albertin et al. 2015), *O. vulgaris* (Zarrella et al., 2019), *O. minor* (Kim et al. 2018), and *O. sinensis* (Li et al. 2020) allowed us to identify the rhodopsin kinase (GRK1) in *O. vulgaris*. This molecule is a member of G protein-coupled receptors that recognizes light. It is found primarily in mammalian retinal rod cells, where it phosphorylates light-activated rhodopsin, and is officially named G-protein-coupled receptor kinase 1, or GRK1.

In the current study, for the first time we found the expression of *O. vulgaris* rhodopsin kinase (Ov-GRK1) in ocular and extraocular tissues. We evaluated its different expression among retina, skin and different type of suckers in adult specimens (Figure 1).

The major novelty of our finding is the Ov-GRK1 mRNA expression at level of arm suckers. Furthermore, we found and showed a significative differential expression in selected suckers (proximal big sucker; proximal large sucker; middle sucker; distal sucker) belonging

to different arm segments respectively. Among arm suckers we found Ov-GRK1 expression up-regulated in proximal large and middle suckers, concerning the proximal big one (Figure 2). On the contrary, there is no significant difference in the gene expression distal sucker and in the skin. This detailed analysis covering arm suckers lets us conclude that Ov-GRK1 is functionally active throughout the arm. Not surprisingly the Ov-GRK1 expression results highly up regulated in retina, about nine fold that PROX-S.

It is known that the epithelium rim of the arm sucker of *O. vulgaris* contains a variety of specialized sensory receptor cells allowing the octopus to perceive in parallel the massive amount of environmental cues. Graziadei and Gagne (Graziadei and Gagne 1973, Graziadei and Gagne 1976) described four types of primary receptors and based on their morphology they assigned them an hypothetic function to each. In their works they described among others an "an-usual receptor" (designated as Cell Type 3 in (Graziadei and Gagne 1976)) that they hypothesize to be a light sensitive receptor. Given the unexpected peculiar expression in the sucker rim of Ov-GRK1, using whole mount in situ hybridization, we interestingly observed that the labeling resemble exactly the photoreceptor type described for the first time by Graziadei and Gagne (Graziadei and Gagne 1973, Graziadei and Gagne 1976) in octopus sucker. Due to the expression of Ov-GRK1 we can finally attribute the photoreceptive function to that structure without any doubt.

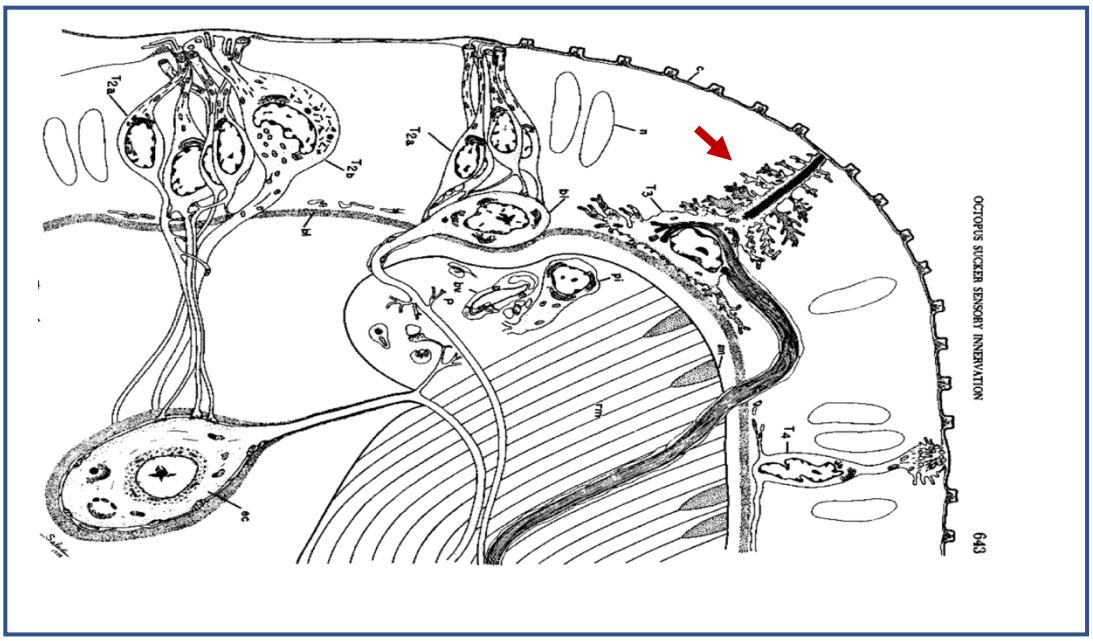

Figure.7. Summary of the four specialized sensory receptor cell types (T2a, T2b, T3 and T4) found in the epithelium rime of the sucker has been investigated by Graziadei and Gagne,1976. The cell type with which we are concerned here is cell Type 3, which occurs in the epithelium over the infundibular muscle (indicated by arrow).

These data led us hypothesize the extension of the distribution of extraocular photoreceptors to octopus suckers; moreover, the regular distribution all around the sucker rim of Ov-GRK1 expression (Fig.3) suggests that octopus could detect light in a steric manner allowing it the improve the reconstruction of the surrounding environment.This capability could enormously affects the predatory performance of octopuses allowing them to track down a hidden prey detecting their presence in dark burrows profiting by their bioluminescence (Herring 1976, Bessho-Uehara, et al. 2020).

Interestingly previous studies of the transcriptome of the light organ in *E. scolopes* have shown that the expression of several genes encoding visual transduction proteins includes the same isoform of opsin that occurs in the retina (Tong et al. 2009). Therefore, we estimated the percent identity and similarity of our Ov-GRK1 protein to the protein sequence of the rhodopsin kinase expressed in the light organ and retina of *E. scolopes* (Fig. 4). The percentage identity and similarity among the amino acid sequences of rhodopsin kinase in the epithelium sucker rim of *O. vulgaris*, the light organ, and retina of *E. scolopes* revealed over 92 % sequence identity.

The phylogenetic tree obtained using the Bayesian method (Fig. 5) clearly shows that Ov-GRK1 appears to be along with another cephalopod rhodopsin kinase branch in the same clade, and not surprisingly results in the nearest neighbor to rhodopsin kinase receptor described in *O. bimaculoides*.

Finally, In an effort to explore the molecular and enzymatic properties of Ov-GRK1, we predict the in silico 3D structure of Ov-GRK1 protein for the first time as an invertebrate enzyme based on homology modeling technique. The availability of structural model of a protein is one of the keys for a deep understanding biological processes of Ov-GRK1 protein at molecular and structural level. Here, homology modeling data showed that the maximum sequence identity (66.32%) to the query protein Ov-GRK1 with GRK2 is an enzyme that in humans is encoded by the ADRBK1 gene and it was initially called Beta-adrenergic receptor kinase (βARK or βARK1) (Fig. 6A). It has been demonstrated that many G protein-coupled receptors such as βARK and rhodopsin kinase (RK) are both known to be phosphorylated in a totally light- or agonist-dependent manner by a member of the specific protein kinase family called G protein-coupled receptor kinases (GRKs) (Benovic, et al. 1986, Haga, et al. 1994). This

stimulus-dependent phosphorylation of the receptors is thought to be involved in the desensitization of these receptors (Lefkowitz 1993, Haga, et al. 1994). In our study, the obtained 3D model is characterized by the presence of three main domains: the RGS domain (Fig. 6B, colored in blue), which comprises two subdomains referred to as the bundle lobe and the terminal lobe; the protein kinase domain (Fig. 6B, colored in yellow), which comprises two subdomains called the small and large lobe; and the PH domain (Figure 6B, colored in red) that is responsible of the interaction with G protein G β γ -subunits and plasma membranes upon phosphorylation of substrate receptors. Our results are similar to those obtained in a previous study in retina of *Octopus dofleini* in which it is reported that octopus rhodopsin kinase (ORK) has markedly enhanced by GTP (Tsuda, Hirata et al. 1992) suggesting that ORK could be activated by G β γ -subunits of a photoreceptor G protein. Furthermore, the predicted molecular weight of the Ov-GRK1 is estimated as 79.6 kD, which is consistent with the molecular mass predicted from the sequence of rhodopsin kinase retinal photoreceptors in *O. dofleini* (80 kDa) (Kikkawa, et al. 1998).

In conclusion, through multidisciplinary approaches using a combination of different techniques, we show the complete characterization of a light-sensing molecule, Ov-GRK1, in several tissues of *O. vulgaris*. The sequence, its phylogenetic relation, the differential expression pattern and localization, together with the 3D structure provide evidence of diffused light-sensing capability in this animal. In particular, the main achievement of this work is the peculiar localization of Ov-GRK1 expression in the sucker rims throughout the octopus arms including these structures among the extraocular light sensing organs of cephalopods. Moreover, our finding support that Octopus suckers have molecular machinery and physiological potential to respond to many different environmental clues indicating that they evolved as multimodal sensory structure able to detect light- meccano- and chemical stimuli.

CHAPTER (5)
Localize and Mapping for Photoreceptor Molecules in The Optic Lobe of *O. vulgaris*

1- Introduction

The optic lobes are the largest brain areas within the central nervous system (CNS) of coleoid cephalopods takes up about two-thirds of the total brain mass (Boycott, 1961; Young, 1962, 1974), and they play important roles in the processing of visual information visuomotor control, the regulation of body patterning, and locomotive behavior (Boycott, 1961; Chichery and Chanelet, 1976, 1978; Chung and Marshall, 2017). The optic lobe is located immediately behind the eyeball and receives visual signals from the retina directly (Boycott, 1961; Young, 1962, 1974). The camera-like eyes of the octopus are a classic example of convergent evolution with vertebrates. Octopus eyes have a cornea and aspherical lens, with excellent visual acuity, that inverts the image of the world onto the retina. The light-absorbing photoreceptors that line the retina employ single rhodopsin (Brown and Brown, 1958; Chung and Marshall, 2016). Processes from these photoreceptors from multiple optic nerves, which leave the orbital cavity and cross dorsoventrally before innervating the optic lobes, thereby reinverting the visual image onto the outer layers of the optic lobe.

The optic lobe is generally divided into two parts, the outer cortex and the central medulla (Boycott, 1961). The cortex also called the deep retina (Cajal, 1917), receives visual signals directly from the retina. It consists of two cell-rich granular layers with a single fiber-rich plexiform zone in-between (Young, 1962, 1974). It covers most of the optic lobe surface except for the optic tract region. In contrast, the medulla can be separated into two major zones, the outer radial column zone and the central tangential zone (Young,1974). The radial column zone is composed of numerous columnar structures of stacked cell somata and neural fibers (Young, 1974). In contrast, the tangential zone is less organized and has many clustered cell soma regions (also called the "cell islands") that are surrounded by neuropils (Young, 1974). The optic lobe then sends the output signals to other brain lobes including downstream motor centers for further information processing and the control of visual behavior (Young, 1974).

In cephalopods, there is a diversity of photoreceptor molecules and their shape, physiology and visual function are different. In the genome of *O. bimaculoides* different types of photoreceptors including rhodopsin, rhabdomeric opsin, peropsin, and retinochrome have

been detected (Albertin et al. 2015). It is suggested that the variety of shapes of the dendritic trees within the optic lobes provides the elements of the coding system by which visual input is classified. The functioning of these photoreceptors are of particular interest.

It has been described as the "simple cephalopod retina" where the major function of the photoreceptor layer was to receive visual inputs, while all visual processing is conducted to the optic lobe (Young, 1963). Organization and localization of the photoreceptor molecules in the optic lobes is thus essential for our understanding of the molecular basis of the visual perception in octopus. Although our overall understanding of optic lobe structure and function, the detailed neural organization of photoreceptor molecules has not been characterized. Also, our understanding of the basis of the vision ability in octopus is still quite limited. In this study, we have mapped and localized the expression of mRNA transcripts for photoreceptor molecules including retina rhodopsin, rhabdomeric opsin, melanopsin, retinochrome, and rhodopsin kinase throughout the entire optic lobe of *O. vulgaris*.

1- Materials and Methods

2.1 Animal acquisition and preparation

Adult specimens of *O. vulgaris* (N=3) (body weight 800g±50g, mean ± SD) were collected from the Bay of Naples (Italy) and transferred to the Di Cosmo's cephalopod facility at the Department of Biology, the University of Naples Federico II (Italy). Octopuses were anesthetized by isoflurane insufflation (Polese et al., 2014) and tissues were dissected under sterile conditions following institutional guidelines.

The brain of octopus was dissected and the optic lobe were isolated then and a fixative 4% PFA (4% Para-formaldehyde in PBS, pH 7.4) was added directly at 4 °C overnight for subsequent whole-mount *in situ* hybridization. Fixed tissues were dehydrated in a graded methanol series (25% MeOH; 50% MeOH; 75% MeOH and 100% MeOH) with 1X PBST (Phosphate-buffered saline with 0.1% Tween-20) for 15 min each and stored in 100% methanol at -20°C until use.

Our research is approved to 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).

2.2 Probe preparation

To generate the Digoxigenin (DIG)-labeled single-stranded RNA probes, we performed PCR standard method using specific primer set (Table 1).The PCR fragments were used as templates for *in vitro* transcription reaction using the T7 RNA polymerase promoter sequence corresponding to forward and reverse primers for the sense and antisense probes, respectively. PCR cDNA fragments were isolated by 1.2% agarose gel and used as templates for *in vitro* transcription reaction. RNA transcription reaction was performed using the DIG-RNA Labelling Kit (SP6/T7) (Roche Applied Sciences, Laval, QC, Canada) following the manufacturer's instructions. Final probes were cleaned up using RNeasy MinElute Cleanup Kit (Qiagen USA), and one microliter was visualized on a 1.5% agarose gel to estimate concentration

Table 1: Primers amplicons used in this study

Gene name	bp
Retina Rhodopsin	**354**
Retinochrome	**428**
Melanopsin-A-like	**353**
Rhabdomeric Rhodopsin	**270**
Rhodopsin Kinase	**518**

2.3 Whole-mount *in situ* hybridization

The procedure for whole-mount *in situ* hybridization was based on our modified protocol, fixed optic lobes were rehydrated by descending methanol series in 75%, 50%, and 25% MeOH in PBST for 15 min each at RT. Completed rehydration was performed twice in 100% PBST for 10 min each with gentle rocking. Tissues were incubated and digested in the detergent mix (20 µg/ml in PBST Proteinase-K) at 37°C for 20 min, post-fixed in 4% PFA for 20 minutes at room temperature, then washed three times in PBST for 5 min each. Tissues were pre-hybridized for 2hr and hybridized overnight at 62°C in hybridization solution (50% formamide, 5X saline-sodium citrate (SSC), 1X Denhardt's solution, 500mg/ml yeast tRNA and 500 mg/ml salmon sperm DNA). After hybridization, the tissues were incubated with 20µg/ml RNAs A (Invitrogen 12091021) for 15 min at 37°C, then subjected to a series of post-hybridization washes in decreasing concentrations of SSC with 0.1% Tween 20. Tissues were blocked in 1X blocking solution (Roche Applied Science 11096176001) in PBST for 1 hr. at room

temperature under gentle rocking, followed by incubation in 1:2500 Anti-Digoxigenin-AP antibody (Roche 11093274910) in blocking solution overnight at 4°C on a rocker.

Tissues washed in PBST five times for 25 min each, equilibrated in alkaline phosphatase buffer (AP) (100mM NaCl, 50mM $MgCl_2$, 100mM Tris, pH 9.5, 0.1% Tween-20) at room temperature. The color reaction was performed in NBT/BCIP stain solution (Roche 11681451001) in AP buffer under the light-resistant environment until the colors reached satisfactory intensity. Control specimens were left in staining solution for the same time interval as those incubated with anti-sense probes. In order to test for nonspecific labeling, negative control experiments were performed for each condition using hybridization buffer only without probe.

2.4 Microscopy and image analysis

After coloration reaction, all tissues were passed through ascending concentrations of ethanol in PBS to remove background and darken the specific signal, re-hydrated in PBS. Whole-mount optic lobes with DIG-labeled probes were observed colourimetrically under a Carl Zeiss Stemi 305 stereomicroscope with Axiocam ERc 5s. Whole images were enhanced for clarity in photos program (Version 3.0). Figures were created using Adobe Illustrator and Microsoft PowerPoint.

3. Results

3.1. Expression of Retina Rhodopsin mRNA in the outer cortex and radial column zone of the optic Lobe of *O. vulgaris*.

Retina Rhodopsin mRNA appear to be highly localized and expressed in the outer cortex (OC) and the radial column zone (R.Z) of the optic lobe of *O. vulgaris*. Expression of Retina Rhodopsin encoding transcript appears to be scattered at radial column zone (R.Z) of the optic lobe (Figure 1A-D).

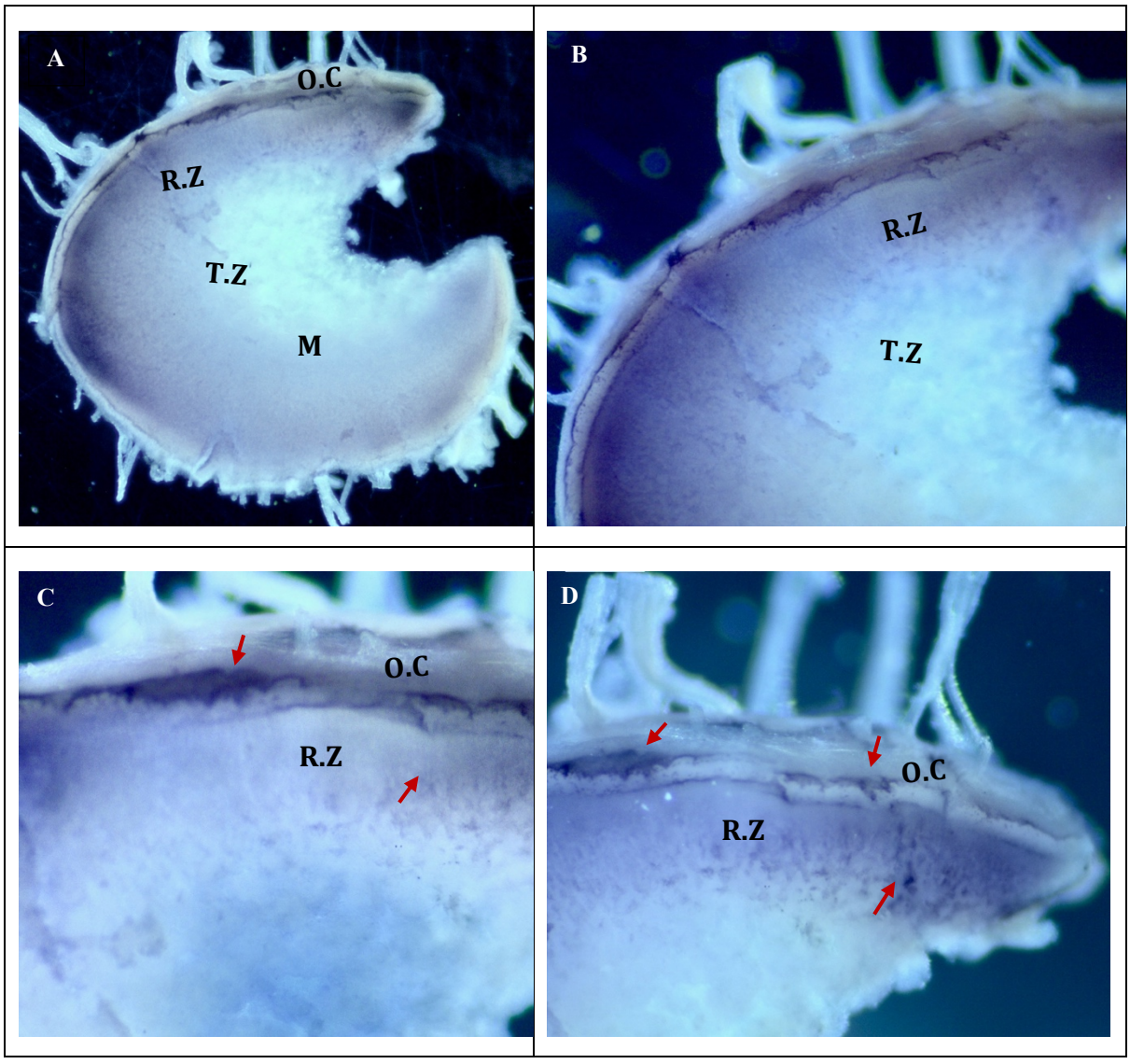

Figure 1: Localization of the expression of Retina Rhodopsin mRNA in in the optic Lobe of *O. vulgaris* by whole-mount *in situ* hybridizations. The expression of Retina Rhodopsin mRNA is observed in the outer cortex (OC) and the radial column zone (R.Z) of the optic lobe, but no positive neurons are visible in the optic tract region (OT) and the central tangential zone(T.Z).

3.2 Expression of Retinochrome mRNA in the outer cortex and the optic tract region in the optic Lobe of *O. vulgaris*

Retinochrome mRNA transcript was detected in the outer regions and the outer cortex (OC) of the optic lobe. Also the optic tract region (OT) of the optic lobe shows positive neurons (figure 2 A-D). Although a few expression of the retinochrome mRNA is visible at the radial column zone (R.Z) of the optic lobe, but no any positive neurons are visible at the central tangential zone of the medulla () in the optic lobe (T.Z).

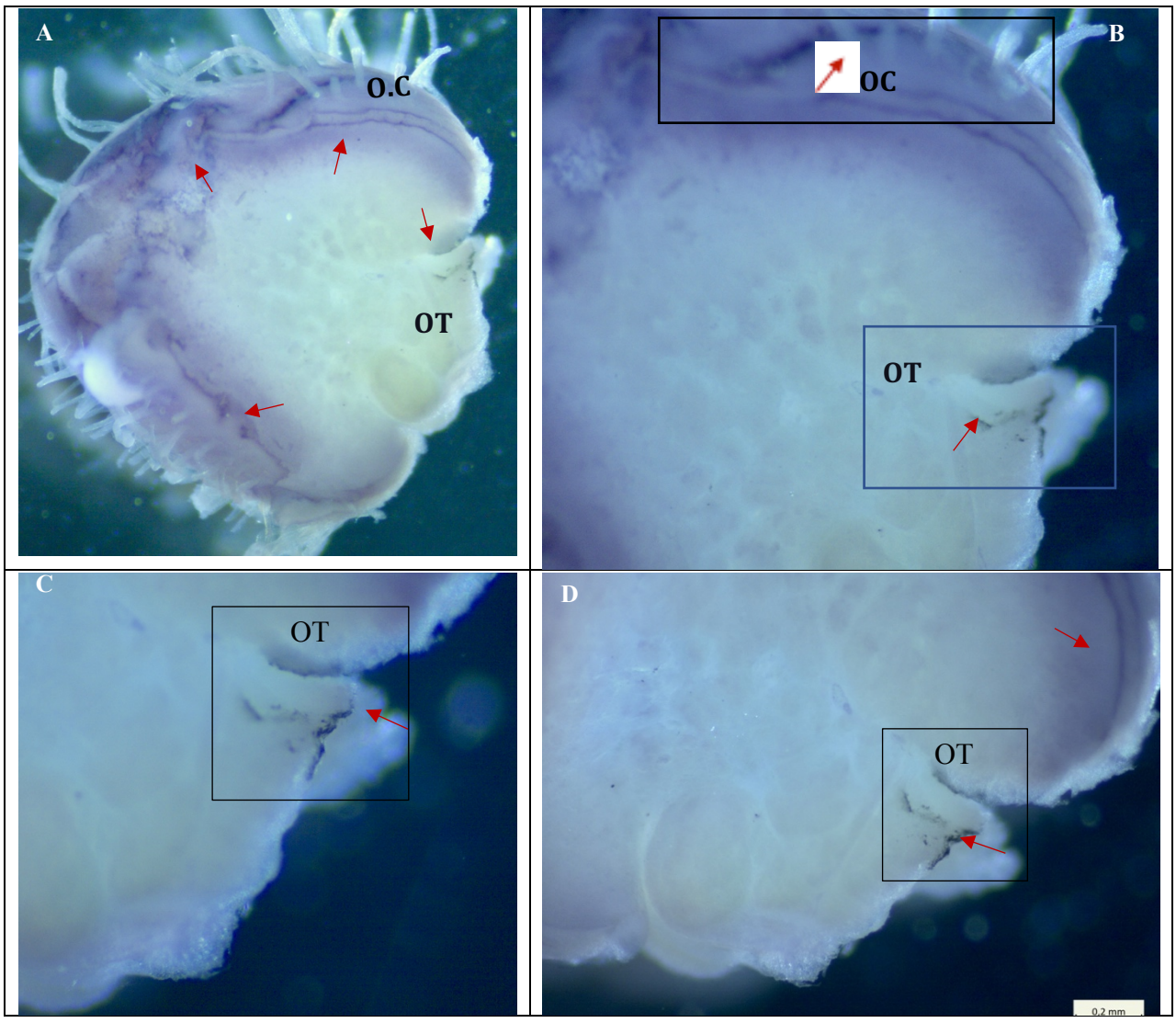

Figure 2: Localization of the expression of Retinochrome mRNA in the optic Lobe of *O. vulgaris* by whole-mount *in situ* hybridizations. The expression of Retina Rhodopsin mRNA is the outer regions and the outer cortex (OC) of the optic lobe. The optic tract region (OT) of the optic lobe shows positive neurons (figure 2 A-D).

3.3 Expression of Melanopsin mRNA highly expressed and localized at the optical tract region in the optic lobe of *O. vulgaris*

The Melanopsin transcript mRNA showed to be located and highly expressed at the optical trace region of the optic lobe. It also found to be mostly distributed on the radial column zone (R.Z) of the medulla of the optic lobe.

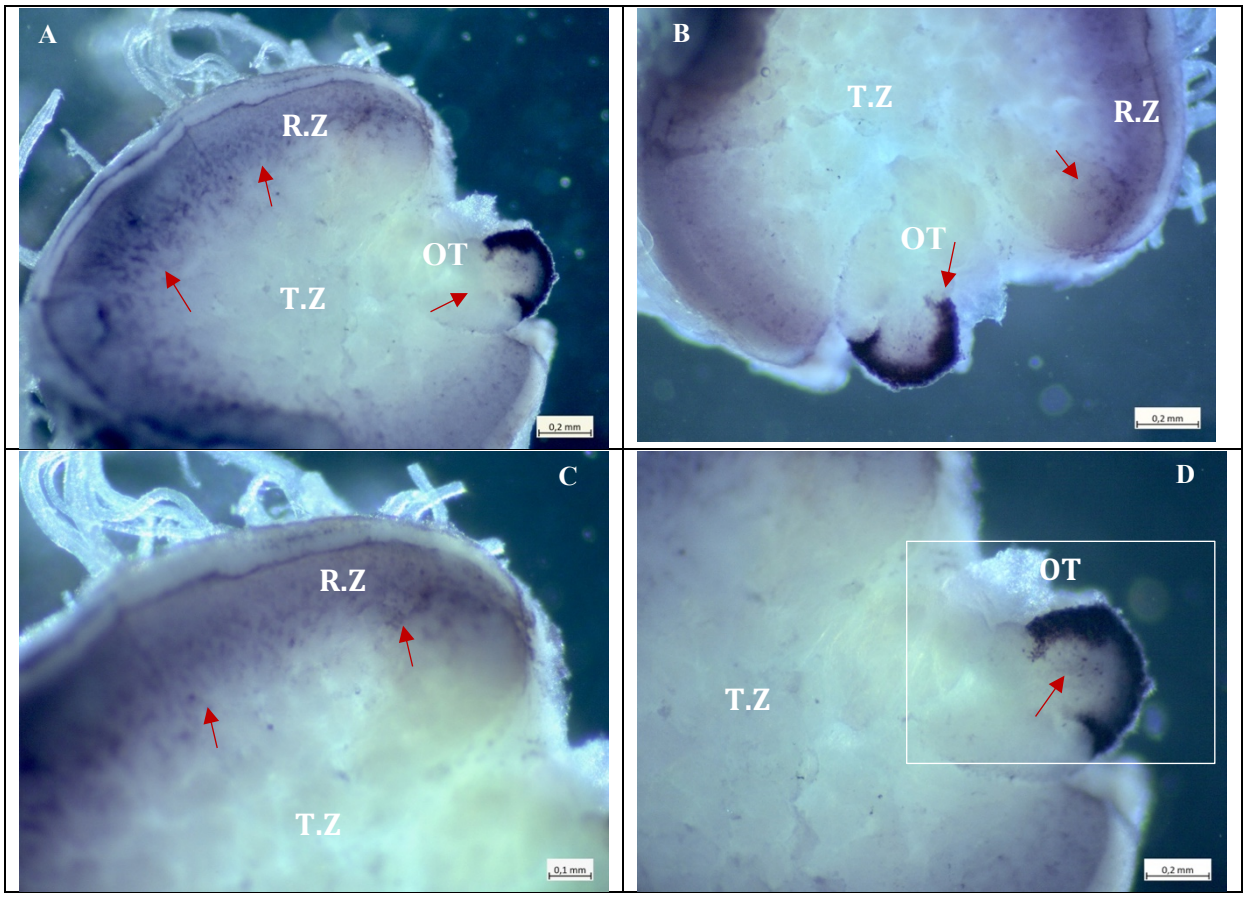

Figure 3: Localization of the expression of Melanopsin mRNA in the optic Lobe of *O. vulgaris* by whole-mount *in situ* hybridizations. The expression of Melanopsin mRNA showed to be highly localized at the optic tract region (OT) of the optic lobe shows positive neurons (Figure 2 A,B,D). It also found to be mostly distributed on the radial column zone (R.Z) of the medulla of the optic lobe (Figure 2C).

3.4 Expression of Rhabdomeric photoreceptor mRNA intensively expressed and localized at the radial column zone (R.Z) in the optic lobe of *O. vulgaris*

The expression of Rhabdomeric mRNA photoreceptor is highly localized in the cortex and the radial column zone of the medulla (R.Z) of the optic lobe. However, no *in situ* hybridization positive signals are found at the inner medulla (the central tangential zone (T.Z)) or the optic tract region of the optic lobe of *O. vulgaris.*

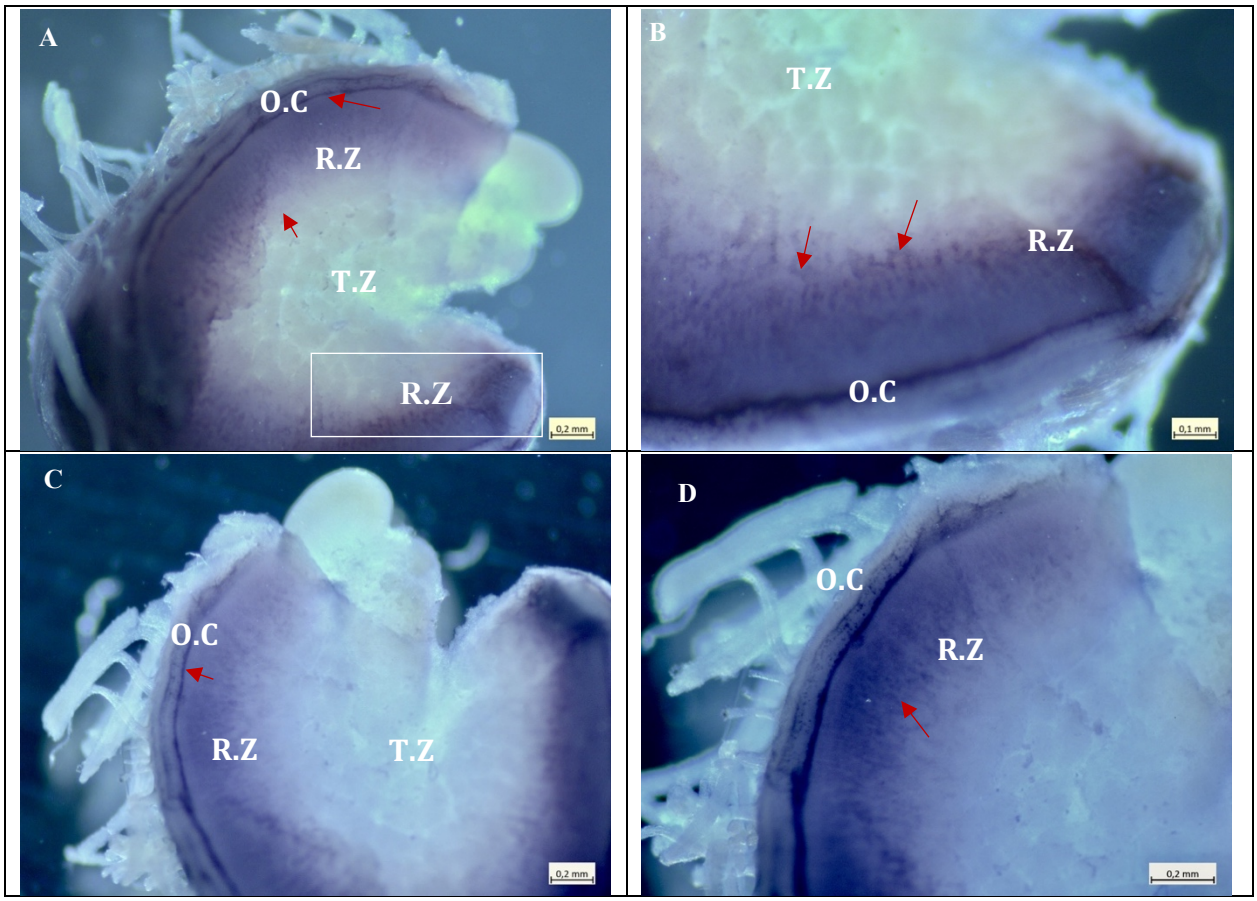

Figure 4: Localization of the expression of Rhabdomeric mRNA in the optic Lobe of *O. vulgaris* by whole-mount *in situ* hybridization. The expression of Rhabdomeric transcript is highly localized in the cortex and the radial column zone of the medulla (R.Z) of the optic lobe (fig. A-D). No ***in situ* hybridizations** signals are visible at the optic tract region (OT) and the central tangential zone (T.Z)of the optic lobe (fig. A-D).

3.5 Expression of Rhodopsin Kinase mRNA intensively distributed , expressed and localized at the radial column zone (R.Z) and the inner medulla in the optic lobe of *O. vulgaris*

The transcript localization of rhodopsin kinase mRNA through whole-mount *in situ* hybridization showed several positive neural cells scattered in the radial column zone of the medulla (R.Z) and the inner medulla (the central tangential zone (T.Z)) (Figure 5). the outer layer appeared rich of intensely positive cells as the most intensely stained areas in the optic lobe (Figure 5). Also the Rhodopsin Kinase mRNA transcript found to be localized at the optic tract region of the optic lobe of *O. vulgaris* (Figure 5).

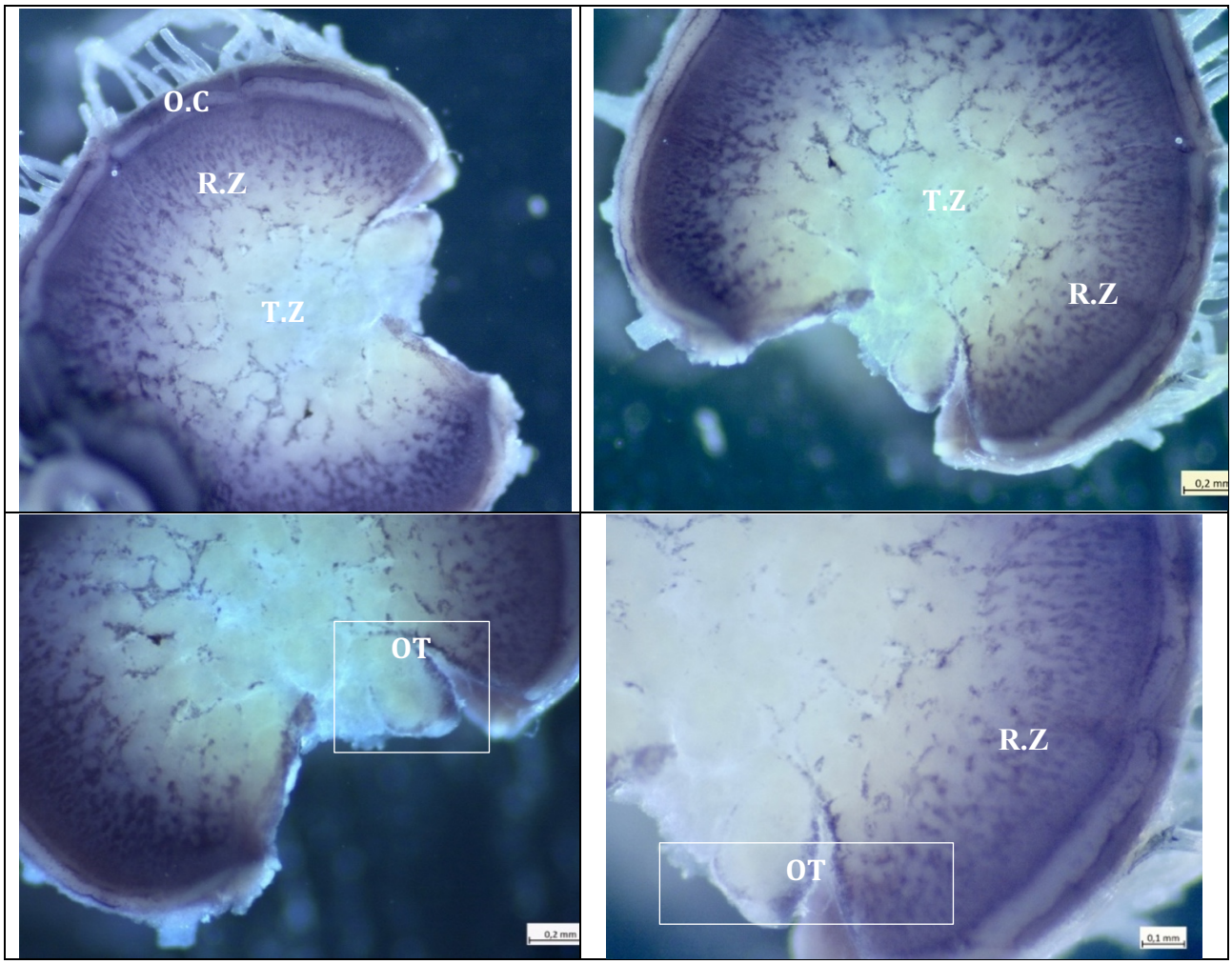

Figure 5: Localization of the expression of Rhodopsin Kinase mRNA in the optic Lobe of *O. vulgaris* by whole-mount *in situ* hybridization. The expression of **Rhodopsin Kinase** transcript is highly localized in the cortex and the radial column zone of the medulla (R.Z) and the central tangential zone (T.Z) of the medulla in the optic lobe (fig. A-D). ***In situ* hybridizations** signals of **Rhodopsin Kinase transcripts** are visible at the optic tract region (OT) of the optic lobe (fig. A-D).

4 Discussion

The vision in cephalopods including squid, cuttlefish, and octopus seems to be one of the major senses for their complex behavioral interactions making them extraordinarily skilled predators for various aspects of life (Hanlon and Messenger, 2018). Octopuses possess sophisticated eyes with keen visual acuity, and complex visual processing in the optic lobes (Hanlon and Messenger, 1996; Grable et al., 2002; Zylinski et al., 2009; Yoshida et al., 2015).

The optic lobes is the largest structure in the octopus brain, a pair of the large nervous structures located outside the cartilaginous capsule of the brain and connected to the retinae of the eyes, reflecting the importance of visual information to the behavior of this animal (Young, 1985; Young, 1960, 1971; Wells, 1966a; Maddock and Young, 1987). The optic lobes provide a system for the computation of visual input, including coding the visual input from the retinal photoreceptors, storing a record of it, and decoding to produce particular motor responses. The visual system of both vertebrates and invertebrates consists of light-sensing photoreceptor neurons in the retina connected to the inner layers of neurons to form a precise retinotopic map for visual information processing (Triplett et al., 2012; Reese, 2011; Chedotal and Richards, 2010). While the general morphology and the neural organization of the optic lobe in adult octopus have been well described (Young, 1962), the localization of light-sensing photoreceptor molecules and examine their expression in the optic lobe of *O. vulgaris* have not been characterized. In the present study, we evidenced expression of different photosensitive molecules (Retina Rhodopsin, Retinochrome, Melanopsin-A-like, Rhabdomeric Rhodopsin, and Rhodopsin kinase- Figure 1-5) in the optic lobe of *O. vulgaris*. Our interest was to determine which of the five known photoreceptors genes are expressed in the optic lobe by localizing the mRNA transcripts through the whole-mount *in situ* hybridization.

In this study, the results showed that Rhodopsin and retinochrome were simultaneously expressed and localized in the outer cortex (OC) and the radial column zone (R.Z) of the optic lobe of *O. vulgaris* (Figure 1; 2). Moreover, Retinochrome mRNA transcript was also detected in the optic tract region (OT) of the optic lobe, showing a few positive neurons (figure 2 A-D). it has been previously demonstrated that cephalopods' vision is frequently found to be controlled by a dual system of photosensitive pigments rhodopsin and retinochrome (Hara et al., 1967). Undoubtedly, Rhodopsin in the rhabdomere of the eye is important as the first photoreceptive molecule for vision. On the other hand, retinochrome is

sufficiently more photosensitive to react to faint light that has passed through the rhabdomeres and the dense layer of black pigment. From the fact that the photopigments are very close to each other, it seems possible that retinochrome may play a supplementary role in visual excitation (Hara and Hara, 1972; Hara et al., 1967). Indeed, rhodopsin and retinochrome are two types of opsin known as r-opsin1 (rhodopsin in the literature, Bellingham et al., 1998). In adult *S. officinalis*, the spatial expression of these two opsins has been showing they are expressed in the eyes and the skin (Mäthger et al., 2010; Kingston et al., 2015a). R-opsins are known for their involvement in vision in many protostomians and retinochromes are thought to work together with them.

Additionally, melanopsin is a type of mammalian photopigment belonging to a larger family of light-sensitive retinal proteins called opsins (Hankins et al., 2008), intrinsically photosensitive retinal ganglion cells (ipRGCs), where it contributes to light entrainment of circadian rhythms, and the pupillary light response (Provencio et al. 1998; Berson et al. 2002; Hattar et al. 2002). Melanopsin is distinct from other vertebrate opsins and is more closely related to rhabdomeric-type opsins than it is to ciliary-type opsins. Melanopsin is thought to function using a rhabdomeric phototransduction pathway, binding Gq G-protein, activating phospholipase C (PLC), and resulting in a depolarization of the cell (Hankins et al. 2014; Graham et al. 2008). While melanopsin-expressing cells are thought to be restricted to a subset of diverse retinal ganglion cells in mammals (Schmidt et al., 2008), zebrafish have five melanopsin genes that are expressed in many cellular layers in the retina (Davies et al. 2011; Matos-Cruz et al. 2011). The expression of melanopsin is not restricted to the retina in zebrafish or other animals, and it has several physiological functions in addition to regulating the circadian clock. Melanopsin has been found in the brains and skin of many animals, and we will discuss melanopsin and another opsin expression outside of the retina in a later section. Here, our results demonstrate that melanopsin transcript mRNA is mostly found to be highly expressed and localized at the optical trace region of the optic lobe (Figures 3A-D). It is also widely presented in the radial column zone (R.Z) of the medulla of the optic lobe where the expression of the retina rhodopsin photoreceptor cells is present (Figures 1, 3A-D).

We also explored the localization of the expression for rhabdomeric opsin in the optic lobe of *O. vulgaris.* Whereas rhabdomeric opsins have been described in many protostome species as the primary opsin for vision (reviewed in (Plachetzki et al., 2005) no previous study

has been so far localized the expression of rhabdomeric opsin in the optic lobe in cephalopods. In *Euprymna scolopes*, rhodopsin and other rhabdomeric phototransduction genes are expressed in the light organ and thought to contribute to light sensitivity (Tong et al. 2009). Our findings provide the first evidence for the localization of rhabdomeric mRNA photoreceptor showing its expression in the cortex and the radial column zone of the medulla (R.Z) of the optic lobe. no *in situ* hybridization positive signals are found at the inner medulla (the central tangential zone (T.Z)) or the optic tract region of the optic lobe of *O. vulgaris.*

In the present study we localized the rhodopsin kinase mRNA transcripts in the cortex, radial column zone of the medulla (R.Z), and the inner medulla (the central tangential zone (T.Z)) of the *O. vulgaris* optic lobe (Figure 5). The outer layer areas appeared to be the most intensely stained areas, with positive cells and extended to the inner medulla in the optic lobe (Figure 5). Early study has been suggested that the cortex is responsible for visual information processing and the medulla is the motor command center for dynamic body patterning (Messenger, 2001). The medulla is also involved in visual learning and memory (Liu and Chiao, 2017). Also, the expression of rhodopsin kinase was found to be localized at the optic tract region of the optic lobe of *O. vulgaris* (Figure 5 A-D).

In conclude, our findings suggest that localization of different photosensitive molecules in the optic lobe of *O. vulgaris* may employ many phototransduction pathways that appear to be correlated with the location of the photoreceptor to better understand their functions.

Chapter (5)

Cognitive Stimulation Induces Differential Gene Expression in *Octopus vulgaris*: The Key Role of Protocadherins

1. Introduction

Octopuses have considerable skills that show them as "intelligent" animals. They express high flexibility in solving demanding problems, and they have been observed using objects as tools. Octopuses also learn very fast when faced with artificial tasks (Richter et al., 2016; Boal et al., 2000; Hvorecny et al., 2007; Gutnick et al., 2011; Kuba et al., 2003; Kuba et al., 2010; Kuba et al., 2014; Mather, 1991; Mather, 2008).

Recently it has been observed that *Octopus vulgaris* cognition and learning abilities are also linked to adult neurogenesis (Bertapelle et al., 2017): animals housed in enriched environment increase adult neurogenesis, using PCNA as marker of cell proliferation and a cytoplasmic isoform of poli (ADP- ribose) polymerase 1 (PARP1) as a marker of neuronal plasticity (Bertapelle et al., 2017; De Lisa et al., 2012). Octopuses, subjected to problem-solving tasks, revealed an increment of cell proliferation in supra-esophageal mass, in particular in the vertical-frontal system and the optic-olfactory lobes, brain areas involved in learning-memory, and sensory stimuli integration respectively (Bertapelle et al., 2017). Moreover, bivariant analysis of flow cytometry using BrdU incorporation allowed to assess the magnitude of adult neurogenesis in those brain lobes, previously identified, characterized by the presence of adult neurogenesis niches, highlighting the amount of cells exhibiting *de novo* DNA synthesis (Di Cosmo et al., 2018). What remains puzzling is what genes are major involved in these intriguing processes in *Octopus vulgaris*.

In cephalopods, assembled transcriptomes reveal a substantial expansion of protocadherin genes (PCDHs) except for Nautilus, which lacks the elaborated Coleoid nervous system (Albertin et al., 2015; Liscovitch-Brauer et al., 2017). The genetic divergence between octopus and squid PCDHs expansions may reflect the notable differences between octopuses and decapodiformes in brain organization. PCDH genes are orthologous in *O. vulgaris* and *O. bimaculoides*, suggesting that the PCDHs' expansion occurred before the speciation (Styfhals et al., 2019). Strikingly, the octopus and squid PCDHs are significantly enriched with RNA editing sites, especially in *O. vulgaris* (Liscovitch- Brauer et al., 2017).

The octopus genome encodes 168 multi-exonic PCDH genes, nearly three-quarters of which are found in tandem clusters on the genome, which is 10 times more than many vertebrates, and more than twice as many as humans and other mammals. PCDHs are expressed in adult mammalian brains, especially in the hippocampus, cerebellum and cortex (Hertel et al., 2008; Hertel et al., 2012; Junghans et al., 2008; Kim et al., 2010; Krishna et al., 2011), suggesting a role in adult brain functioning, beyond the establishment of neural connectivity (Goodman et al., 2017; Peek et al., 2017). The expression of PCDHs in octopus's neural tissues and the high number of editing sites are consistent with a central role for these genes in development and maintenance coleoids nervous system organization as they do in vertebrates (Albertin et al., 2015; Liscovitch-Brauer et al., 2017).

Additionally, *Pax* genes encode for a family of metazoan transcription factors, which are essential for cell specification and tissue differentiation including the nervous system (Blake et al., 2008; Blake and Ziman, 2014; Scherholz et al., 2017; Wollesen et al., 2015). In vertebrates, *pax3* and *pax7* contribute to the development of the nervous system (Ericson et al., 1997; Monsoro-Burq, 2015; Thompson and Ziman, 2011), their homologs in *Drosophila* (Breitling and Gerber, 2000; He and Noll, 2013) are essential segment-polarity genes (Kilchherr et al., 1986) and are involved in neurogenesis (Colomb et al., 2008). In Lophotrochozoa studied so far, Platyhelmintha (Callaerts et al., 1999; Salo et al., 2002), Annelida (Quigley et al., 2007), Mollusca (Buresi et al., 2014; Gehring, 2005; Gehring and Ikeo, 1999; O'Brien and Degnan, 2000; Tomarev et al., 1997; Wollesen et al., 2015), Brachiopoda (Passamaneck et al., 2011), and Nemertea (Loosli et al., 1996), the expression patterns of each *Pax* gene suggests conserved and consistent roles for *pax3/7*, *pax2/5/8* and *pax6* in the nervous system development, in the sensory structure formation, and in the eye morphogenesis respectively. In particular, among Cephalopods, in *Sepia officinalis*, *Sof-pax6* expression was largely distributed in central nervous system (CNS) and in the brachial nervous chord (Navet et al., 2017). Like-wise, the restriction of *Sof-pax6* expression at the distal tip of growing arms, which is described as a growing/proliferation region in octopus (Nodl et al., 2015) and Euprymna (Nodl et al., 2016), suggests a role of *pax6* in the promoting proliferation mechanisms underlying the growth of the arms. Furthermore *Sof-pax3/7* neural expression occurs later, suggesting that these genes are not involved in early neurogenesis, but it is restricted to the ventral brain in "motor" areas controlling the arms (Navet et al., 2017). Moreover, *Sof-pax2/5/8* might be involved in early

steps of locomotor structures development derived from the ancestor mollusk foot (Navet et al., 2017).They could be implicated in the formation of the whole nervous circuitry controlling arms and funnel muscles (Navet et al., 2017).

In annelid and *Drosophila*, ELAV is the earliest marker for neural cells as they just exit the cell cycle and start to differentiate into neurons (Berger et al., 2007; Meyer and Seaver , 2009). The ELAV protein is present exclusively in all immature and mature neurons (Robinow, 1991).In addition, its upregulation in rodent hippocampal neurons, having learned a spatial discrimination paradigm, suggests a role in memory storage (Quattrone et al., 2001). In *Drosophila*, ELAV is necessary for neuronal differentiation (Samson and Chalvet, 2003), and it is expressed pan-neurally in all stages of development. In *Sepia*, *Sof-elav* appears to be involved in neurogenesis during embryogenesis (Buresi et al., 2013).

ZIC family members, instead, play key roles in early neural patterning and the development of the neural crest, visual system, and cerebellum in mammals (Brown et al., 1998; Inoue et al., 2004). *zic* homologs have been identified in chordates, arthropods, and nematodes(Aruga, 2018). Moreover, *zic* family genes are expressed in a subset of the developing brain and mesoderm derivatives in annelids (Aruga, 2018).

In *O. vulgaris*, many genes have been hypothesized to have a role in its cognitive abilities, but up to date, their roles have been suggested just based on genomic and transcriptomic analysis (Albertin et al., 2015). Nerveless, it has never been demonstrated if and how their expressions fluctuate after cognitive stimulations.

We than sequenced two isoforms of Oct-PCDH18 and one of Oct-PCDH15. The two isoforms of PCDH18 found in zebrafish play a crucial role in the brain: PCDH18a has a role in cell adhesion and migration (Aamar and Dawid, 2008), whereas PCDH18b interacts with Nap1, an important regulator of actin dynamics, to control motor axon growth and arborization in primary motoneurons (Biswas et al., 2014). PCDH15 expression was described in the adult brain in mice (Hertel et al., 2012), and it is expressed in inner ear hair cell stereocilia and retinal photoreceptors in humans (Alagramam et al., 2000).

As potential co-actors of PCDHs in octopus cognitive processes, we sequenced *Oct-pax2/5/8*, *Oct-pax3/7*, *Oct-pax6, Oct-elav*, and *Oct-zic1*. We then analyzed for all these selected genes their differential expression patterns under different behavioral cognitive stimulations.

2. Materials and Methods

2.1. Experimental Model and Subject Details

O. vulgaris specimens (female n = 33, weight 800 ± 50 g), collected in Bay of Naples, were transferred to the Department of Biology (Di Cosmo et al., 2015). Animals were housed in individual tanks to prevent aggressive social interactions and cannibalism. They were housed in PVC tanks (50 × 50 × 50 cm), covered with a Plexiglas lid to avoid animals' escape, equipped with a den, natural sand, and shells. Water and room temperature were maintained at 18 °C, and the light/dark cycle was set to the natural photoperiod. Water was treated with biological filters and protein skimmers. First days of captivity were considered as the acclimatization period, during which several physiological and behavioral parameters were monitored to verify the welfare and healthiness of the octopuses (Di Cosmo et al., 2015; Maselli et al., 2020). During the acclimatization phase, animals were fed by experimenters with their natural prey: Crabs (*Carcinus mediterraneus*) or mussels (*Mytilus galloprovincialis*) once a day.

Octopuses were anaesthetized by isoflurane insufflation (Polese et al., 2104) and brains were dissected in sterile conditions, isolating the central part of the supraesophageal mass, the subesophageal mass, and the optic lobes including olfactory and peduncle lobes on the optic tracts (OOP, Figure 1). Dissected samples were stored at −80 °C for further experiments.

Our research conformed to 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).

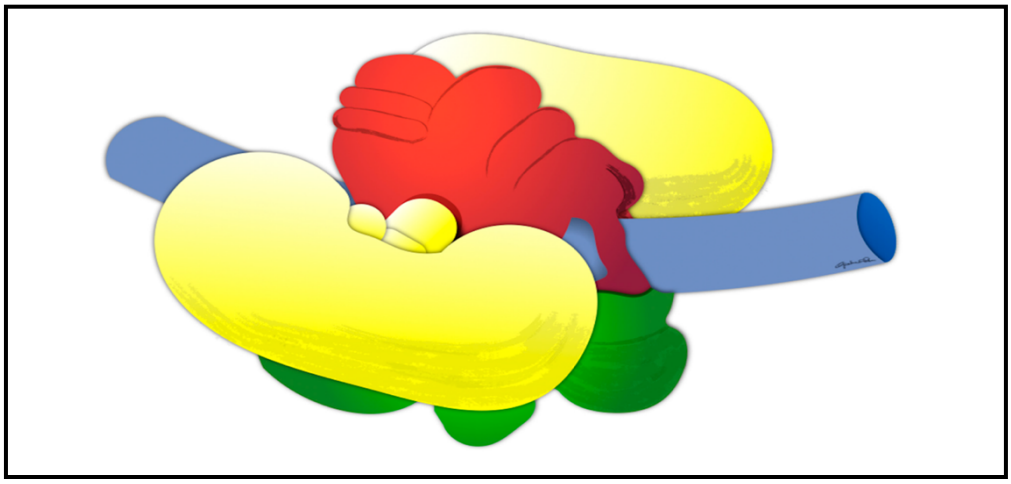

Figure 1 :Schematic view of *Octopus vulgaris* brain anatomy, highlighting the tissues sampled for analysis: the optic lobes with olfactory and peduncle lobes on the optic tracts (OOP, yellow), the central part of the supraesophageal mass (red), and the subesophageal mass (green) that enwraps the esophagus (blue, not analyzed).

2.2. Acclimatization Experiment

In order to experimentally quantify the acclimatization time required for octopuses, we performed an initial experiment to determine the time needed by octopuses to feel comfortable and ready to face the challenge of opening two jars containing food.

Octopuses (N = 3 for 5 groups, total N = 15) were tested at different times after they arrived in the lab for 3 consecutive days, once a day, with two plastic jars closed with a screw lid containing a live prey. During experimental days, octopuses had no feeding opportunities except to open a jar. The first group was tested since day 1, the second starting on day 2, the third on day 6, the fourth on day 14, and the fifth on day 19, measuring the average of opened jars during the training period. Jar position was constant through the experiments, and behavioral quantification was restricted to measuring the average number of opened jars.

Following the results of the acclimatization experiment, we set up a period 14 days for the next experiments.

2.3. Cognitive Stimulation

Novel animals (N = 18) were used in three experimental groups: Control, tested, and wild. The control animal group (N = 6) was not tested, and they were fed regularly without any task for 17 days (14 acclimatization + 3 experimental days). The tested animal group (N = 6) was tested for the consecutive 3 days after the acclimatization period (14 days).

We altered the standard housing conditions providing a cognitive challenge. During 3 experimental days, once a day, octopuses were tested with two jars containing a live prey and closed with a screw lid. During experimental days, octopuses had no feeding opportunities except to open the jars to reach the prey (Bertapelle et al., 2017). The wild animal group (N = 6) was captured and directly sacrificed. All experiments (acclimatization and cognitive stimulation) were conducted once per day and recorded for at least 1 h with a digital camera (GoPro Hero 5, GoPro, Inc. CA, USA) positioned on the front of the aquarium (20 cm), to analyze the octopus's choice and behavioral responses, such as exploring, opening the jar, and eating.

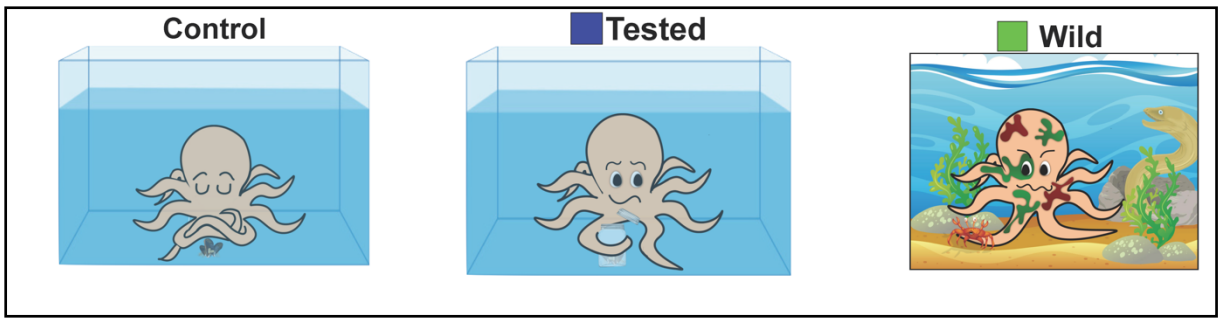

Figure 2: The standard housing conditions providing cognitive stimulations in three experimental groups: The control animal group (N = 6) was not tested, and they were fed regularly without any task for 17 days (14 acclimatization + 3 experimental days). The tested animal group (N = 6) was tested for the consecutive 3 days after the acclimatization period (14 days). The wild animal group (N = 6) was captured and directly sacrificed.

2.4. RNA Extraction, Selection and Primer Design, and Gene Expression Pattern

Total RNA was extracted using the RNeasy minikit (Qiagen, Valencia, CA, USA). The quality and amount of purified RNA were analyzed spectrophotometrically with Qubit 3.0 (Thermo Scientific Inc., Waltham, MA, USA). RNA of 1000 ng was reverse transcribed with the QuantiTect® Reverse Transcription Kit (Qiagen). Three members of the PCDH family (PCDH15, PCDH18a, and PCDH18b), pax2/5/8, pax3/7, pax6, elav, and zic1 were characterized in an EST library and initial amplification primers for polymerase chain reaction (PCR) were synthesized using the software Geneious 9.1 (Table 1).

PCRs were performed in a final volume of 20 µL, with 0.2 µL of Pfu DNA polymerase (Thermo Scientific), 4 µL of 4× Tris buffer with $MgCl_2$, 1.6 µL of dNTPs (each dNTP 2.5 µM), 0.2 µL of 50 µM of each primer, and 100 ng of cDNA template under the following conditions: An initial denaturing step of 98 °C for 3 min; 35 cycles of 10 s at 98 °C; 30 s at 55–60 °C and 1 min at 72 °C; and a final extension step of 5 min at 72 °C. PCR products were purified from unincorporated primers using Exonuclease I and Fast Alkaline Phosphatase (Thermo Scientific). The sequencing reaction was performed using the BigDyeTM Terminator Cycle Sequencing chemistry (Applied Biosystems, Foster City, CA, USA). Sequences were purified using AutoSeq G-50 (Amersham, Uppsala, Sweden) spin columns and analyzed by an ABI 3100 automated sequencing instrument (Perkin-Elmer, Genetic Analyzer, Foster City, CA, USA). Chromatograms were assembled and analyzed using software Geneious version 9.1

(Biomatters, Auckland, New Zealand, available from http://www.geneious.com). PCR products were analyzed with GenBank BLASTn and BLASTx (BLAST, basic local alignment search tool). The analysis of the sequencing confirmed the identity of the fragments. All sequence data generated in this study were deposited in GenBank (accession numbers MN138036-43).

Additionally, we performed a real-time PCR using the QuantiTect SYBR Green PCR Kit (Qiagen). PCR was performed in a final volume of 25 µL, with 50 ng of cDNA, 1 mM of each primer, and 12.5 µL of QuantiFast SYBR Green PCR Master Mix (2×). The PCR cycling profile consisted of a cycle at 95 °C for 5 min, 40 three-step cycles at 95 °C for 15 s, at 60 °C for 20 s, and at 72 °C for 20 s. Quantitative RT-PCR analysis was conducted by using the 2-(ΔΔCt) method (Livak and Schmittgen, 2001). RT-PCR was performed in a Rotor-Gene Q cycler (Qiagen).

Specific primers were designed (Table 1) for seven target genes and the ubiquitin gene was used for normalization of the relative expression. At the end of each test, a melting curve analysis was done (plate read every 0.5 °C from 55 to 95 °C) to determine the formation of the specific products. Each sample was tested and run in duplicate. The control and the treatment groups in various assays were compared and analyzed using a Wilcoxon two group test and data with p-values < 0.05 were considered statistically significant.

Table1: Primers used in this study

Genes	Primer sequences (5′→3′)
Ubiquitin (113bp)	F: TCAAAACCGCCAACTTAACC
	R: CCTTCATTTGGTCCTTCGTC
Oct-PCDH15 (115bp)	F: GACAGAGACAGCAGGCAGAA
	R: AAACTGGCCGAGAGAAGGAC
Oct-PCDH18a (147bp)	F: AGGCTCGCCTCCTCAAAATG
	R: GCCGACAGCTTGACAATTGG
Oct-PCDH18b (135bp)	F: GCAAGTTTGGCACGCTTACA
	R: TCCCTCAGTTGTTGCCTGAC
Oct-pax2/5/8 (103bp)	F: ACAGCTCCGCGTATCTCATG
	R: TACCTTCGGCTTGGAACCAC
Oct-pax3/7 (136bp)	F: GAAACCTCGCGTTGCTACAC
	R: ACTAGGTACGGTACTGCGGT
Oct-pax6 (122bp)	F: TTTTGTAAATGGACGGCCGC
	R: TGCTCACACAACCATTGGAGA
Oct-elav (182bp)	F: GCACGAAATGCATCAACCGATGCGG
	R: CTGCAGGCCCCT TTAATGCTT TCACT
Oct-zic1 (129bp)	F: TCATGGACACATCACACGGG
	R: CGTTCGGTTGGGTTCCAAAC

3. Results

3.1. Acclimatization Experiment

We measured the efficiency of the acclimatization process as the average of jars opened after different numbers of days (Figure 3).The first animal group tested after the 1 day of acclimatization completely ignored the jars standing at the corner for most of the time. This happened during all 3 trial days (3TD). The second animal group that started to be tested on the second day was able to open just one jar on average during the 3TD. The third animal group was able to open 1.3 jars on average during the 3TD. The following fourth animal group opened 1.7 jars on average during the 3TD. The last animal group that started to be tested on day 19; in the following 3TD, they were able to open both jars. The number of days of acclimatization had a significant influence on the establishment of testing, as it occurred mostly on days 14-20 of the experiment, raising the level of performance in the tests after day 14 in captivity.

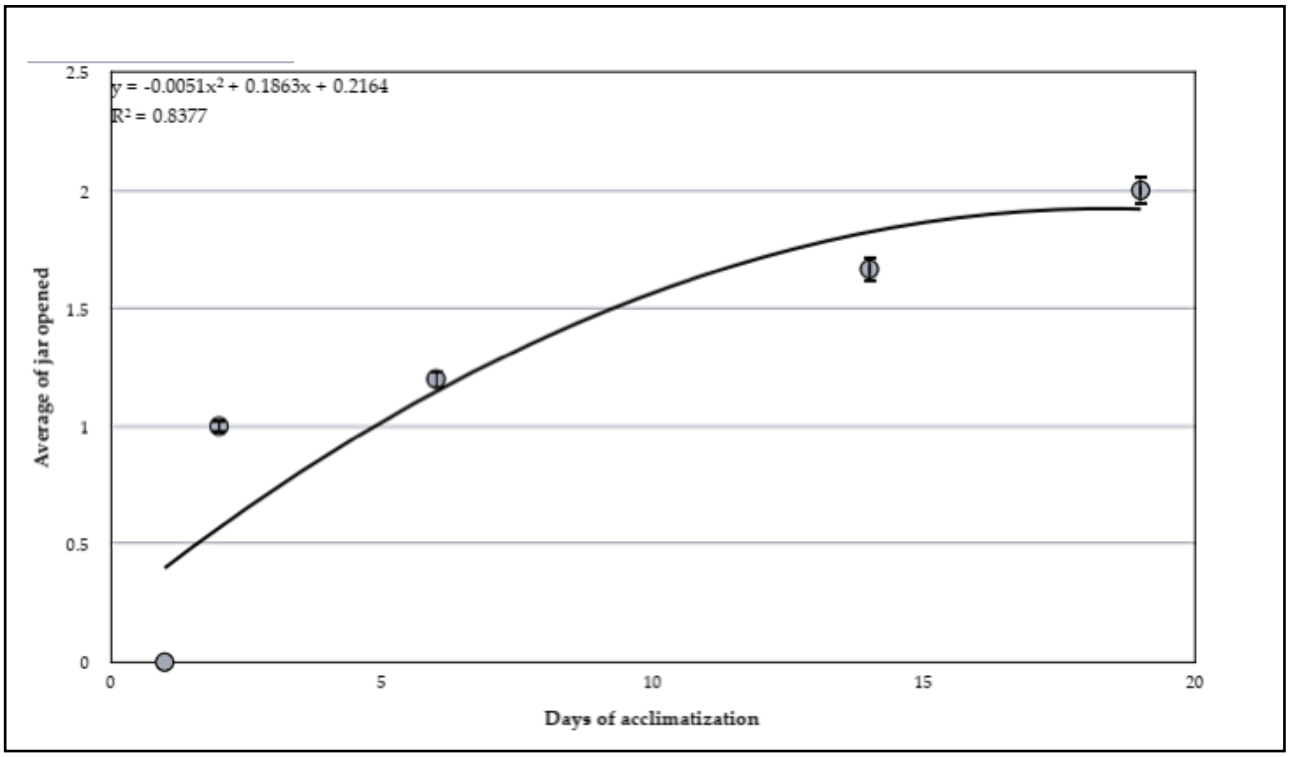

Figure 3 : Efficiency of the acclimatization process evaluated as the number of jar opened.

To evaluate the differential gene expression patterns avoiding any kind of stress effects due to the octopus fishing and transport procedures, our first result shows the optimal acclimatization period for an octopus, before any behavioral experiment in captivity (Malham et al.,1998; Walker et al.,1970). We assessed that 14 days of acclimatization, in which the animals were left without any kind of stress, resting and being feed ad libitum, is the minimum period that allows octopuses to solve a test appropriately at the very first time (**Figure 3**).

For the gene expression experiment, challenged animals, acclimatized for 14 days, responded appropriately to the problem-solving stimulation opening the two jars at the very first day of training. The main goal of this work was to find the genetic basis that underlies the adult neurogenesis in *O. vulgaris*. To do that, we used a combination of ethological and genetic approaches to evaluate the gene expression patterns in the specific brain areas previously identifies ad neuronal proliferation sites (Bertapelle et al., 2017; Di Cosmo et al., 2018).

3.2. Gene Expression Patterns

3.2.1. Control Animal Group

Of the control group animals, we evaluated the transcription activities of the selected genes (Oct-PCDH15, Oct-PCDH18a, Oct-PCDH18b, Oct-pax2/5/8, Oct-pax3/7, Oct-pax6, Oct-elav, and Oct-zic1 genes) in three brain regions, the central part of the supraesophageal mass, the subesophageal mass, and the optic lobes, including the olfactory and peduncle lobes (OOP) on the optic tracts (Figure 4).

The subesophageal mass, compared to the supraesophageal mass, showed a higher expression for Oct-PCDH18a and b, Oct-pax2/5/8, Oct-pax3/7, and Oct-elav genes, and lower gene expression for Oct-pax6 and Oct-zic1 genes; there were no significant differences for the Oct-PCDH15 gene (orange bar in Figure 4). The OOP compared to the central supraesophageal mass showed a higher expression for Oct-PCDH18a, Oct-zic1, and Oct-elav genes, and a significantly lower expression was observed only for the Oct-pax2/5/8 gene (red bar in Figure 4). In particular, the Oct-PCDH18a gene showed a substantial increase in the subesophageal mass (about 70 times higher) and in the OOP (about 90 times higher; Figure 4).

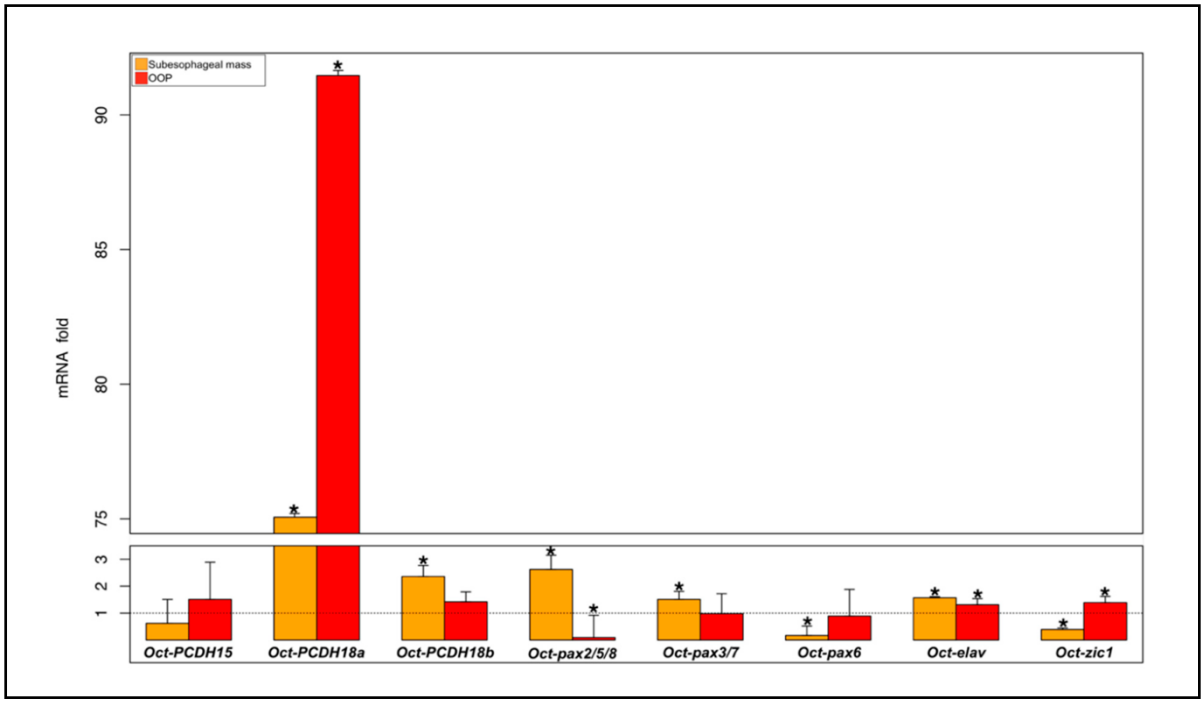

Figure 4 : Gene expression analysis of different brain regions of control animal group (N=6). Relative mRNA fold change in gene expression in subesophageal mass (orange) and OOP (red) compared to supraesophageal mass (dot line set y=1). *asterisk indicates that the difference *vs* supraesophageal mass expression is statistically significant (t-test, $p<0.05$). Error bars represent the SEM.

3.2.2. Tested and Wild Animal Groups

The tested animal group (N = 6) was acclimatized for 14 days and animals were trained for 3 consecutive days using two plastic jars. All octopuses were considered to have the same behavioral performance, responding to the test stimulation, opening both jars from the first day of the experiment, progressively decreasing the time spent opening the jar during the 3 days of training.

Comparing the results among wild, tested, and control animals, it is possible to observe that in the supraesophageal mass (Figure 5), tested and wild animals showed an upregulation for Oct-PCDH15, Oct-PCDH18a, Oct-PCDH18b, and Oct-pax2/5/8 genes (Figure 5). The Oct-pax3/7 gene showed a downregulation in both tested and wild animals. Meanwhile, the Oct-zic1 gene is significantly downregulated in wild octopus. Interestingly, the Oct-elav gene in the supraesophageal mass is downregulated in tested animals and it is upregulated in wild animals (Figure 5).

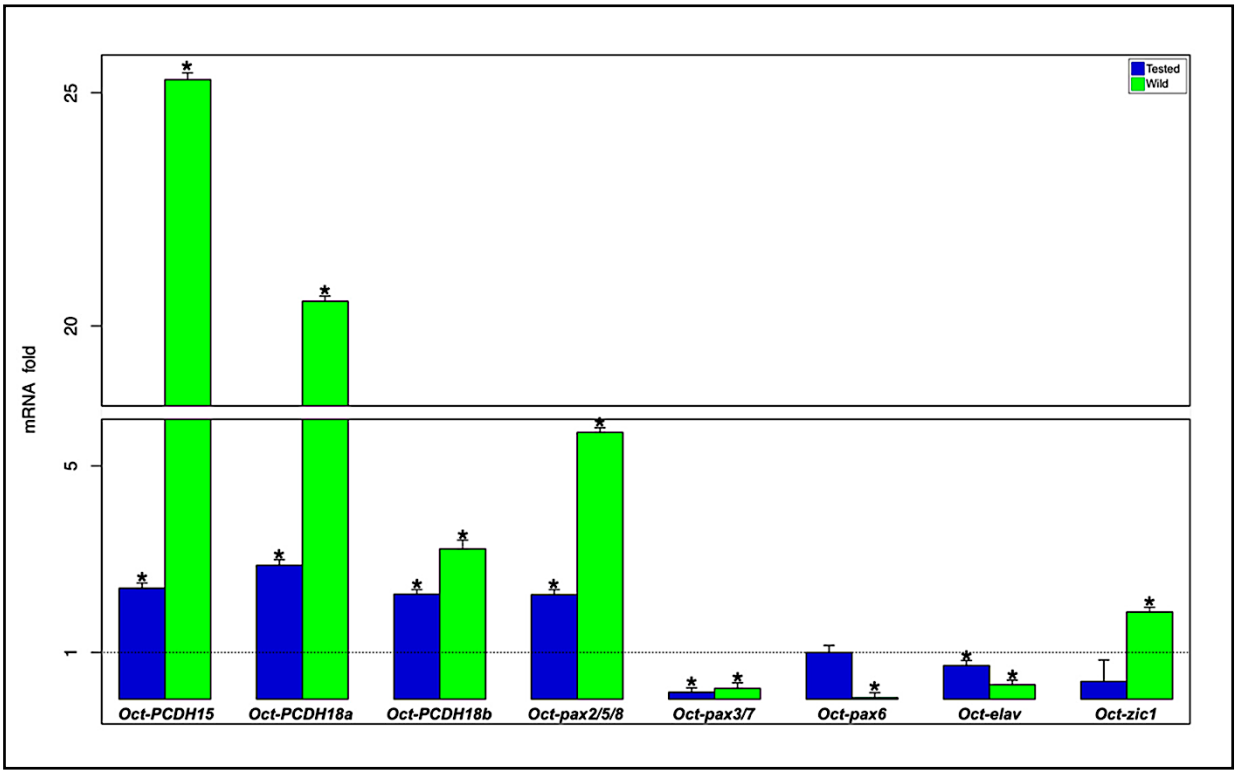

Figure 5 : Gene expression analysis in supraesophageal mass. Relative mRNA fold change in gene expression in tested (blue) and wild (green) animal groups are compared to control (N=6/group; dot line set y=1). * asterisk indicates that the difference vs control group is statistically significant (t- test, $p<0.05$). Error bars represent the SEM.

In the OOP, tested and wild animals showed an upregulation for Oct-PCDH18a, Oct-PCDH18b, Oct-pax6, and Oct-zic1 genes (Figure 6). Oct-PCDH15 and Oct-elav genes showed an upregulation in tested animals and no significant differences in the wild ones (Figure 6). Quite the opposite, the Oct-pax2/5/8 gene showed lower expression in wild animals and no significant difference was found in the tested ones(Figure 6).

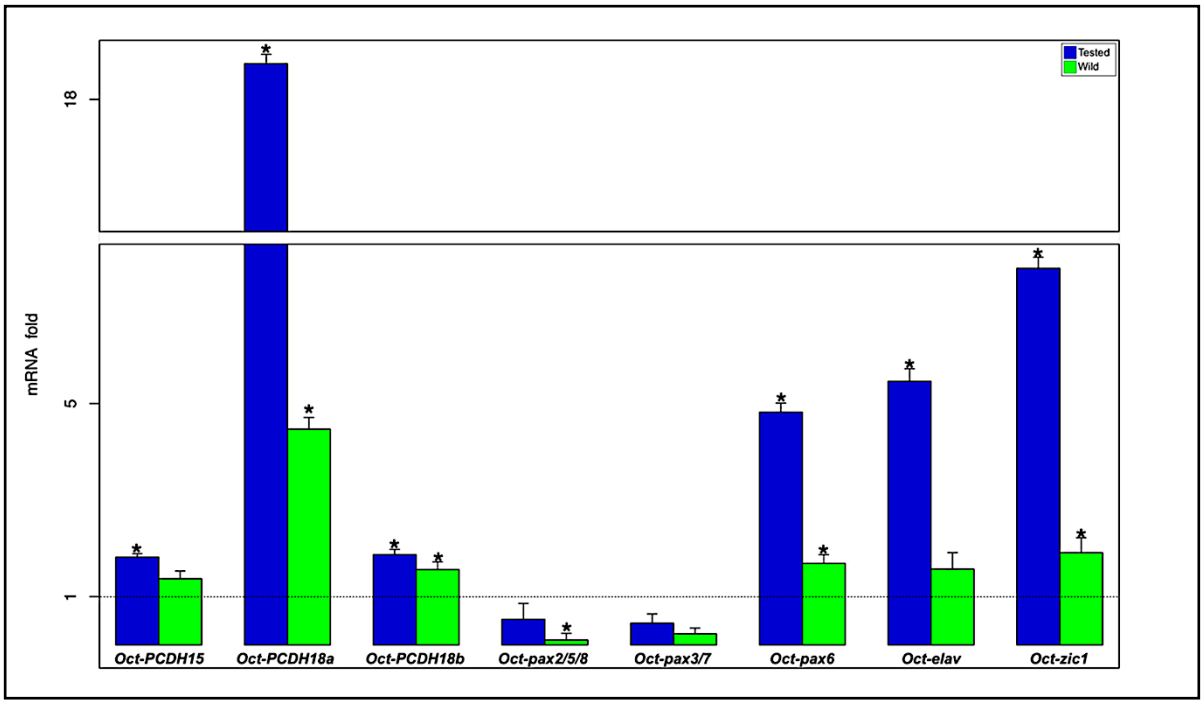

Figure 6 : Gene expression analysis in OOP. Relative mRNA fold change in gene expression in tested (blue), and wild (green) octopus are compared to control (N=6/group; dot line set y=1). * asterisk indicates that the difference vs control group is statistically significant (t-test, $p<0.05$). Error bars represent the SEM.

In the subesophageal mass, tested animals showed a synergistic upregulation of all genes considered, except for Oct-pax3/7 (Figure 7). This pattern is confirmed for Oct-PCDH15, Oct-PCDH18a, Oct-PCDH18b, and Oct-zic1 genes, which showed a strong upregulation in wild animals compared not only with control animals but also with tested octopuses (Figure 7). Wild animals showed a significantly high level of expression also for the Oct-pax3/7 gene (Figure 7). Oct-elav gene expression was higher in tested animals and downregulated in wild ones (Figure 7). Moreover, Oct-pax2/5/8 and Oct-pax6 genes did not show significant differences in the wild octopus compared to the control animals (Figure 7).

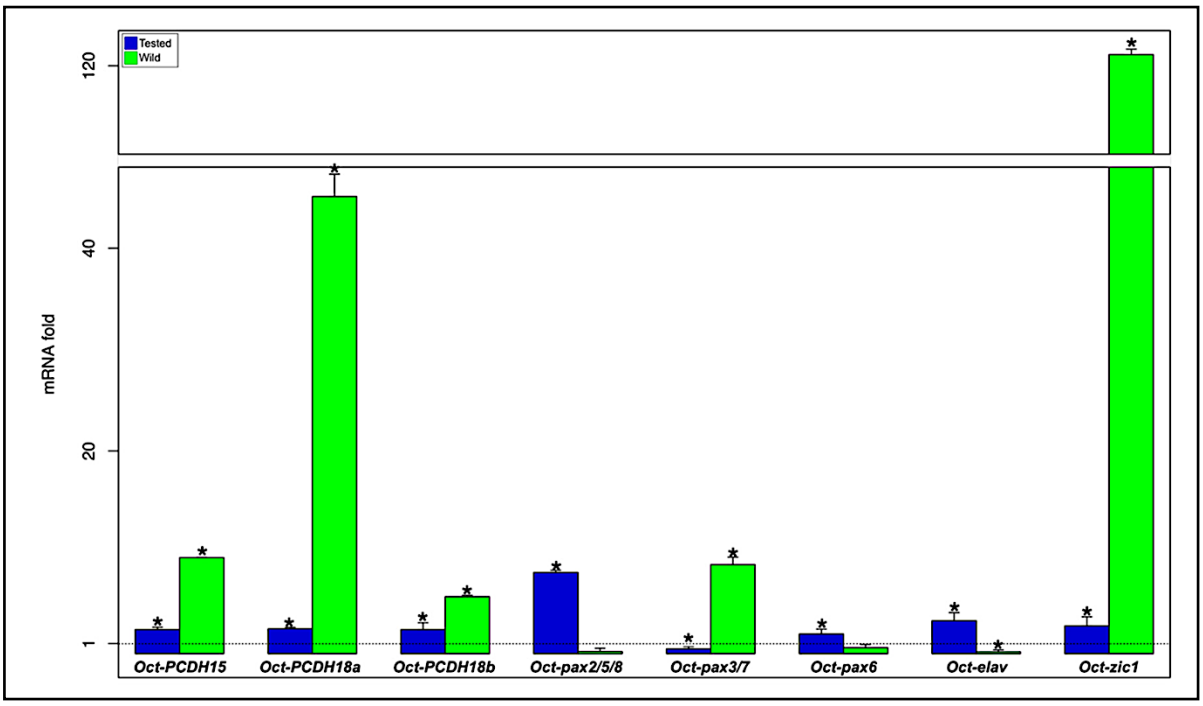

Figure 7: Gene expression analysis in subesophageal mass. Relative mRNA fold change in gene expression in tested (blue), and wild (green) octopus are compared to control (N=6/group; dot line set y=1). * asterisk indicates that the difference vs control group is statistically significant (t-test, $p<0.05$). Error bars represent the SEM.

4. Discussion

Adult neurogenesis can be considered as an intrinsic factor to the maintenance of a healthy brain and to memory assessment (Konefal et al., 2013). *O. vulgaris* possesses high cognitive capabilities, and just recently, it has never been shown that they have adult neurogenesis (Bertapelle et al., 2017; Di Cosmo et al., 2018). We now extend the knowledge to the genes involved in this process.

4.1. Selected Gene Expression Analysis in Octopus Brain Areas

Our study is the first to describe how the enhancement of the environmental conditions, in wild and tested octopuses, affects the RNA expression level in genes involved in cell proliferation and synaptogenesis, compared to control animals, that lack any environmental stimulation.

Interestingly, we observed that, among the analyzed genes possibly involved in adult neurogenesis, wild animals show higher PCDH expression either than tested or control animals. In all examined brain areas, their expression level showed a positive trend from the control, tested, and wild animal groups (Figure 7). Since environmental/cognitive stimulation enhances adult neurogenesis in *O. vulgaris* (Bertapelle et al., 2017; Di Cosmo et al., 2018), these results strongly support the suitability of PCDHs' expression level as a marker for this process.

4.2. Learning centers of the octopus's brain

The supraesophageal mass contains the vertical lobe and the superior frontal lobe (VL-SFL), this system plays an important role in learning and memory. Furthermore the supraesophageal mass is also the site of visual learning and tactile memory, respectively localized in the vertical/subvertical/superior-frontal lobes and inferior-frontal lobe (Wells, 1978). These brain regions showed an increase of adult neurogenesis in animals cognitively stimulated (Bertapelle et al., 2017). In our study, the Oct-PCDHs and Oct-pax2/5/8 showed a different level of expression in accordance to the stimulations to which the animals are exposed: lower in control, higher in tested and the highest in wild octopuses. The upregulation of Oct-PCDHs is supported by the key role that these genes play in the nervous system and by the wide expansion of this family gene in the Octopus sp (Albertin et al., 2015; Liscovitch-Brauer et al., 2017; Styfhals et al., 2019). In particular, PCDH18 (a and b) genes are involved the essential steps of neurogenesis, as neuron migration, adhesion, and arborization (Alagramam et al., 2000).

For the other genes considered we do not observe a similar trend (Figures 6 and 8). Oct-pax6 and Oct-zic1 expressions in tested animals not only show no difference with the control, but even they are down-regulated in wild animals. This finding can be explained by the fact that PAX6 is mainly involved in the neural development of the brachial nervous chord in cephalopods (Nodl et al., 2015). Otherwise ZIC1 gene is involved in the visual system and cerebellar development (Merzdorf and Sive, 2006; Nakata et al., 2000).

Furthermore, the Oct-pax3/7 gene is down-regulated in both tested and wild animals. In Sepia officinalis this gene and PAX6 gene do not seem involved in neurogenesis of the supraesophageal mass, but they are restricted to the "motor" areas controlling the arms in the subesophageal mass (Navet et al., 2017). The Oct-elav gene is instead down-regulated in tested animals and up-regulated in wild ones. This divergent behavior found in the two stimulated groups (tested and wild) suggests that Oct-elav gene as well as Oct-pax 3/7, Oct-pax 6, and Oct-zic 1are not suitable as adult neurogenesis markers.

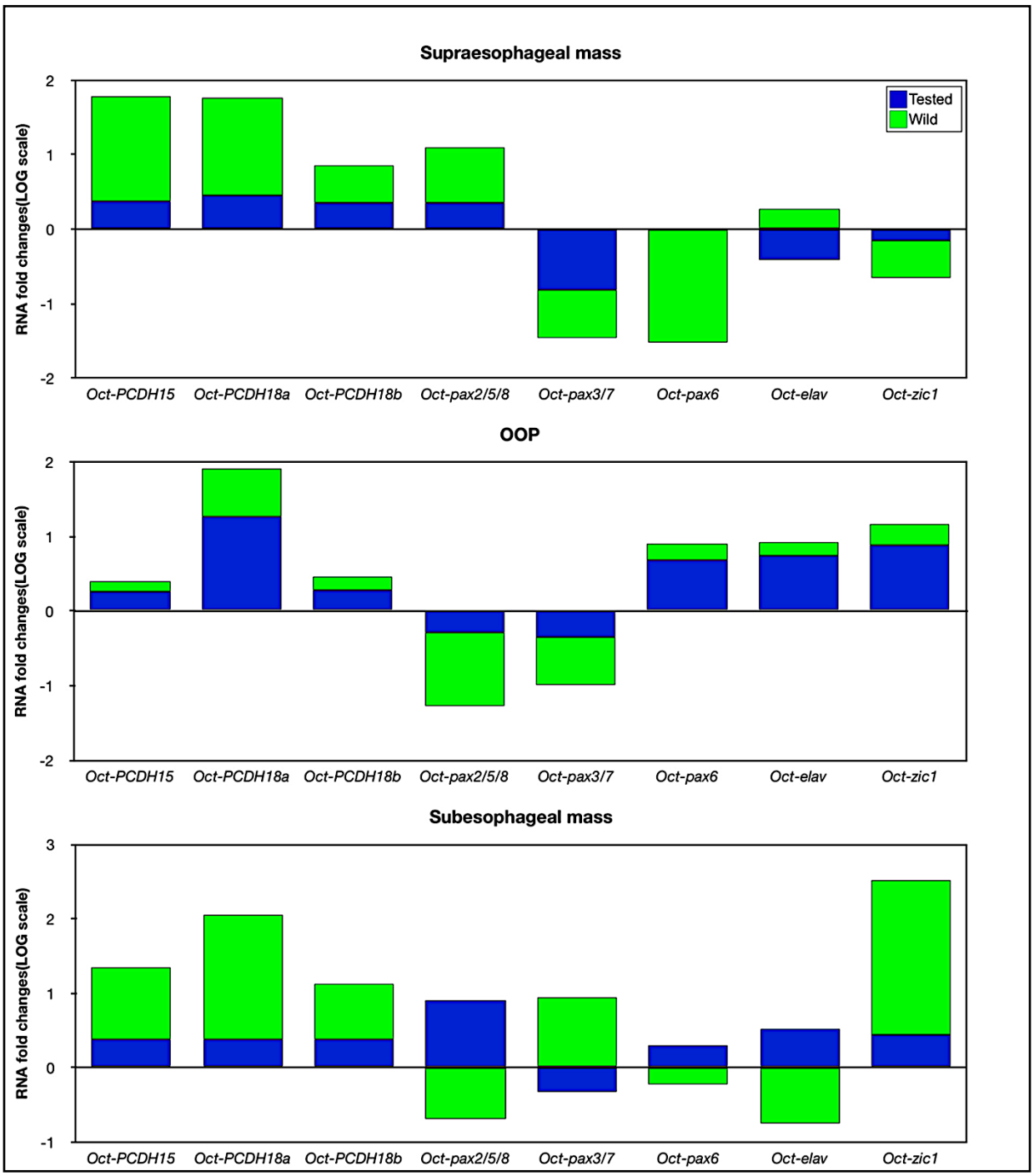

Figure 8: Map of gene expression detected in supraesophageal mass, OOP and subesophageal mass in *Octopus vulgaris* under different degree of cognitive stimulation. Relative mRNA fold change in gene expression was in logarithmic scale, tested and wild octopuses were compared to control (N=6/group; line set y=1).

4.3. Lower motor centers of the octopus's brain

The lower motor centers of the octopus's brain, responsible for accurate motor control and coordination, are the anterior part of the subesophageal mass that is involved in the actions of arms and suckers, while the posterior part is involved in the actions of the mantle and viscera (Messenger, 1967; Nixon and Young, 2003; Young, 1971). In these lobes, neuronal proliferative activity was found in adult octopus (Bertapelle et al., 2017; Di Cosmo et al., 2018). In tested animals we observe a synergistic effect exerted by an up-regulation of all genes considered, except for Oct-pax3/7, suggesting that they are all stimulated by the problem solving task that implies a complex motor coordination. This pattern is confirmed in wild animals for Oct-PCDHs and Oct-zic1 genes that show a higher level of up-regulation compared not only with control animals, but also with tested octopus.

Interestingly, wild animals show a significantly higher expression level for Oct-pax3/7 gene, probably because they face more stimuli coming from natural environment than control and tested animals, it does not appear to be affected by the specific proposed task. On the contrary, Oct-pax2/5/8 and Oct- pax6 genes resulted upregulated only in tested octopus, suggesting a specific involvement in the implementation of nervous network necessary for tuning fine movements, as those needed to open screw-lid jars (Bertapelle et al., 2017).

In this brain area also the Oct-elav gene expression result upregulated in tested animals while significantly down-expressed in wild ones. Differently from what observed in supraesophageal mass, in tested octopus, the higher expression of Oct-elav allows us to hypothesize that this gene is specifically involved in the interneuron proliferation. This contributes to the fine motor coordination, supporting our previous finding on adult neurogenesis in subesophageal mass (Bertapelle et al., 2017). Wild octopus showed an opposite trend, with lower Oct-elav gene expression. This latter situation could be due to the multiple options offered by the natural environment in which animal is not forced to solve a specific problem.

4.4.The multi-sense integration area

The optic and olfactory lobes integrate sensory stimuli detected by sensory organs from the environment while the spine of the peduncle lobe is considered an analog of vertebrate cerebellum. These lobes are involved in the coding of the visual and chemical inputs as well as the coordination of voluntary movements such as posture resulting in smooth and balanced

muscular activity supporting the neurophysiological bases of octopus sophisticated behavior (De Lisa et al., 2012; Di Cosmo et al., 2018; Di Cosmo and Polese, 2017; Di Cosmo et al., 2015; Polese et al., 2015; Polese et al., 2016; Young, 1971).

In wild and tested animals we found up-regulation for Oct-PCDHs, Oct-pax6, Oct-elav, and Oct-zic1 genes suggesting that these genes are related to sensory integration that is strictly connected to the increasing of the neuronal structure at adult neurogenesis as previously found in this area (Bertapelle et al., 2017; Di Cosmo et al., 2018). On the other hand, the Oct-pax2/5/8 and Oct-pax3/7 genes appeared down regulated in wild animals while no significant differences are found in their expression level in tested octopus suggesting that in these brain areas they do not have any role in the adult neurogenesis regulation.

5. Conclusion

After the discovery that cognitive stimulation positively affects adult neurogenesis in *O. vulgaris'* brain (Bertapelle et al. 2017), we propose for the first time a link between the process of adult neurogenesis and transcriptomic expression using a large set of genes classified as markers for neurogenetic mechanisms.

With a detailed analysis of expression levels for the selected genes, we found a clearer pair of adult neurogenesis occurring after cognitive stimulation with Oct-PCDHs, which appeared to be upregulated in both tested and wild octopuses. Our study showed that the gene expression level of PCDHs could represent a valuable tool to detect and measure adult neurogenetic processes, including synaptic plasticity. The selected three non-clustered PCDHs are known to be involved during early stages, such as axon outgrowth and path finding (Styfhals et al., 2019; Goodman et al., 2017; Peek et al., 2017).

Furthermore, the next steps could be directed to expand the gene marker panel in order to deeply understand the adult neurogenesis mechanism. We then proposed a novel approach that combining cognitive stimulation and evaluation of the pattern of differential gene expression could give information about remote and proximate causes of animal behavior

Chapter (6)
Final Conclusion

The common octopus (*Octopus vulgaris*) is the most studied of all octopus species. They have complex nervous systems with remarkable cognitive abilities and keen senses that perform reliably in a variety of visual and chemo-tactile learning tasks (Sanders, 1975; Wells, 1978; Mather, 1995; Boal, 1996; Anderson and Mather, 2010; Hochner, 2008; Mather and Kuba, 2013; Hanlon and Messenger, 2018). The nervous system of *O. vulgaris*, is comprised of central lobes surrounding the esophagus and a pair of optic lobes that together contain approximately a third of the neurons, with the remaining two-thirds distributed within the arms (Young, 1971).The octopus's arms are lined with hundreds of tactile and chemosensory structures, known as suckers that interact with the environment for achieving the level of motor and sensory information processing necessary for their sophisticated behaviors (Graziadei and Gagne 1976; Nixon and Young, 2003, Shigeno et al., 2018; Zullo et al. 2009; Packard 1972).

In cephalopods, particularly octopus, the chemical and visual perceptions are most likely the dominant sensory modality. When both chemical and visual information is available, octopuses combine information from all sensory inputs that they perceive and then the animals can camouflage themselves, escape a predator, or chase prey or a partner in the wild, or open jars for food in captivity (Richter et al., 2016; Anderson and Mather, 2010; Bertapelle et al., 2017). However, the relative contribution of each sense remains poorly understood.

For searching and selection of food behavior, octopuses use multiple sensory modalities, in particular, chemical and visual cues. However, the question of which sensory cues are thought to be more involved in food selection ability in *O. vulgaris* remains open. In the first experiment of this study, we designed behavioral trials to investigate the priority given to chemical vs. visual perception, establishing the sensorial hierarchy in food choice by *O. vulgaris.* The behavioral results clearly showed that *O. vulgaris* integrates sensory information from chemical and visual cues during food choice. Nevertheless, food choice resulted in being more dependent on chemical cues than visual ones (88.9%, Friedman test $p < 0.05$), with a consistent decrease of the time spent identifying the preferred food. These results define the role played by the senses with a sensorial hierarchy in food choice, opening new perspectives on the *O. vulgaris'* predation strategies in the wild, which until today were considered to rely mainly on visual cues.

Furthermore, octopuses evolved many unique organs that allow them to sense and explore diverse environments such as arm suckers that function as specialized tactile and chemosensory organs, as well as an elaborate chromatophore system under direct neural control that enables rapid changes in appearance (Hanlon and Messenger, 1996; Kröger et al., 2011). It has been demonstrated that octopuses widely use their suckers for sensing and exploring the environment (Kier and Smith, 1990). Octopus have chemoreceptors on their suckers (Graziadei, 1958 Graziadei and Gagne 1976), which are thought to facilitate a taste-by touch ability (Van Giesen et al., 2020; Wells, 1963; Wells et al., 1965). However, many insoluble molecules that are detected from distance on land known as "odors" (smaller than 300 Da), must be perceived by touch in aquatic systems and octopuses can "smell" them by touch using suckers, exhibiting a peculiar behavior that has been described recently as "smell by touch" based on the kind of molecules that they can perceive. (Polese et al., 2015; Di Cosmo et al., 2018; Di Cosmo and Polese 2017). To investigate the molecular bases for the tactile form of olfaction process in the sucker of *O. vulgaris*, I utilized the biomolecular approaches to investigate the presence of olfactory receptors on octopus' suckers. I have localized the expression octopus-TAARs (Trace Amine Associated Receptors) in the sucker of *O. vulgaris* and *O. bimaculoides* using a whole-mount *in situ* hybridization, and real-time qPCR. With personalized experimental protocols I was able to determine the exact localization and the expression levels of three octopus TAARs. Furthermore, bioinformatic methods for phylogenetic analysis combined with behavioral experiments allow us to provide a comprehensive view on the evolution of chemoreception in the common octopus, *O. vulgaris*.

In addition, the octopus sucker epithelium has been shown to contain a variety of specialized sensory receptors, giving them unique features to perform a remarkable variety of sensory functions (Guerin, 1908; Martoja and May, 1956; Rossi and Graziadei, 1958 Graziadei and Gagne 1976; Packard 1988). However, studies related to identifying the function of these sensory receptors at the molecular levels are still scarce. In the current study, for the first time, besides to state the presence of *O. vulgaris* GRK1(G- Rhodopsin kinase1, Ov-GRK1) gene expression in eyes and skin, we localized its expression in the suckers rim epithelium. Furthermore, we also quantify the relative mRNA in different sucker types at several arm levels. We sequenced the Ov-GRK1 gene defining a phylogenetic tree and performing a 3D structure model prediction using homology modeling. Taking together our data extend the

touch/chemo sensations of these structures (Maselli et al. 2020, van Giesen et al. 2020) to the light-sensing ability suggesting the sucker of *O. vulgaris* as extra-ocular photoreceptive system.

Furthermore, while all visual processing is conducted to the optic lobe (Young, 1963), organization and localization of the photoreceptor molecules in the optic lobes is thus essential for our understanding of the molecular basis of the visual perception in octopus. Although our overall understanding of optic lobe structure and function, the detailed neural organization of photoreceptor molecules has not been characterized. Also, our understanding of the basis of the vision ability in octopus is still quite limited. In this study, I have mapped and localized the expression of mRNA transcripts for photoreceptor molecules including retina rhodopsin, rhabdomeric opsin, melanopsin, retinochrome, and rhodopsin kinase throughout the entire optic lobe of *O. vulgaris*.

Last but not least, besides their sensory systems, my attention was focused on the cognitive system that in octopuses is considered as integrated, adaptive system able to perform a myriad of cognitive functions in the brain achieving sophisticated vertebrate-like plasticity and neural control (Shomrat et al. 2008; Edelman and Seth 2009, Young 1991). Octopus show an extraordinary learning ability, cognitive function, and adaptability are linked to the increments of the adult neurogenesis in the neuro-genic zones of the octopus brain (Bertapelle et al. 2017; Di Cosmo et al., 2018). In this study, we used a combination of ethological and genetic approaches to evaluate the gene expression patterns in the specific brain areas previously identifies ad neuronal proliferation sites (Bertapelle et al., 2017; Di Cosmo et al., 2018).

Firstly, we evaluated the optimal acclimatization period needed for an *O. vulgaris* before starting a cognitive stimulation experiment. Subsequently, we analyzed differential gene expression in specific brain areas in adult animals kept under different cognitive stimulations: tested (enriched environment), wild (naturally enriched environment), and control conditions (unenriched environment). Then, we selected and sequenced three protocadherin genes (PCDHs) involved in the development and maintenance of the nervous system; three Pax genes that control cell specification and tissue differentiation; the Elav gene, an earlier marker for neural cells; and the Zic1 gene, involved in early neural formation in the brain. In this study, we found a clearer pair of adult neurogenesis occurring after cognitive

stimulation with Oct-PCDHs, which appeared to be upregulated in both tested and wild octopuses. Our data shows that Oct-PCDHs genes are upregulated in the learning and lower motor centers in the brain of both tested and wild animals (higher in the latter). Combining these results with our previous studies on *O. vulgaris* neurogenesis (Bertapelle et al., 2017; Di Cosmo et al., 2018), we proposed that PCDH genes may be involved in adult neurogenesis processes, and related to their cognitive abilities. The next steps could be directed to expand the gene marker panel in order to deeply understand the adult neurogenesis mechanism.

My PhD had the ambitious aim to provide a comprehensive view on the genetic bases for the tactile form of olfaction, extraocular photoreception, localization of photoreceptors molecules in the optic lobe of *O. vulgaris*, as well as define the major genes are involved in the adult neurogenesis and then the cognitive system in *O. vulgaris*. I have applied a developed whole-mount *in situ* hybridization, real-time qPCR, and bioinformatic methods, supported by behavioral analysis to provide a comprehensive view on these processes in the common octopus, *O. vulgaris*, highlighting how genomic innovation translates into organismal organization novelties. I believe to have contributed to some extent, and think I have also promoted interest in the study. I hope that future studies may contribute in the above lines and that my work, will assist future students.

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APPENDIX

Abbreviations
(alphabetical order)

ANC	Axial Nerve Cord
Anti-dig	Anti-Digoxigenin
AP	Alkaline Phosphatase Buffer
AR	Acetabulum
AS	Anti-sense
BLAST	Basic local alignment search tool
CNS	Central Nervous system
CR	Chemotactile Receptors
DIG	Digoxigenin
DIS	Distal part of the octopus' arms
FP	Food Preference
GMQE	Global Model Quality Estimation
GPCRs	G-Protein-Coupled Receptors
GRK1	G-Protein-Coupled Receptor Kinase 1
HHBlits	Lightning-Fast Iterative Protein Sequence Searching by HMM-HMM alignment
HM	Homology modeling
IF	Infundibulum
L1	Left. 1 of the octopus' arms
LINE	Long interspersed element
lncRNAs	Non-coding RNAs
MgCl2	Magnesium Chloride
MID	Middle part of the octopus' arms
ML	Maximum Likelihood
NBT/BCIP	Nitro Blue Tetrazolium chloride/5-Bromo-4-Chloro-3-Indolyl Phosphate
NJ	Neighbour Joining
OB	*Octopus bimaculoide*
OC	Outer Cortex

OL	Optic Lobe
OR	Olfactory Receptors
OT	Optic Tract Region
OV	*Octopus vulgaris*
Ov-GRK1	G- Rhodopsin Kinase 1
PBS	Phosphate Buffer Saline
PCDH	Protocadherins
PCR	Polymerase chain reaction
PFA	Para-Formaldehyde
PNS	Peripheral Nervous System
PROX	Proximal part of the octopus' arms
R.Z	Radial Column Zone
R1	Right. 1 of the octopus' arms
RHO	Phosphorylation of Rhodopsin
RIM	Epithelium Rim
S	Sense
SMTL	SWISS-MODEL Template Library
SSC	Saline-Sodium Citrate
T.Z	Tangential Zone
TAARs	Trace Amine-Associated Receptors
TEs	Transposable Elements
WM-ISH	Whole -Mount *In Situ* Hybridization
WOOM	Whole Olfactory Mucosa
Znf	C_2H_2 zinc fingers
3D	Three-Dimensional Structure
7TM	7-Transmembrane

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1. Sensorial Hierarchy in Octopus vulgaris's Food Choice: Chemical vs. Visual. Maselli, V.; Al-Soudy, A.S+.; Buglione, M.; Aria, M.; Polese, G.; Di Cosmo, A. Animals 2020, 10, 457.

2. Cognitive Stimulation Induces Differential Gene Expression in Octopus vulgaris: The Key Role of Protocadherins. Maselli, V.; Polese, G.; Al-Soudy, A-S.; Buglione, M.; Di Cosmo, A. Biology 2020, 9, 196.

3. See through the arms: extra-ocular photoreceptive system in the sucker of Octopus vulgaris. Al-Sayed Al-Soudy; Valeria Maselli; Stefania Galdiero; Michael J. Kuba; Gianluca Polese; Anna Di Cosmo. Submitted, Under revision.

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