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Studi sull'ecologia del nudibranco aeolide *Spurilla neapolitana* (Mollusca, Heterobranchia)

Studies on the Ecology of the Aeolid Nudibranch *Spurilla neapolitana* (Mollusca, Heterobranchia)

Tesi di Laurea Magistrale di:

Elisa Fasano

Relatrice:

Chiar.ma Prof.ssa Cristina Gioia Di Camillo

Correlatrice:

Dott.ssa Agnese Riccardi

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ITALIAN SUMMARY

Questo studio mira ad analizzare le interazioni ecologiche del nudibranco aeolide *Spurilla neapolitana*, concentrandosi sul suo ciclo vitale, sulle preferenze alimentari e sulla reazione simbiotica con l'anemone *Aiptasia couhcii* e le zooxantelle che condividono. Gli esemplari sono stati raccolti nella Riviera del Conero (Mar Adriatico) e allevati in condizioni controllate per studiarne strategie riproduttive e comportamento trofico. Esperimenti di laboratorio hanno valutato la risposta del nudibranco a diverse specie di anemone, la selezione e immagazzinamento di nematocisti e il ruolo dei metaboliti rilasciati dalla sua preda nell'induzione della metamorfosi larvale. I risultati evidenziano una stretta e complessa interazione tra il nudibranco e la sua preda, mostrando una risposta all'anemone solo dopo un contatto tra i due apparentemente casuale. *S. neapolitana* trattiene selettivamente alcuni tipi di nematocisti nei cerata, in particolare basitrichi e p-mastigofori, mentre espelle spirocisti tramite le feci, suggerendo un meccanismo di selezione finalizzato alla difesa. L'analisi delle feci ha inoltre rivelato la presenza di zooxantelle espulse, sollevando interrogativi sul loro possibile ruolo nella dispersione dei simbionti. Nonostante il notevole impegno, le fasi del ciclo vitale di *S. neapolitana* non sono state del tutto chiarite tramite l'osservazione in laboratorio, evidenziando le difficoltà nell'allevamento di questa specie. Questo studio fornisce nuove conoscenze sul ruolo ecologico di *S. neapolitana* e sottolinea la necessità di ulteriori ricerche per comprendere meglio le sue relazioni trofiche e simbiotiche negli ecosistemi costieri mediterranei.

ABSTRACT

This study investigates the ecological interactions of the aeolid nudibranch *Spurilla neapolitana*, focusing on its life cycle, feeding preferences, and symbiotic relationship with the sea anemone *Aiptasia couchii* and their shared zooxanthellae. Specimens were collected from the Conero Riviera (Adriatic Sea, Italy) and reared in controlled conditions to examine reproductive strategies and feeding behavior. Laboratory experiments assessed the nudibranch's response to different prey species, its ability to selectively store nematocysts, and the role of prey-derived metabolites in larval metamorphosis. Results indicate that *S. neapolitana* exhibits a complex interaction with its prey, recognising the anemone only after what appears to be a casual contact. The nudibranch retains specific nematocysts in its cerata, primarily basitrichs and p-mastigophores, while expelling spirocysts in its feces, suggesting a selective retention mechanism for defensive purposes. Additionally, fecal analysis revealed the presence of expelled zooxanthellae, raising questions about their potential role in symbiont dispersal. Attempts to close the nudibranch's life cycle under laboratory conditions were unsuccessful, highlighting challenges in rearing this species. This study contributes to the understanding of *S. neapolitana*'s ecological role and underscores the need for further research on the dynamics of its symbiotic and trophic relationships.

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INTRODUCTION

1.1 Spurilla neapolitana

Nudibranchs, often referred to as "sea slugs", are a fascinating group within the Heterobranchia subclass of gastropod molluscs (Ponder *et al.*, 2019). Originally grouped within the now obsolete taxon Opisthobranchia, nudibranchs were reclassified through molecular analyses, highlighting the convergent evolutionary traits shared with other Heterobranchia groups (Haszprunar, 1985; Grande *et al.*, 2004). Renowned for their striking coloration and extraordinary morphological diversity (Agersborg, 1923; Rudman, 1991b; Layton *et al.*, 2018; Lisova & Vortsepneva, 2022), these animals inhabit marine environments worldwide, even in harsh habitats, where they play vital ecological roles (Lambert, 2013; Riccardi *et al.*, 2022).

There are several superfamilies within the order Nudibranchia, including Aeolidioidea, with its distinguishing features and characteristics. The systematics of this taxon is still not completely clear, as new species have been constantly described over the past few years and due to the frequent presence of cryptic species (Edmunds, 2016; Valdés *et al.*, 2018; Schillo *et al.*, 2019; Soon *et al.*, 2023; Galià-Camps *et al.*, 2024). Aeolidaceans are characterized by their predation on cnidarians, the presence of numerous dorsal cerata, and the possession of a large jaw. *Spurilla neapolitana* (Delle Chiaje, 1841) was first found in the Naples Gulf, hence the specific epithet, and attributed wrongly to the *Eolis* genus (Delle Chiaje, 1841; *WoRMS - World Register of Marine Species - Spurilla neapolitana* (Delle Chiaje, 1841), s.d.). In 2006, Willan defined the species as a “globe-trotting nudibranch” because of its wide

diffusion. It's been reported first in the Mediterranean (Trinchese, 1878; Vayssière, 1885; Sánchez-Tocino *et al.*, 2000), then both the western and the eastern Atlantic (Garcia & Cervera, 1985; Domínguez *et al.*, 2008), the Caribbean Sea (Valdés *et al.*, 2006) and also the Pacific Ocean (Gosliner, 1979; Uribe & Pacheco, 2012). In contrast, Carmona and her collaborators in 2014 published a paper describing the geographical range, endorsed by molecular data, going from the whole Mediterranean Sea to the Atlantic Ocean, between France and Senegal, whilst the other report could be of other species of the same genus (*Fig. 1*) (see also Carmona *et al.*, 2013).

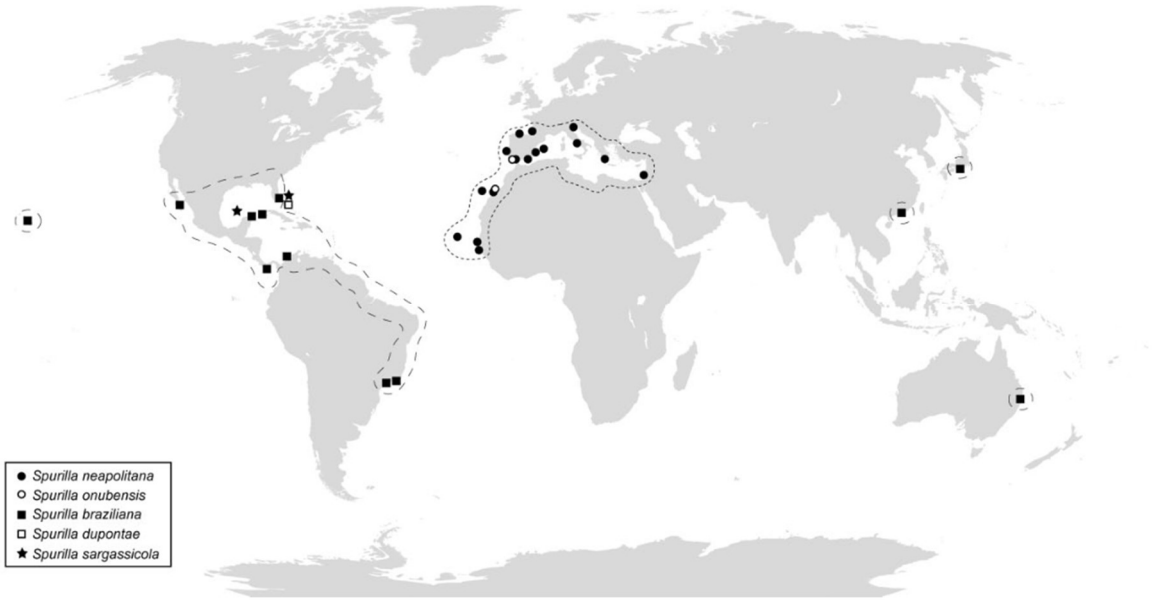


Fig. 1: map of the distribution of the different species of *Spurilla* (Carmona *et al.*, 2014)

Because of its alleged worldwide presence and its variable colour, from pink to orange to brown, based on its diet (Trainito & Doneddu, 2014), to *S. neapolitana* have been attributed different synonym, nowadays all unaccepted according to WoRMS: *Eolis neapolitana* Delle Chiaje, 1841; *Eolis alderiana* Deshayes, 1865; *Eolis conspersa* P. Fischer, 1869; *Eolis*

neapolitana Delle Chiaje, 1841; *Flabellina inornata* A. Costa, 1866; *Spurilla mograbina* Pruvot-Fol, 1953; *Spurilla vayssierei* García-Gómez & Cervera, 1985.

In the years following the discovery, R. Bergh carried out the first anatomical research and put the nudibranch in the genus *Spurilla* (Bergh, 1864, 1874). In 1878, S. Trinchese integrated the descriptions of the anatomy of the animal, reporting for the general body structure that *Spurilla neapolitana* exhibits the elongated, soft body typical of nudibranchs, with bilateral symmetry. The body is divided into a cephalic region, the mantle, and a muscular foot used for locomotion. It possesses prominent perfoliated rhinophores, located dorsally on the head, that represent the only morphological synapomorphy of the genus *Spurilla* (Carmona *et al.*, 2014). These are lamellate or annulated, serving as primary sensory structures for olfaction and environmental navigation. Between the rhinophores are present two little eye spots. The nudibranch also presents narrow, elongated oral paired tentacles near the mouth, that aid in tactile sensing and food localization, and dorsal ceras or cerata (*Fig. 2*). These last ones are a defining structure for this species, with its recurved shape, arranged in packed rows along the body, longer in the dorsal section rather than the lateral ones. Ceras are multifunctional, serving roles in respiration, digestion, and defence. They harbour the digestive gland extensions, allowing the sequestration of nematocysts from prey in a specialised cnidosac (Trinchese, 1878; Trainito & Doneddu, 2014; Valdés *et al.*, 2018). Between the first and second group of cerata there is the cardiac region, where heart contractions, and the separation between the ventricle and atrium, can be observed in live animals (*Fig. 2B*) (*Spurilla neapolitana*, 2012).

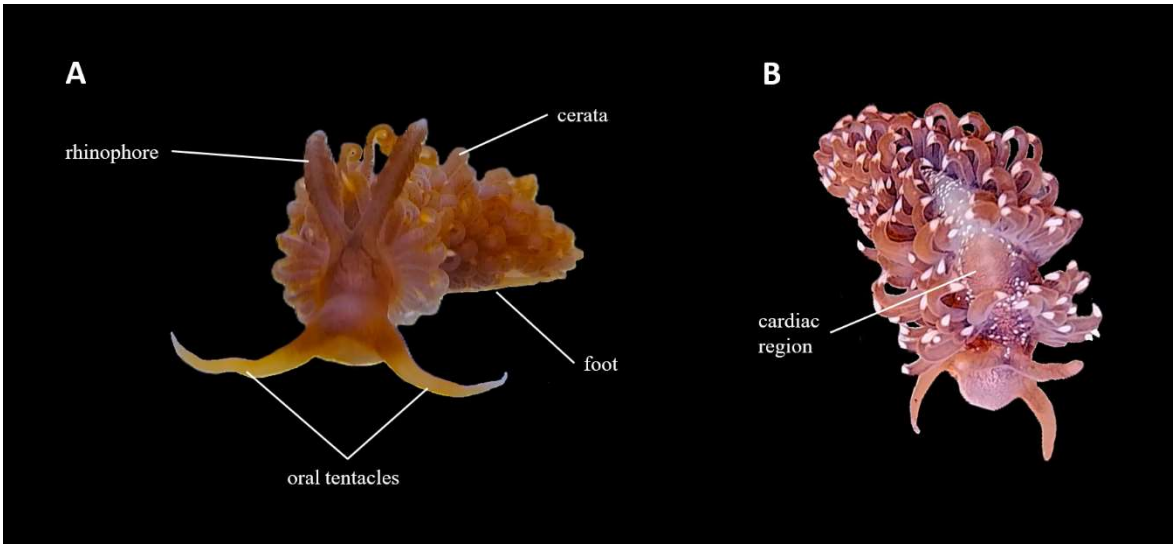


Fig. 2: photos of two reared *Spurilla neapolitana* with the main morphological features. A) frontal view; B) top view

Internally, the nudibranch presents a digestive system adapted for its carnivorous diet and is visible by transparency. The apparatus begins with a very large, muscular buccal bulb, that contains the most adapted structure, the radula, a toothed structure within the mouth, essential for scraping or consuming prey (Valdés *et al.*, 2018). The jaws have a smooth or finely denticulate masticatory edge and the radular teeth become smaller towards the back of the radula. Each of the 10-13 curved tooth is bi-arched, with 10–60 long, sharp denticles on either side of a small triangular central cusp. Large oral glands are located dorsolaterally to the buccal bulb, while salivary glands are absent (Trinchese, 1878; Domínguez *et al.*, 2008; Carmona *et al.*, 2014). The digestive gland extends into the cerata, where it processes food and stores defensive structures. The anus is cleioproctic, meaning that is in lateral position, surrounded by the certain the anterior part of the left lobe of the digestive gland (*Glossary*, 2020). The respiration occurs primarily through the cerata, which increase the surface area for gas exchange. This adaptation is particularly suited for the species' intertidal and shallow-water habitats. The central nervous system is well-developed, featuring

cerebral, pleural, and pedal ganglia (Trinchese, 1878). The rhinophores and tentacles are highly innervated, emphasizing the species' reliance on sensory input (Arey, 1918). Like other nudibranchs, *Spurilla neapolitana* is hermaphroditic, possessing both male and female reproductive organs. The reproductive system is very elongated and convoluted, ending with the female gland complex next to the prostate opening. It includes structures for internal fertilization and the production of gelatinous egg masses (Trinchese, 1878; Valdés *et al.*, 2018).

1.1.1 Cerata

A cerata is a hollow, dorso-ventrally flattened structure that houses diverticula from the digestive gland. These diverticula serve as conduits for ingested nematocysts to travel to the cnidosacs located at the tips of the cerata. A sphincter muscle separates the cnidosac from the rest of the ceras. Once within the cnidosac, nematocysts are organized into structures known as cnidocysts, likely facilitated by phagocytic cells. These cnidocysts store the nematocysts until they are needed for use (*Fig. 3*) (Vayssière, 1885; Goodheart *et al.*, 2018).

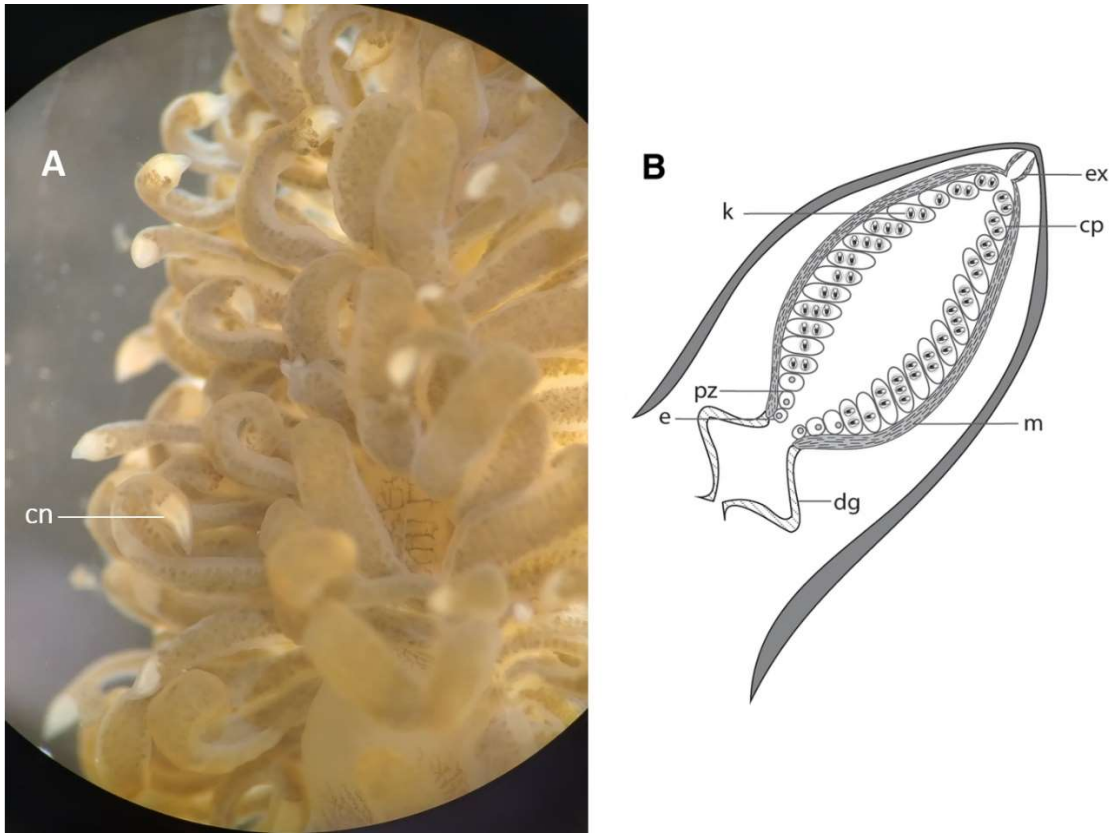


Fig. 3: morphology of the cerata. A) stereoscopic microscope photo of *Spurilla neapolitana*; B) main morphological features of a cnidosac from Goodheart *et al.*, 2018. Legenda: cn, cnidosac; cp, cnidophage; dg, digestive gland; e, entrance (or ciliated channel in some cases); ex, exit (or pore in some cases); k, kleptocnides; m, musculature; pz, proliferation zone

Most nematocysts are incorporated into cnidocysts, which are subsequently stored in the cnidosac. Within the cnidosac, the nematocysts are oriented so their functional ends point outward—an arrangement similar to their positioning in the tentacles of the anemones from which they were originally derived. Conklin and Richard already observed in 1977 that both individual nematocysts and those within cnidocysts can be observed discharging upon contact with the surrounding medium after being expelled from the cnidosac. At the tip of the cerata a cnidopore is present, through which nematocysts are released, a small opening controlled by a sphincter muscle. Near the cnidopore are the openings of the defensive glands, which are thought to play a role in predator defence (Edmunds, 1966).

The surface of the cerata is covered with large bundles of motile cilia. These cilia are more abundant near the base of the cerata and can move small particles or possibly a mucous sheet over the surface. In contrast, smaller, non-motile ciliary bundles are primarily located toward the ceras tip. These sensory bundles are positioned at the junctions of three or more cells and may be formed by contributions from multiple cells (Edmunds, 1966; Vorobyeva *et al.*, 2021). The surfaces of the cerata cells are covered by a dense layer of short microvilli. Some of these microvilli appear to form fine interconnections and may be arranged in rosette-like patterns. Finally, extruded and discharged nematocysts are sometimes found near the cnidopore on the ceras tip (Conklin & Mariscal, 1977).

1.1.2 Rhinophores

It is not completely clear where the term *rhinophore* is derived, though it has always been clear that their purpose is deputed to multiple sensory activities (Arey, 1918). In 1975, Audesirk demonstrated how another nudibranch, *Aplysia californica* J. G. Cooper, 1863, primarily use rhinophores and tentacles to distance food localization, using a Y-maze. In the following years, the presence and importance of chemosensory capacities, including both contact and distance chemoreception (olfaction), has been proved by different authors (Boudko *et al.*, 1999; Wyeth & Willows, 2006; Göbbeler & Klussmann-Kolb, 2007). They demonstrated how rhinophores are critically important for their behavioural coordination and orientation to prey, conspecific individuals, and potential predators in the environment. Studies also suggest that rhinophores are more effective in detecting distant chemical cues than oral tentacles, which are primarily involved in contact chemoreception.

The sensory cells in rhinophores are typically arranged in subepidermal clusters, extending, through dendrites, to the outer surface. These clusters often contain various types of receptor cells, including subepidermal sensory cells, intraepidermal sensory cells, and ciliated cells, each potentially specializing in specific sensory functions (*Fig. 4*). The first ones are primarily associated with olfactory processes, while intraepidermal sensory cells are mediate the contact chemoreception stimuli. Ciliated dendrites help in mechanoreception or the detection of water currents. These adaptations allow nudibranchs to navigate using chemical and hydrodynamic cues, underscoring the rhinophores' essential role in the nudibranch's sensory and ecological interactions (Boudko *et al.*, 1999; Göbbeler & Klussmann-Kolb, 2007).

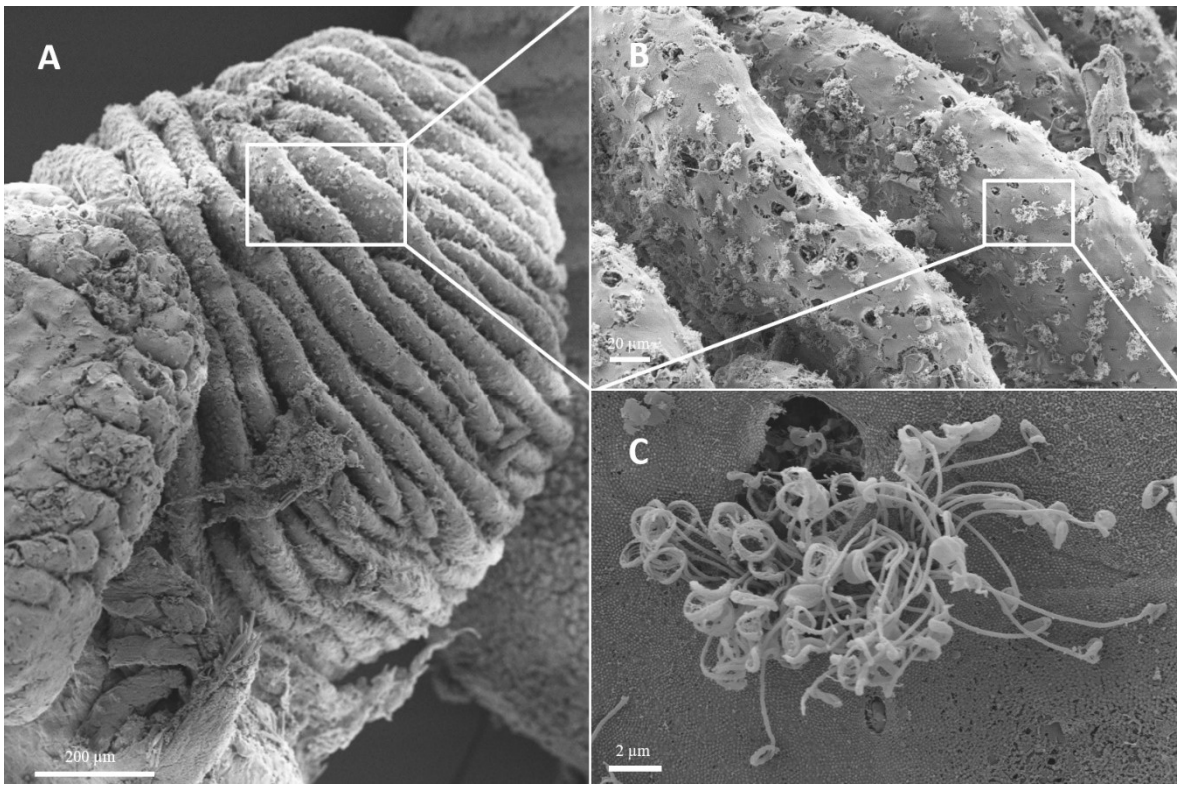


Fig. 4: Scan Electron Microscopy of the rhinophore of *Spurilla neapolitana*, with B) detail of the surface of the epidermis and C) of the ciliated cells

1.1.3 Life cycle

As most marine heterobranchs, *Spurilla neapolitana* is semelparous, undergoing an annual life-cycle with a discrete spawning period and total post-reproductive mortality (Todd, 1981; Havenhand & Todd, 1989). Fertilization is internal and, as for all opisthobranchs, individuals are simultaneously hermaphrodite and are obligatory outbreeders (Todd *et al.*, 1991). When two sexually mature individuals meet, they assume the typical mating position, juxtaposing the genital pores. To do so, the nudibranchs will oppose the direction of the bodies, making coincide the head region of one with the posterior end of the other and vice versa (Costello, 1938).

Virtually nothing is known of the life history of *S. neapolitana*. The only work that discusses and successfully carried out a laboratory culture is from Schlesinger and colleagues (2009). They sampled adults and reared them to collect the embryos, from which the colture started. They observed the main morphologic stages such as embryonic, planktonic, metamorphic, juvenile and adult stages. The nudibranch lay its eggs, throughout the entire year, in the shape of a white, coiled spiral cylindrical string strongly attached to the substratum on which it is laid. The eggs, and then the embryos, are covered by membranes that protect them, from both physical damages and possible pathogens, and the dimension of the cylindrical string is directly correlated with the adult's dimensions and the number of embryos. Spawning of unfertilized oocytes may occur in nudibranchs held in isolation and prevented from copulating. In such cases the spawn mass appears more or less normal, with the oocytes contained within capsules and chains of these embedded in the typical spawn mass stroma,

but unfertilized oocytes do not undergo cleavage (Todd *et al.*, 1997). The development of the larvae is indirect, through a free-living planktotrophic veliger larva. The larval phase of this nudibranch consists of a 30-day growing phase followed by an 18-day non-growth phase in which the larvae are metamorphically competent. Larvae then settled and metamorphosed in seawater containing metabolites from sea anemone prey species, because it appears that larvae of *S. neapolitana* respond to cues that induce settlement and ultimately recruit to several sea anemone species, of both fouling and inter-tidal rocky shore communities. Schlesinger's study showed that a crawling post-larva (250 μm long) must find a food source within 4 days. During the first 2 weeks after metamorphosis, juveniles appeared to feed upon and coat themselves with the mucous secretion of their sea anemone prey, probably acting as a chemical camouflage. Sea anemones appeared to be consumed by 17 days post-metamorphic juveniles and the reproductive. Copulation was first observed 37 days post-metamorphosis, and oviposition occurred 2 days later (Schlesinger *et al.*, 2009).

1.1.4 Feeding behaviour

Spurilla neapolitana is a carnivorous nudibranch that primarily preys on sea anemones. Its diet includes various actinian species, whose reported are (with the actual accepted names, according to *WoRMS*): *Actinia equina* (Linnaeus, 1758); *Actinothoe sphyrodeta* (Gosse, 1858); the genus *Aiptasia* Gosse, 1858 with the species *A. couchii* Gosse and *A. mutabilis* (Gravenhorst, 1831); *Aiptasiogeton hyalinus* (Delle Chiaje, 1822); *Anemonia sargassensis* Hargitt, 1908, *A. sulcata* (Pennant, 1777) and *Anemonia viridis* (Forsskål, 1775); *Anthopleura ballii* (Cocks, 1851) and *A. krebsi* Duchassaing & Michelotti, 1860; *Bunodactis rubripunctata* (Grube, 1840) and *B. verrucosa* (Pennant, 1777); *Bunodeopsis* sp.,

Bunodeopsis antilliensis Duerden, 1897; *Calliactis parasitica* (Couch, 1842); *Cereus pedunculatus* (Pennant, 1777); *Condylactis gigantea* (Weinland, 1860); *Diadumene lineata* (Verrill, 1869) and *Diadumene cincta* Stephenson, 1925; *Exaiptasia diaphana* (Rapp, 1829); *Lebrunia neglecta* Duchassaing & Michelotti, 1860; *Metridium senile* (Linnaeus, 1761); *Paractinia striata* (Risso, 1826); *Paranemonia cinerea* (Contarini, 1844); *Sagartia lacerata* (Dalyell, 1848), *S. undata* (Müller, 1778) and *S. viduata* (Müller, 1778); *Telmatactis forskalii* (Hemprich & Ehrenberg in Ehrenberg, 1834); *Urticina felina* (Linnaeus, 1761) (Conklin & Mariscal, 1977; McDonald & Nybakken, 1997; Marín & Ros, 1991; Betti *et al.*, 2017). Given the number of anemone species it feeds on, *Spurilla neapolitana* is considered a generalist species (Todd *et al.*, 2001). This nudibranch predominantly consumes the oral disk and tentacles of its prey (Conklin & Mariscal, 1977). Based on the colour of the anemone it consumes, the nudibranch takes the cryptic coloration that provides effective camouflage, blending it into its environment.

Conklin and Mariscal (1977) described the predation process of *S. neapolitana*: before feeding, the nudibranch undergoes an acclimation phase similar to that of anemonefish. During the initial minutes of an attack, *S. neapolitana* is stung by the anemone's nematocysts. However, it eventually becomes immune to the stings. Nudibranchs produce species-specific chemicals to inhibit nematocyst discharge (Greenwood *et al.*, 2004). Each prey species elicits a unique chemical inhibitor, enabling *S. neapolitana* to neutralize the defences of multiple anemone species effectively. Once acclimated, the nudibranch can consume significant portions of its prey, feeding for up to an hour and ingesting 25–100% of the anemone depending on its size (Conklin and Mariscal, 1977).

1.1.5 Symbiotic relationship

Another intriguing and fascinating aspect of *Spurilla neapolitana* is its ability to combine the carnivorous diet with the utilization of zooxanthellae, symbiotic dinoflagellates of the genus *Symbiodinium* LaJeunesse, 2017 (Dinophyceae) acquired horizontally from its cnidarian prey (Rudman, 1982; Burghardt & Wägele, 2014; Ciavatta *et al.*, 2017). These algae, once ingested, are transported from the digestive lumen into the cells of the nudibranch's digestive gland, where they remain photosynthetically active and are able to reproduce asexually (Marín & Ros, 1991). This mechanism is called kleptoplasty (Marshall, 2006). Over time, the zooxanthellae are expelled from the host's body through the feces, displaying varying levels of cellular integrity, ranging from healthy to significantly degenerated states (Kempf, 1984; Parker, 1984). The host benefits from symbionts that act as a sink for potentially toxic nitrogenous waste compounds while the symbionts benefit from access to the N source for growth and find a protected environment (Douglas, 2008). Moreover, the host can regulate the relationship by ingesting or not cnidarian preys. This kind of association plays a significant role in nutrient cycling, and thus in the conservation of marine ecosystems, particularly in oligotrophic waters (Muscatine & Porter, 1977), like the ones in the Mediterranean Sea (Siokou-Frangou et al., 2010; Fuerst-Bjeliš, 2017). This is made possible because the nudibranch would profit from the metabolites produced by the endosymbiotic zooxanthellae and allow the animal to look for food sources in periods of food shortage. To facilitate the creation of photosynthetic products created by zooxanthellae, the flattened cerata serves the purpose of increasing the surface area, for gas exchanges, and sunlight absorption (Marín & Ros, 1991). For this reason, aeolids are often referred as solar-powered sea slugs. As proposed by Rudman (1991a), and confirmed by Burghardt in 2007,

a higher branching grade of the digestive gland is positively correlated with a more effective symbiosis and this, together with the morphology of the cerata, can give insights about the evolution of the zooxanthellae-symbiosis (Burghardt *et al.*, 2008). But according to Burghardt, the availability of photosynthetic products would be of second advantage, compared to colour camouflage. Analyses on another nudibranch species included in the same family – but originally belonging to the same genus – *Baeolidia australis* (Rudman, 1982), showed a stable number of zooxanthellae even after 22 days of starvation, highlighting a somehow advanced mutualistic association for this species (Burghardt, 2007). An advanced state is represented in forms with a retarded digestion or excretion of the zooxanthellae (Burghardt *et al.*, 2008). Analyses on the nudibranch *Phyllodesmium* indicated that *Symbiodinum* might switch to a heterotrophic lifestyle when photosynthesis is prohibited. This, together with the protection from excessive solar ultraviolet radiation, could be the mechanisms at the base of the habit of *Spurilla neapolitana* to be found during the day under rocks or at nighttime (Cockell & Knowland, 1999). These strategies not only maximize the benefits of the symbiosis but also ensure protection against potential stressors like UV radiation. *Spurilla neapolitana* thus exemplifies how intricate biological relationships and adaptations enable survival and ecological success in challenging marine environments.

1.1.6 Defence mechanisms

As regards the predators of *S. neapolitana* the literature is basically non-existent. Edmunds, in 1966, refers that eolids are rejected as food by several species of fish – with *Aeolidia papillosa* (Linnaeus, 1761) being the sole exception – and some potential defensive

strategies against crustaceans are described. Finally, *Navanax inermis* (J. G. Cooper, 1863) is mentioned as a typical predator of *Hermissenda crassicornis* (Eschscholtz, 1831) and another aeolid nudibranch but still, an updated or specific list of *Spurilla neapolitana* is lacking.

Primary defences are mechanisms that decrease the probability that an interaction will occur between a prey animal and a potential predator, and function regardless of whether a predator is present or not (Field & Glasgow, 2001). It's been already described as the different colourations of *Spurilla neapolitana*, based on its diet, helps the nudibranch in camouflaging, making it inconspicuous and cryptic when in their normal habitat. But it is not the only defence mechanism that it possesses.

Ceratal autotomy is the process in which ceras are voluntary detached from the body wall along an autotomy plane located at its basal constriction, releasing copious amounts of mucus (Edmunds, 1966). The detached ceras regrow back in a way that becomes indistinguishable from the other ceras (Miller & Byrne, 2000). This process helps the nudibranch to escape predation, breaking free from a predator's grasp or to distract the predator (Emberts *et al.*, 2017). Given that the cost of autotomy depends on the function of the lost organ, this species shows minimal short-term and no long-term costs to autotomy (Fleming *et al.*, 2007). Anyhow, the ceratal autotomy is less readily done in the Aeolidiidae family, to whom *Spurilla* belongs, than in other aeolid families (Edmunds, 1963).

Moreover, Aeolids show a peculiar structure of the epidermis, packed with empty-looking ellipsoidal vesicles. These supposedly have the function to cushion the body against contact, and hence presumably damage, from hard bodies (Graham, 1938). Internally, they can

protect the digestive system from the occasional nematocyst explosions, because while the nudibranch is likely immune to the toxic and penetrative effects of these nematocysts, it is not entirely unaffected (Edmunds, 1966). Edmunds (1966) also described the defensive glands, or repugnatorial glands, thought to be also involved in defence against predators. These glands are unicellular, surrounded by fine muscle fibres and secrete a mucopolysaccharide. Epidermal or subepidermal glands are involved in cleaning the epidermis from micro-organisms by producing a slime cover, which must be renewed occasionally (Wägele *et al.*, 2006). In *Spurilla neapolitana*'s cerata, two types of ceratal gland are present, both being distributed over the entire ceratal surface (*Fig. 5*). One of the two is an osmiophil gland, that is unicellular, flask-shaped with coiled contents and give its name by the fact that can be coloured by an osmium-base fixative. Most opisthobranchs possess more than these two types of glands and the foot is particularly highly glandular, but are primarily involved in other function such as locomotion (Wägele *et al.*, 2006).

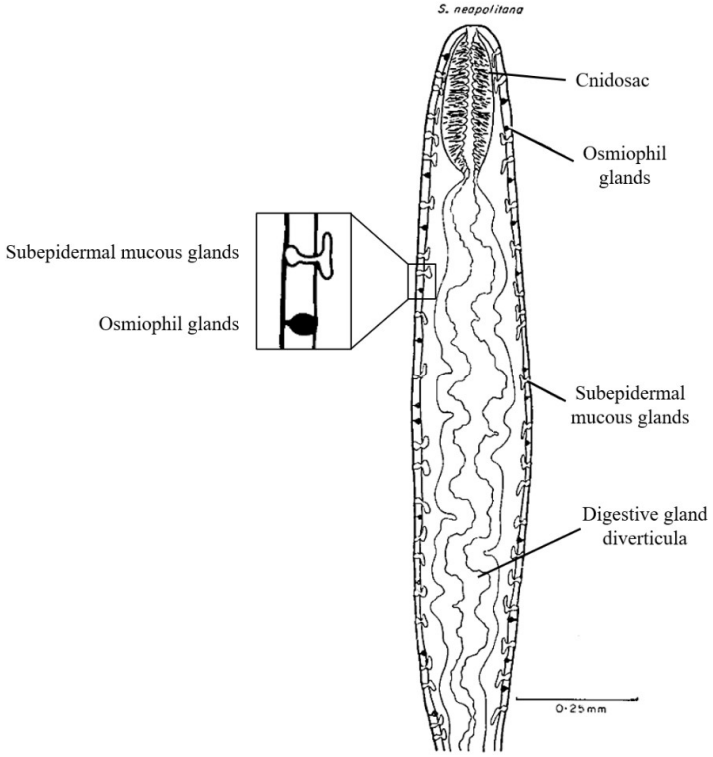


Fig. 5: defensive ceratal glands of *Spurilla neapolitana*. Modified from Edmunds, 1566

As discussed above, the cerata of *Spurilla* also contain zooxanthellae derived from the preys' tissues, as such this gives the nudibranch the same colouration of the anemones with which it lives, enhancing the camouflaging characteristic of the animal.

Ciavatta and her colleagues in 2017, isolated a secondary metabolite biosynthesised by this species. This compound, named spurillin A, is a terpenoid whose origin remains debated. It is unclear whether spurillin A is produced *de novo* by the nudibranch or derived from dietary sources, potentially not linked to cnidarians but rather to symbiotic microorganisms, such as zooxanthellae (Ciavatta *et al.*, 2017). This terpenoid may function as a defensive strategy of the nudibranch, potentially acting as a toxic or unpalatable compound to deter predators (Winters *et al.*, 2018, 2022).

Another very unique tactic typical of aeolid nudibranchs is kleptocnidiosis, a specialized biological process that involves the sequestration and utilization of nematocysts from its cnidarian prey (Garese *et al.*, 2013). This process begins when *S. neapolitana* consumes anemones, ingesting nematocysts along with the prey's tissues. This stinging cells, which cnidarians use for predation and defence, are transported through the nudibranch's digestive tract without being triggered. As the prey tissues are processed, immature nematocysts are selectively transported from the lumen of the digestive system into the gland's specialized cells, the cnidophages. There are several membranous structures within the cnidophages, but it appears that only the original nematocyst membrane remains, following the fact that they break down shortly after the inclusion of the nematocysts. This avoid the digestion of the cells and allows their keeping and maturity (Greenwood & Mariscal, 1984). Once sequestered, the nematocysts are referred to as kleptocnidae (Greenwood, 2009). Studies, such as those by Greenwood *et al.* (2004), suggests that *S. neapolitana* produces specie-specific inhibitory compounds that neutralize the firing mechanism of the nematocysts. allowing it to incorporate nematocysts from diverse anemone species effectively. Moreover, cells of epithelium and stomach contains intracellular chitin, as a protective layer against the deleterious effects of nematocysts (Martin *et al.*, 2007). Once inside the digestive gland, the nematocysts are encapsulated and stored in specialized aggregations called cnidocysts. This encapsulation preserves the nematocysts' structural and functional integrity while preventing accidental activation that could harm the nudibranch. Encapsulation ensures that the nematocysts retain their toxic capabilities, enabling the nudibranch to repurpose them for its own defence (Greenwood, 2009). By maintaining these nematocysts in a ready-to-use state, the nudibranch can deploy them against predators when needed. Nematocysts are a product of an actinian giant post-Golgi vesicle and consist of several different proteins that

assembles into a large capsule, containing a long spiny tubule. When triggered by the cnidocil, the sensory receptor of the nematocyte, the mature nematocyte discharge its tubule in less than 3 milliseconds, delivering a sting to its prey (*Fig. 6*) (Fautin, 2009; Özbek *et al.*, 2009).

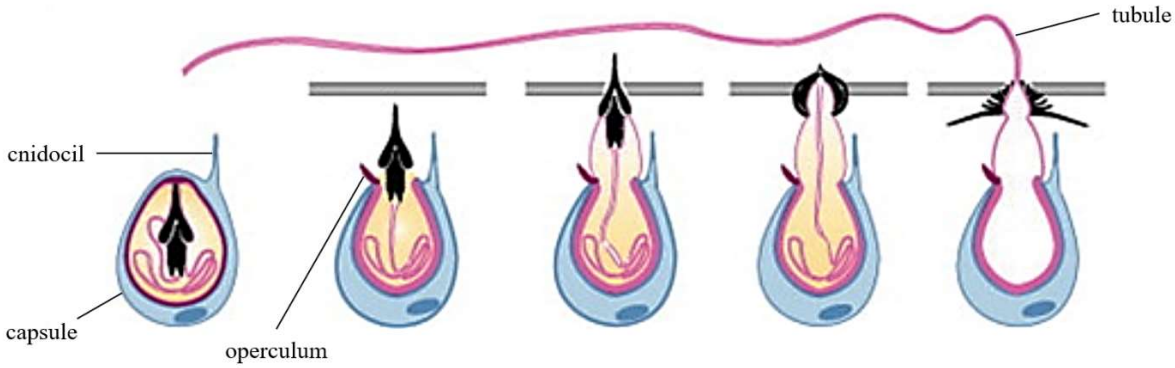


Fig. 6: Morphodynamics of nematocyst discharge, modified from Özbek *et al.*, 2009

Secondary defence mechanisms operate when a prey animal detects a predator, or when under attack (Edmunds, 1974). When the animal is disturbed, *S. neapolitana* shows a deimatic behaviour, contracting the body and rhinophores whilst erecting and waving the ceras. This is aimed to distract or confuse the potential predator, exposing the unpalatable and expendable parts of its body (Edmunds, 1963; Drinkwater *et al.*, 2022). If the predator still attempts to eat the nudibranch, it will likely get a cluster of the easily detached cerata whilst the body of the animal remains unharmed.

All these defensive strategies, both in behavioural and anatomical adaptations, reflect the advanced evolutionary adjustment of *S. neapolitana* to its ecological niche, taking into account the lack of a protective shell (Faulkner & Ghiselin, 1983).

1.1.7 Threats and conservation

Spurilla neapolitana faces a range of ecological and anthropogenic threats that jeopardize its survival and ecological role. Being typical of intertidal zones, it is particularly vulnerable to the pressures this dynamic environment endures, especially when habitat loss occurs. Intertidal ecosystems, located between the high and low tide marks, are among the most dynamic and ecologically significant environments on Earth (Raffaelli & Hawkins, 1996). These zones host a rich biodiversity, supporting numerous species of algae, invertebrates, and vertebrates adapted to fluctuating conditions. The constant exposure to tidal cycles creates a unique environment where organisms must endure changes in temperature, salinity, oxygen levels, and moisture (Helmuth, 1999; Helmuth & Denny, 2003). Additionally, intertidal zones play a crucial role in nutrient cycling, sediment stabilization, and as nurseries for marine species (Raffaelli & Hawkins, 1996). Despite their importance, intertidal ecosystems face significant threats. Their accessibility makes them susceptible to various anthropogenic impacts, originating from both the land and the sea (Thompson *et al.*, 2002). Coastal development, including construction of harbours and seawalls, leads to habitat loss and fragmentation. Tourism, particularly in regions with rocky shores and tide pools, increases trampling of sensitive organisms like anemones and sponges. Industrial activities, such as oil spills, cause catastrophic damage to intertidal zones by smothering organisms and contaminating sediments. Additionally, invasive species introduced through shipping and aquaculture compete with native species, altering community structures (Mieszkowska *et al.*, 2013).

The most wide-spread threat to invertebrates is climate change (Hughes *et al.*, 2017; Pandori & Sorte, 2019) and its impacts are exacerbated in intertidal habitats (Mieszkowska *et al.*, 2013). Intertidal invertebrates, which occupy lower trophic levels, are particularly sensitive to climate alterations, often exhibiting the earliest responses in a chain of effects through the food web (Johnson *et al.*, 2011). Beyond temperature, other climate-related drivers, including sea-level rise, increased wave height and storm intensity, and secondary impacts from adaptation and mitigation strategies in coastal areas, are affecting intertidal habitats (Mieszkowska *et al.*, 2013). Ocean acidification, caused by CO_2 emissions and subsequent absorption into oceans, is particularly concerning. This process shifts seawater chemistry toward acidity, impacting organisms that rely on calcium carbonate structures. Between others effects, chronic exposure to ocean acidification alters vital physiological processes such as respiration and metabolism (Doney *et al.*, 2020). Because nudibranchs heavily rely on chemoreception, they are sensitive to the change in pH associated to acidification (Seroy & Grünbaum, 2018).

Indirectly, the species is also subjected to other threats, present on a wider spatial scale. Since the distribution range of nudibranchs frequently coincides with that of their preys, the disappearance of nudibranchs from a given area is very often due to the unavailability of prey organisms (Todd *et al.*, 2001). Despite being considered a generalist species, *S. neapolitana* is still specialised at a higher taxonomic level, in being obligately associated with cnidarians. For this reason, the disappearance of anemones will bring to the disappearance of the nudibranch too. Cnidarians are also facing numerous threats, between the others all of the listed before. Moreover, the concomitance of different stressors on the cnidarian component of ecosystems can facilitate the diffusion of diseases and the formation

of outbreaks (Behringer *et al.*, 2020). In the Mediterranean Sea mass mortality events have been reported from various authors and they can be driven by different causes, but primarily by heatwaves (Garrabou *et al.*, 2009; Camillo & Cerrano, 2015; Hughes *et al.*, 2017; Garrabou *et al.*, 2022). These, contrarily to a gradual temperature change, are sudden events and as such could be of greater concern (Smale *et al.*, 2019). Additionally, climate change infers also with the interactions between the different components of the ecosystem: in this case, it can result in the disruption of the synchronisation of life cycles between nudibranchs and cnidarians (Damien & Tougeron, 2019).

Because changes in abiotic conditions affect the life histories of organisms across all trophic levels, ultimately altering biotic interactions, community stability and ecosystem functioning (Blois *et al.*, 2013), monitoring of climate change indicators will be fundamental to formulate and inform better adaptive strategies to address the inevitable consequences of climate change (Philippart *et al.*, 2011). In 2011, Goddard & Pearse demonstrated that Californian nudibranchs are good indicators of nearshore ocean climate, and that the more conspicuous species would be appropriate for monitoring in fixed areas by personnel and citizen scientists. Other studies have also demonstrated how climate affects the distribution of these animals (J. H. R. Goddard *et al.*, 2016; Nimbs & Smith, 2016), but literature that specifically assess the climate change problem on Heterobranchia in the Mediterranean Sea is still lacking. A virtuous case of monitoring is the Sea Slug Census program, in Australia, that made proper use of citizen scientists to record the diversity and distribution of heterobranchs across multiple locations (Smith & Nimbs, 2022). Citizen Sciences is based on the involvement of citizens in the collection of scientific data, allowing both an expanded spatial and temporal range of data collection and the increase in society perception and

knowledge of the environment (Thiel *et al.*, 2014). Knowing that *S. neapolitana* is particularly vulnerable to changes that disrupt prey availability and life cycle synchronization, it become essential to monitor these ecosystems, with nudibranchs serving as effective indicators of climate impacts. Citizen science initiatives, like Australia's Sea Slug Census, demonstrate the value of public involvement in biodiversity monitoring, bridging data gaps and raising environmental awareness.

1.2 Aiptasia couchii

Sea anemones of the family Aiptasiidae (Cnidaria, Anthozoa, Actiniaria) are conspicuous components of tropical and subtropical shallow and intertidal waters worldwide, characterised by their symbiosis with *Symbiodinium*, that facilitate their rapid growth rate, combined with asexual reproduction via pedal laceration (Clayton Jr, 1985; Presnell *et al.*, 2022). The symbionts reside within the anemone's gastrodermal cells, where they perform photosynthesis, producing glucose and other organic compounds that sustain the host. This relationship is mutualistic, as the anemone provides the zooxanthellae with protection and access to sunlight, as well as nutrients like nitrogen and phosphorus derived from its metabolic waste (Muscatine *et al.*, 1981; Davy *et al.*, 2012). In addition to symbiotic nutrition, *Aiptasia couchii* is a proficient carnivore. It captures prey, such as plankton and small invertebrates, using its nematocyst-laden tentacles. This dual feeding strategy allows the anemone to survive in nutrient-poor environments and contributes to its ecological success.

Adult polyps of *Aiptasia* Gosse, 1858 exhibit radial symmetry, featuring multiple tiers of tentacles encircling the oral disc, which houses a central mouth leading to a muscular

pharynx. Within the inner gastrodermal epithelial layer, longitudinal mesenteries are radially arranged along the central axis. These structures contain gonads and muscles, providing structural support to the body column (*Fig. 7*). The pedal disc is used for locomotion and helps adhere the polyp to surfaces (Shick, 2012). This renders the organisms relatively incapable of rapid locomotion as a method for prey capture, so anemones have several adaptations that aid in this process. Between these, they present nematocysts throughout the body, particularly packed in structures called acontia, thread-like structures located in the gastrovascular cavity of the anemone (Lam *et al.*, 2017). To protect themselves from predator attacks, the anemone ejects acontia by contracting the body column and extending its tentacles in self-defence (Edmunds *et al.*, 1976).

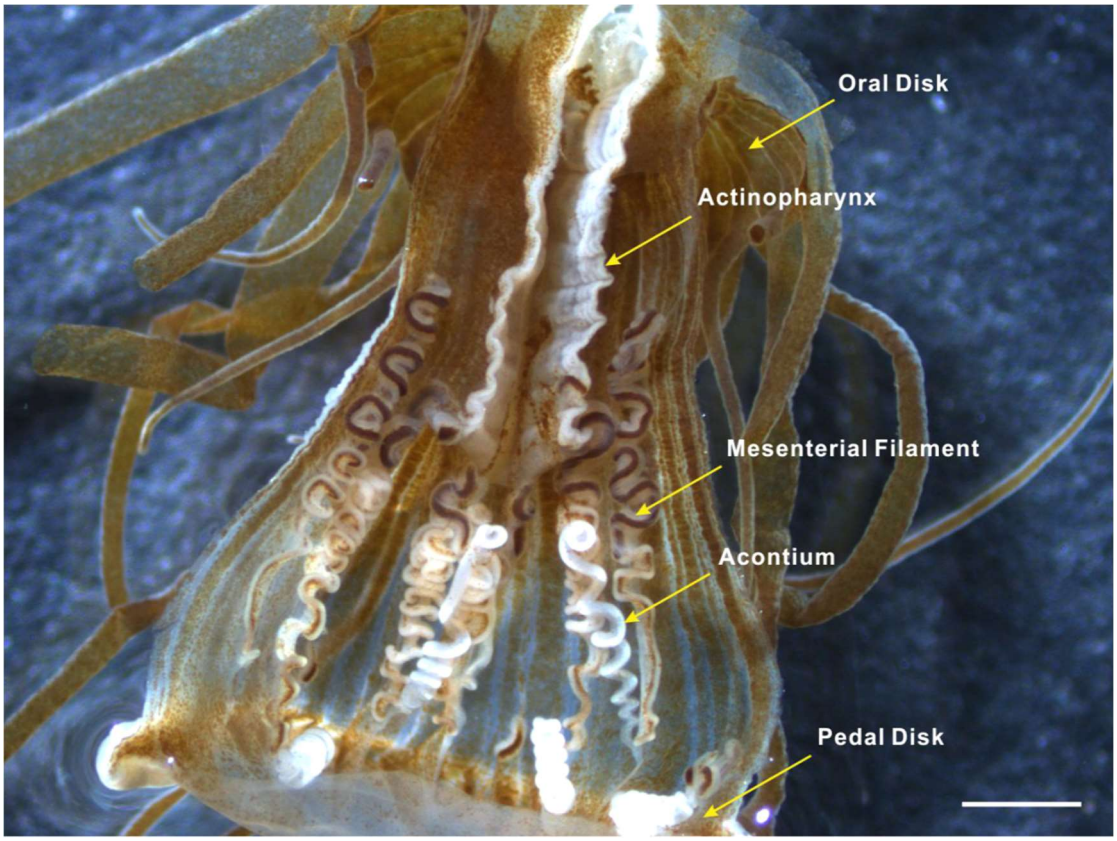


Fig. 7: longitudinal section of an Aiptasiidae, *Exaiptasia* sp., from Lam *et al*., 2017. Scale bar of 1 mm

A. couchii exhibits both sexual and asexual reproduction, enabling rapid population growth and resilience. Sexual reproduction occurs through external fertilization, where gametes are released into the water column. The resulting planula larvae eventually settle and develop into juvenile anemones (Schlesinger *et al.*, 2010). Asexual reproduction, primarily through pedal laceration, is a hallmark of this species. During this process, fragments of the anemone's pedal disk detach and grow into genetically identical clones. This ability to clone itself enhances its colonization potential and ensures survival in fluctuating environmental conditions (*Fig. 8*) (Hunter, 1984; Grawunder *et al.*, 2015).

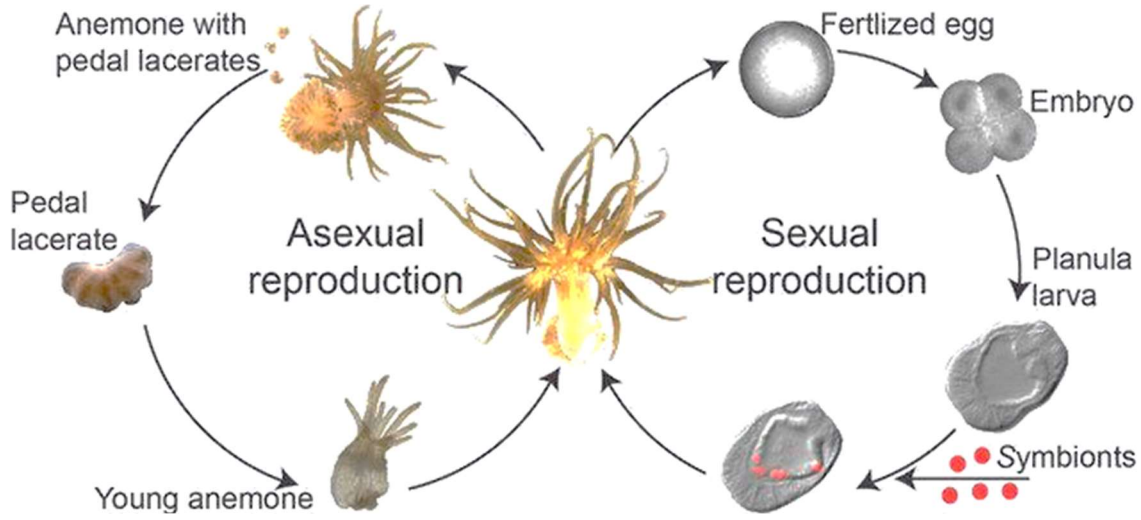


Fig. 8: Overview of *Aiptasia* life cycle showing dual reproductive modes, modified from Grawunder *et al.*, 2015

Aiptasia couchii is widely used as a model organism in scientific research, particularly in studies related to coral biology, symbiosis, and climate change (McDonald & Nybakken, 1997; Weis *et al.*, 2008; Grawunder *et al.*, 2015). Its symbiotic relationship with zooxanthellae mirrors that of reef-building corals, making it an invaluable proxy for understanding coral bleaching, stress responses, and symbiotic dynamics, major threats to anemones and corals (Hoegh-Guldberg *et al.*, 2007). Furthermore, its capacity for asexual

reproduction and ease of maintenance in laboratory conditions make it ideal for experimental studies.

While *Aiptasia couchii* is a resilient species, it is not immune to the impacts of climate change and anthropogenic pressures. Ocean acidification, rising temperatures, and habitat destruction pose threats to its populations, as I have already mentioned for *Spurilla neapolitana* and its preys. The disruption of its symbiotic relationship with zooxanthellae, akin to coral bleaching, could have significant ecological consequences. Efforts to conserve intertidal and coastal habitats indirectly benefit *A. couchii* and the broader marine ecosystems it inhabits. Protecting water quality, mitigating climate change, and establishing marine protected areas are essential steps to ensure the survival of this species and its ecological functions.

Aiptasia couchii exemplifies the adaptability and ecological importance of intertidal cnidarians. Its symbiotic relationships, feeding strategies, and reproductive versatility highlight its role in marine ecosystems and its value as a model organism in scientific research. Understanding and preserving this species is crucial, not only for its intrinsic value but also for the broader insights it provides into the health and functioning of marine environments.

1.3 The Study Area

The Mediterranean basin is one of the very important biodiversity hotspot in the world (Coll *et al.*, 2010; Gerovasileiou & Voultsiadou, 2012; Damalas *et al.*, 2022; Gallozzi *et al.*, 2024). The Conero Riviera is one of its unique environments: it presents a coastal marine ecosystem along the Adriatic Sea, a 15-km long stretch of rocky shore located South of the city of

Ancona, on the central Adriatic shore of Italy. It provides the sole rocky interruption in the otherwise sandy and gravelly coastline characteristic of the northern and western Adriatic shores. The depth does not exceed 14 m, although on most of the Riviera is approximately 5–6 m (Masini *et al.*, 2007; Rindi *et al.*, 2020). A large amounts of nutrients and sediments discharged by the rivers, especially Po river, are carried towards the Riviera by surface currents, making the waters near Ancona nutrient-rich and turbid (Tesi *et al.*, 2013). The seabed fosters larval dispersal and provides habitats for rare and specialized species adapted to confined spaces, including heterobranchs (Gasteropoda, Heterobranchia) (Trainito & Doneddu, 2014; Rindi *et al.*, 2020; Knapič *et al.*, 2024). The state of marine conservation in the Conero Riviera is a growing concern due to the area's ecological importance and the anthropogenic pressures it faces. This distinctiveness of its characteristics, combined with its biodiversity, makes it a priority for conservation. The Conero Riviera is protected under the Habitats Directive 92/43/EEC (Habitat Directive, 1992) with three Sites of Community Importance (SCI IT5320006 “Portonovo e falesia calcarea a mare”, SCI IT5230005 “Costa tra Ancona e Portonovo” and SCI IT5230007 “Monte Conero”). All the three sites are furthermore included in the protection under the Birds Directive 2009/147/EC (Birds Directive, 2009) in a Special Protection Area (SPA IT5320015 “Monte Conero”). Even though these European designations highlight the area’s value and provide a regulatory framework for biodiversity protection, some zones that host important habitats are actually outside the borders of these protection.

Within the Conero Riviera the rocky tide pools at Passetto Beach represent a distinct and ecologically valuable habitat and its Heterobranchia community has been exhaustively described by Riccardi *et al.* in 2022. These tide pools span approximately 750 m² and are formed along two primary rock fissures perpendicular to the coastline. Although pools can

offer refuge from the harsh conditions encountered on emergent rock (e.g. temperature and desiccation stress) (Schonbeck & Norton, 1978; Moran, 1985), they may also become stressful environments, with large fluctuations in temperature, salinity, pH, carbon dioxide and dissolved oxygen (Goss-Custard *et al.*, 1997; Firth & Williams, 2009). Of these physical factors, depth is known to strongly influence diversity end community composition (Martins *et al.*, 2007; Browne & Chapman, 2014). The tide pools host diverse biocenosis, influenced by the physical characteristics and the seasonality. Between these, macroalgal communities are dominated by *Ulvales* and *Cystoseira sensu lato* (Rindi *et al.*, 2020; Bianchelli *et al.*, 2023), while faunal diversity encompasses cnidarians such as *Aiptasia couchii* (Cocks, 1851) and *Anemonia viridis* (Forsskål, 1775), biocenosis of *Mytilus galloprovincialis* (Lamark, 1819), crustaceans, and various vertebrates and invertebrates, forming a rich and complex ecosystem (Camillo & Cerrano, 2015; Arossa *et al.*, 2021). These pools are essential in maintaining biodiversity, supporting macroalgae, cnidarians, crustaceans, and other species uniquely adapted to the dynamic and sometimes stressful conditions of these environments. The peculiar characteristics of this environment could represent a nursery habitat for both vertebrates and invertebrates and refuges from stressful environmental condition (Metaxas & Scheibling, 1993). However, the location of Passetto Beach in an urban setting presents challenges, as the proximity to the city of Ancona increases the pressure from human activities. Despite these challenges, the accessibility of Passetto Beach makes it an excellent site for marine education and public engagement. Its location within a densely populated area allows for significant opportunities to raise awareness about marine biodiversity and conservation. Tide pools, with their rich and visible biocenosis, serve as natural laboratories, capturing public interest and fostering a deeper understanding of the importance of protecting these ecosystems. Initiatives such as citizen science projects and educational

programs can leverage this accessibility to involve the local community in monitoring biodiversity, collecting data, and supporting conservation efforts (Thiel *et al.*, 2014). This combination of ecological significance and public engagement potential makes the tide pools at Passetto Beach not only a unique marine habitat but also a crucial site for promoting environmental stewardship and sustainability.

OBJECTIVE

Despite adult aeolid nudibranch have become premier models for investigations in various fields, including mutualism and kleptoplasty (Garese *et al.*, 2013; Grawunder *et al.*, 2015; Monteiro *et al.*, 2019), neurobiology and learning (McPhie *et al.*, 1993), chemical ecology (Faulkner & Ghiselin, 1983), climate change and environmental stress (Nimbs & Smith, 2016; Damien & Tougeron, 2019), some species within the Heterobranchia taxon have not been studied and in some cases very little to none information are available. Significant gaps remain in our understanding of symbiont nudibranchs, such as the aeolid *Spurilla neapolitana*. This species and one of its preys — *Aiptasia couchii* — thrive at the Conero Riviera (Ancona, Italy), a good place to conduct *in situ* observations and to collect samples for laboratory analysis.

This thesis aims to fill critical knowledge gaps about the ecology, behavior, and interactions of this species. It focused particularly on the relationship between the aeolid nudibranch *S. neapolitana*, its preferred food source, the sea anemone *Aiptasia couchii* and their symbiotic algae. In this context, no information about how their life cycles are synchronized is available.

Therefore, main objectives of this work are:

- To shed light on reproductive and feeding strategies of *S. neapolitana;*
- To interpret the behaviour of wild non-aposematic aeolid nudibranch;
- To contribute to the understanding of the coevolved predator-prey relationship among two animal partners and the shared algal symbiont.

Several experiments at lab or observations in the field have been carried out to achieve specific objectives and to answer the following research questions:

OBJECTIVE	QUESTION	HYPOTHESIS
To elucidate the life cycle of *Spurilla neapolitana*	What are the main phases of *S. neapolitana*'s life cycle, and when do they occur?	The life cycle of *S. neapolitana* mirrors descriptions by Schelsinger *et al.* (2009), with consistent developmental stages under varying environmental conditions, such as temperature
To assess the interaction between *S. neapolitana* and its prey, the sea anemone *Aiptasia couchii*, investigating the nature of their predator-prey relationship and the potential role of the prey in triggering metamorphic processes	What kind of interaction is established between the predator and its prey?	The presence of prey may act as a metamorphic trigger for larvae, as already shown for other species (Carroll & Kempf, 1990; Chia & Koss, 1988; Pawlik, 2019), ensuring food availability for juveniles Adults are hypothesised to recognize prey even without direct contact
To identify the food preferences of *S. neapolitana*, exploring the nudibranch's handling of nematocysts from its prey	What is the food preference of *S. neapolitana*?	*S. neapolitana* selects prey based on olfactory cues, favouring *Aiptasia couchii* due to its ecological relevance and previously reported importance (McDonald and Nybakken, 1996)
	How does *S. neapolitana* handle nematocysts from its prey?	*S. neapolitana* selectively isolates and stores cnidocysts, retaining only those beneficial for its defence while expelling others

Table 1: objectives, research questions and hypotheses of this study

This research contributes to a deeper understanding of *Spurilla neapolitana*, shedding light on its ecological role and coevolved relationship with its prey and symbiotic algae. Insights from this study will advance knowledge about aeolid nudibranchs, particularly in predator-prey dynamics, metamorphic triggers, and the use of prey-derived defences. Furthermore, it emphasizes the importance of detailed ecological studies in addressing broader marine biodiversity and conservation challenges.

MATERIALS AND METHODS

To conduct the observations in aquaria on the living nudibranchs, eighteen adults of *Spurilla neapolitana* were collected in the "Piscinetta" within Passetto Beach in Ancona in snorkelling operations (*Fig. 9*). The collection of the individuals was always carried out between h 20.30 and 22.30, with respect to the nocturnal habits of the animal. The biggest sampling activity was carried out on June 17th in high tide conditions, with a waxing gibbous moon. On that occasion, eleven individuals were sampled, and of all the operations the maximum occurrence of the species was noted. Three more adults were collected on farm ropes of mussels *Mytilus galloprovincialis* from Sene Gallica Soc.Coop. fishmonger, located in Senigallia (AN).

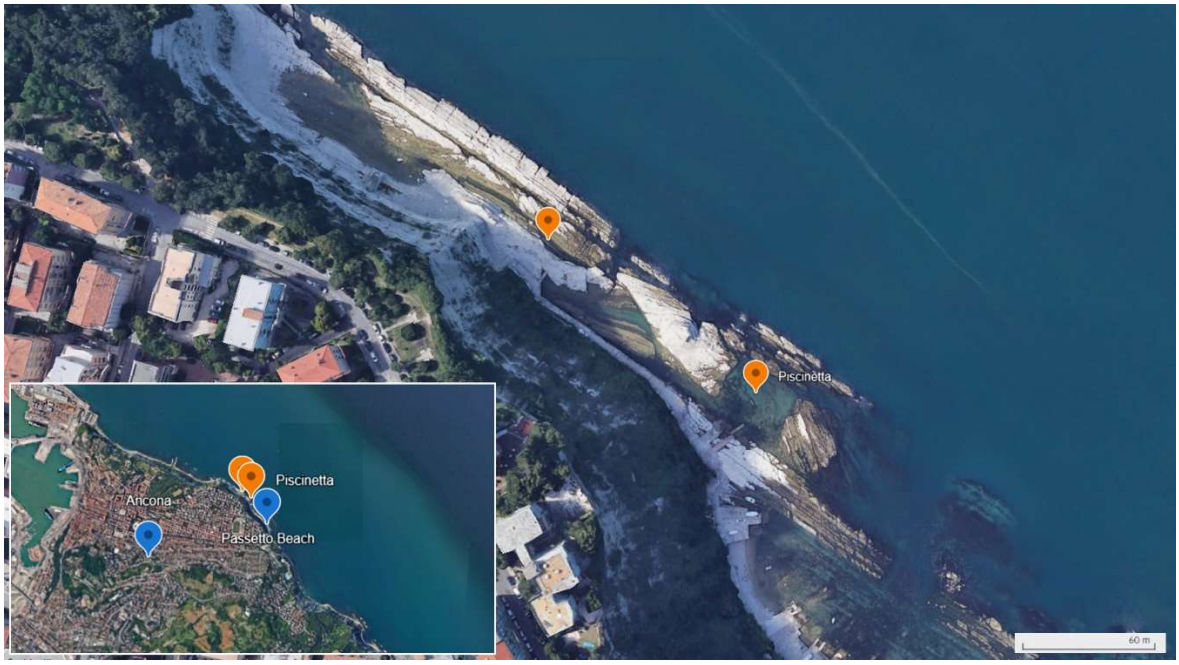


Fig. 9: Collecting sites of both the nudibranchs and the anemones, within Passetto Beach

Individuals of *Aiptasia couchii* were sampled at the same sites throughout the whole duration of the experiments to feed the adults of the *S. neapolitana* and to assess the nudibranch's food preference (*Fig. 10*). The species is selected among all those listed as possible prey in literature (Conklin & Mariscal, 1977; Betti *et al.*, 2017) and based on its presence on the study area.

Fig. 10: Sampling operations of *Aiptasia couchii*

The nudibranchs were housed in delivery rooms, to facilitate the embryos collecting, immersed in a recirculating 200L tank with a Berlinese filter type, with a temperature of 18°C and salinity of 36 PSU. In the tank were present a community of different sea anemones and sponges (*Fig. 11*). The animals were maintained on a 12:12-h light:dark cycle and fed twice a week with individuals of *Aiptasia couchii* with an average pedal disc of 1,5 cm diameter.

Fig. 11: A) Transport system after the sampling operations of *Spurilla neapolitana*; B) *S. neapolitana* in its natural habitat; C) One of the sampled individuals (white arrow) in the aquarium

3.1 Experiment I – Life cycle

To elucidate the life cycle of *Spurilla neapolitana* from the Adriatic Sea, daily observations of brood-stock to monitor egg laying, hatching, larval development, and metamorphosis were carried out. Larvae and adults were tracked to calculate population history and analyse developmental patterns. When the adults laid at least three egg ribbons, these were collected, gently scraped from the delivery room walls, and its content estimated. To do so, the volume of the whole egg ribbons was estimated through image analysis with the software ImageJ (Schneider *et al.*, 2012), a small portion per each was cut, their volume was estimated in the same way, and the number of individuals inside the eggs counted. The obtained number was then reported to the total volume of the egg mass, to get an estimate of the total number of eggs. The limit presented by this method, for this nudibranch species, is related to the stage of development of the individuals inside the eggs: at the beginning of the development only

a cluster of cells is visible per each capsule, but as time passes by the cluster begin to differentiate into one, two or three different individuals. Based on this, if the count happened after the first hours after the deposition, there was an underestimation of the total number of individuals. To resolve this issue, the density of individuals has been calculated each day during the whole experiment, so that the estimated number of individuals inside the egg ribbon could be compared to the estimated number of individuals after the hatching. After the estimation, each egg cylinder has been cut into eighteen portions of similar dimensions. Then, one casual fragment of each was moved inside the system made for the growing larvae. Daily observations were conducted to assess the state of the eggs until their complete hatch.

The system in which the eggs are inserted into is created to assess the triggering of the metamorphosis of competent larvae and whether it is of a chemical or physical nature. For this reason, the anemone *Aiptasia couchii*, is chosen to be put together with the larvae. The single apparatus is composed of a jar with a suspended smaller cup with mesh, to allow water to flow, and an aerator tube inside it. To not affect the larvae, the effect of bubbling from the aeration is reduced by putting a micropipette tip at the end of the tube and positioning it against the wall of the cup. Clamps are used to keep the placement of both the cup – through a cable tie – and the aerator tube stable. Jars are divided into three different conditions: INSIDE (IN), OUTSIDE (OUT) and CONTROL (CTR). INSIDE: one anemone per jar is placed in direct contact with the larvae; OUTSIDE: the anemone is set inside the smaller cup, with the 30 μm mesh preventing the larvae from having contact with it; CONTROL: is left without anemones. To further assess the possible impact of the mesh on the experiment, the same three conditions are repeated both with a 30 μm mesh filter and a >500 μm. Each

environment for both conditions is repeated three times, reaching a total of eighteen jars (*Fig. 12* and *13*).

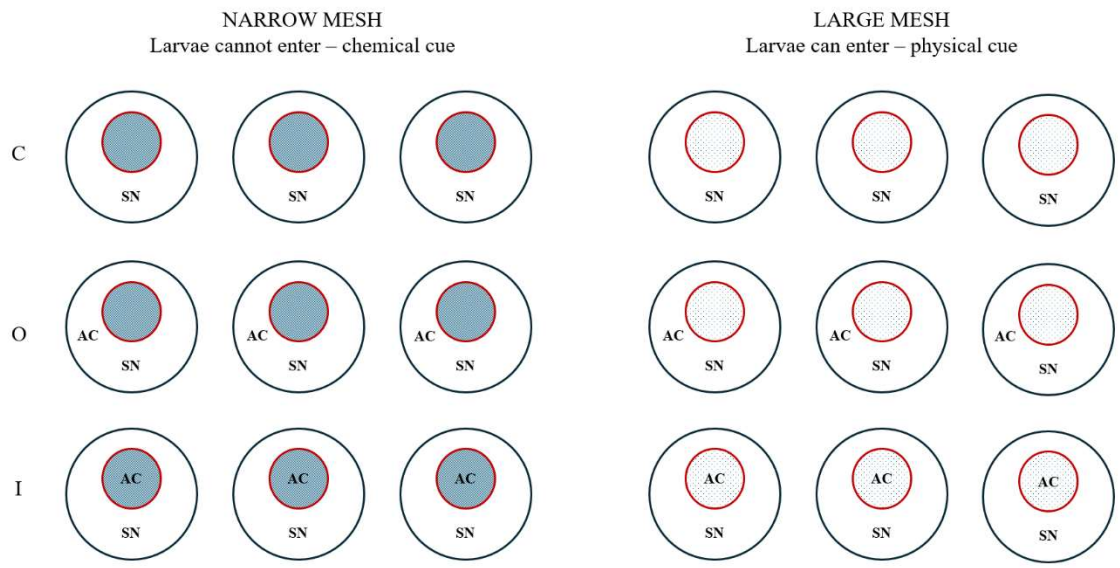


Fig. 12: model of the COI system. Legenda: blue circle, jar; red circle, small cup; SN, *Spurilla neapolitana*; AC, *Aiptasia couchii*

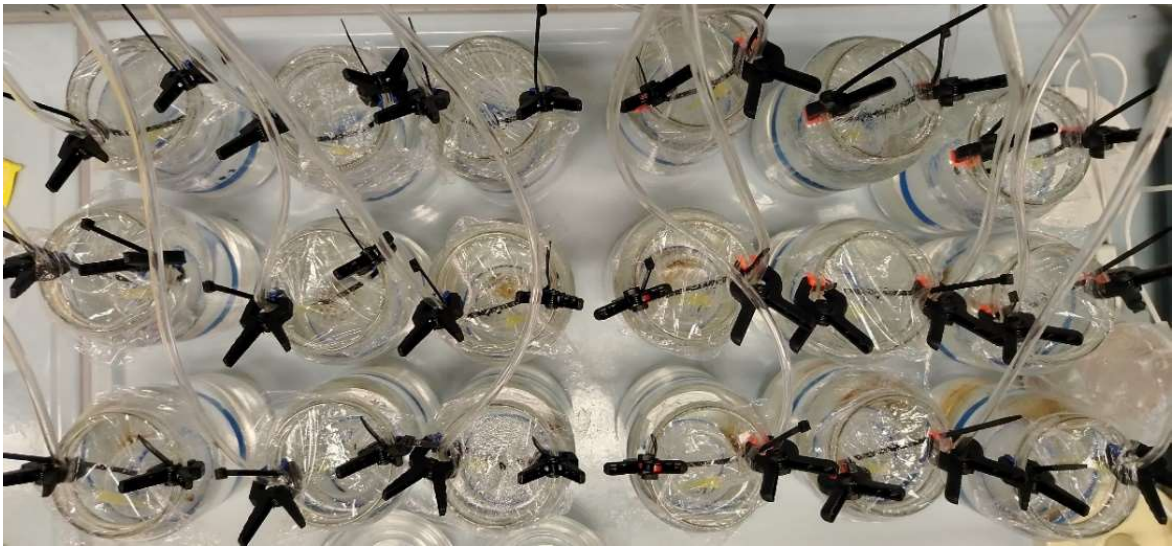


Fig. 13: photo of the complete setup

The water used was artificial seawater (ASF) with a salinity of 37 PSU and a mean room temperature of 23° C, without recirculation. To keep the water clean, 25% of the total was changed three times a week. When the larvae hatched, they were fed with microalgae. To test different diets on the larval development, the whole experiment was repeated so the veliger could be fed with:

- Trial 1: phytoplankton from Easy Reefs, Easy Booster 25, following the indicated dosages which gradually increase over the days;
- Trial 2: phytoplankton from Easy Reefs, Easy Booster 25, following the highest dosage from day one;
- Trial 3: sea water collected on the nudibranchs' sampling site, to replicate the same environmental food conditions.

To be sure the phytoplankton was fully available to the larvae, it has been filtered with a 30 µm mesh from time to time. The anemones instead were fed once a week with few alive individuals of *Artemia salina*, to minimize the dirtying of the water. The eggs, and then the larvae, underwent daily monitoring under a Leica optical microscope, taking photos with its software Leica LAS EZ to keep track of the different developing stages. Days of survival are defined as period from the start of the experiment until the count for the larval density was <1 per jar.

Finally, to best reproduce the natural environment, at the end of the three trials the adults were put in tank with the same system as the one they were kept. Here, they were free to roam, reproduce and lay their eggs. To prevent both the nudibranchs and the larvae to end up inside the filter, a panel with the 30 µm mesh was prepared and used to halve the tank. In this way, the further half to the filter was dedicated to the nudibranch, while the other half

contained the filter and a community of animals composed by *Mytilus galloprovincialis, Anemonia viridis*, urchins and shrimps. The panel was put in the middle of the tank to reduce to the minimum the pressure of the filter suction to not disturb the larvae.

In addition to these tests, information on the life cycle of *Spurilla* in the coastal area of the Conero Riviera was obtained. Through observations conducted during this study and previous research, the aim was to provide insights into the seasonal patterns of occurrence and reproductive activity of the species. Presence data derived from visual and photographic census as well as human observation. The obtained records were collected via Citizen Science, Web Ecological Knowledge, Local Ecological Knowledge or direct observations.

3.2 Experiment II – Prey-predator interaction

To assess the kind of interaction established between *Spurilla neapolitana* and *Aiptasia couchii* the system of experiment I is reused (*Fig. 12*), but only using the nine jars with the larger mesh and the control with the narrower mesh. In this case, the experiment was carried out particularly to observe the movement pattern of the nudibranch in relation to the prey presence and, after a potential contact, the mechanics of feeding on the prey. The experiment involved partitioning starving adult nudibranchs into the three environments: one with prey in direct contact, one with prey confined but in the same water, and a control without prey. Time-lapse video covering a 2.30-hour period were made and then re-examined to observe the nudibranchs' behaviour.

During this experiment the behaviour of detached cerata is also observed. A cerata was cut from a healthy individual and observed daily, keeping it in a microcentrifuge tube in ASW changed every other day.

3.3 Experiment III – Food preferences

To assess the preferred food choice of *Spurilla neapolitana,* a T-maze chamber was created, made of white PVC, of measures reported in *Fig. 14*. The passage of nudibranchs is allowed by two carved doors between the longer segment and the arms.

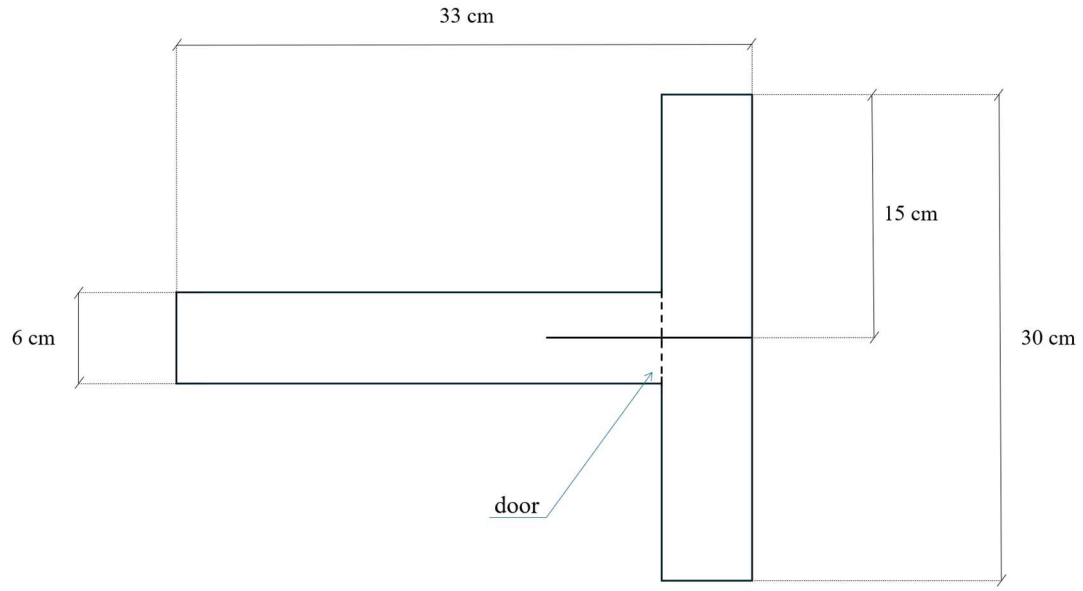


Fig. 14: T-maze chamber model

On top of the maze was placed a carton box to maintain a shadowed environment, to reduce the illumination. A weak continuous flow was created putting at the further extremities of the opposite arms of the maze two 0.5 cm of diameter tubes with a constant ASW apport while the water outflow was permitted by a 0.5 cm diameter hole. This setup allowed the released metabolites of the anemone to be brought and perceived by the nudibranch. With this system is also possible to assess the sensitivity of the chemical perception of *Spurilla neapolitana*. 17 starving nudibranch individuals were used, with a starvation period of 2-5

days. Between runs the whole water was discharged and the T-maze chamber cleaned with running freshwater, to remove every possible trace of mucus.

Three types of runs were carried out inside the T-maze chamber (*Fig. 15*): i) RUN A: AC – nothing: in the opposite arms were placed a healthy individual of *Aiptasia couchii* and nothing on the other side, to assess the effective attraction of the nudibranch to the prey; ii) RUN B: AC – AV: in the opposite arms were placed an individual of *Aiptasia couchii* and an individual of *Anemonia viridis*; iii) RUN C: AC – AC': in the opposite arms were placed a healthy individual of *A. couchii* and a previously pricked individual of *A. couchii*, to check out a possible different response or an augmented release of metabolites. This also simulates the presence in the environment of an anemone that was previously attacked by another nudibranch.

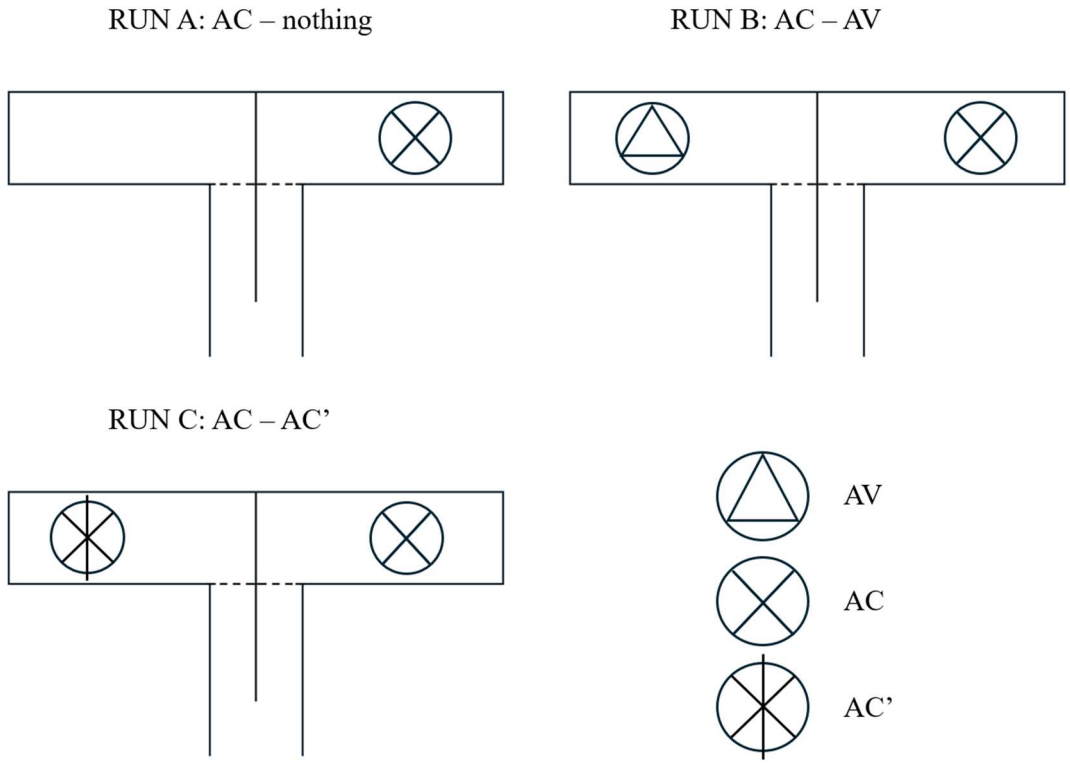


Fig. 15: different runs schematic. Legenda: AV, *Anemonia viridis*; AC, healthy *Aiptasia couchii*; AC', pricked *Aiptasia couchii*

A nudibranch was placed at the base of the longest arm and left to acclimate for five minutes, isolated by the rest of the maze by a removable mesh. After the acclimatation, the mesh was removed, and the nudibranch was free to roam in the environment for 25 minutes. As for the method, it was chosen the continuous choice, meaning that the individuals were free to pick a side and eventually change it. But when first contact was made with the prey then the run was considered finished, to avoid the alimentation of the nudibranchs. If the run ended with the nudibranch in one of two side, but without making contact with the prey – or reaching the end in the case of empty arm – that was considered a partial choice. RUN A was repeated 13 times, RUN B 7 times and C 11 times, for a total of 35 runs. Data were collected through

direct observation and taking videos with a camera mounted on a structure to obtain a top view of the maze.

An additional run, RUN D, was carried out in a 30x20 cm container with static water with only a pricked individual of anemone and a nudibranch to verify the distance it needs to react to the prey. This was estimated by moving the anemone closer, progressively at 10 cm, 8 cm, 5 cm, 3 cm and finally at 1 cm. The same process was carried out with the only mucus produced by the anemone when pricked (RUN E) (*Fig. 16*). The focus was on determining the sensitivity of *S. neapolitana* to the metabolites released by a pricked anemone and its mucus outside the T-maze chamber. The experimental design aimed to assess the distances at which nudibranchs initiated a response in static waters.

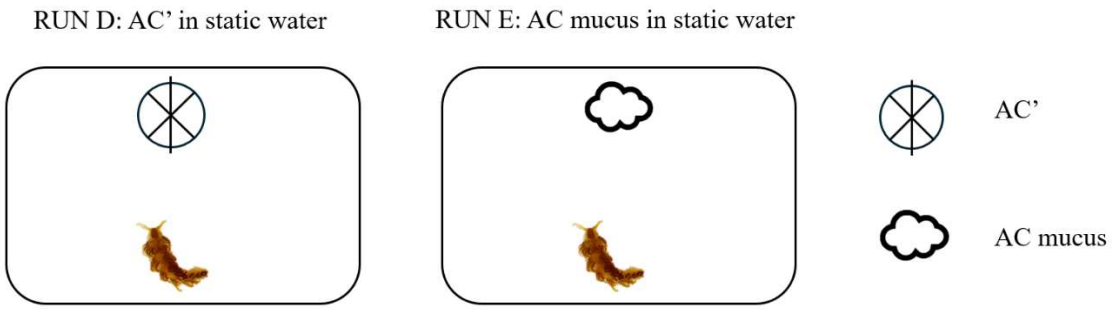


Fig. 16: RUN D and RUN E models

3.4 Experiment IV – Cnidome

To understand how *S. neapolitana* handle nematocysts from its prey, its cnidome is reconstructed. "Cnidome" is the term used to indicate the total complement of cnidocytes

within a cnidarian specimen, including their cnidocyst size, abundance and distribution into the body of the specimen (Heins *et al.*, 2015). Samples of cerata and feces are taken from healthy adults and observed under the light microscope. Here, using the same Laica microscope and software from experiment I, different photos are taken and examined with the ImageJ software. A dataset is created to register type and the measures of the first 50 nematocysts per sample. From this data, the maximum width-maximum length ratio of the measured cnidocysts and the relative abundance of the types are calculated. Details photos of the cnidae are taken with an oil-immersion 100x objective. To further analyse the morphology of the cnidocysts and cerata, some samples are arranged for Scanning Electron Microscopy examination. Samples were fixed in 4% formalin, dehydrated through a graded ethanol series and dried with the Critical Point Dryer, coated with gold-palladium in a Quorum Q150T Plus and examined with a Zeiss SUPRA 40 SEM.

The same process is repeated to describe the cnidome of *Aiptasia couchii*, examining its acontia, tentacles and column. In this way is possible to compare the respective cnidomes, and to understand if *Spurilla neapolitana* selectively isolates and stores cnidocysts, from which anemone's body district it retrieves the retained ones and which ones it expels.

RESULTS

The experiments took place over several months, from April till August. This is due to the technical times required by the sampling operations and to implement and carry on the different experiments. Given their duration, the sampled individuals met the natural end of their life cycle, sometimes before retrieving all the necessary data. This caused some differences on the dimensions of the data sets of each experiment, so some inconsistencies can be traced back to this. Here follows the results obtained through the different experiments.

4.1 Experiment I – Life cycle

Adults of *Spurilla neapolitana* used to lay their eggs just after the sampling, even if they were in a container to be transported from the collecting site to the university facilities. A total of 17 cylindrical string of eggs have been collected, measured and estimated. Even if the number of embryos showed a weak correlation with the body length of the adult who laid the eggs (*Fig. 17*) ($R^2 = 0.2725$) likely due to the data scarcity, it is possible to see how the bigger layings belong to the bigger individuals. An adult of mean dimension (body length from oral tentacles to the end of the foot) of 39.21 ± 6.51 mm (mean ± SD, $n = 16$) laid $1.05 * 10^5 \pm 4.87 * 10^4$ ($n = 16$), in a mass with volume 124.31 ± 84.04 mm^3 ($n = 16$).

Moreover, the smallest deposition, counting only $2.68* 10^4$ eggs, was collected in a moment in which the embryos where not yet visibly separated due to the characteristics of polyembryonic development. Since the division of individuals within each egg capsule was

not distinguishable before gastrulation, it is highly like this count represent an underestimation, as previously explained (chapter 3.1).

Fig. 17: dispersion plot of the correlation between the body length of the adult nudibranch and the number of embryos it laid, with the logarithmic trend line

Egg deposition (*Fig. 18*) occurred in a variable number per individual, with the difference being found based on at what point in the life cycle the individuals were sampled, so is not possible to give information about the maximum number of depositions made per individual. The time required for a complete deposition is short, falling within half an hour. Contrary to what observe in the work of Schlesinger *et al*. (2009), adults of *Spurilla neapolitana* laid their eggs in whichever container they were put, even when clean and in absence of biofouling. The delivery rooms have proven to be ideal for egg sampling, as the mass can be detached, using a slide, without breaking it.

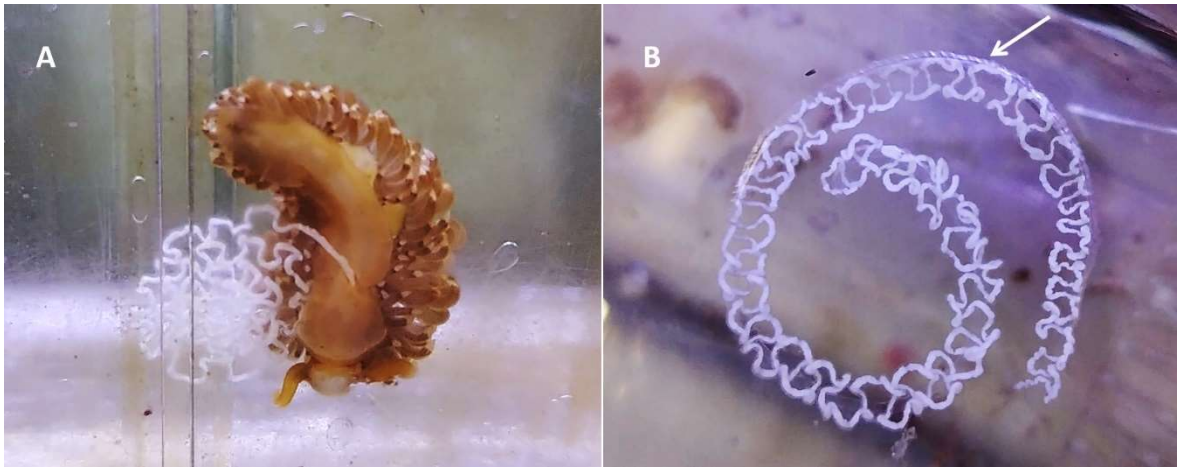


Fig. 18: A) a nudibranch in the act of laying its egg; B) the laid mass, with the mucous filament (white arrow)

The laid eggs where in a coiled cylinder shape, strongly attached to the substratum. Once detached from the substrate, its compact morphology caused the mass to take the shape of a helix. The eggs are immersed in a mucous matrix (*Fig. 19A*) and along the internal axis of the helix is present a firmer mucous filament, that maintains the shape of the cylinder and presumably aid in the adhesion to the substratum (*Fig. 18B* and *Fig. 19B*).

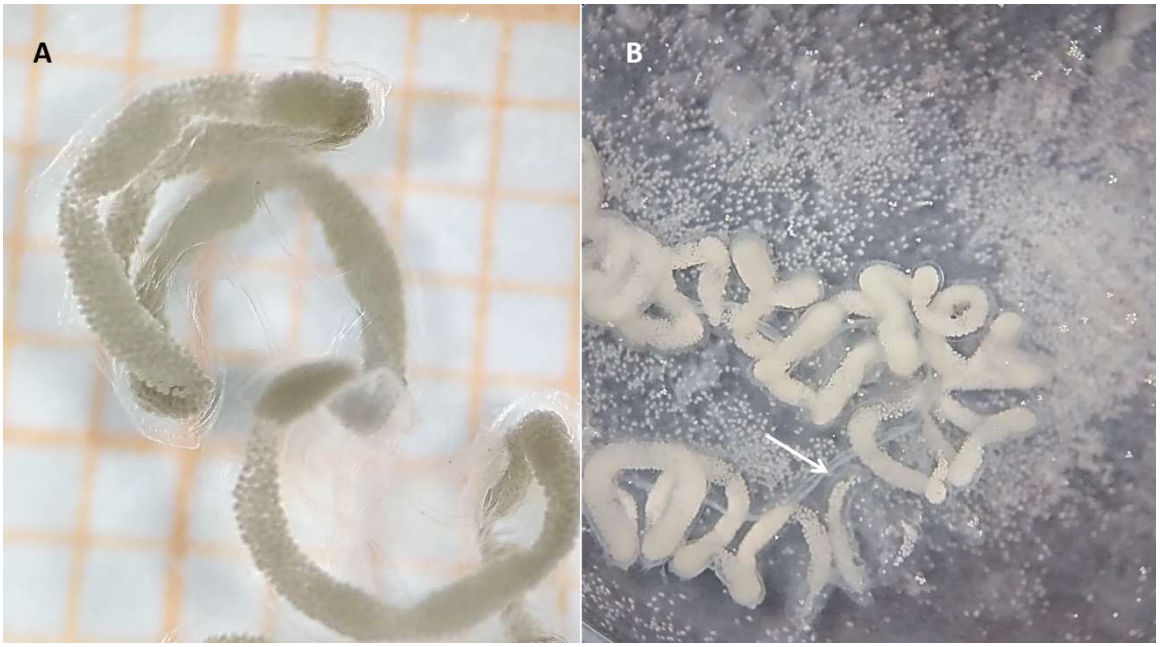


Fig. 19: an egg cylinder observed under the stereoscopic microscope, in which is possible to see: A) the eggs immersed in the matrix and B) the mucous filament (white arrow) and the scattered eggs in the background

Observing an egg mass under a light microscope, ciliates and algae were often observed immediately after the collection, especially on the central filament of the cylinder. In one case, deposition on the water surface was observed (*Fig. 18B*). When a fragment of the mass was sampled, the change in pressure forced the eggs proximal the cut to exit the cylinder (*Fig. 19B*). This phenomenon was easily observed when preparing a slide, particularly when a cover slip was placed on the sample, and this allowed a clear observation of the embryos and its developing stages (*Fig 20*). The mean length of the egg mass was 440,85 ± 191,21 mm (n = 16) and per unit of volume were present a mean of 1108,25 ± 622,91 embryos/mm^3 (n = 16).

During the first trial of this experiment, dividing the egg mass as described previously (chapter 3.1), some of the masses turned pink in colour on the second day of observation (*Fiig. 21B*). Under the microscope it was possible to see an intense activity of ciliated

organisms and after two to three days most of them returned white in colour. One of them on the sixth day of observation post-oviposition had become the most intense pink of all and the majority of egg capsule was still present, but with dead embryos inside (*Fig. 20A*).

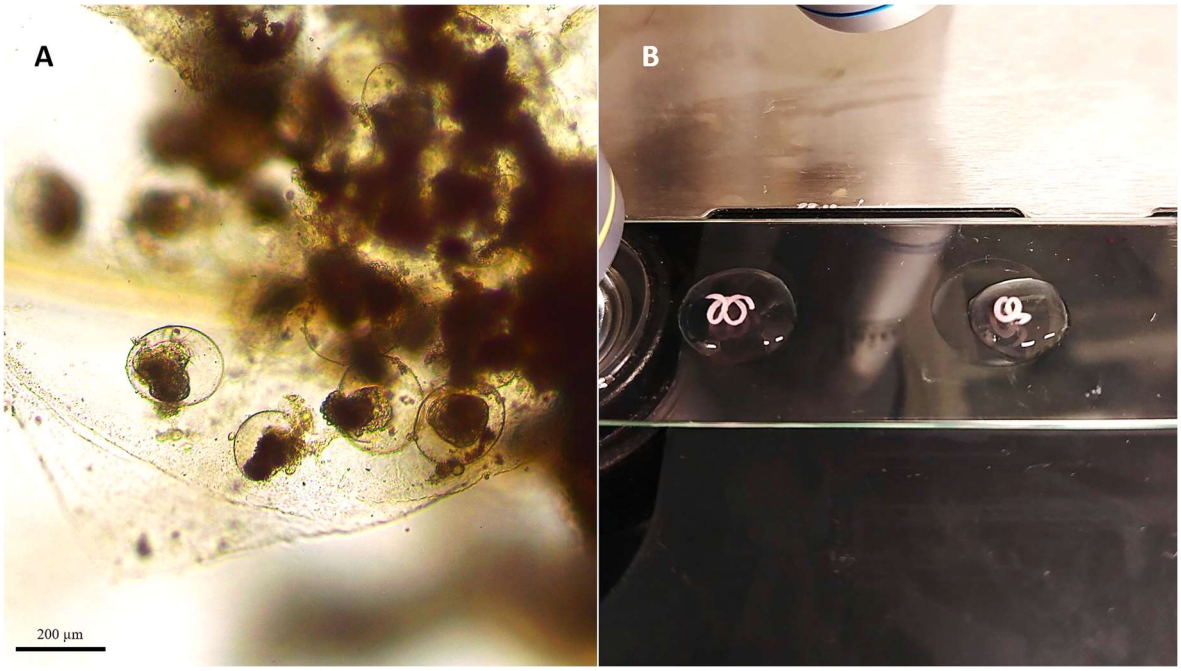


Fig. 20: photos of A) not-hatched dead larvae inside the egg mass; B) fragment of the mass turned pink in colour

This case, alongside other observation made during the hole duration of the experiments, highlighted the importance in breaking the egg mass to help the larvae to spawn, after the beginning of the hatching. This led to a change in the method used up to that moment: the eggs were put to hatch in a 700 mL filled with ASW beaker, with an aeration tube to keep the water flowing. This system helped greatly to the complete hatching of the larvae for the second and third trial (*Fig. 21*). A Pasteur pipette was also used in aiding the breakage. To add the larvae to the jar system, the volume of the water in the beaker was equally divided into the 18 jars.

The estimated number of embryos was compared to the maximum number of counted larvae of the same eggs cylinder to evaluate the hatching success (*Fig. 21*). The day at which the maximum number of larvae is counted correspond at the fifth one, since the hatching of the eggs happens in the span of a few days and because the different eggs cylinders used on a same experiment weren't laid on the same moment (*Fig. 22*).

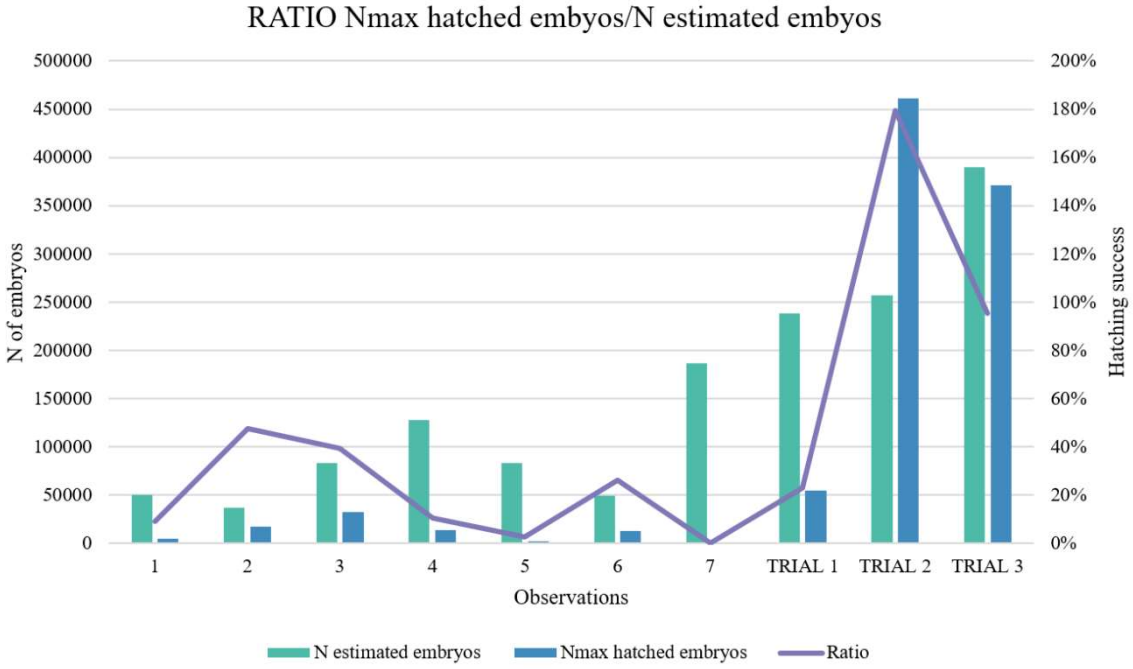


Fig. 21: plot of the ratio between the maximum number of hatched embryos on the number of estimated embryos inside the cylinder throughout the duration of the experiments

Regarding the three different administered diets and their associated treatment larvae density, it's not possible to appreciate a probable difference because after the fourth day of rearing a drastic population decline occurred in every environment. Because larvae were obligatory planktotrophs, i.e., veligers did not develop or survive without an algal food source, high mortality of individuals during the experiment can be attributed to an inadequacy of the administered diets. Anyway, because a one-way ANOVA was performed

on the collected data, but no statistically significant differences were observed among the groups, to illustrate the results, a plot was created showing the mean larval density across all treatments (*Fig. 22*).

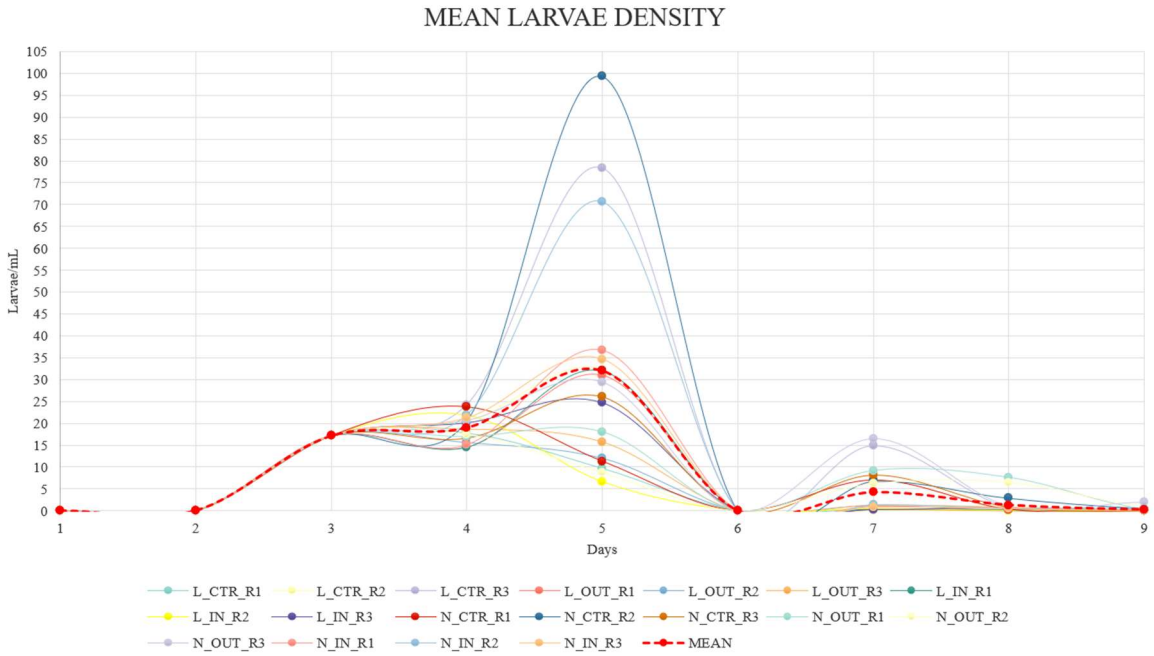


Fig. 22: plot of the mean larvae density of all trials

As already described in chapter 1.1.5, in this study was also observed the horizontal transmission of zooxanthellae: the embryos and the hatched larvae didn't show any algal symbiosis.

Regarding the last part of this experiment, which involved leaving the nudibranchs free and allowing contacts between them, no significant data were obtained.

The presence of *Spurilla* in the Conero Riviera was recorded across different months, as illustrated in *Table 2*. The species was observed primarily from March to September, with peak occurrences during June, in the day corresponding to the sampling of individuals for

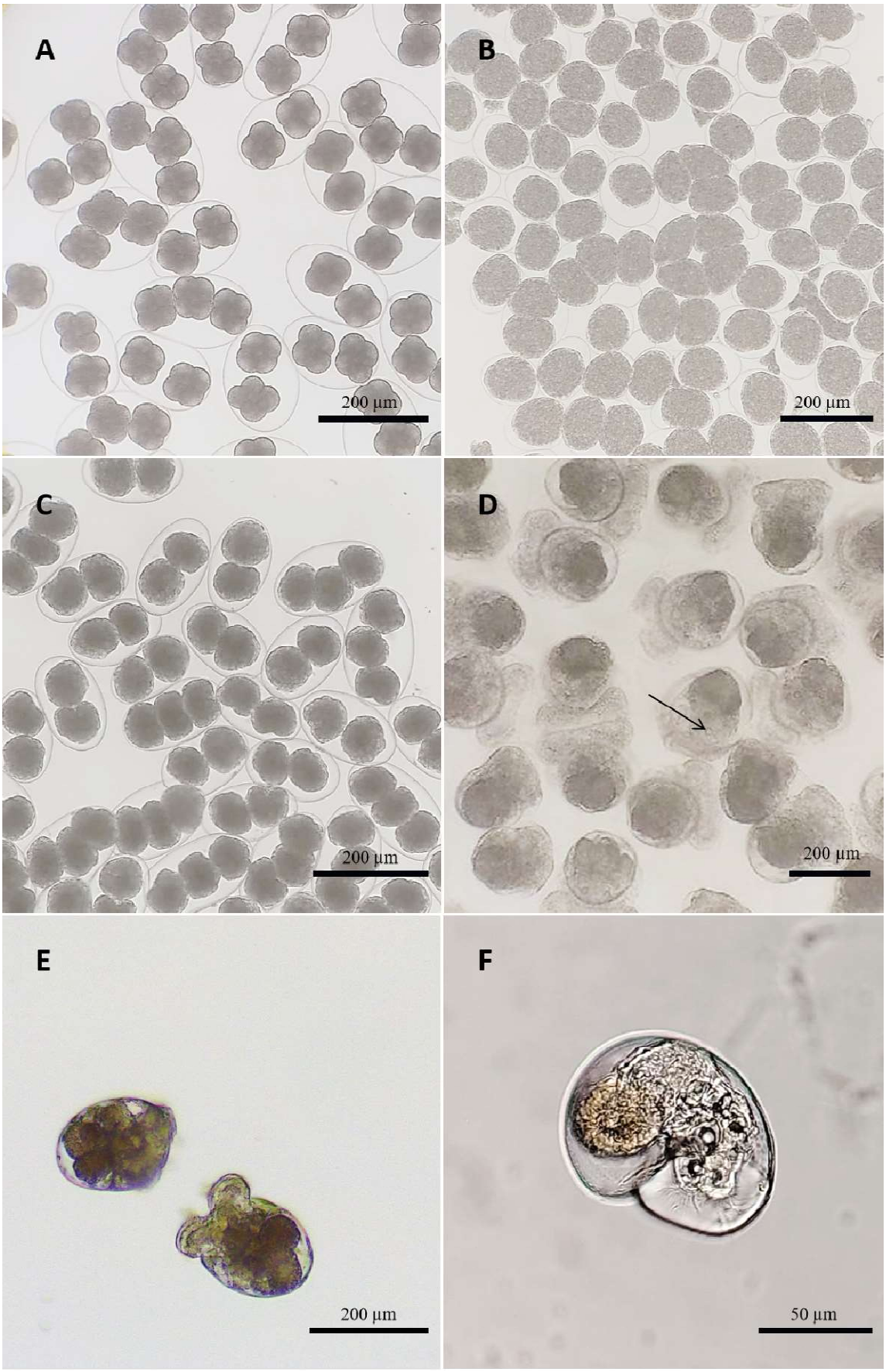
the current study. In the period between June and September the presence of egg masses was observed.

Tab. 2: seasonal presence of *Spurilla neapolitana* in the Conero Riviera. Legenda: green colour points record of presence in the month; green colour signals the co-presence of egg masses; question marks corresponds to data gaps

Conero Riviera	J	F	M	A	M	J	J	A	S	O	N	D
Presence	?	?		?			?			?	?	?

However, it is true that the compiled dataset shows a lack of data in the winter period, suggesting that the apparent absence of the species may be due to data scarcity rather than an actual absence.

4.1.1 Embryos and larvae morphology


A
200 µm
B
200 µm
C
200 µm
D
200 µm
E
200 µm
F
50 µm

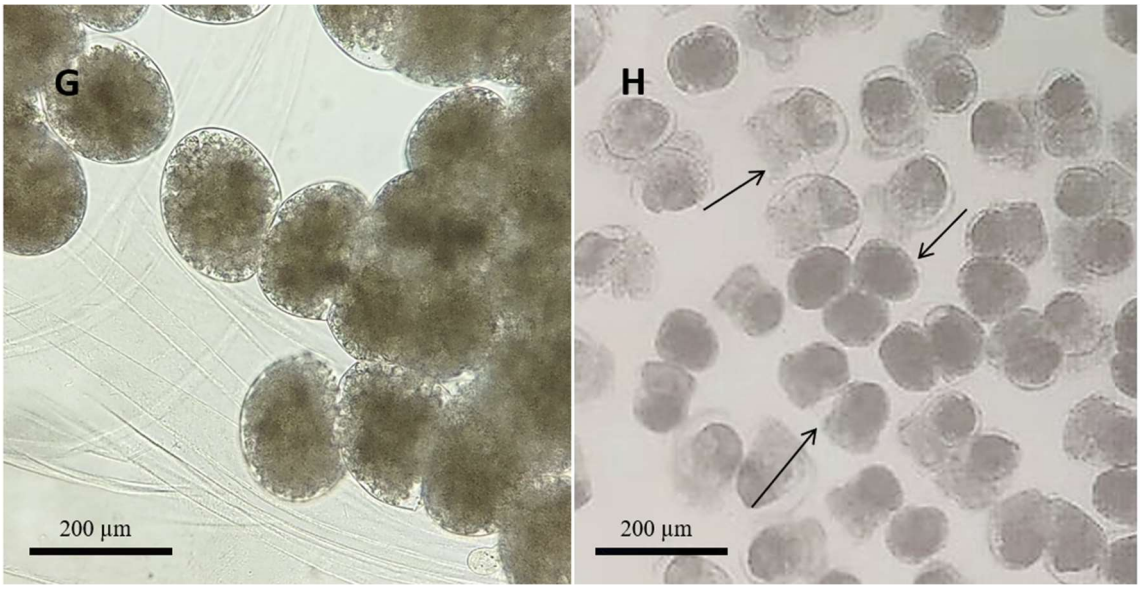


Fig. 23: the different stages of embryo development: A) four-cell stage; B) advanced state of cell division; C) beginning of cells organization into tissues; D) pre-hatching larvae, is possible to see the oil droplets (black arrow); E) hatched larvae; F) larvae aged 7 days post-oviposition; G) non-fertile eggs; H) non-synchronous development, the black arrows point different larval stages

Each capsule in the egg mass contained 1-4 embryos (*Fig. 23B*) and hatched in 5 days. The embryos that didn't hatch in this period remained trapped inside their capsule and dyed shortly after. The development was typically not synchronous, with a polarity towards the first laid extremity (*Fig. 23H*).

Micrographs by SEM allowed to better observe the characteristics of the larvae and its capsule (*Fig. 24*).

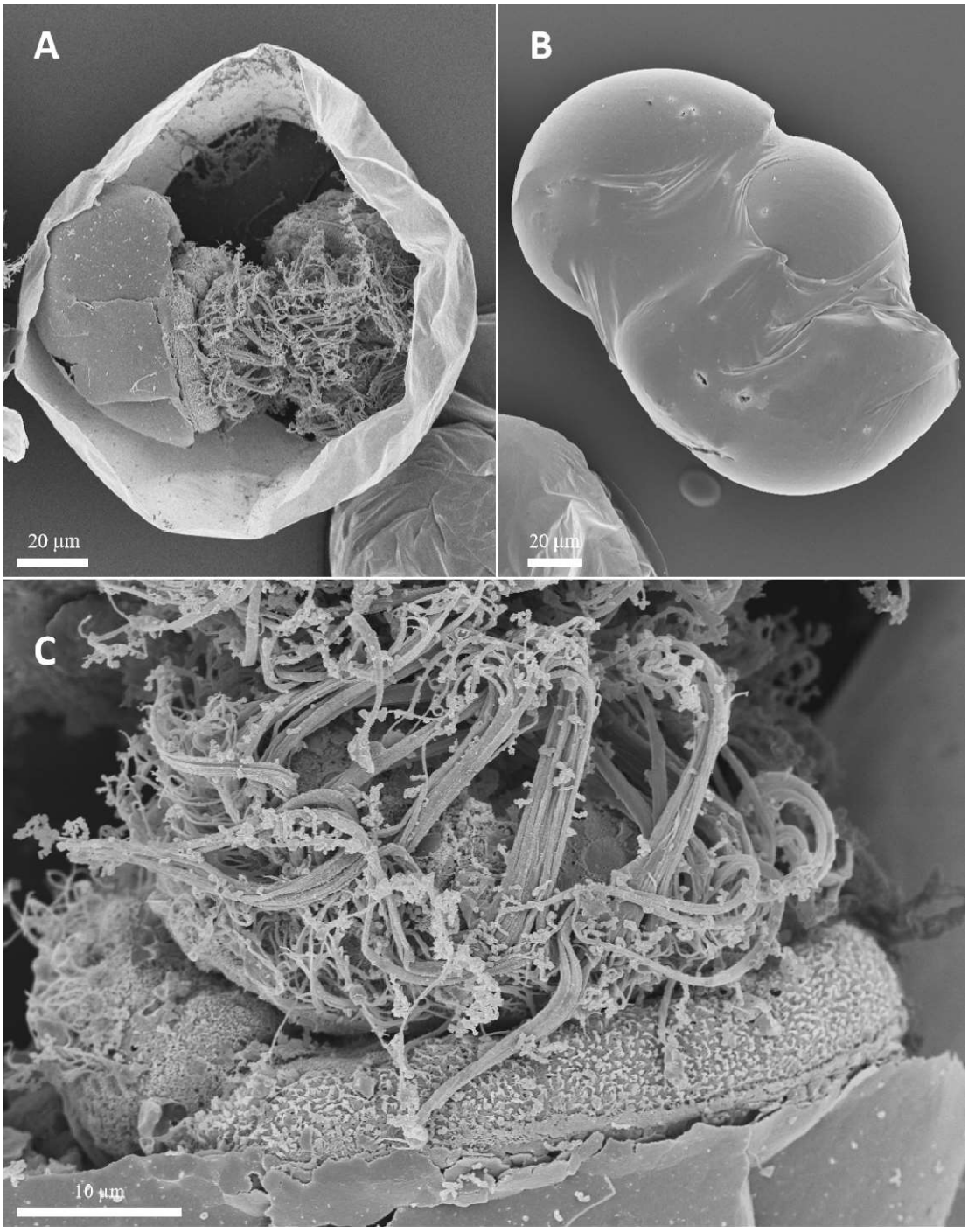


Fig. 24: SEM micrographs of A) a broken capsule with a larva inside; B) a capsule egg with at least three embryos inside; C) detail of the larvae in A, in which is possible to observe its cilia

4.2 Experiment II – Prey-predator interaction

Direct observation and reanalysis of the time laps allowed to describe the behavioural pattern of *Spurilla neapolitana* towards its prey *Aiptasia couchii*, especially regarding the search and recognition of the anemone.

For the INSIDE environment, on average the nudibranch found the anemone in 21.30 ± 16.28 SD minutes and fed on the anemone for 42.06 ± 29.13 SD minutes. The mean length of the five observed nudibranchs was 33.66 ± 5.49 SD mm while the *Aiptasia couchii* used for the experiment had a pedal disc of Ø = 5-10 mm. The individual who found its prey the fastest did so in 5 minutes, whilst another individual never approached the anemone during the experiment. To approach the anemone, the nudibranch extended its buccal apparatus to initiate an attack. Upon initial contact, which occurs preferentially on the pedal disc, the nudibranch exhibited a reaction to the nematocyst stings delivered by *A. couchii*. However, after repeated attempts, the individual acclimated to the stings, as previously reported by Conklin and Mariscal (1977). During the attack, the tentacles of the anemone exhibited a marked colour change, turning black as tissue necrosis set in. Following significant predation, all tentacles of the anemone became necrotic and darkened in appearance.

Regarding the OUTSIDE environment, the nudibranch roamed free in the jar, seemingly without noticing the anemone. Only one of the eight nudibranchs used in this condition reacted to the prey. After 50 minutes of wandering, the nudibranch interacted with the anemone, triggering the releasing on the acontia, that were visible hanging trough the openings of the mesh (*Fig. 25*). To be able to have a contact with its prey, the individual had to be upside-down, sliding on the mesh, and 9.30 minutes after triggering the release of the acontia the nudibranch fell and sank to the bottom. Afterwards, the individual didn’t come

back to the anemone, even after passing by it again. About the first contact though, is remarkable to notice how it happened at the 50th minute of the experiment, but the nudibranch was already on the mesh, aligned with the anemone, already after 39.30 minutes and there it stayed, until minute 50.

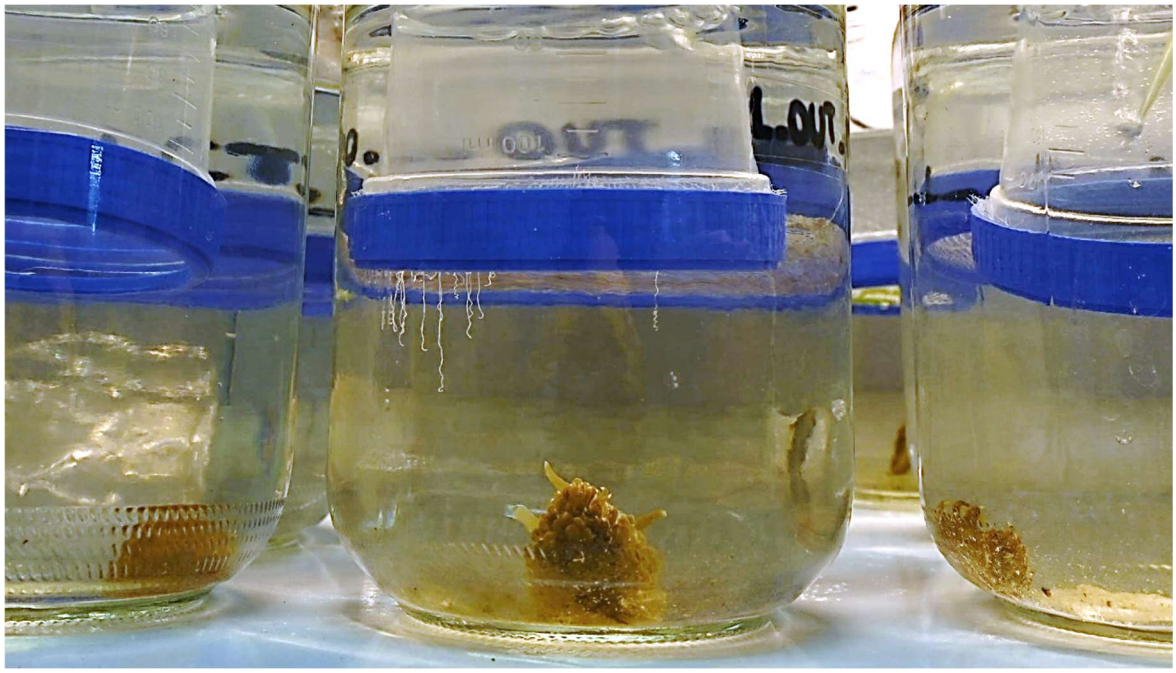


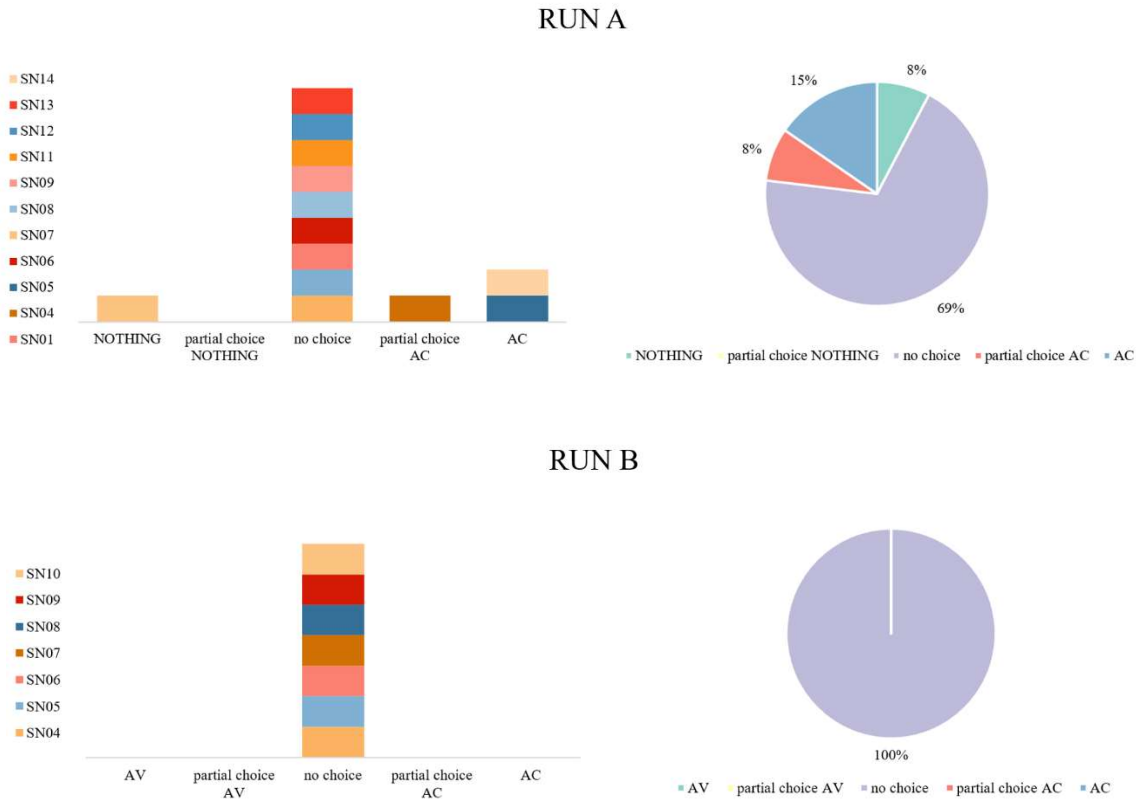
Fig. 25: nudibranch falling to the bottom of the jar after triggering the release of the acontia

About the two CONTROL environments, with both large and narrow mesh, no relevant observations were made.

Additionally, a detached cerata was observed to remain motile for up to one-week post-detachment. Its movement, although progressively slowing over time, exhibited a continuous writhing motion. Throughout the observation period, a gradual loss of zooxanthellae was also noted. This depletion may be attributed to active physiological process, escaping a portion of the body destined to die, or to passive leakage driven by a pressure gradient.

4.3 Experiment III – Food preferences

The food preference experiment aimed to evaluate the ability of *Spurilla neapolitana* to distinguish between prey types and assess its chemical sensory perception in controlled environments. Using a T-maze chamber, various runs were conducted (RUN A, B, C, D, and E) to simulate different prey scenarios and analyse the behavioural responses of the nudibranchs. The results are plotted in *Fig. 26*.



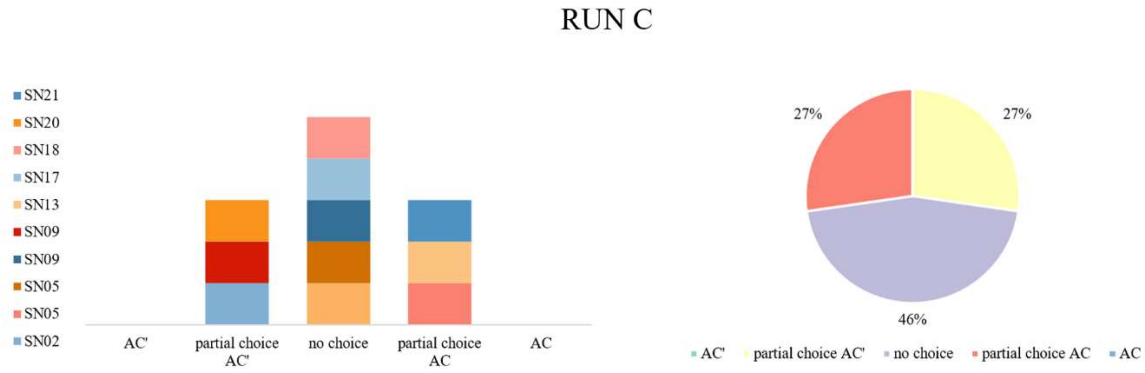


Fig. 26: plotted results of RUN A, B and C

The most relevant result to notice in these runs is how in 90.32% of the cases the nudibranch didn't make a clear choice during the experiment.

Regarding RUNS D and E, no behavioural reaction was observed at any distance, including 10 cm, 8 cm, 5 cm, or 3 cm. It was only upon direct contact with the pricked anemone or its mucus that the nudibranch displayed a response, with the behavioural pattern observed in earlier experiments. During this moment of contact, the nudibranch extended its buccal apparatus, demonstrating exploratory behaviour followed by an attempt to attack the prey, even when not present, i.e. in presence of its mucous.

4.4 Experiment IV – Cnidome

4.4.1 Aiptasia couchii

Aiptasia couchii's cnidome was characterised by the presence of basitrichs (type I and II), p-mastigophore (type I and II), and spirocysts (*Tab. 3*). *Table 4* shows the type of cnidocytes found into the different body districts of the species, the mean size of the capsule in length and width and their ratio, to give and insight at their conformation.

The acontias of *Aiptasia couchii* were densely packed with p-mastigophores I (*Fig. 27A*), explained by the fact that this nematocytes, a microbasic mastigophora, is used in defence and prey capture, as it is involved in envenomation (Endean et al., 1991). Basitrich isorhizae type II were also prevalent in the acontia (56.21%), whereas spirocysts were nearly absent. The tentacles, on the other hand, were characterized by a predominance of spirocysts (85.54%), reflecting their primary role in adhesion rather than envenomation.

Table 3: cnidocysts present in *Spurilla neapolitana* and *Aiptasia couchii,* each in their unreleased state and after the triggering

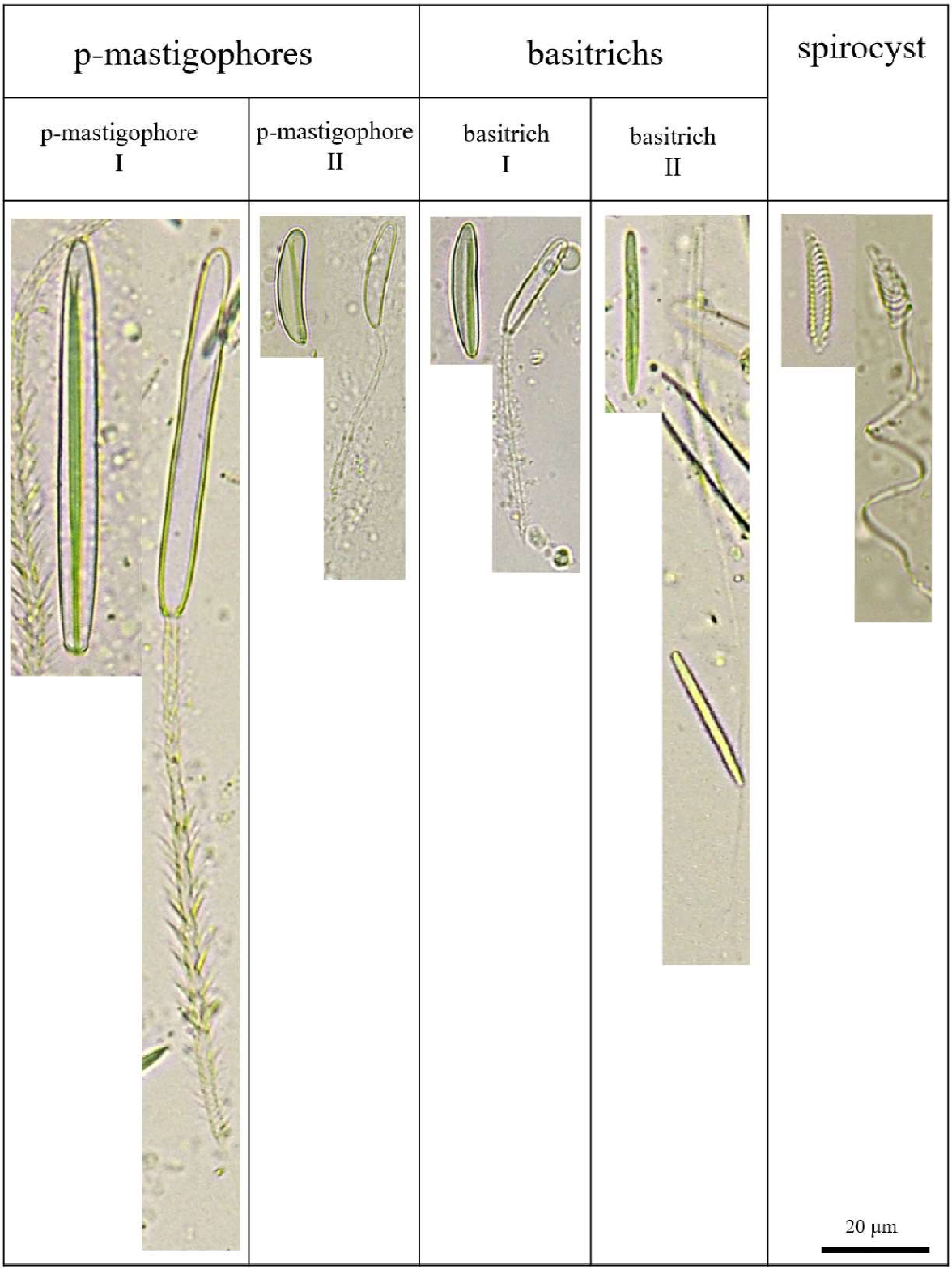


Table 4: a summary of data concerning *Aiptasia couchii*'s cnidome, including mean and standard deviation of capsule length and width of cnidae, their ratio and frequency in the samples. Datas of the categories with <1% frequences are not reported, as they are considered a possible contamination from other tissues

Tissue/ **Cnidae type**	**Capsule length** Mean ± SD (µm)	**Capsule width** Mean ± SD (µm)	**Length/width ratio** Mean ± SD	**Frequency percentage**
Acontia				
basitrich II	28.23 ± 2.70	2.29 ± 0.56	12.95 ± 3.04	56.21
p-mastigophore I	79.41 ± 5.23	7.14 ± 0.89	11.25 ± 1.30	43.20
spirocyst				0.59
Column				
basitrich I				0.83
basitrich II	20.67 ± 4.69	2.91 ± 0.73	7.43 ± 2.40	58.68
p-mastigophore II	21.35 ± 1.53	5.06 ± 1.26	4.45 ± 1.07	40.50
Tentacles				
basitrich I	24.72 ± 4.81	3.52 ± 0.67	7.04 ± 0.78	5.31
p-mastigophore I				0.48
p-mastigophore II	26.29 ± 2.87	4.21 ± 0.59	6.39 ± 1.31	9.66
spirocyst	21.10 ± 4.77	3.11 ± 0.96	7.10 ± 1.58	85.54

4.4.2 Spurilla neapolitana

Only four of the five nematocysts' types of *A. couchii* are acquired by the nudibranchs through predation. The cnidome of *Spurilla neapolitana* includes basitrichs (type I and II) and p-mastigophore (type I and II), while spirocysts are present only in feces (*Tab. 5*).

Table 5: nematocysts acquired by *Spurilla neapolitana* feeding upon *Aiptasia couhcii*, including mean and standard deviation of capsule length and width of cnidae, their ratio and frequency in the samples

Tissue/ Cnidae type	**Capsule length** Mean ± SD (μm)	**Capsule width** Mean ± SD (μm)	**Length/width ratio** Mean ± SD	**Frequency percentage**
Cerata				
basitrich I	26.28 ± 5,83	4.16 ± 0.83	6.44 ± 1.45	28.02
basitrich II	29.30 ± 2.77	1.95 ± 0.44	15.96 ± 4.63	25.66
p-mastigophore I	78.99 ± 5.65	7.18 ± 1.26	11.29 ±1.81	9.44
p-mastigophore II	23.55 ± 5.60	4.12 ± 0.97	5.97 ± 1.81	36.87
Feces				
basitrich I	25.69 ± 6.03	3.21 ± 1.02	8.68 ± 3.43	13.37
basitrich II	27.80 ± 3.60	1.89 ± 0.45	15.53 ±4.16	65.43
p-mastigophore I	77.48 ± 5.49	7.03 ± 0.94	11.23 ± 1.87	16.50
spirocyst	21.07 ± 4.70	2.70 ± 0.43	7.78 ± 1.12	4.69

The ceras of the nudibranch were characterized by the presence of basitrichs, type I and II, (53.68%) and p-mastigophores, both type I and II, (46.31%), with the former being the dominant class, though not by much. The least represented class was p-mastigophore I, with mean size, mirrored by maximum length reached by the capsules, of 78.32 ± 5.58 SD μm and a length/width ratio of 11.26 ± 1.82. The highest ratio though was reached by basitrich II, with a value of 13.28 ± 4.85, but an average size of 26.85 ± 4.65 μm of length by 2.22 ± 0.66 μm of width. Excluding p-mastigophores I, the mean sizes of cnidocytes present in ceras ranged between 23.55 ± 5.60 um and 29.30 ± 2.77 um.

The cnidome composition of feces showed a marked shift, with basitrich type II (65.43%) becoming dominant and spirocysts being exclusively found in this fraction, albeit at a low frequency (4.69%).

Several cnidocysts were triggered when mounting the slide with the samples, and it was possible to observe some of them in a semi-discharged state and the fired shaft (*Fig. 27C* and *Fig. 27D*). In the feces were found some deformed nematocysts and healthy and reproducing zooxanthellae (*Fig. 27B*), as was observed in cerata.

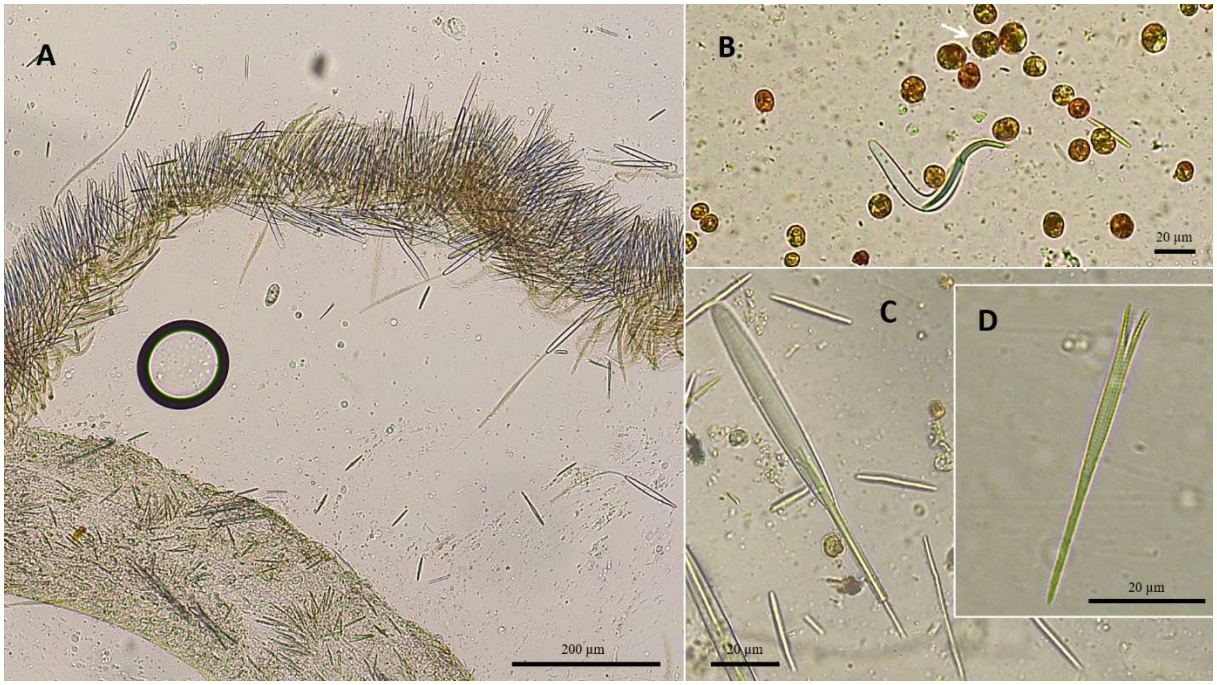


Fig. 27: details of some nematocysts structure: A) packed p-mastigophores I came out from the acontia; B) deformed p-mastigophore I and healthy and reproducing zooxanthellae (white arrow); C) semi-discharged p-mastigophore I and its fired shaft in D. B, C and D are photos of feces samples' content

The SEM analyses allowed to better observe the different cnidocyst type (*Fig. 28*).

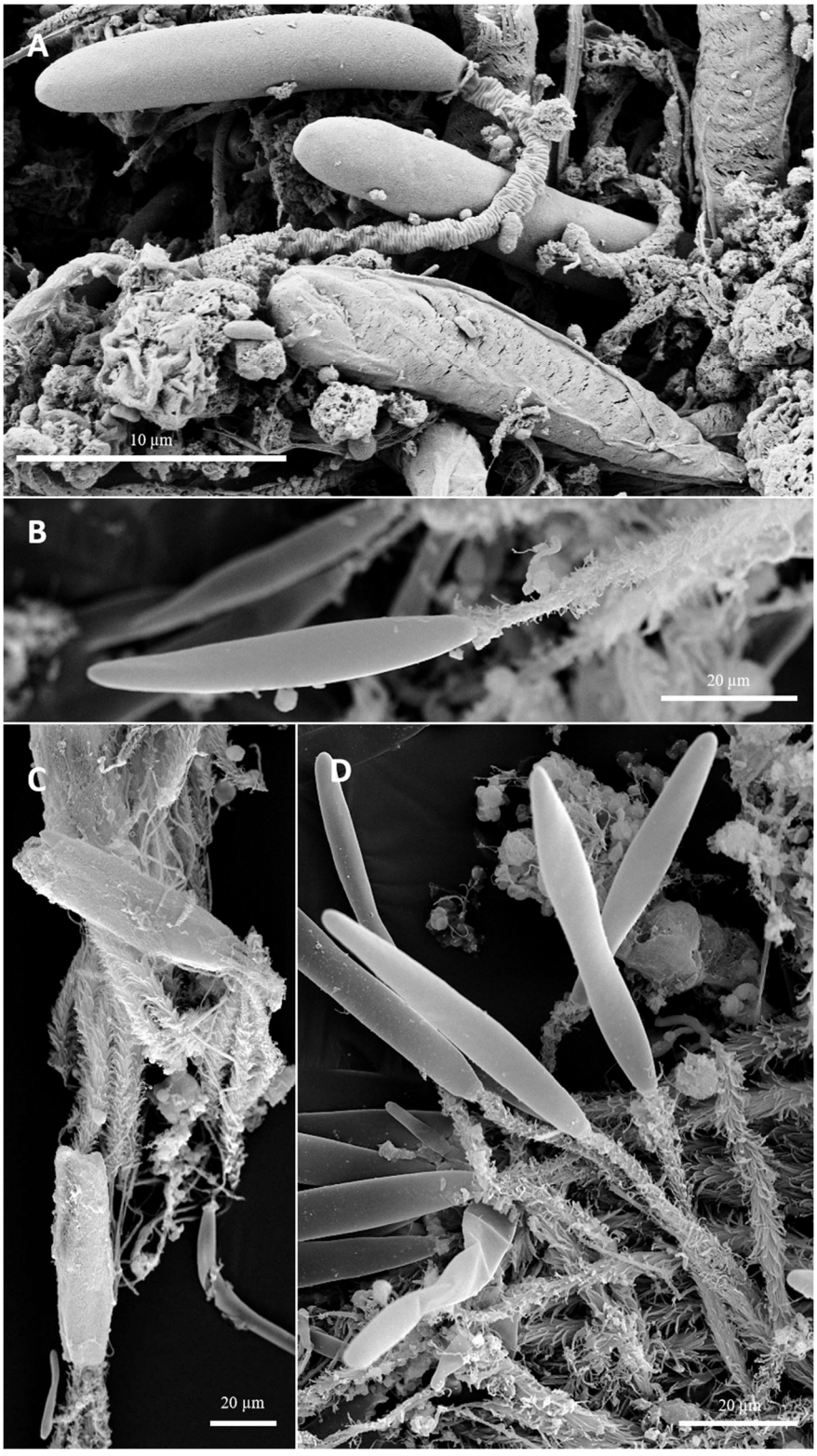


Fig. 28: micrographs by Scanning Electron Microscopy (SEM) of some nematocysts in *Spurilla neapolitana*: A) two spirocysts and two basitrichs I; B) basitrich isorhiza; C) discharged packed p-mastigophores I; D) detail of p-mastigophores I tubule. Photos A and B are from feces samples, C and D from analyses of the cerata

DISCUSSION

The present study provides insights into the ecology, feeding behavior, and symbiotic interactions of *Spurilla neapolitana*, with a particular interest on its relationship with the anemone *Aiptasia couchii* and the kleptoplastic integration of zooxanthellae and nematocysts. Through a combination of laboratory experiments and field observations, key aspects of the nudibranch's life cycle, feeding preferences, and cnidome composition have been elucidated.

This study was conducted to supply new indications to facilitate the rearing of *Spurilla neapolitana* as other species of nudibranchs often have simpler life cycles that are easier to complete in captivity. However, these species are not always readily available, making it essential to investigate the life cycles of a broader range of local nudibranchs to better understand the ecological dynamics of the region. The recorded seasonal presence of *Spurilla* in the Conero Riviera shows a peak occurrence during late spring-summer in conjunction with the observation of egg masses, potentially indicating the mating season. Despite efforts to rear the studied species under controlled conditions, closing its life cycle proved to be challenging, ultimately resulting in failure. This highlights the complexity of its developmental stages and the need for further research to refine rearing techniques and enhance our knowledge of its reproductive and larval ecology. Regarding the sampling of the individuals, I found that *S. neapolitana* is more frequently encountered during spawning events, suggesting a seasonal pattern that may influence its availability. The experimental assessment of this species' reproductive strategy confirmed that egg deposition is directly correlated with the adult's body length, supporting prior observations that larger individuals

produce more eggs. However, the weak correlation coefficient suggests that additional factors, such as physiological condition or environmental influences, may also play a role. The asynchronous development of embryos within the same egg ribbon further indicates a plasticity in larval development, possibly as an adaptive response to environmental variability. Hatching success was inconsistent, but improved throughout the experiment, and the occurrence of pink discoloration in certain egg masses, in both cut pieces and whole mass, could suggests microbial contamination or oxidative stress, requiring further investigation.

Observations on the feeding behavior of *S. neapolitana* toward *A. couchii* confirmed that initial contact elicits defensive nematocyst discharge from the anemone, followed by an acclimatization phase where the nudibranch becomes tolerant to the stings. The necrosis and subsequent blackening of *Aiptasia* tentacles post-attack indicates degradation of the anemone's tissues, which aligns with previous descriptions of nudibranch digestive mechanisms. These findings reinforce the notion that *S. neapolitana* has evolved specialized adaptations to neutralize the prey's defences and efficiently extract nutrients. The second experiment about the sensitivity of *S. neapolitana* to the metabolites released by a pricked anemone and its mucus in static waters didn't remark a strong reliance on olfactory clues to find the prey, as the nudibranch didn't directly approach the anemones that were behind the large mesh, if not in one isolated case.

Despite the data reported in literature shows that nudibranchs strongly rely on olfactory stimulus to find preys and react to prey's metabolites alone, such as demonstrated for *Berghia stephanieae* (Á. Valdés, 2005) (Fischer, 2021) and *Aplysia californica* J. G. Cooper, 1863 (T. Audesirk & Audesirk, 1985), the T-maze experiment provided evidence consistent with

those of the previous experiment, regarding the chemosensory capabilities of *S. neapolitana*. The nudibranch didn't show a marked attraction to either anemone prey (*Aiptasia couchii* and *Anemonia viridis*), but a possible inadequacy of the system has been denied by the subsequent runs. As a matter of fact, the lack of response to pricked anemones or mucus at a distance suggests that close-range chemical or chemotactic signals, rather than diffuse exudates, could be the primary triggers for foraging behavior. This contrasts with other aeolids that rely on long-distance olfactory cues, highlighting potential ecological specializations of *S. neapolitana* within its habitat. In the Passetto's tide pools in fact, *Aiptasia couchii* lives in densely packed population, so that a chance meeting during the roaming of the nudibranch in highly probable. However, further *in situ* observation would be helpful to clarify to this aspect, also because the nudibranch's sensitivity could be influenced by its age.

The cnidome analysis revealed that *S. neapolitana* selectively retains certain types of nematocysts, with a predominance of basitrichs and p-mastigophores II in the cerata, while spirocysts were largely expelled. Both found basitrichs are isorhizae, meaning that they possess an isodiametric tubule, and release of their venom content has been linked to prey capture (Daly, 2017). The two p-mastigophore types are nematocysts with a well-defined isodiametric shaft with prominent spines, a v-notch at shaft end and a terminal tubule. Both types are microbasic, with a discharged shaft less than one and a half times capsule length, with terminal tubule. While basitrichs and mastigophores are penetrant nematocysts, with established role in venom delivery, spirocysts have not been shown to contain toxins. Upon discharge into seawater, the tubules of spirocysts solubilize and adhere to substrates or prey. Contact with surfaces causes the everting thread to form a fine web of microfibrils, enhancing adhesion (Mariscal et al., 1977). This selective sequestration aligns with the

functional requirements of defence, as basitrichs and p-mastigophores are more effective in penetration and toxin delivery. The significant presence of undigested spirocysts in the feces further supports the hypothesis that these nematocysts are not useful for the nudibranch's defence and are thus eliminated. Furthermore, the discharge of p-mastigophores I could be attributed to their bigger size, being the other retained class of similar size between them. In fact, p-mastigophores I have a mean dimension in ceras of 78.99 ± 5.65 SD μm, while the other classes present in ceras range between 23.55 ± 5.60 SD μm and 29.30 ± 2.77 SD μm. The selection of nematocysts in nudibranchs is function-driven and carried out by cnidophages, internalizing nematocysts while excluding other structures like spirocysts, contributing to the defensive power of the nudibranch (Goodheart & Bely, 2017; Goodheart et al., 2022). Even if a size-driven selection of nematocysts has not been demonstrated, studies observed how the cnidophages differ in dimensions along the same cnidosac (Ekimova et al., 2022), but this detail was not appreciable in the present work, so further investigations are needed.

One of the most remarkable aspects of *S. neapolitana*'s ecology remains that of its ability to integrate and temporarily retain zooxanthellae from its anemone prey *A. couchii*, creating a three-way symbiotic relationship (*Fig. 29*). The presence of symbiotic algae in the digestive gland, their photosynthetic activity, and the prolonged retention in the absence of food suggest a mutualistic interaction that contributes to the nudibranch's energy budget. This aligns with previous findings in other kleptoplastic nudibranchs, where zooxanthellae-derived metabolites supplement the host's nutrition, that could be particularly incident in oligotrophic waters like the Mediterranean Sea. While this study does not directly assess photosynthetic contributions, the behavioural tendency of this nudibranch to hide under rocks during the day may indicate a limited reliance on light-dependent energy production.

Other research on photosynthetic rates, such as the measurements of photosynthetic activity (PAM), and metabolic integration of algal-derived products is necessary to explain this aspect. This type of data is highly species dependant, as it's been demonstrated how for *Aeolidia papillosa* (Linnaeus, 1761) the retention time of symbionts in ceras is of 11 days in starving conditions (McFarland & Muller-Parker, 1993) and *Berghia stephanieae* undergoes complete loss of endosymbionts within 8 days, affecting its somatic growth (Silva *et al.*, 2021), while after a 22-days period of starvation the zooxanthellae count in *Baeolidia australis*'s ceras remained consistent (Burghardt, 2007). Moreover, the fate of the expelled zooxanthellae remains uncertain. Once released, they are no longer directly associated with the anemone's tissues and may be dispersed by the nudibranch, trough the feces. It is possible that expelled zooxanthellae encyst or become available for uptake by other cnidarians, during the processes of shuffling and exchange (Curran & Barnard, 2021; Grupstra *et al*., 2021). This mechanism could play a role in dispersal and redistribution of symbionts trough the ecosystem, as the free-living stage of the *Symbiodinium* could last years, based on the clade it belongs to (Davin & Brannet, 2009).

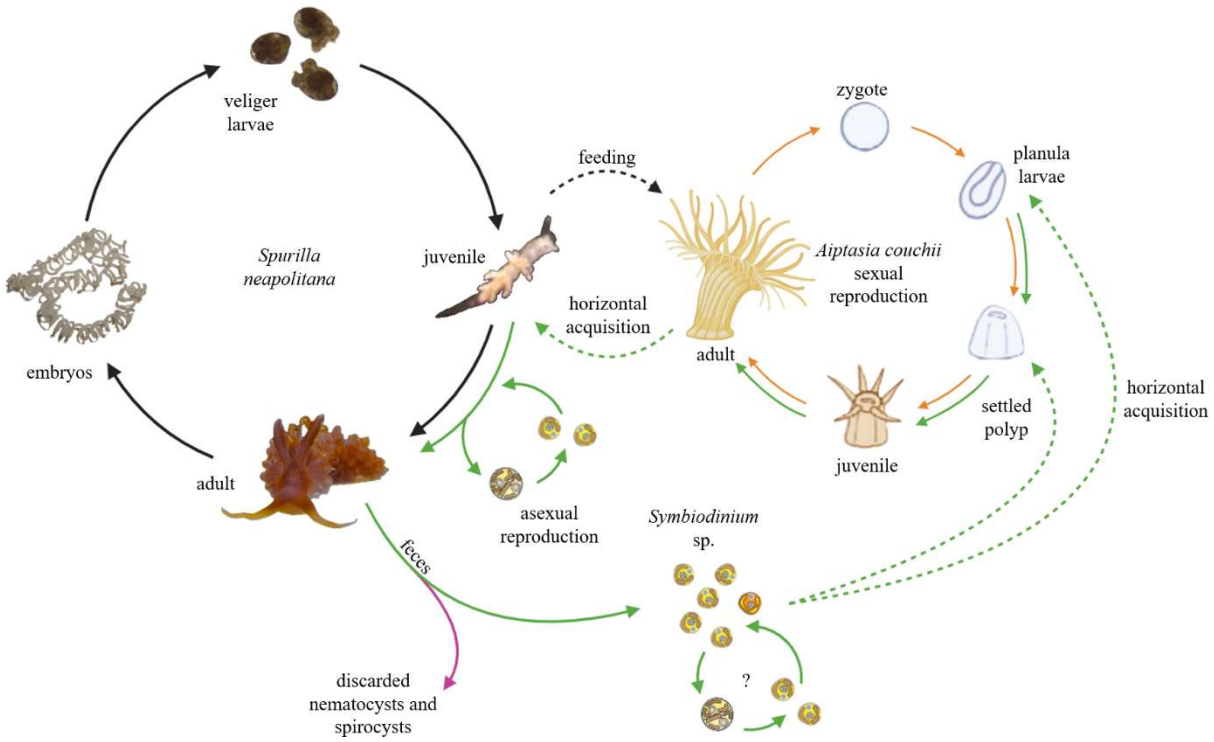


Fig. 29: diagram of the three-way symbiotic relationship between *Spurilla neapolitana*, *Aiptasia couchii* and *Symbiodinium*, with their interaction through the different life cycles. Legenda: black arrows: *S, neapolitana*'s life cycle; orange arrows: *A. couchii*'s life cycle; green arrows: *Symbiodinium*'s interactions with its hosts; purple arrow: discarded nematocyst and spirocysts

The results of this study highlight the ecological vulnerability of *S. neapolitana* due to its obligate relationship with cnidarian prey. The sensitivity of anemones to environmental stressors, such as ocean acidification and rising temperatures, poses an indirect but significant threat to the nudibranch's survival. The disruption of synchronized life cycles between predator and prey, as documented in other marine invertebrates (Damien & Tougeron, 2019), could have cascading effects on population stability.

This study advances our understanding of the ecological interactions and physiological adaptations of *Spurilla neapolitana*, emphasizing the intricate balance between predator-prey dynamics and symbiotic relationships. The findings underscore the importance of continued research on nudibranch-anemone interactions, particularly in the context of

climate change-driven habitat alterations. Future investigations should aim to understand factors promoting the larval metamorphosis and the mechanisms driving the molluscs towards their preys. Furthermore, the assessment of the metabolic contributions of zooxanthellae and the observation of reared/wild nudibranch juveniles, would help explore the potential of the three-ways relationships an ecological sentinel in marine conservation efforts. According to the results of this study and given its sensitivity to abiotic changes and its reliance on a limited prey spectrum, *S. neapolitana* represents an excellent bioindicator for monitoring the health of Mediterranean intertidal ecosystems.

CONCLUSIONS

The results obtained throughout this study highlight the complexity of the predator-prey interaction, regarding the mechanisms by which *S. neapolitana* acclimates to the nematocysts of its prey and incorporates symbiotic dinoflagellates into its tissues. This study also emphasizes the potential role of fecal expulsion in zooxanthellae dispersal, suggesting that nudibranchs could contribute to symbiont transfer within marine ecosystems.

In the broader ecological context, this research contributes to our understanding of intertidal ecosystem dynamics and highlights the importance of monitoring symbiotic and predator-prey interactions in response to environmental changes. Given the increasing pressures on coastal habitats, such as climate change and habitat degradation, species like *S. neapolitana* can serve as valuable indicators of ecosystem health.

Overall, this study advances knowledge on the ecological role of *S. neapolitana*, its trophic interactions, and its symbiotic strategies. Further research should focus on optimizing rearing conditions, understanding the biochemical mechanisms underlying kleptoplasty, and assessing the broader implications of symbiont dispersal facilitated by nudibranchs. Such efforts will provide essential contributions to marine conservation and the sustainable management of intertidal ecosystems.

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