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Reproductive biology and ecology of the sea cucumber

***Holothuria tubulosa* Gmelin, 1788**

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Abstract

Sea cucumbers play a key role on the benthic ecosystem functioning and marine biodiversity. They bioturbate the seafloor sediment reducing the organic load, recycling nutrients, and redistributing surface sediments assuming the role of keystone species in the bioremediation process in marine ecosystems. Many sea cucumbers have also a remarkable food interest particularly in Asia as traditional food product and object of traditional folk medicine, a food market resulting in an international demand. The increasing of uncontrolled international harvesting led to the overexploitation of many holothurians species, including the Mediterranean sea cucumber *Holothuria tubulosa*, the species I focused on in the present PhD thesis. The Italian government in 2018, adopting a precautionary approach, declared a moratorium on all fishing of sea cucumbers with the intention to understand the biological characteristics and the available stock, although this latter objective remained only theoretical. The Mediterranean sea cucumber *H. tubulosa*, although one of the most common sea cucumber species, showed a knowledge gap in many research areas starting from its life history traits, those characteristics that play a crucial role in the species management and assessment. A careful preliminary review allowed me to focus on some important questions to face the gaps in the life history, biology and ecology of *H. tubulosa* that then resulted in the structuring of the doctoral project. My key questions were: When and How does this species spawn in the central Mediterranean? Which are the environmental cues that lead the spawning? Can the early larval stages be affected by density-dependent factors? Moreover, what are the stimuli related to food availability affecting settlement and metamorphosis of larvae of *H. tubulosa*? Finally, I dealt with the important issue of the feeding behavior of adults of this deposit feeder species. My last question regarded the ecological role of *H. tubulosa* and its effect on the sedimentary organic matter, in particular: Can *H. tubulosa* modify the trophic status of the marine sediment? Can *H. tubulosa* select the sediment particles during the food ingestion and can it concentrate the protein-rich particles in the digestive tract?

I sought to answer all these issues with different ecological approaches, by using experimental manipulations that have been coupled with documentation of natural processes, and field work, that required the development of complex sampling and

experimental designs and evaluation in the field of experimental and statistical procedures. Four developments have followed from these research lines. First, *H. tubulosa* showed a unique reproductive event during the year in the central Mediterranean, corresponding to a relatively short time interval during the end of the Summer season. Second, the most effective trigger for spawning of *H. tubulosa* was the bubbling, which acted in the increasing of the water turbulence. The third development is that the algal biofilm that allowed the maximum settlement success was the green algae *U. lens*, also ensuring high survival of the post-settlers, a crucial issue that can help the feasibility of sea cucumbers aquaculture. The fourth development shed light on the trophodynamyc of *H. tubulosa*, revealing that this sea cucumber can select the protein-rich particles during the feeding action and can modify, locally and temporally, the trophic status of the sediment by producing feces rich in the most labile components of the organic matter.

Thesis outline

The body of this doctoral thesis is divided into six chapters, beginning with a general introduction followed by four chapters each structured with a “scientific article style”, i.e. Introduction, Material and Methods, Results, Discussion and Conclusion. In the last Chapter, are reported the general conclusions and future perspectives.

In **Chapter 1**, I reported a general review on the biology and ecology of commercial sea cucumbers identifying major critical issues. I focused on the Mediterranean sea cucumber *Holothuria tubulosa*, the target species of the present study. The chapter's contents are fully published in Pasquini *et al.* (2021).

In **Chapter 2**, the reproductive biology of *H. tubulosa* was investigated. I described the seasonal variation of *H. tubulosa* body length and body mass, which, together with the analysis of macroscopic and microscopic features of its gonads, allowed to identify the reproductive period of specimens inhabiting two sites in the central-western Mediterranean Sea. The study of the temporal patterns of the reproductive biology of *H. tubulosa*, provided evidence that this species has a synchronous development of the gonads, with an apparently unique reproductive event during the year. Through a new macroscopic approach based on the gonads' color and their features, it was revealed the presence of five different stages of gonads development (Recovery, Growing, Mature, Partly spawned and Spent), which was also confirmed by the histological analyses.

In **Chapter 3**, the spawning process and early embryonal development were discussed. I first explored the effect of different triggers on the spawning mechanism of *H. tubulosa* broodstock, and secondly, I investigated the effect of different stocking densities on the hatching rate of fertilized eggs. Among the treatments considered, which included the thermal shock, mechanical stress, addition of conspecific sperms, addition of an excess of food and the air bubbling, the last one was the most effective. Indeed, bubbling led to the emission of about 60% of the specimens tested. The results of early embryonal development showed that the hatching rate decreases with the increasing of the stocking density of fertilized eggs.

In **Chapter 4**, the mechanism of settlement and metamorphosis of larvae of *H. tubulosa* were described. I used different marine algal biofilms to foster the

metamorphosis of larvae from the doliolaria stage to the pentactula stage. I used the diatom *Amphora spp.* and the green algae *Ulvella lens* as algal biofilms. Results showed that both biofilms were able to induce the settlement and the metamorphosis, but *U. lens* was the most effective.

In **Chapter 5**, the trophodynamic of *H. tubulosa* was investigated. Particularly I explored the effect of *H. tubulosa* feeding behavior on the quality and quantity of sedimentary organic matter, with focus on the digestion process of the sedimentary protein content. Through a biochemical approach, I compared the sediment and sea cucumbers feces. Moreover a comparison of protein contents in ambient sediment, digestive tracts and feces, allowed to describe the selective ability of *H. tubulosa*. The results showed that firstly, sea cucumber can influence the trophic status of the sediment by changing the content of the most labile portion (proteins and lipids), increasing temporally and locally the nutritional quality of the sediment. Secondly, *H. tubulosa* can select the sediment particles during the food ingestion and can concentrate the protein-rich particles in the esophagus.

In **Chapter 6**, I reported the main concluding remarks of the thesis results and the possible future perspectives on the use of sea cucumbers breed in captivity.

Chapter 1- Introduction and Aims

Published as: **Pasquini V**, Giglioli AA, Pusceddu A, Addis P. 2021. Biology, ecology and management perspectives of overexploited deposit-feeders sea cucumbers, with focus on *Holothuria tubulosa* (Gmelin, 1788). *Advances in Oceanography and Limnology*. 12(2).

1.1 Holothurian's exploitation

The worldwide consumption of fish food products from 1961 to 2017 increased at an average annual rate of 3.1%, with a consumption per capita of fish food rising from 9.0 kg to 20.5 kg in the same period (FAO, 2020). Although the wild catches have been followed by the development of fish farming, the state of the wild stocks has continued to decline with less than 66% of the stocks harvested in a sustainable way (FAO, 2020). In response to the over-exploitation of wild finfish stocks, the invertebrate fisheries rapidly increased, being a new available source of seafood proteins and socio-economic opportunities (Berkes *et al.*, 2006, Anderson *et al.*, 2008). Many of the new target species now belong to low trophic levels, as a response to the overall down effect of trophic webs caused by top predators (Pauly *et al.*, 2002; Anderson *et al.*, 2008). In many cases, the pressure on stocks within low trophic levels increased faster than their management policies (Anderson *et al.*, 2011a, b), causing the spread of unregulated fishery and raising concerns for the possible consequences on ecosystem functioning and the sustainability of the fishery (Andrew *et al.*, 2002; Leiva and Castilla 2002; Berkes *et al.*, 2006; Anderson *et al.*, 2008; FAO, 2008).

Sea cucumbers, marine invertebrates belonging to the Echinodermata Phylum, include more than 1500 species (Horton *et al.*, 2018) and, mainly being deposit feeders, represent a good example of low trophic level organisms. Their fisheries had rapidly grown and expanded since 1980 because of the increasing demand from international markets, aquaculture and biomedical research programs (Bordbar *et al.*, 2011).

Holothurians are present in almost all the marine biotopes, from the littoral to hadal depths (Purcell *et al.*, 2012). Holothurians are part of the Chinese culinary tradition, are considered gourmet and luxury seafood and are generally sold as a dried product called *bêche-de-mer* or *trepang* (Wen *et al.*, 2010; Yang and Bai, 2015). The market price of this product depends on the quality (grade low, medium, high) (Ram *et al.*, 2014), with

some particularly valuable species as *Apostichopus japonicus* Selenka, which holds the highest price of 2950 US\$ dried kg⁻¹, followed by *Holothuria scabra* Jaeger, 1833 (115-640 US\$ dry kg⁻¹), *Holothuria lessoni* Massin, Uthicke, Purcell, Rowe and Samyn, 2009 (240-790 US\$ dry kg⁻¹) (Purcell *et al.*, 2012).

The presence of high-value nutrients such as Vitamin A, Vitamin B1 (thiamine), Vitamin B2 (riboflavin), Vitamin B3 (niacin), and minerals (i.e., calcium, magnesium, iron and zinc) indicate that sea cucumbers are suitable tonic and restorative products, also rich in crude proteins (range 41-63%) (Wen *et al.*, 2010, Bordbar *et al.*, 2011).

Moreover, sea cucumbers, containing a number of biological and pharmacological bioactive compounds, have attracted attention for their potential medical value (Bordbar *et al.*, 2011). Sea cucumbers contain numerous bioactive and anti-age substances that are already exploited in the cosmetic and pharmaceutical industries (Fredalina *et al.*, 1999; Zhao *et al.*, 2007; Bordbar *et al.*, 2011; Purcell, 2014). All these properties and the high market price led to the overexploitation and decline of sea cucumbers Indo-Pacific populations and the expansion of the fishery to reach new virgin stocks in Galapagos Islands, Mexico, North America and the Mediterranean Sea (Conand, 2006; Purcell *et al.*, 2012; González-Wangüemert *et al.*, 2018). The estimated sea cucumbers harvest, from Asia and Pacific regions, ranges from 20.000 to 40.000 t per year of the dry product (FAO, 2012). Fisheries from African and Indian Ocean regions also contribute to the complex amount with the range of 2000 2500 t per year (FAO, 2012).

Less information is available about sea cucumbers fisheries in the Mediterranean Sea, in particular for *Holothuria tubulosa* Gmelin, 1788, *Holothuria mammata* Grube, 1840, *Holothuria sanctori* Delle Chiaje, 1823, *Holothuria forskali* Delle Chiaje, 1823, *Parastichopus regalis* Cuvier, 1817, and *Holothuria arguinensis* Koehler and Vaney, 1906 (Çakly *et al.*, 2004; Antoniadou and Vafidis, 2011; Sicuro and Levine 2011; González-Wangüemert and Borrero-Perez, 2012; Mezali and Thandar 2014; Gonzalez-Wangüemert *et al.*, 2014a, 2015). Presently, more than half of global sea cucumber fisheries are considered depleted or overexploited to the extent that governments (including the Italian Government) have banned their harvesting (Andreson *et al.*, 2011; González-Wangüemert *et al.*, 2014, 2018) (Fig. 1.1; 1.2). With the 38% of sea cucumber fisheries currently unregulated and an unknown level of illegal catches, this fishery is

considered unsustainable and far from being adequately managed (Choo, 2008; Toral-Granda, 2008; Anderson *et al.*, 2011).

The unregulated exploitation of sea cucumbers is a rising concern for their conservation, with 16 species worldwide now classified as “vulnerable” or “endangered”, according to the IUCN Red list (Conand *et al.*, 2014, Ramírez-González *et al.*, 2020). Concern also raises because, most of the harvested sea cucumbers are deposit-feeders, thus playing an ecological key role due to their feeding behavior (Uthicke, 2001; Roberts *et al.*, 2000), their decline could have severe consequences on sedimentary biogeochemistry and benthic ecosystem functioning.

Here we reviewed the available information about the ecological role of sea cucumbers, with a focus on the Mediterranean *H. tubulosa*, their breeding, fishery management issues, main gaps of knowledge and future perspectives for their use as remediation of eutrophicated sediments.

1.2 Life history and population dynamics of sea cucumbers

The increasing interest towards sea cucumbers and their use for food, medical and habitat remediation purposes, stimulated exploration about their reproductive cycle and population dynamics, both crucial aspects for the assessment of wild stocks and their eventual management.

Almost all sea cucumbers are broadcast spawners with external fertilization that present an annual or bi-annual maturation season (Mercier and Hamel, 2009; Mohsen and Yang, 2021). With a few exceptions of hermaphrodite species, they are generally gonochoric that lack in sexual dimorphisms (Smiley *et al.*, 1991; Mercier and Hamel, 2009). The life cycle of sea cucumbers is characterised by one or more planktonic larval stages starting with a feeding auricularia (early, mid and late), a non-feeding doliolaria and then a feeding pentactula that settle on the substrate (Strathmann, 1975; Ito and Kitamura, 1997; Yanagisawa, 1998).

Doliolaria actively explore the surrounding environment to identify the best place to settle and made the last metamorphosis into the pentactula. If the conditions are not suitable for settlement, the larvae will keep swimming for several days (Mercier *et al.*, 2000). The pentactula lose the ability to swim but can continue to explore the surrounding

environment with the buccal podia, moving by small jumps (Mercier *et al.*, 2000). Although rarely, Evans and Palmer (2003) reported the ability of the pentactula larvae of *Parastichopus californicus* Stimpson, 1857, to clone, forming a bud that, after separation, will normally develop into an auricularia larvae.

The pentactula larvae will start to feed and grow, becoming a juvenile in a variable time lag (Mercier *et al.*, 2000; Agudo, 2006; Mercier and Hamel, 2009; Rakaj *et al.*, 2018, 2019). Information about the mechanisms of settlement, physiology and cue that can stimulate the larvae to settle are poorly explored and understood, so far. Studies conducted in mesocosm investigated the success of the larval settlement, which can strongly depend on the larval nutrition state and the capacity to accumulate lipids (Peters-Didier and Sewell, 2019). In the late auricularia stage of *H. scabra*, the development of the hyaline spheres indicates an adequate feeding, and their size is a reliable indicator for subsequent performance (Duy *et al.*, 2016). The settlement and the last metamorphosis, as for other echinoderms, represents a survivorship bottleneck that can lead to high mortality rates. The early juvenile stage (<5 mm length) is also vulnerable and a critical phase with substantial mortality rates (Agudo, 2006; Rakaj *et al.*, 2018).

The holothurians recruitment has been studied mainly on historically exploited species, and information about post-settlers and juveniles in the field is scarcely recorded in the literature and, even, referred to sporadic occasions. For instance, the recruitment of *H. scabra* has been found to occur on a monthly time scale on seagrasses, with adult specimens mainly observed in sandy sediments and juveniles in organic matter (OM) enriched muddy sediments (Mercier *et al.*, 2000). The lack of other information about holothurians recruitment can also be ascribed to the potential misidentification of the species because they can have a considerably different morphology when compared with that of adults. Besides this, juveniles might occupy different habitats and can be obscured from the researchers' view because of their cryptic behaviour (Shiell, 2004). *H. scabra* juveniles can also be affected by predation-mediated mortality by fish belonging to the Balistidae, Labridae, Lethrinidae and Nemipteridae families (Dance *et al.*, 2003), sea stars, and crustaceans (Kinch *et al.*, 2008). Holothurians' recruitment can also be affected by geographic distances, the duration of the larval period and to the hydrodynamic retainment in coastal areas (Uthicke, *et al.*, 1998, 1999, 2001; Uthicke and Purcell, 2004).

Most studies about holothurians' population dynamics explored species with a long history of exploitation, including *A. japonicus*, *Cucumaria frondosa* Gunnerus, 1767, and *Isostichopus fuscus* Ludwig, 1875, (Herrero-Pérezrul *et al.*, 1999; Reyes-Bonilla and Herrero-Pérezrul, 2003; Hamel and Mercier, 2008; Anderson *et al.*, 2011; Purcell *et al.*, 2011; Yang *et al.*, 2015; Glockner-Fagetti *et al.*, 2016). Unfortunately, the absence of a rigid structure in sea cucumbers and the high plasticity of the body wall make it difficult to investigate the growth rates of holothurians. Alternative methods proposed include marking the calcareous (epi-pharyngeal) ring, chemical marking of spicules, external and internal tagging (Kinch *et al.*, 2008). However, all of these methods are affected by wide methodological biases but also by the bio-ecological traits of holothurians. In fact, the body size of holothurians can vary as a response to changing environmental conditions (Tolon *et al.*, 2017b), the occurrence of asexual reproduction through fission (Purwati and Dwiono, 2005; Uthicke and Conand, 2005; Laxminarayana, 2006; Purwati and Dwiono, 2007; Purcell *et al.*, 2012; Dolmatov, 2014,2021) or the evisceration of their internal organs (intestine, gonads and respiratory trees) through autotomy, in response to predation and other environmental stressors (Shukalyuk and Dolmatov, 2001; Wilkie, 2001; Spirina and Dolmatov, 2003; Zang *et al.*, 2012). The evisceration is a typical behavioural trait of holothurians that does not lead to the death of the organism, rather is followed by the re-growth of the internal organs (Dawbin, 1949; Murray and García-Arrarás, 2004; García-Arrarás *et al.*, 2006; Dolmatov and Ginanova, 2009). Interestingly, after evisceration, the respiratory function shifts to the body wall for the time necessary for the respiratory trees' regrowth. During this period, sea cucumbers will consume endogenous substances, which causes a significant body weight loss (Zang *et al.*, 2012, Zhang *et al.*, 2017). Because of the multiple factors regulating holothurians body size, small individuals are not necessarily the youngest ones (Kinch *et al.*, 2008).

1.3 Breeding of sea cucumbers

The development of sea cucumbers *ex situ* breeding protocols derived from the need to reduce the pressure on wild overexploited stocks. Breeding sea cucumbers can be used for restocking activities (Purcell and Kirby, 2006) as already explored for other exploited echinoderms (Couvray *et al.*, 2015; Giglioli *et al.*, 2021). Moreover, producing and releasing juveniles sea cucumbers reared in “conservation hatchery”, could be a useful

tool for bioremediation of eutrophicated sediments or in Integrated Multi-Trophic Aquaculture systems (see below) without burden on wild populations. The experimental reproduction of sea cucumbers has been carried out for many species and the aquaculture is now established for largely exploited Indo-Pacific species like *H. scabra* (Agudo, 2006) and *A. japonicus* (Purcell *et al.*, 2012; Shi *et al.*, 2013, 2015; Pietrak *et al.*, 2014).

China, the largest consumer and producer country, is breeding annually about 10 000 t of dry weight *A. japonicus* from aquaculture to supply the local demand, while in other countries this activity is still in a pilot scale or in early development stages (Choo, 2008). It has been estimated that once released in the field *H. scabra* can reach the commercial size of 700 g ind⁻¹ in about 2 - 3 years, with a survivorship of 7 - 20% (Purcell and Simutoga, 2008). In the last decade, new attempts have been also made with the Mediterranean species *H. tubulosa* (Fig. 1.3) and *Holothuria polii* Delle Chiaje, 1823, (Rakaj *et al.*, 2018, 2019); *H. arguinensis* (Domínguez-Godino *et al.*, 2015); *H. mammata* (Domínguez-Godino and González-Wangüemert, 2018).

1.4 Feeding behavior and ecological role of sea cucumbers

Deposit-feeders holothurians acquire food by swallowing large volumes of sediment (Ramon *et al.*, 2019). They sift through the sediment with tentacles and feed on detritus, organic matter, sand and the relative grown-over biofilm, expelling sandy pellets after digestion (Hartati *et al.*, 2020).

The feeding starts with capturing the sedimentary food particles with tentacles and their release into the pharynx through the circum-oral tentacles. Once inside the mouth the particles are mixed with the digestive enzymes and compressed into a plug which moves throughout the gut following a plug-flow reactor model. The plug is then transported by peristalsis along the simple digestive system that ends in the posterior part of the animal (Zamora and Jeffs, 2011).

Sea cucumbers predominantly feed on sedimentary organic detritus associated with micro-organisms and small benthic organisms (Roberts *et al.*, 2000). In the gut mineral and organic particles are found along with fragments of shell, barnacles, seagrasses, echinoderms ossicles, faecal pellets, foraminifera shells, with a highly variable size (Roberts *et al.*, 2000).

Information about the potential selectivity of shallow-water holothurians is controversial. Some holothurians are able to choose organic matter (OM) enriched particles, whereas others appear not to be (Moriarty 1982; Hammond, 1983; Uthicke and Karez, 1999; Battaglione *et al.*, 1999; Slater *et al.*, 2011; Navarro *et al.*, 2013; Sun *et al.*, 2015; Lee *et al.*, 2018; Hartati *et al.*, 2020). The selective ability can be related to how sea cucumbers feed on the sediment, which is highly variable among species, depending on their tentacles dimension, the size and gut morphology (Roberts *et al.* 2001, Dar and Ahmad, 2006; Ramón *et al.*, 2019). The selection of smaller organic-rich particles might be due to the greater ease of being caught and held by the tentacles, or to the potential chemo-selection ability of holothurians (Schneider *et al.* 2013; Lee *et al.*, 2018). The presence of a higher OM content in the gut compared to the one present in the sediment can be a consequence of a passive selection of the finest grain size of the particles which can be more easily ingested. This, in turn, can be explained because smaller grain size particles can have a higher OM content due to the wider surface available for microbial colonization (Hargrave, 1972; Levinton, 1972; Dale, 1974; Yamamoto and Lopez, 1985; Manini and Luna, 2003).

Considering their feeding behaviour, sea cucumbers are great seafloor bioturbators, able to rework large amounts of sediments via ingestion and excretion (9–82 kg ind⁻¹ year⁻¹) which can extensively blend and reform seafloor substrata (Coulon and Jangoux, 1993; Uthicke and Karez, 1999; Mangion *et al.*, 2004). Bioturbation intensity can influence the sediment permeability, oxygen concentration, water content and chemical gradients in pore water, affecting the rate of remineralization and the inorganic nutrient flux and, finally, can redistribute food resources for the other benthos (Reise, 2002; Lohrer *et al.*, 2004; Solan *et al.*, 2004; Meysman, 2006a). Bioturbation carried out by sea cucumbers can be circumscribed to the upper layer of the sediment or reach up to ten centimeters depth based on the habits of the species whether they are fossorial or not (Uthicke and Karez, 1999; Purcell, 2004a; Amaro *et al.*, 2010).

The role of holothurians in recycling the sedimentary OM is considered one of their main ecosystem functions (Purcell *et al.*, 2016). The ability to reduce the OM content in the sediment has been recently investigated (Dar and Ahmad, 2006; İsgören-Emiroğlu and Günay, 2007; Slater and Carton, 2009; Zamora and Jeffs, 2011; Tolon *et al.*, 2017a; Neofitou *et al.*, 2019; Hartati *et al.*, 2020). The sea cucumber *Australostichopus mollis*

Hutton, 1872, can significantly reduce total organic carbon (TOC), chlorophyll-a and phaeopigments contents of sediments impacted by green-lipped mussel biodeposits (faeces and pseudofaeces) (Slater and Carton, 2009). MacTavish *et al.* (2012) reported that *A. mollis* suppressed benthic microalgae and facilitated bacterial activity, causing a shift in the balance of benthic production and decomposition processes. Juveniles of the same species decreased their ingestion rate with the increasing of the total sedimentary organic matter (TOM), showing the ability of this species to use different amounts of TOM, changing their feeding behaviour and digestive physiology (Zamora and Jeffs, 2011). *H. tubulosa* reduced the sedimentary OM and organic carbon (OC) by 31-59%, with an absorption rate of 43 and 55% respectively, both in manipulative laboratory and field experiments (Neofitou *et al.*, 2019).

The functioning of the digestive system of holothurians has been modelled and defined as a sort of ‘bioreactor’, where the ingested nutrients are quickly extracted and assimilated (Penry and Jumars, 1986, 1987; Jumars, 2000; Amaro *et al.*, 2010). The grazing of holothurians could increase the exchange flux of nutrients across the sediment-water interface and promote nutrient regeneration (Zhou *et al.*, 2006; Yuan *et al.*, 2013; Slater and Carton, 2009; Slater *et al.*, 2011; Zamora and Jeffs, 2011, 2012a,b). On the other hand, other species, like *A. japonicus*, could not affect TOC and total nitrogen (TN) sedimentary contents, but can cause OM particles redistribution and inhibit microphytobenthos (Michio *et al.*, 2003).

1.5 The Mediterranean sea cucumber *Holothuria tubulosa*

A new target species candidate for sea cucumbers aquaculture is *Holothuria tubulosa* (Gmelin 1788), one of the most common and widespread holothurians in the coastal areas of the Mediterranean Sea and the Eastern Atlantic Ocean (Tortonese, 1965; Koukouras *et al.*, 2007). In the last few years, *H. tubulosa* has been actively harvested in Turkey, Greece, Italy, Spain and the increasing of illegal and unregulated fishing is one of the main issues for its management (Rakaj *et al.*, 2019). Overexploitation of this species led the Italian Ministry of Agriculture, Food and Forestry (MIPAAF) to ban sea cucumbers fishing along the entire national coastline (Ministerial decree 156/2018), as a precaution for the conservation of the species.

H. tubulosa is a continuous deposit-feeder, generally encountered in organic matter enriched soft bottoms and seagrass meadows (Bulteel *et al.*, 1992; Gustato *et al.*, 1982). Coulon and Jangoux (1993) reported that large individuals of *H. tubulosa* might ingest up to 17 kg of dry weight sediment $\text{ind}^{-1} \text{y}^{-1}$. Using the data provided by Costa *et al.* (2014) it can be estimated that the quantity of seagrass detritus potentially ingested by *H. tubulosa* ranges between 12 and 28 g dry weight $\text{m}^{-2} \text{y}^{-1}$.

The reproductive cycle of *H. tubulosa* was studied in specimens from the Adriatic Sea, Oran coast (Algeria) and Dardanelles Strait (Turkey). The development stages of male and female gonads showed a clear annual pattern and all authors agreed that the spawning period was set between June and October with minor local differences, and a resting period from October to January (Despalatović *et al.*, 2004; Ocaña and Tocino, 2005; Dereli *et al.*, 2015; Tahri *et al.*, 2019). Rakaj *et al.* (2018) successfully bred and reared *H. tubulosa* in the laboratory, completing the larval development in 27 days, which, however, was followed by high mortality shortly after the settlement. A recent study reported the use of *H. tubulosa* larvae as new model for embryo-larval bioassays to assess marine pollution (Rakaj *et al.*, 2021), but, to date, rearing techniques of this species remain still not very efficient.

1.6 Sea cucumbers in integrated Multi-Trophic Aquaculture (IMTA)

In the last two decades, to satisfy the demand for seafood product, aquaculture activities increased and the need to mitigate its impacts on the environment became an urgent need, especially in the presence of vulnerable habits like seagrass beds (Pusceddu *et al.*, 2007; Holmer *et al.*, 2008). Wastes coming from mariculture plants can affect sediments biochemistry, increasing the organic contents, ultimately exacerbating eutrophication (David *et al.*, 2009; Keeley *et al.*, 2014). In fact, wastes from mariculture can cause benthic hypoxia and anoxia, hydrogen sulphite enrichment and, in extreme cases, also led to rising of methanogenic bacteria populations, which, in turn, can significantly impact the abundance and biodiversity of benthic organisms (Karakassis *et al.*, 2000; Angel *et al.*, 2002; Mirto *et al.*, 2002; Burford *et al.*, 2003; La Rosa *et al.*, 2004; Fodelianakis *et al.*, 2015).

The conceptual approach of Integrated Multi-Trophic Aquaculture (IMTA) is to use different trophic-levels organisms in the same system: those belonging to the highest

trophic level (generally fish) are fed artificially and those belonging to the lowest trophic level (extractive species) feed on waste released by the specimens of the highest trophic level (Troell, 2009; Granada *et al.*, 2015). The extractive species commonly used in IMTA include molluscs, seaweeds or detritivorous species (Zhou *et al.*, 2006; Slater and Carton, 2007; Yuan *et al.*, 2013; Slater *et al.*, 2009; Zamora and Jeffs, 2011, 2012a,b; Lamprianidou *et al.*, 2015; Shpigel *et al.*, 2018). Among detritivorous species, considering their feeding habits, sea cucumbers appear to be ideal candidates as extractive species for IMTA systems.

Commercially valuable holothurians species most used in IMTA systems include *A. japonicus* (Zhou *et al.*, 2006; Yuan *et al.*, 2013; Kim *et al.*, 2015), *A. mollis* (Slater and Carton, 2007; Slater *et al.*, 2009; Zamora and Jeffs, 2011, 2012a,b), and *P. californicus*, (Paltzat *et al.*, 2008), mainly fed with scallops and mussels' biodeposits alone, or mixed with powdered algae (Yuan *et al.*, 2006). Other small-scale experiments used *Actinopyga bannwarthi* Panning, 1944 (Israel *et al.*, 2019) and *H. scabra* (Mathieu-Resuge *et al.*, 2020).

The IMTA feasibility in the Mediterranean Sea is still in an experimental scale, whereas either pilot or commercial scale activities have been carried out in other regions (MacDonald *et al.*, 2013; Marinho *et al.*, 2013; Lamprianidou *et al.*, 2015). To our best knowledge, only two studies investigated the use of *H. tubulosa* in IMTA systems in the Mediterranean Sea.

Beneath fish cages, Tolon *et al.* (2017b) observed a biomass increase of holothurians ranging from 9 to 31 g ind⁻¹ in just 90 days and suggested that these animals are ideal candidates to mitigate in IMTA the benthic eutrophication generated by fish farming. Neofitou *et al.* (2019) during an experiment carried in the field beneath farming cages of the sea bream *S. aurata* and the sea bass *Dicentrarchus labrax* Linnaeus, 1758, reported that the maximum extractive capacity of holothurians is reached at a density of ca. 10 individuals m⁻². Such a density allowed abating OM and OC contents in sediments beneath the cages by 31 and 59%, respectively. These results, though spatially and temporally fragmented, corroborate the idea of using sea cucumbers beneath fish cages, in IMTA systems, to mitigate the impacts of biodeposition on the sediment, at the same time providing a commercially important by-product, without any additional feed. With

these assumptions, it can be envisaged that sea cucumbers in IMTA will increase the environmental sustainability of aquaculture and will also generate an important economic advantage, due to the high value of sea cucumbers.

1.7 Holothurians' management perspectives

The ecological consequences of holothurians overexploitation include a loss in bioturbation and a consequent reduction of benthic biomass, biodiversity, and ecosystem functioning (Lohrer *et al.*, 2004; Solan *et al.*, 2004; Meysman *et al.*, 2006b). Therefore, sea cucumbers' overexploitation claims for urgent measures to preserve natural populations and their ability to provide reproductive adults for either natural or artificial breeding.

On the one hand, the peculiar biological and ecological traits of holothurians and the lack of reliable stock assessments make a scientific based management of this resource still far to be reached. Management and regulation of sea cucumbers fishery are currently being implemented in some countries, using different approaches. Among these, for example, a rotational zone strategy has been applied to the multispecies sea cucumber fishery in Australia's Great Barrier Reef Marine Park, where this approach led to a substantial reduction of the risk of localized depletion, higher long-term yields, and improved economic performance (Plagányi *et al.*, 2015).

To guarantee significant recruitment in an acceptable timeframe, future management policies of sea cucumbers should set a minimum population density threshold, below which exploitation should be banned (Battaglene and Bell, 2004), also to avoid the Allee effect, which occurrence has been reported for overexploited populations of *H. scabra* in the Warrior Reef, Australia (Skewes *et al.*, 2000), *I. fuscus* in the Galapagos Marine Reserve, Ecuador (Toral-Granda and Martinez, 2007), and *H. nobilis* (Selenka 1867) in the Great Barrier Reef, Australia (Uthicke and Benzie, 2000) and *Holothuria mexicana* Ludwig, 1875, (Rogers *et al.*, 2018).

Ultimately, we notice that adequate protocols of holothurians' populations management still need large amount of quantitative information about their population dynamics, recruitment success, rates of growth and natural mortality (Romero-Gallardo *et al.*, 2018), mechanisms allowing larval settlement. Concurrently, studies aiming at

identifying new and more efficient *ex situ* rearing protocols, also to feed restocking actions and to preserve the natural genetic pools (Purcell, 2004b, Purcell and Kirby, 2006; González-Wangüemert *et al.*, 2015) are also needed.

1.8 Aims

The recent increase of the fishing pressure led, recently, to a worldwide over-exploitation of sea cucumbers, including the Mediterranean *Holothuria tubulosa*. This raised concern for their conservation status and for the possible consequences on coastal ecosystem functioning.

The general aim of my PhD thesis is to improve the knowledge about the biology and ecology of the sea cucumber *H. tubulosa* and to optimize the current protocols for its breeding in captivity, to foster the use of reared specimens in restocking practices in the field.

The specific objectives of the thesis are:

- 1) To fill some of the gaps of knowledge about the seasonal variability of *H. tubulosa* body size and weight, with focus on its reproductive biology and, more specifically, on the micro- (histologic) and macroscopic identification of the different gonadal development stages (**Chapter 2**).
- 2) To explore the relative importance and efficacy of different stimuli putatively triggering the emission of the gametes and to evaluate the hatching rate of fertilized eggs at different densities in mesocosms (**Chapter 3**).
- 3) To understand the settlement mechanisms of *H. tubulosa* planktonic doliolaria larvae and its metamorphosis into the benthic pentactula as influenced by different algal biofilms (**Chapter 4**).
- 4) To explore the trophodynamics of *H. tubulosa* in different environmental settings, with a focus on the digestion process of sedimentary proteins (**Chapter 5**)

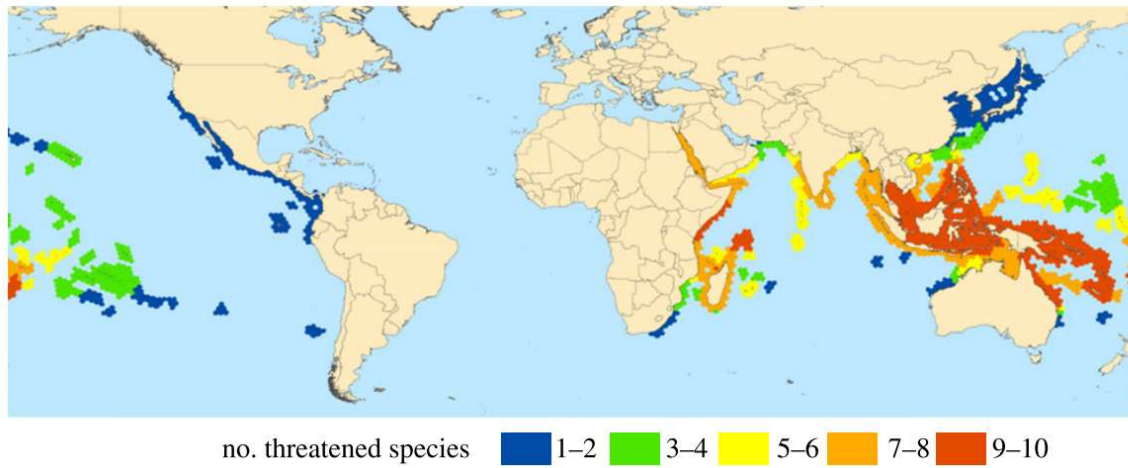


Fig. 1.1: Global prevalence of threatened sea cucumbers. Number of sea cucumber species within the deposit-feeders (order Aspidochirotida) that were classed as EN or VU among locations worldwide. (Source: Purcell *et al.*, 2014).

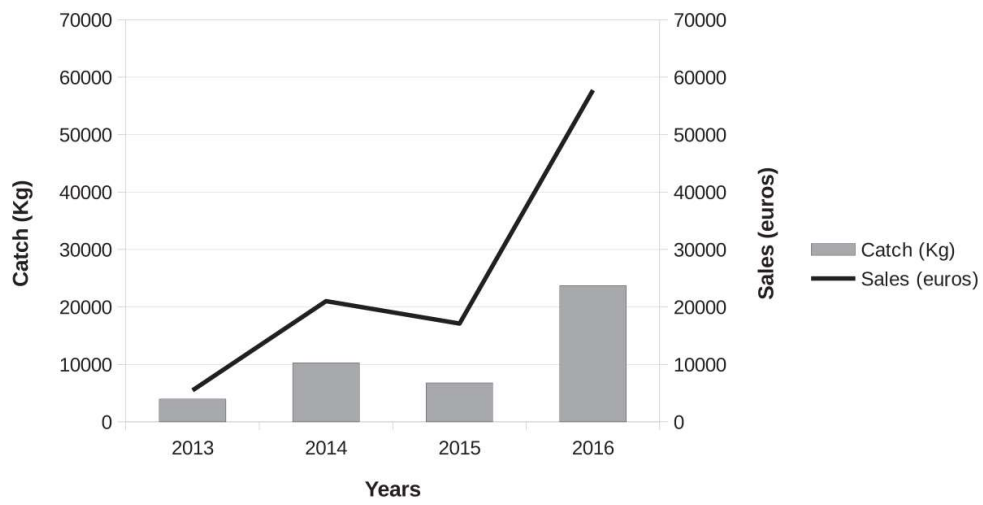


Fig. 1.2: Evolution of *Holothuria forskali* catches (grey bars) and sales (black line) in Galicia (NW Spain) since 2013 to 2016. (Source: González-Wangüemert *et al.*, 2018).



Fig. 1.3: Juveniles of *H. tubulosa* reared in captivity at the experimental hatchery of the University of Cagliari.

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Chapter 2

Seasonal variations in length and body mass of the sea cucumber *Holothuria tubulosa* Gmelin 1788, in different environmental settings of the Central Western Mediterranean Sea with cues on reproductive biology

2.1 Introduction

Holothurians, known as sea cucumbers, are common benthic marine invertebrates that belong to the Phylum Echinodermata, Class: Holothurioidea, and are represented by more than 1500 species worldwide (Horton *et al.*, 2018). Most sea cucumbers are strong deposit-feeder bioturbators and, as such, are thought to play a key ecological role in benthic biogeochemistry (Roberts *et al.*, 2000; Uthicke, 2001; Mangion *et al.*, 2004; Amaro *et al.*, 2010; Purcell *et al.*, 2016; Neofitou *et al.*, 2019).

Sea cucumbers are part of the culinary culture of Eastern Asian countries and are largely used in traditional Chinese medicine (Yang and Bai, 2015). They are a valuable source of bioactive compounds used in the pharmaceutical and cosmetic industry and are also considered a gourmet seafood called *trepang* or *bêche de mer* (Kinch *et al.*, 2008; Bordbar *et al.*, 2011; Purcell *et al.*, 2012; Janakiram *et al.*, 2015; Yang and Bai, 2015). The high cost of *trepang* (generally between 50 to 600 US\$ dry kg⁻¹) (Ram *et al.*, 2014; Purcell *et al.*, 2012, 2014), and the numerous medical applications of sea cucumbers, led to the overexploitation of the Indo-Pacific species (Lovatelli *et al.*, 2004; Conand, 2006; Choo *et al.*, 2008; Purcell *et al.*, 2011; Conand *et al.*, 2014). In response, the fishery of sea cucumbers shifted to new target species as the Mediterranean ones (González-Wangüemert *et al.*, 2014; 2018). In many cases, sea cucumber's harvesting increased without an adequate management, which, coupled with the illegal catches, caused the collapse of several populations (Toral-Granda *et al.*, 2008; Anderson *et al.*, 2011; Purcell *et al.*, 2014, González-Wangüemert *et al.*, 2018; Ramírez-González *et al.*, 2020). The main cause of the failure of sea cucumbers management can be also a reflection of severe

gaps of knowledge about their basic biological and ecological attributes and their reproductive biology. On the other hand, the current lack of information about sea cucumbers' population dynamics depends also upon their peculiar morphology: indeed, the lack of a rigid skeleton and the high plasticity of the body wall, that can vary in response to environmental conditions (Bulteel, Jangoux and Coulon, 1992; Kinch *et al.*, 2008; Zang *et al.*, 2012, Prescott *et al.*, 2015 Tolon *et al.*, 2017a; Ramírez-González *et al.*, 2020), limit practically the possibility to estimate appropriately their age and growth rates.

Sea cucumbers species are generally gonochoric, only sporadically hermaphrodites, and are mainly broadcast spawners (Hyman, 1955; Smiley 1998, 1990; Smiley *et al.*, 1991; Mohsen and Yang, 2021). The reproductive apparatus of sea cucumbers consists in a single gonad ramified in several tubules and a gonoduct that lead to the gonopore situated in the anterior part of the organism (Mercier and Hamel 2009). The maturation process of gametes seems to be controlled by either exogenous or endogenous factors. Temperature, light intensity, photoperiod, lunar cycle, tidal flux, food quality and its availability are among the most influential exogenous factors (Ramofafia *et al.*, 2000; Tan and Zulfigar, 2001; Hamel and Mercier, 2004; Mercier and Hamel 2009), but their different role in modulating the reproduction is still far to be fully accomplished.

Since an accurate knowledge of the reproductive cycle is a crucial step for breeding practices in captivity, more insights about the biology and ecology of sea cucumbers are needed to ultimately produce valuable biomass and reduce the fishing pressure on wild stocks (Morgan, 2000; Agudo, 2006; Purcell *et al.*, 2012; Dominguez-Godino and González-Wangüemert, 2018; Rakaj *et al.*, 2018, 2019).

In the Mediterranean Sea, one of the most common sea cucumbers species is *Holothuria tubulosa* Gmelin 1788, which inhabits organic matter-rich soft bottoms and *Posidonia oceanica* meadows, where it plays a prominent role in recycling sedimentary organic detritus (Bulteel, Jangoux and Coulon, 1992; Mezali, Zupo and Francour, 2006; Mezali and Souali, 2013; Costa *et al.*, 2014; Boncagni *et al.*, 2019). In the last few years, this species has been one of the most commercially exploited in the whole Mediterranean basin (González-Wangüemert *et al.*, 2014, 2015, 2018; Dereli and Aydin 2021).

The sea cucumber *H. tubulosa* has an annual reproductive cycle and the spawning period occurs typically at the end of summer (Bulteel, Jangoux and Coulon, 1992) from late July to October in the Kastela Bay (Adriatic Sea) as well as in the Dardanelles Strait and Oran coast, Algeria (Despalatović *et al.*, 2004; Dereli *et al.*, 2016, Tahri *et al.*, 2019). Nevertheless, very little information was reported about the spawning period of *H. tubulosa* in the Central Western Mediterranean Sea.

Studies about the reproductive period of *H. tubulosa* have been mainly focused on the histological and microscopic observation of the gonads but have almost entirely ignored their macroscopic characteristics, as instead done for other species (e.g., *H. fuscogilva*; Ramofafia *et al.*, 2000). Furthermore, studies on the reproductive cycle and population dynamics conducted so far on *H. tubulosa* were related to reduced temporal and spatial scales and, as such were not exhaustive.

The purposes of this part of the thesis were to study the size characteristics of the sea cucumbers *H. tubulosa* inhabiting two different sites in the central-western Mediterranean Sea, and to identify their reproductive period, based on the seasonal analysis of macroscopic (i.e., gonad weight, gonado-somatic index and color) and microscopic (i.e. histological determination of oocytes and size of germinal layers) features of gonads.

2.2 Materials and methods

2.2.1 Study areas and sampling

The sites selected for the study were the Oristano (39°52'50''N; 8°28'54''E), and Teulada (38°55'46''N; 8°43'17''E) Gulfs (Sardinia, Italy - central-western Mediterranean Sea) both characterized by the presence of abundant populations of *H. tubulosa* (Fig. 2.1).

The Oristano Gulf is characterized by muddy-sandy sediment and hosts offshore mariculture and suspended mussels farming plants, close to the sampling area; the Teulada Gulf is characterized by either sandy sediment or wide *Posidonia oceanica* meadows.

A total of 91 (13 samplings from May 2018 to December 2020) and 42 (7 samplings from April 2019 to July 2020) specimens were collected from the Oristano and the

Teulada Gulfs, respectively. At each sampling date, surface temperature and salinity were recorded using a multiparametric probe.

Specimens (n= 6-10 per sampling) were placed into a 3L plastic bag and transported to the laboratory inside a cooler box (at *in situ* temperature). In the lab, sea cucumbers were wet weighted (± 0.01 g), and their length (± 0.1 cm) and width (± 0.1 cm) were recorded. Prior to body weight and size measurement, specimens were gently squeezed to remove the excess water from the respiratory tree (Costa *et al.*, 2014). The wet weight, length and width were used to establish the Length-Wet Weight, Width-Wet Weight and the Length-Width relationships.

2.2.2 Macroscopic observation of the gonads and reproductive period

The gonad was separated from the gut and the respiratory tree and weighted (± 0.01 g). The Gonado-Somatic Index (GSI) was calculated as follow:

$$(\text{Gonad Weight} / \text{Wet Weight}) \times 100$$

Whenever possible, the sex of the specimens was assigned observing the gonads' color according to Despalatović *et al.* (2004): white for males and pink for females. The number of branches ramification of the gonad tubules (from 1 to 5, used as a descriptor of the gonad maturation stage; the high the number of ramifications, the more developed the gonad; Ramofafia *et al.*, 2000) were considered as reported in Fig. 2.2, and the highest ramification level recorded.

2.2.3 Colorimetric analysis of the gonads

The gonad color is one of the common macroscopic clues to identify sea cucumbers' sex (Ramofafia *et al.*, 2000, 2003; Despalatović *et al.*, 2004). In this study, the color was defined using a reference palette with 10 main colors and the respective RGB scores and Pantones® codes (Pantone Inc. USA) in natural daylight (Tab. 2.1) (Prato *et al.*, 2018). Since the capacity to assign colors may vary between observers, we also used an analytical method to assign the color to the gonads (Addis *et al.*, 2014). The gonads' colorimetric analyses were conducted using a digital colorimeter (Chroma meter CR-400, Konica Minolta, Tokyo, Japan), which specifies the color according to the Commission Internationale de l'Eclairage (CIE, Vienna, Austria) as L*a*b* color space (CIELAB).

The Chroma meter CR-400 measures color with six silicon photocells and a standard luminant D65. The light source is a pulsed xenon lamp, used to take readings calibrated against a white plate. The color formula defines color in terms of three coordinates (L^* , a^* , b^*), which locate the color of an object within a 3-dimensional color space. The L^* component represents the lightness; a^* is the component from green (negative values) to red/magenta (positive values); b^* is the component between blue (negative values) and yellow (positive values) (CIE, 1978). The colorimetric analysis of the gonads was conducted only when the gonad's dimension was adequate to fit in the instrument chamber, which only happened when the gonads were in Growing and Mature stages.

2.2.4 Length at first maturity (LFM) and Length at maturity (L_{50})

Specimens were ordinated in different size (length) classes and the Length-frequency distribution was defined separately for females and males. The length at first maturity (LFM; length of the smallest mature individual) and length at maturity (L_{50} ; the length at which 50% of the individuals are mature) were estimated separately for females and males. Non-linear least squares regression was used to estimate the following parameters: $p = 100\{1 + \exp [a+bL]\}^{-1}$, where p is the proportion of mature individuals by any length measure and a and b are the fitted parameters, which can change during the life cycle (King, 1995).

2.2.5 Histological analyses

One portion of the gonads of a sub-sample of 50 specimens was fixed for the histological analysis in 5% buffered formaldehyde (0.1 M, pH 7.4) for a maximum period of 48 h and stored in 70% ethanol (Cerri and Sasso-Cerri 2003). Prior to coloration, the whole gonad portions were dehydrated through a progressively higher series of alcohol concentrations (70% - 100%), embedded in a synthetic resin (GMA, Technovit 7100, Bio-Optica), and cut with a rotative microtome (Histo-line ARM3750) into 3.5 μm slides. Slides were then stained with haematoxylin and alcoholic eosin. All sections were then observed using a Nexcope NE600 optical microscope equipped with a digital camera (MD6iS) at different magnifications (40 \times , 100 \times , 400 \times), and edited with Adobe Photoshop CS6.

The microscopic maturity stages of females were determined based on the gonad wall thickness, the position of the oocyte in the gonad section and the oocyte size (Cameron and Fankboner, 1989; Smiley, 1988; Eckelbarger and Young, 1992; Morgan *et al.*, 2000; Ramofafia *et al.*, 2000; Despalatović *et al.*, 2004). The oocytes diameter was measured using the tpsDIG v.2.12 software (Rohlf, 2009). Since oocytes migrate towards the internal lumen of the gonad during their development (Despalatović *et al.*, 2004) the gonad frontal section was divided into 3 concentric circles; the external monolayer including the youngest oocytes attached to the germinal epithelium, the intermediate one, and the internal layer including all the most developed oocytes within the lumen (Fig. 2.3).

To assess the maturity stage of males' gonad the following clues were considered: the thickness of the gonad, the extension of the germinal layers and its folds and the abundance (by rank) of spermatozoa filling the gonad lumen.

2.2.6 Statistical analyses

To test the adequacy of the models used to assess the Length-Wet Weight, the Width-Wet Weight and the Length-Width relationships, an ANOVA using the P value for Lack-of-Fit was performed on previously transformed (ln) data. The ANOVA was conducted with the software STATGRAPHICS PLUS 5.1 professional edition (Statistical Graphics Corp., Rockville, MD, USA).

To assess differences between in Wet Weight, Length, Width, Gonad Weight and the GSI of the specimens between sites and sampling times a univariate permutational analysis of variance (PERMANOVA) was applied to each variable separately, using Site (Oristano, Teulada) and Season (Winter, Spring, Summer, Autumn) as fixed orthogonal factors. Prior to the statistical analysis, the GSI data were arcsin transformed ($x' = \arcsin\sqrt{x}$).

The univariate 2-way PERMANOVA was also performed to test differences:

- in the oocytes' diameters, as obtained from microscopic observations, using Season (Winter, Spring, Summer, Autumn) and Oocyte position (1st, 2nd, Other) as fixed and orthogonal factors;

- in gonad colors spaces L^* , a^* , b^* , using sex (Female, Male) and gonadal stage of maturation (Growing, Mature) as fixed and orthogonal factors.

All PERMANOVA tests were based on Euclidean distances of previously normalized data, using 999 random permutations of the appropriate units. When significant differences were observed, pairwise tests were also performed. P values in the PERMANOVA and pairwise tests were obtained from Monte Carlo asymptotic distributions (Anderson and Robinson, 2003).

All the statistical analyses were performed using the routines included in the PRIMER 6+ software (Clarke and Gorley, 2006).

2.3 Results

2.3.1 *Environmental parameters*

The surface temperature varied in both sites accordingly to the variation of the seasons, and was higher in summer in both sites. The salinity followed a different trend in the two sites, in Oristano the variation was between 34.5 in winter and 35.5 in summer, in Teulada was minimum in summer (35.0 salinity) and maximum in autumn (37.1 salinity) (Fig. 2.4).

2.3.2 *Sea cucumbers' body size and weight*

Wet Weight, Length and Width of the sampled specimens are reported in Fig. 2.5. The results of the PERMANOVA test and the relative pairwise comparisons are reported in Tables 2.2 and 2.3. The Wet Weight, Length and Width of the sampled specimens show a significant effect of the Site \times Season interaction. The pairwise comparisons show that all variables varied among seasons only in the Oristano gulf (Fig. 2.5, Tab. 2.3).

The Length-Wet Weight relationship (Fig. 2.6), with a slope ($b=1.76$) lower than 3, suggests a negative allometric growth. The Width-Wet Weight relationship follows a significant ($R^2=0.719$) exponential curve (Fig. 2.6), whereas the Length - Width relationship approximates a significantly ($R^2=0.500$) linear fit (Fig. 2.6). The results of the Analysis of Variance with Lack-of-Fit of the three relationships ($P>0.05$) indicate that the models used to describe the three relationships fit with the observed data.

2.3.3 Reproductive period assessment with gonads' macroscopic features

The Gonad Weight varies significantly among seasons (Tab. 2.4) only in the Oristano gulf (Tab. 2.5), where it increases in Spring and shows a relative maximum in Summer (Fig. 2.7).

The GSI varies significantly among seasons in both sites (Tab. 2.4; 2.5). In both gulfs, the GSI increased progressively from Winter to Summer, when it reaches the highest value, then drops in Autumn, when it reaches the lowest value (Fig. 2.8). The GSI in female specimens reached higher values than that in males, in Winter, Spring and Summer (Fig. 2.9).

2.3.3.1 Branches ramification of the gonad's tubules

In both Winter and Autumn, the gonad branches' ramifications could not be identified. Only a small number of specimens show a visible ramification (2-3 branches) of gonad branches in Spring. In Summer, the gonads show the largest size and the ramification of branches varies from 2 to 5.

2.3.3.2 Sea cucumbers sex from gonads' color

The color of the gonads in Summer, and in certain cases in Spring, allowed identifying the sex of the specimens, which were red and coral-red in females and pale yellow in males (Tab. 2.1, Fig. 2.10 A, A1, B, B1).

2.3.3.3 Macroscopic maturity stages

Based on the Gonad Weight, the GSI, the number of gonad branches ramification and the assigned color, five gonadal stages can be identified.

- 1) **Stage 1: Recovery.** This stage occurs in Autumn and Winter. The gonad weight is < 1 g and the GSI varies between 0 and 1. Branches' ramifications nor sex are visible or recognizable. The gonad color is pale (Color ID 454 and 461) and transparent.
- 2) **Stage 2: Growing.** This stage occurs mainly in Spring and Summer. The gonad weight ranges between 2 and 25 g and the GSI varies between 1 and 5. The number of branches varies between 2 and 3. Males have a matt pale yellow (Color ID 461,

- 454) gonad, whereas females have a translucent red–coral red (Color ID: 151, 157) gonad.
- 3) **Stage 3: Mature.** This stage occurs mainly in Summer. The gonad weight varies between 20 and 150 g; the GSI is comprised between 4 and 31. The branches' ramifications varies between 3 and 5. Males have a matt pale yellow (Color ID 157, 461) gonad, whereas females have a translucent red-coral and red (Color ID: 165, 172, 485) gonad.
 - 4) **Stage 4: Partly spawned.** This stage is observed in late Summer and Autumn. The gonad weight is between 2 and 25 g; the GSI is between 1 and 15. The branches' ramifications varies between 3 and 4. Males have a matt pale yellow (Color ID 461) gonad, whereas female gonads lose their translucency and appear dull coral-red (Color ID: 157).
 - 5) **Stage 5: Spent.** This stage is observed in both Autumn and Winter. The gonad weight is between 0 and 2. The GSI is between 0 and 1. Branches' ramifications nor sex are visible or recognizable. The gonads presented segments with different colors and was not possible distinguish between females and males.

2.3.3.4 Colorimetric analysis of the gonads in Growing and Mature stages

The color of the gonads differs significantly between Growing and Mature stages in both females and males (Tab. 2.6). The pairwise comparison shows that in mature females, when compared to the Growing ones, the L* (lightness) component decreases, the a* (redness) one increases, and the b* (blues) one does not change. Males' gonad color is much less variable and significant differences occur between Growing and Mature males only for the a* (redness) component (Fig. 2.11).

2.3.4 Length – frequency distribution, LFM and L₅₀

The sea cucumbers' length at different maturity stages (pooling together females and males) is higher in the Mature stage and minimum in the Recovery and Spent (Fig. 2.12) ones.

The Length – frequency distributions of females and males show similar trends in the Oristano Gulf, whereas in the Teulada one largest specimens are, surprisingly, only males (Fig. 2.13).

The LFM of females is 15 cm, with a L_{50} of 21 cm. The LFM of males is 11 cm with a L_{50} of 20cm.

2.3.5 Histology and gonad morphology

2.3.5.1 Oocytes population

Oocyte's diameter varies significantly among seasons, with minimum values in Winter, and much higher values in all other seasons. Values increase progressively in Spring and Summer. In Autumn, the oocyte diameter is greatly variable, showing oocytes in all the vitellogenesis stages. (Tables 2.7, 2.8; Fig. 2.14).

The oocyte diameter varies significantly among oocyte position in the layers (OoP; External, Intermediate, Internal layers; Fig. 2.15) and Seasons (Se), and shows a significant effect of the interaction $Se \times OoP$ (Tab. 2.9). In winter, the oocyte diameter in the external monolayer is significantly smaller than that in the lumen, whereas it does not vary among the other layers (Tab. 2.10A; Fig. 2.16). In Spring and Summer, the oocyte diameter increases from the external to the intermediate and internal layers. In Autumn, the diameter of the oocytes in the internal and intermediate layers were significantly smaller than those in the internal layer (Tab. 2.10A, Fig. 2.16).

The oocyte diameter in the external layer shows minimum values in Autumn and in Winter and significantly increases in Spring and Summer. The oocyte size in the intermediate layer varies significantly in all the seasons, showing smaller diameter in Autumn, which significantly increase in Winter, Spring and Summer. The oocyte diameter in the internal layer varies significantly in all the seasons except Spring and Autumn, showing the highest values in Summer. (Tab. 2.10 B, Fig. 2.16).

2.3.5.2 Microscopic identification of the gonadal stages

Based on the histology of the gonads (Fig. 2.17) and the size distribution of mature oocyte diameter (Fig. 2.18), five female gonadal stages can be recognized as follows:

- 1) **Stage 1: Recovery** (Fig. 2.17a, a1). Ovaries show thickened walls; previtellogenic and early vitellogenic oocytes are present in the germinal (external) layer; most oocytes are close to the tubule wall (external layer) and the tubule lumen is almost empty. The oocyte diameter is $35 \pm 11.9 \mu\text{m}$ (Mean \pm SD).
- 2) **Stage 2: Growing** (Fig. 2.17b). The wall of the tubule is still thickened; oocytes in the germinal (external) layer show different vitellogenesis levels, the mature oocyte migrate towards the gonad lumen (intermediate and internal layers) and have a clearly visible nucleus (germinal vesicle), a nucleolus and follicular cells. The follicular membrane surrounds the oocyte. The oocyte diameter is $71 \pm 35.0 \mu\text{m}$ (Mean \pm SD).
- 3) **Stage 3: Mature** (Fig. 2.17c). Oocytes reach their maximum size in all of the gonad layers ($110 \pm 20.3 \mu\text{m}$) (Mean \pm SD) and are surrounded by the follicle membrane. Mature oocytes completely fill the gonad lumen (internal). In the germinal (external) layer, some oocytes are in mid vitellogenesis. In this stage, the oocyte population shows a unimodal distribution, indicating that *H. tubulosa* females have a synchronized development of the gonad (Fig. 2.18).
- 4) **Stage 4: Partly spawned** (Fig. 2.17d). The gonad has a thinned wall. The number of mature oocytes inside the tubules decreases, and empty spaces due to the partial gametes spawn are visible. Oocytes are in the gonad lumen only (internal layer). Oocytes without the follicular membrane and without a visible germinal vesicle are also present. The oocyte diameter ($109 \pm 28.4 \mu\text{m}$; Mean \pm SD) is similar to that in the Maturity stage but the oocytes show clear signs of morphological degradation.
- 5) **Stage 5: Spent** (Fig. 2.17e). The gonad wall is thickened with abundant connective tissue that fills the lumen. Due to the absence of gametes, females and males are indistinguishable.

Five male gonadal stages have been identified based on the gonad histology as follows:

- 1) **Stage 1: Recovery** (Fig. 2.19a). The gonad wall is thickening. The germinal layer shows numerous folds and active spermatogenesis. Spermatogonia is visible in the germinal layer and the gonad lumen is almost empty.

- 2) **Stage 2: Growing** (Fig. 2.19b). The gonad wall is thinner than that in the Recovery stage. Spermatogenesis is highly active. Spermatozoa are migrating towards the lumen and begin to fulfil the gonad. Numerous folds of the germinal epithelium are still present.
- 3) **Stage 3: Mature** (Fig. 2.19c, c1). The gonadal wall begins to thin, the folds are reduced and less visible. In this phase, spermatozoa fill the lumen of the tubule. Spermatogenesis continues and spermatocytes are present in the germinal layer (Fig. 2.19c1).
- 4) **Stage 4: Partly spawned** (Fig. 2.19d). After the partial emission of the gametes, the density of spermatozoa in the gonad decreases, leaving empty areas in the lumen.
- 5) **Stage 5: Spent** (Fig. 2.19e). The wall of the gonad is very thick and is characterized by the presence of abundant connective tissue. As for the female, the absence of gametes in this stage does not allow the sex identification.

The cross-inspection of the gonadal stages as determined using separately macroscopical and histological cues provided evidence of a correspondence of the two different approaches. A summary of the two approaches is provided in Tab. 2.11.

2.3.6 Frequency of the gonad stages

Based on the classification reported in Tab. 2.11, seasonal variations in the frequency of the different gonadal stages have been also investigated (Fig. 2.20).

As far as the females are concerned (Fig. 2.20), in Winter only the Recovery and the Growing Stages are observed (about 50 % each). In Spring, the Growing stage frequency increases (63%) and is accompanied by the appearance of the Mature stage (36%), which increases (up to 88%) in Summer. Mature and Growing stages disappear in Autumn, when the Partly spawned and the Recovery stages codominate.

As far as the males are concerned (Fig. 2.20), in Winter all investigated specimens are characterized by the exclusive presence of the Recovery stage; in Spring the Growing and Mature stages almost codominate (70% and 30% respectively), whereas in Summer the frequency of the latter increases and the former decreases. In Autumn, no males were retrieved.

Unassigned specimens (ND) in the Spent stage appear only in Winter and Autumn (Fig. 2.20).

2.4 Discussion and conclusion

2.4.1 Body size metrics of *H. tubulosa* and their temporal variations

Several field and modelling studies have been conducted so far on the population dynamics of historically exploited sea cucumbers such as *A. japonicus*, *Cucumaria frondosa*, and *Isostichopus fuscus* (e.g., Herrero-Pérezrul *et al.*, 1999; Reyes-Bonilla and Herrero-Pérezrul, 2003; Hamel and Mercier, 2008; Purcell *et al.*, 2011; Glockner-Fagetti *et al.*, 2016; Ramírez-González *et al.*, 2020). However, Gonzalez-Wangüemert *et al.* (2016) put the baseline for the study of population dynamics of the new target species including *Holothuria polii*, *Holothuria mammata*, *Holothuria arguinensis* and *H. tubulosa*. The analysis of sea cucumbers population dynamics based on common tagging methods is typically biased by the high morphological plasticity of the body wall (Prescott *et al.*, 2015), which makes poorly effective the use of tags (Kinch *et al.*, 2008). At the same time body size of holothurians can vary in response to environmental conditions, food availability and to the stress level of the organism (including the evisceration of the internal organs) (Bulteel, Jangoux and Coulon, 1992; Wilkie, 2001; Spirina and Dolmatov, 2003; Zang *et al.*, 2012 Tolon *et al.*, 2017a). Consequently, most studies on population dynamics of sea cucumbers considered weight measurements alone more reliable than the length (Kazanidis *et al.*, 2010; González-Wangüemert *et al.*, 2014; Prescott *et al.*, 2015). Other studies used the gutted weight and length to reduce the intra-specimen variability (Marquet *et al.*, 2017; Aydin, 2020, Dereli *et al.*, 2016) or gently squeezed the specimens to remove the excess water from the respiratory tree (Sewell, 1990; Costa *et al.*, 2014), the same approach used in the present study.

We report here that both the Length-Wet Weight and the Width-Wet Weight relationships are significant, thus suggesting that the combined use of those metrics (length or width vs. wet weight) can be reliably used as proxy for investigating the size structure of *H. tubulosa* and compare populations under different environmental settings of exploitations rates. Noticeably, the slope of the Length-Wet Weight relationship of *H. tubulosa* ($b = 1.76$) indicates a negative allometric growth of the investigated populations,

a characteristic also reported previously even using other couples of metrics like drained weight vs. the total length (Kazanidis *et al.* 2010) or the gutted length vs. the gutted weight (Aydin 2020).

The results of our study carried out in two different sites characterized by different environmental settings (in terms of sediment grain size, organic matter contents) show that temporal variations (on a seasonal scale) in *H. tubulosa* individual wet weight, length, and width are significant only in the muddy asset (Oristano), where larger, heavier and reproductive individuals were observed in Spring and Summer. In the sandy asset (Teulada), temporal variations in sea cucumbers' body size, if any, were much less evident. Such a discrepancy is difficult to explain, but we infer that the smaller size of the population investigated in the sandy asset could have masked body size temporal changes.

2.4.2 *Reproduction stages of H. tubulosa: methodological considerations and reproduction period assessment.*

To identify the different maturity stages of *H. tubulosa* gonads we used a combination of macroscopic (Gonad weight, GSI, tubules ramification degree, and the gonads' color; Conand, 1981; Smiley, 1988; Ramofafia *et al.*, 2000; 2003) and microscopic (oocyte diameter in females, histological features of the gonads) (Despalatović *et al.* 2004) approaches. Both approaches have proven to be reliable in identifying five different gonadal stages that were fully overlapping.

Seasonal variations in the gonad weight, GSI and tubules ramification reported here altogether indicate that the reproductive period of *H. tubulosa* in the study sites is concentrated in Summer. This result is in good agreement with those reported earlier by Despalatović *et al.* (2004) for the same species in the Adriatic Sea and by Dereli *et al.* (2016) in the Dardanelles Strait. Moreover, the oocytes' diameter shows a minimum in Winter, when most of the gonads are in the Recovery and/or Growing stage, and progressively increases in Spring, when most gonads are in the Growing stage and reaches the highest value in Summer, when oocytes are characterized by the presence of clearly visible germinal vesicles and follicular membranes (Smiley, 1988, 1990). These microscopic features progressively disappear in Autumn when most of the mature gametes have been released.

The gonad colors assigned using the RGB color palette (Tab.1) allowed us to distinguish between sexes with a good match in developed (Growing, Mature and Partly spawn) gonads, but not in gonads in the Recovery or Spent stages, in which gonads are pale and white or show a heterogeneous color with rust blotches, respectively (Marquet *et al.*, 2017). In Growing and Mature stages, the female gonad is typically red–coral translucent and the male one is matt pale yellow. The discrimination between the two stages in males and females is not always clear because of the numerous nuances of the gonads color and the observer’s sensitivity, which can lead to different color assignments. Thus, to overpass such a subjective assignment of the color and the consequent erroneous assignment to a certain gonadal stage (Addis *et al.*, 2014), we enhanced the colorimetric analysis of *H. tubulosa* gonads using the CIELAB color space. This analysis allowed us to show that female gonads in the mature stage show a reduction of Lightness (L^*) and an increase of the red/magenta (positive value of a^*) component, which is consistent with the progressive fulfilling of the gonads lumen with mature oocytes. The analysis shows also that male gonads in mature stage are characterized by an increase of the red/magenta (positive value of a^*) component only.

Noticeably, the presence of five recognizable gonadal stages in the Mediterranean sea cucumber *H. tubulosa* resembles that reported for the tropical *Holothuria fuscogilva* and *Holothuria scabra* (Ramofafia *et al.*, 2000, 2003), thus suggesting that this pattern is a recurrent feature of the sea cucumbers, at least of those of the genus *Holothuria*.

In conclusion, the results of this study provided evidence that the sea cucumber *H. tubulosa* has a synchronous development of the gonads, with an apparently unique reproductive event during the year, corresponding to a relatively short time interval during the end of the Summer season. Our results show also that either the macroscopic or microscopic approach used to identify the different gonadal stages provide overlapping information. Despite both approaches are destructive (in both cases specimens need to be sacrificed), the one using macroscopic proxies is much faster than and similarly reliable to the one that uses microscopic proxies. Additionally, the macroscopic approach can be (more) easily used in the field, allowing even not-expert observers to expeditiously determine sex and maturity stage of natural populations of sea cucumbers.

Tab. 2.1: Color palette selected for sea cucumber's gonads and Pantone® codes are reported with the relative RGB coordinates.

ID color	Pantone® code	R	G	B
485	485 C	218	41	28
172	172 C	250	70	22
165	165 C	255	103	31
151	151 C	255	130	0
157	157 C	236	161	84
137	137 C	255	163	0
123	123 C	255	199	44
120	1205 C	248	224	142
461	461 C	233	223	151
454	4545 C	213	203	159

Tab. 2.2: Results of the univariate PERMANOVA carried out to test for differences in Wet Weight (g), Length (cm), and Width (cm) between Sites and among Seasons. DF = degrees of freedom; MS = mean square; Pseudo-F = F statistic; %EV = percentage of explained variance; P(MC) = probability level after Monte Carlo simulations. (***) = $P < 0.001$; (**) = $P < 0.01$; (*) = $P < 0.05$; ns = not significant.

Variable	Source	DF	MS	Pseudo-F	P(MC)	%EV
Wet Weight (g)	Site (Si)	1	0.027	0.032	ns	0
	Season (Se)	3	4.636	5.478	**	14
	Si×Se	3	2.916	3.445	*	15
	Residual	125				71
Length (cm)	Site (Si)	1	0.172	0.194	ns	0
	Season (Se)	3	4.215	4.753	**	12
	Si×Se	3	2.708	3.053	*	13
	Residual	125	0.887			74
Width (cm)	Site (Si)	1	2.574	3.122	ns	4
	Season (Se)	3	4.934	5.985	**	14
	Si×Se	3	3.019	3.662	*	15
	Residual	125	0.824			66

Tab. 2.3: Results of the pairwise tests contrasting Wet Weight (g), Length (cm) and Width (cm) between Site and Season. t = t value; P(MC) = probability level after Monte Carlo simulations. (***) = P < 0.001; (**) = P < 0.01; (*) = P < 0.05; ns = not significant.

Variable		Wet Weight (g)		Length (cm)		Width (cm)	
Site	Contrast	t	P (MC)	t	P (MC)	t	P (MC)
Oristano	Spring, Summer	1.935	ns	1.829	ns	0.252	ns
	Spring, Autumn	2.924	**	1.708	ns	2.020	ns
	Spring, Winter	3.425	**	3.410	**	3.238	**
	Summer, Autumn	2.277	*	0.919	ns	3.378	**
	Summer, Winter	3.250	***	3.722	**	5.099	***
	Autumn, Winter	3.036	**	4.171	**	2.348	*
Teulada	Spring, Summer	0.305	ns	0.018	ns	1.285	ns
	Spring, Autumn	0.166	ns	0.103	ns	0.096	ns
	Spring, Winter	1.027	ns	0.721	ns	0.180	ns
	Summer, Autumn	0.085	ns	0.124	ns	1.156	ns
	Summer, Winter	1.350	ns	1.125	ns	0.929	ns
	Autumn, Winter	1.288	ns	0.736	ns	0.321	ns

Tab. 2.4: Results of the univariate PERMANOVA carried out to test for differences in Gonad Weight (g) and GSI between Site and among Seasons. DF = degrees of freedom; MS = mean square; Pseudo-F = F statistic; %EV = percentage of explained variance; P(MC) = probability level after Monte Carlo simulations. (***) = $P < 0.001$; (**) = $P < 0.01$; (*) = $P < 0.05$; ns = not significant.

Variable	Source	DF	MS	Pseudo-F	P(MC)	%EV
Gonad Weight (g)	Site (Si)	1	0.408	0.514	ns	0
	Season (Se)	3	6.841	8.607	***	25
	Si×Se	3	0.236	0.297	ns	0
	Residual	125	0.795			75
GSI	Site (Si)	1	2.175	0.053	ns	0
	Season (Se)	3	1104.9	27.064	***	53
	Si×Se	3	18.244	0.447	ns	0
	Residual	125	40.826			47

Tab. 2.5: Results of the pairwise tests for Gonad Weight (g) and GSI among seasons in each Site. t = t value, P(MC) = probability level after Monte Carlo simulations; (***) = $P < 0.001$; (**) = $P < 0.01$; (*) = $P < 0.05$; ns = not significant.

Variable		Gonad Weight (g)		GSI (%)	
Site	Contrast	t	P(MC)	t	P(MC)
Oristano	Spring, Summer	1.473	ns	4.571	***
	Spring, Autumn	1.688	ns	2.318	*
	Spring, Winter	1.416	ns	2.634	*
	Summer, Autumn	4.118	***	6.170	***
	Summer, Winter	3.435	***	5.780	***
	Autumn, Winter	2.659	*	3.580	**
Teulada	Spring, Summer	1.876	ns	2.429	*
	Spring, Autumn	1.865	ns	2.236	*
	Spring, Winter	1.867	ns	2.292	*
	Summer, Autumn	1.861	ns	3.932	**
	Summer, Winter	1.862	ns	3.950	**
	Autumn, Winter	0.042	ns	0.261	ns

Tab. 2.6: Results of the univariate and multivariate PERMANOVA carried out to test for differences in the L*, a*, and b* values between different maturity stages (Growing, Mature) and Sex (Female; Male). DF=degrees of freedom; MS=mean square; Pseudo-F = F statistic; %EV = percentage of explained variance; P(MC) = probability level after Monte Carlo simulations; (***) = P < 0.001; (**) = P < 0.01; (*) = P < 0.05; ns = not significant.

Variable	Source	DF	MS	Pseudo-F	P(MC)	%EV
L*	Maturity stage (Ma)	1	0.181	1.007	ns	0
	Sex (Se)	1	42.799	238.710	***	89
	Ma×Se	1	0.281	1.568	ns	0
	Residual	78				11
a*	Maturity stage (Ma)	1	6.241	16.908	***	15
	Sex (Se)	1	24.160	65.454	***	59
	Ma×Se	1	0.229	0.619	ns	0
	Residual	78				27
b*	Maturity stage (Ma)	1	1.656	2.342	ns	3
	Sex (Se)	1	15.173	21.461	***	40
	Ma×Se	1	0.863	1.221	ns	1
	Residual	78	0.707			57

Tab. 2.7: Results of the univariate PERMANOVA carried out to test for differences in the Oocyte diameter among Seasons. DF = degrees of freedom; MS = mean square; Pseudo-F = F statistic; %EV = percentage of explained variance; P(MC) = probability level after Monte Carlo simulations; (***) = $P < 0.001$.

Source	DF	MS	Pseudo-F	P(MC)	%EV
Season (Se)	3	218.72	435.230	***	61
Residual	1313	0.503			39

Tab. 2.8: Results of the pairwise tests contrasting the Oocyte diameter (μm) between Seasons. t = t value, P(MC) = probability level after Monte Carlo simulations; (***) = $P < 0.001$; (**) = $P < 0.01$; (*) = $P < 0.05$; ns = not significant.

Contrast	t	P(MC)
Autumn, Spring	6.2844	***
Autumn, Summer	16.075	***
Autumn, Winter	4.150	***
Spring, Summer	14.842	***
Spring, Winter	21.848	***
Summer, Winter	53.083	***

Tab. 2.9: Results of the univariate PERMANOVA carried out to test for differences in the oocyte diameter (μm) among Seasons and the Oocyte position in the gonad (External, Intermediate, Internal). DF = degrees of freedom; MS = mean square; Pseudo-F = F statistic; %EV = percentage of explained variation; P(MC) = probability level after Monte Carlo simulations; (***) = $P < 0.001$; (**) = $P < 0.01$; (*) = $P < 0.05$; ns = not significant.

Variable	Source	DF	MS	Pseudo-F	P(MC)	%EV
Oocyte diameter	Season (Se)	3	170.79	428.180	***	51
	Oocyte position (OoP)	2	29.894	74.946	***	19
	Se×OoP	6	4.677	11.725	***	4
	Residual	1305	0.398			27

Tab. 2.10: Results of the two pairwise tests contrasting the oocyte diameter (μm) between Seasons and Oocyte position (External, Intermediate, Internal). t = t value; P(MC) = probability level after Monte Carlo simulations; (***) = $P < 0.001$; (**) = $P < 0.01$; (*) = $P < 0.05$; ns = not significant.

A	Season	Contrast	t	P(MC)
Winter		External, Intermediate	0.931	ns
		External, Internal	2.149	*
		Intermediate, Internal	1.623	ns
Spring		External, Intermediate	7.767	***
		External, Internal	8.894	***
		Intermediate, Internal	0.923	ns
Summer		External, Intermediate	6.725	***
		External, Internal	14.036	***
		Intermediate, Internal	1.293	ns
Autumn		External, Intermediate	0.279	ns
		External, Internal	7.122	***
		Intermediate, Internal	5.848	***

B	Oocyte position	Contrast	t	P(MC)
External		Autumn, Spring	6.929	***
		Autumn, Summer	11.840	***
		Autumn, Winter	0.939	ns
		Spring, Summer	7.989	***
		Spring, Winter	8.708	***
		Summer, Winter	16.050	***
Intermediate		Autumn, Spring	5.098	***
		Autumn, Summer	11.548	***
		Autumn, Winter	2.306	*
		Spring, Summer	5.124	***
		Spring, Winter	17.050	***
		Summer, Winter	36.429	***
Internal		Autumn, Spring	0.592	ns
		Autumn, Summer	2.708	*
		Autumn, Winter	15.195	***
		Spring, Summer	8.644	***
		Spring, Winter	17.150	***
		Summer, Winter	58.926	***

1

Tab. 2.11: Summary of the macroscopic and microscopic features of *H. tubulosa* gonads in different stage of development.

Stage	Season	Macroscopic characteristics						Microscopic characteristics				
		Sex	Gonad weight (g)	GSI	Branches ramification	Observed color	ID Color	SEX	Gonad Walls	Description	Gonad lumen	Mean oocytes diameter
Stage 1: Recovery	Autumn - Winter	ND	< 1	< 1	ND	Pale and transparent	454 461	F	Thickened wall	Germinal layer with previtellogenic and early vitellogenic oocytes.	Mainly empty	35 ± 11.9 µm
								M	Thickened wall	Germinal layer with numerous folds and active spermatogenesis.	Mainly empty	
Stage 2: Growing	Spring - Summer	F	2 - 25	1 - 5	2 - 3	Translucent red-coral red	151 157	F	Less thickened wall	Oocytes have different levels of vitellogenesis, mature oocyte show the nucleus and follicular cells.	Progressively filled with mature oocytes	71 ± 35.0 µm
		M						Less thickened wall	Spermatogenesis is highly active. Numerous folds of the germinal epithelium are still present.	Progressively filled with spermatozoa		
Stage 3: Mature	Summer	F	20 - 150	3 - 5	3 - 5	Translucent red-coral red	172 485	F	Thin wall	In the germinal layer oocytes are in mid vitellogenesis. Mature oocytes are the most abundant.	Completely filled with mature oocytes	110 ± 20.3 µm
		M						Thin wall	The folds of the germinal epithelium are reduced and less visible.	Completely filled with spermatozoa		
Stage 4: Partly spawned	Summer - Autumn	F	2 - 25	1 - 15	3 - 4	Matt red-coral red	157	F	Very thin wall	Oocytes are in the gonad lumen only. Oocytes show signs of degradation.	Empty space and relict oocytes are visible	109 ± 28.4 µm
		M						Very thin wall	After the partial spawn of the gametes, the density of the spermatozoa decreases.	Empty areas are visible after the release of the gametes		
Stage 5: Spent	Autumn - Winter	ND	< 2	< 1	ND	Different colors and rust blotches	ND	ND	Very thick	No gamete is observed, and abundant connective tissue is visible.	Empty	

2

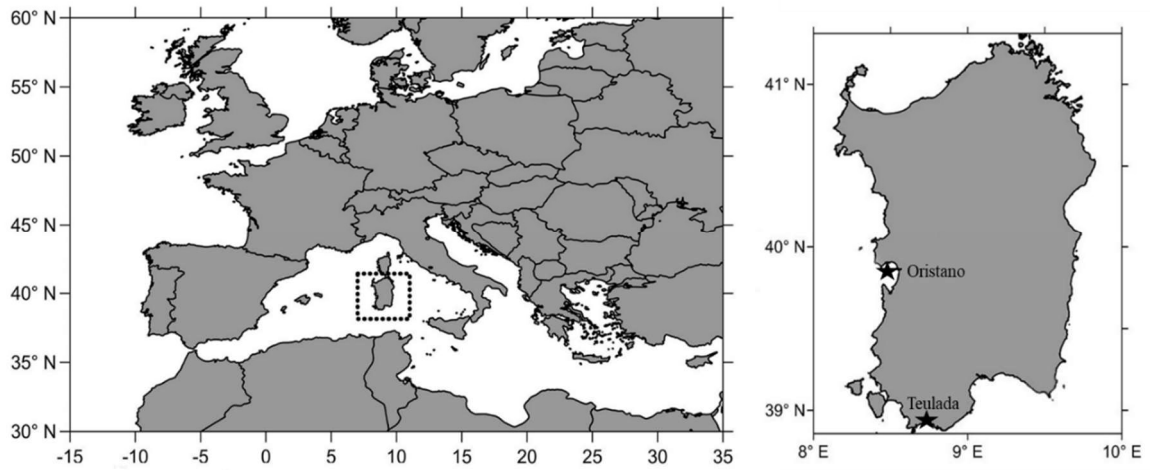


Fig. 2.1: Sampled sites of Oristano Gulf and Teulada Gulf.

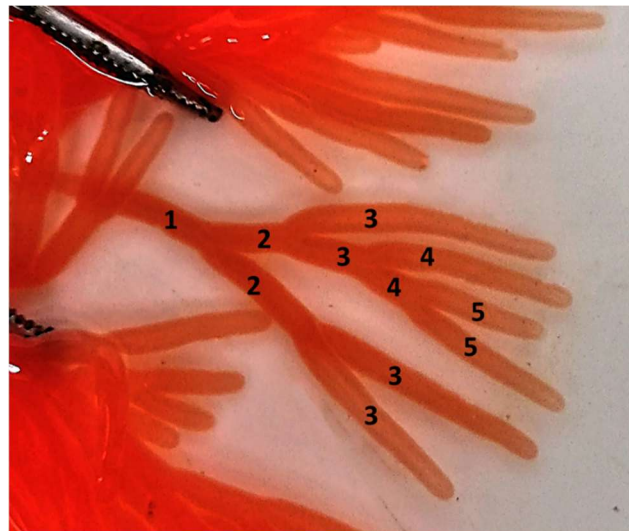


Fig. 2.2: Example of branches ramification in the gonad's tubule of a *H. tubulosa* female. In this case the maturation stage, associated to the 5th ramification, was the highest possible.

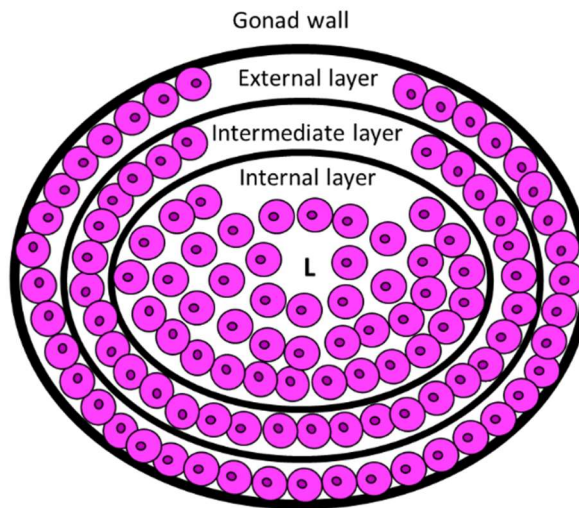


Fig. 2.3: Schematic representation of the oocyte's stratification inside the gonadal tubule. Illustrated are: the external layer of oocytes, the intermediate layer of oocytes and the internal layer. L = lumen of the gonads.

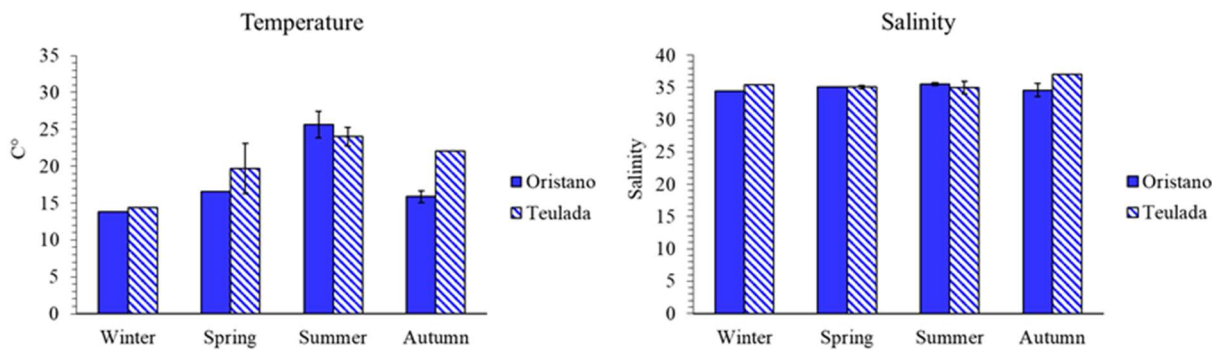


Fig. 2.4: Seasonal variation of temperature (°C) and salinity in Oristano and Teulada.

Holothuria tubulosa body size and weight

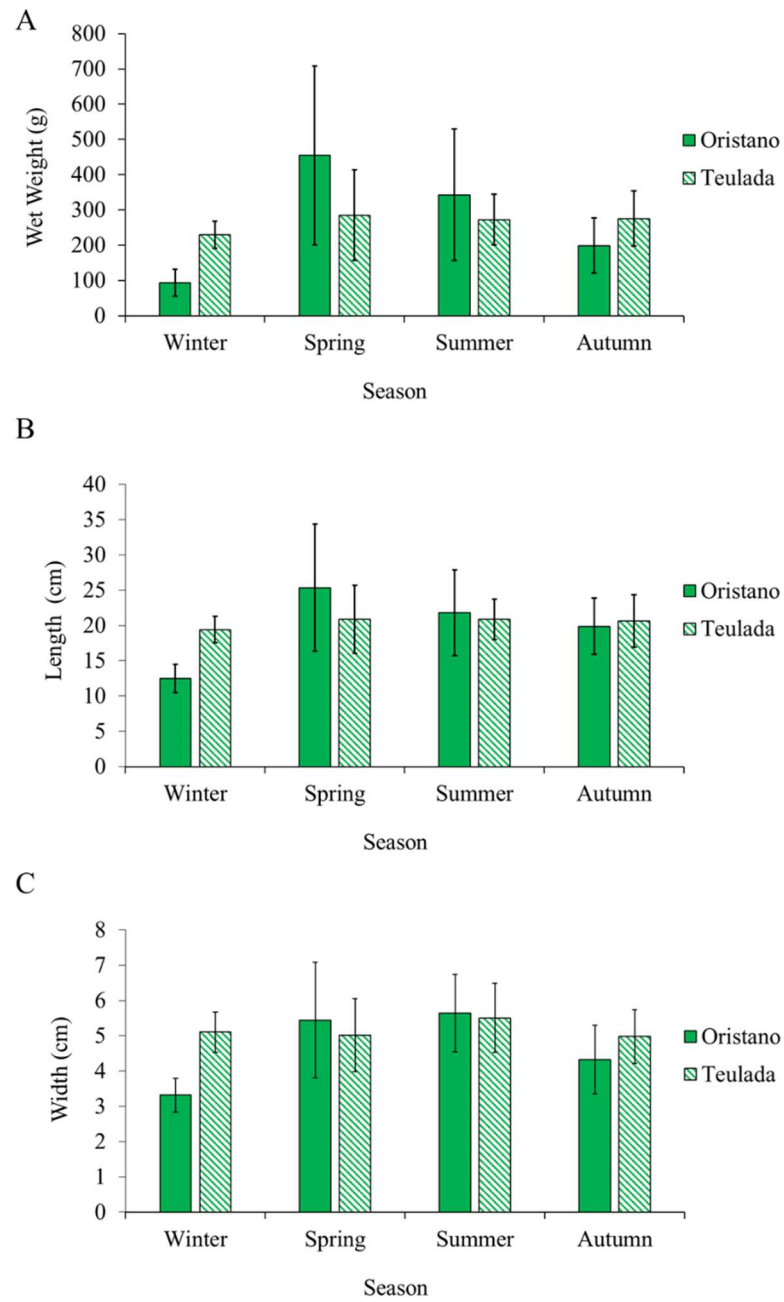


Fig. 2.5: Wet Weight (A), Length (B) and Width (C) of the sampled specimens collected in different seasons in the sites of Oristano and Teulada. The error bars indicate the standard deviation.

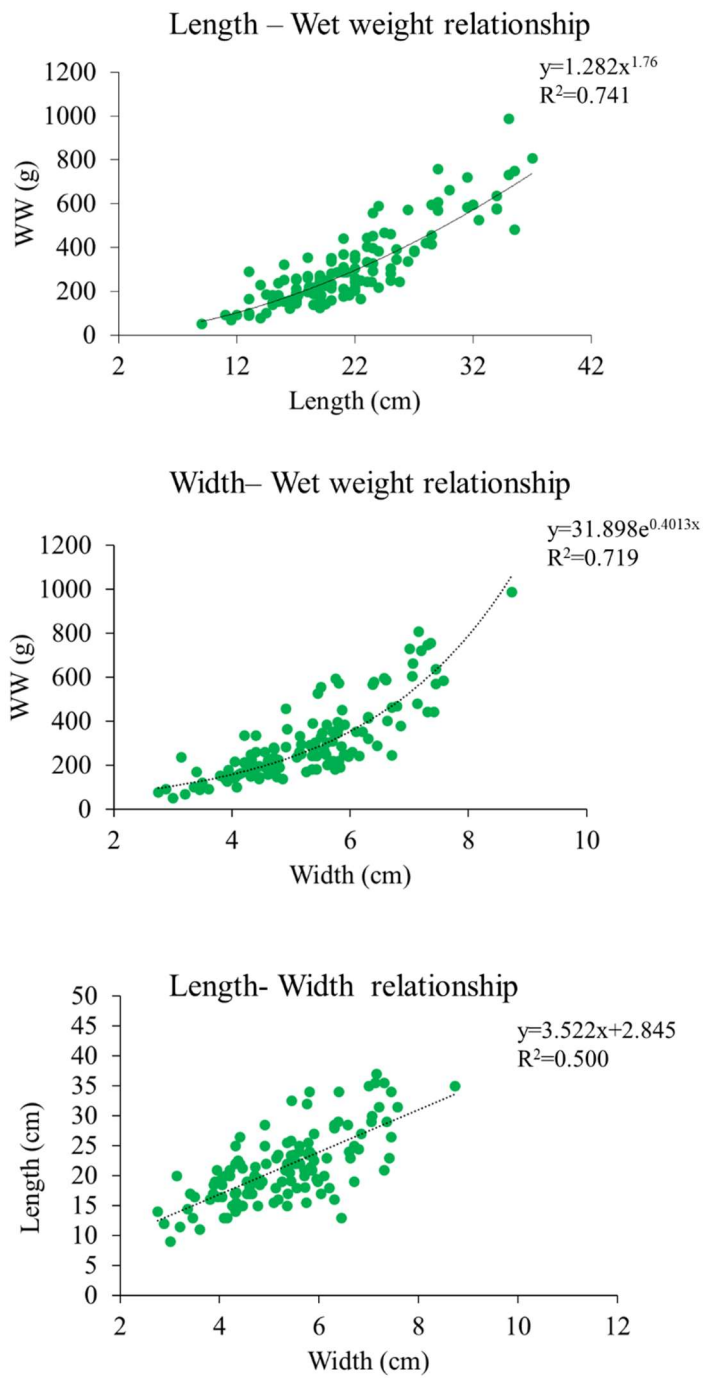


Fig. 2.6: Length – Wet Weight (A); Width – Wet Weight (B) and Length- Width (C) relationships. (WW = Wet Weight).

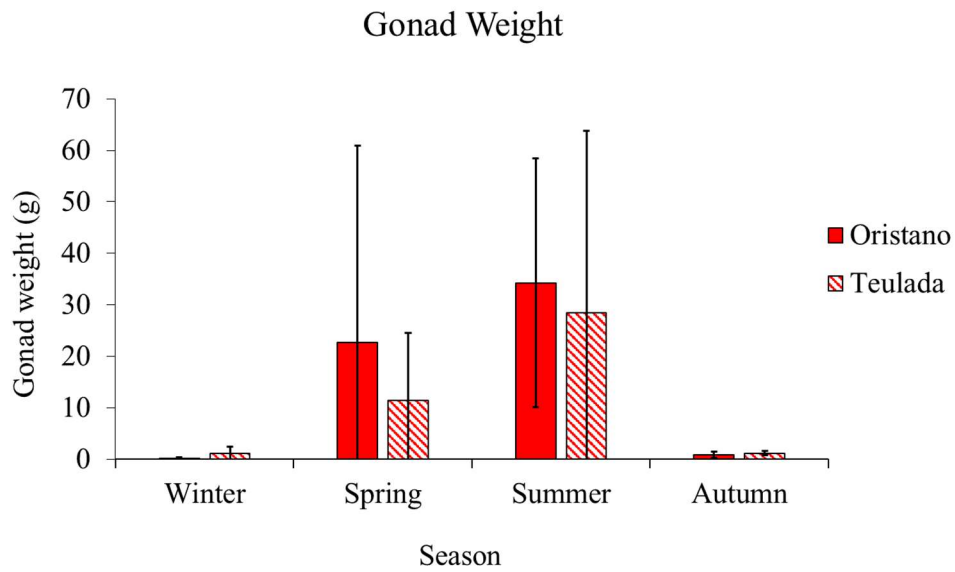


Fig. 2.7: Seasonal changes in Gonad Weight in the Oristano and Teulada gulfs. Error bars indicate the standard deviation.

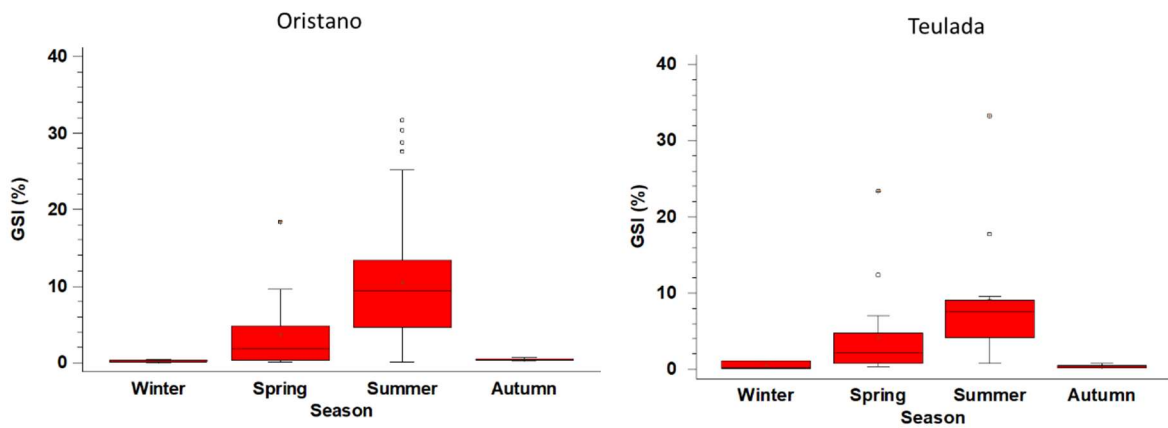


Fig. 2.8: Seasonal changes in the gonado-somatic index (GSI) of *H. tubulosa* in Oristano and Teulada gulfs. The boxes show the interquartile range, with the median value indicated by the horizontal line and the '+' sign indicating the mean; whiskers show the range. Individual symbols show outliers.

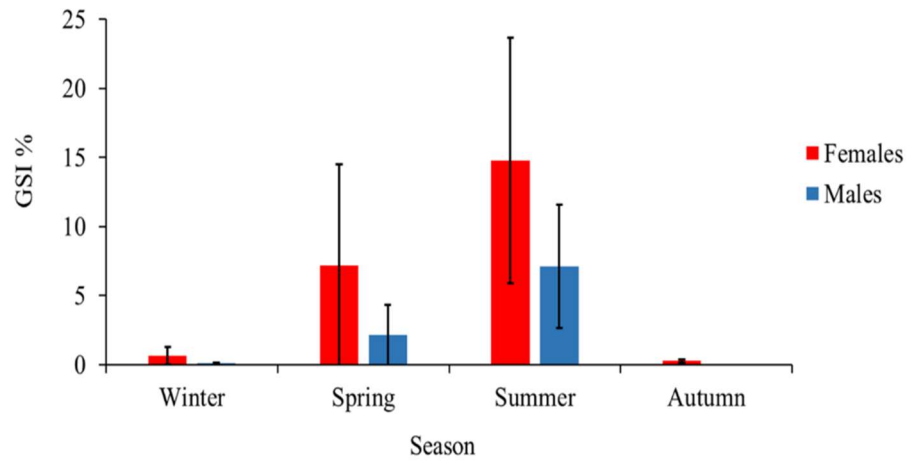


Fig. 2.9: GSI of females and males *H. tubulosa* in different seasons. The error bars indicate the standard deviation. Males were not present in the Autumn samples.

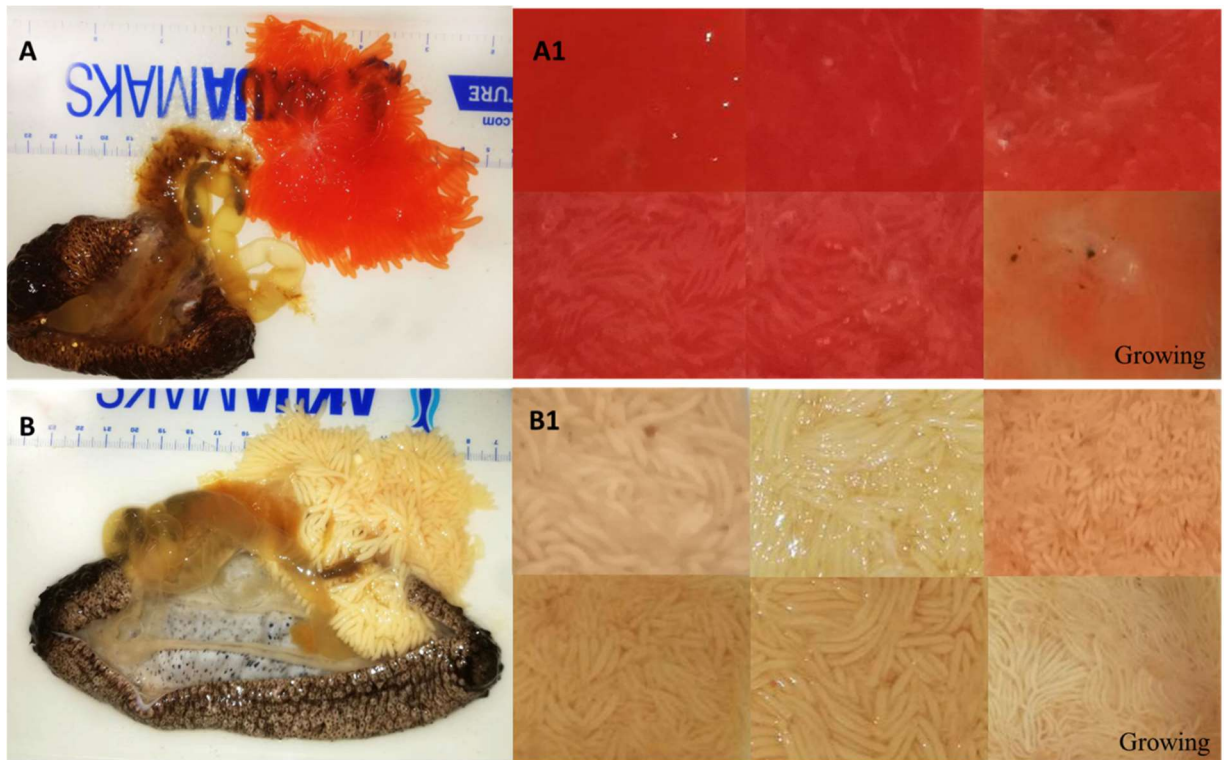
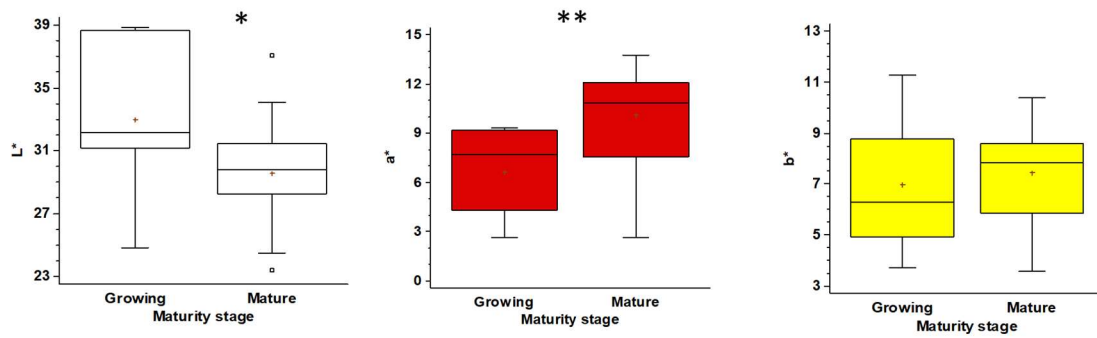


Fig. 2.10: Examples of female (A, A1) and male (B, B1) sea cucumbers gonads in growing (bottom left right) and mature stages.

Females



Males

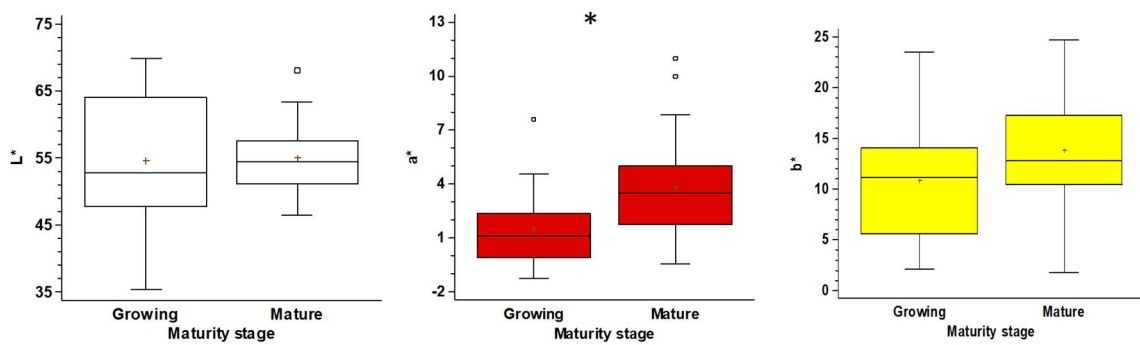


Fig. 2.11: Boxplot of the color space of the gonads of *H. tubulosa*, for female and male at different maturity stages. The boxes show the interquartile range, with the median value indicated by the horizontal line and the '+' sign indicating the mean; whiskers show the range. Individual symbols show outliers. The asterisk indicate the results of the probability level after Monte Carlo simulations in the pairwise comparison of the $L^*a^*b^*$ value between Maturity stages; (**) = $P < 0.01$; (*) = $P < 0.05$.

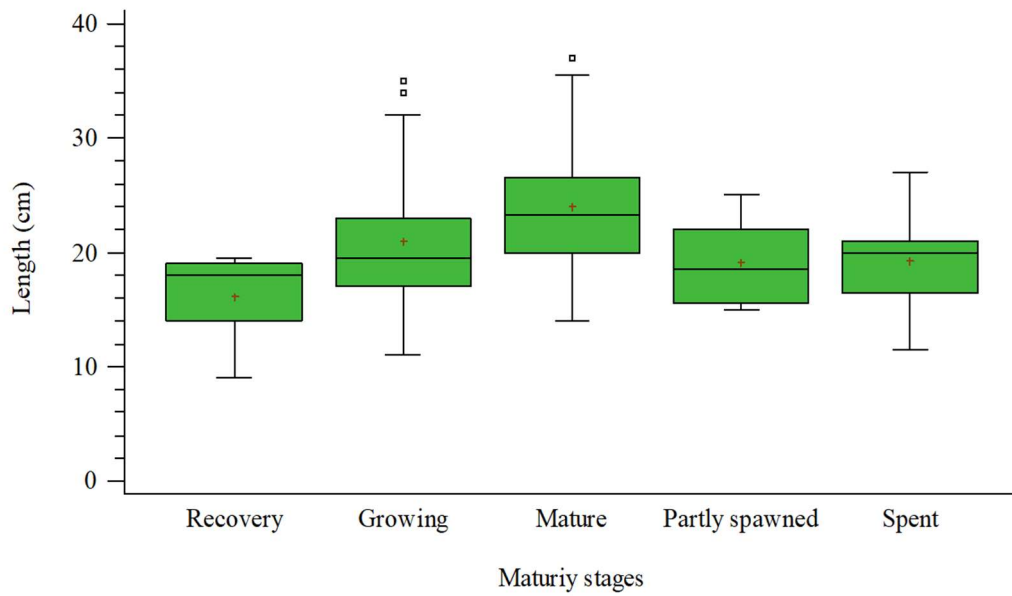


Fig. 2.12: Length of sea cucumbers at different maturity stages. The boxes show the interquartile range, with the median value indicated by the horizontal line and the '+' sign indicating the mean; whiskers show the range. Individual symbols show outliers.

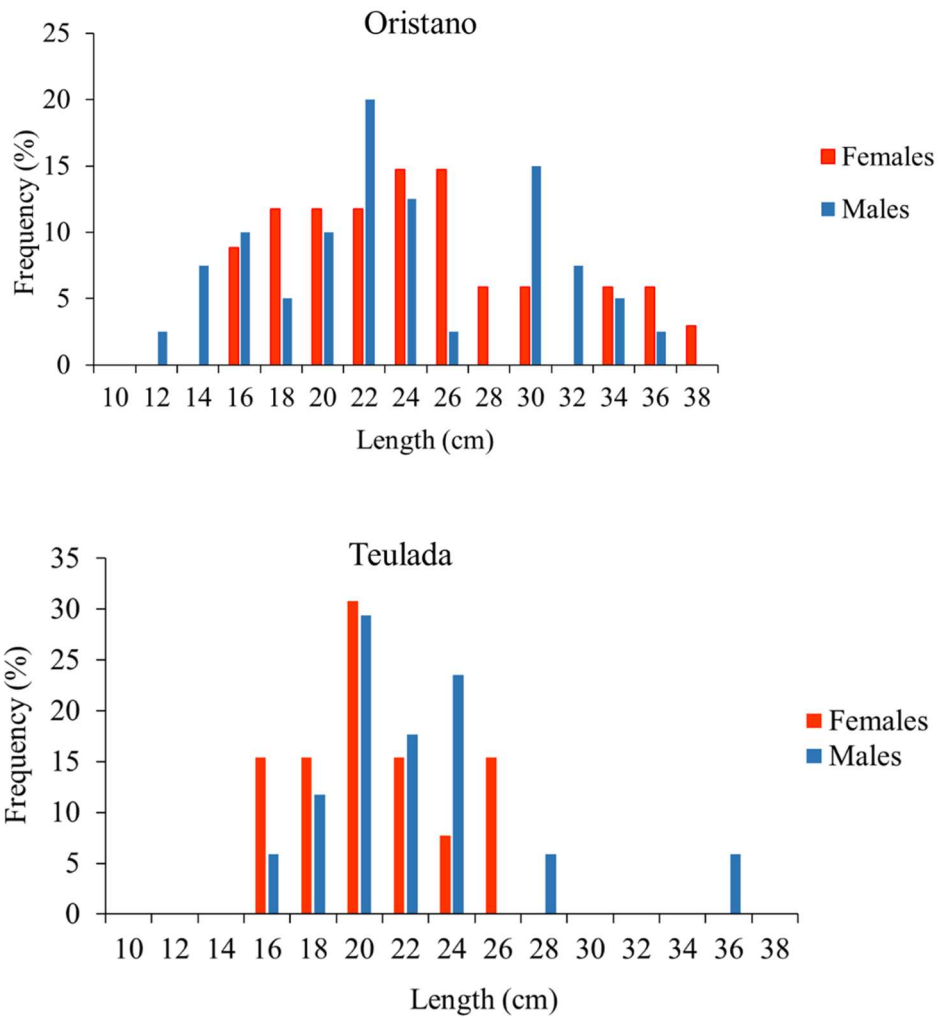


Fig. 2.13: Length – frequency distribution of the different size classes of females and males in the Oristano and Teulada gulfs.

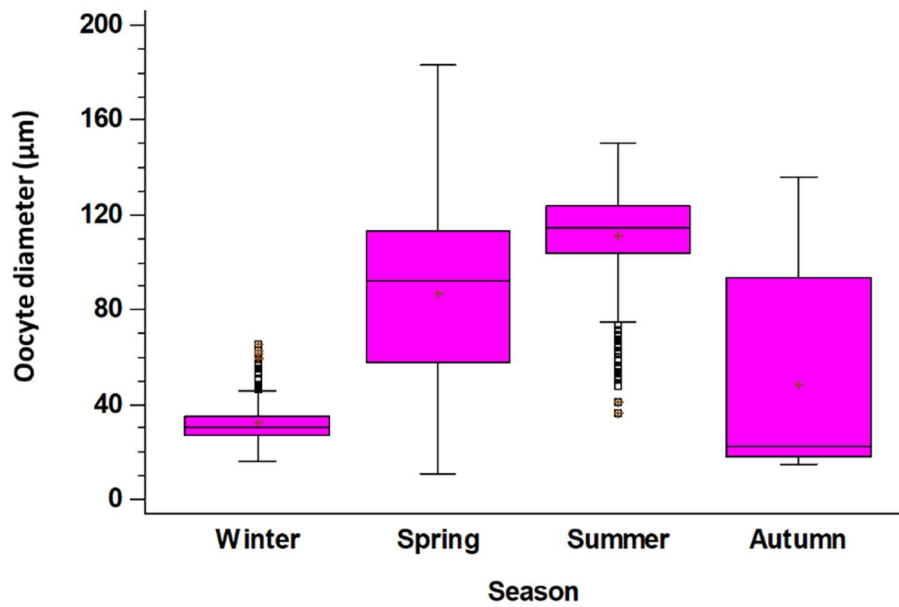


Fig. 2.14: Oocyte diameter in the different seasons. The boxes show the interquartile range, with the median value indicated by the horizontal line and the '+' sign indicating the mean; whiskers show the range. Individual symbols show outliers.

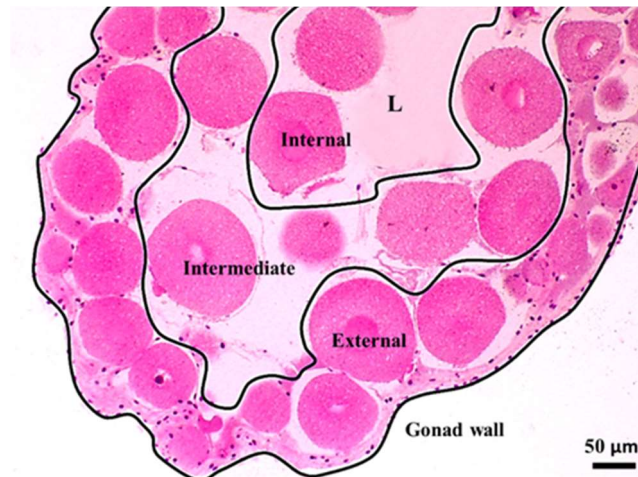


Fig. 2.15: Example of the division of the gonad's tubule in different layers. L = lumen.

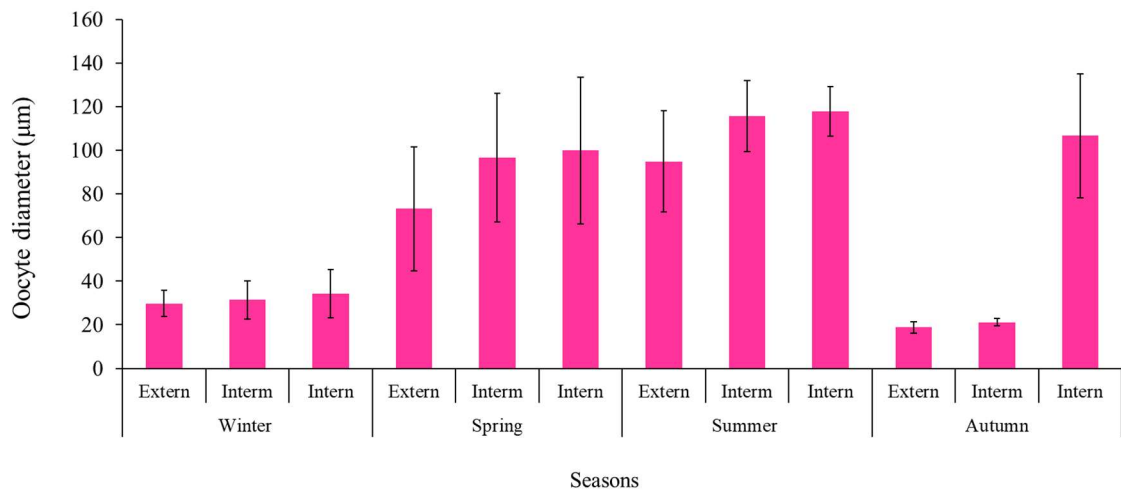


Fig. 2.16: Oocyte diameter in different gonad layers (External, Intermediate, Internal) in different seasons. Bars indicate the standard deviation.

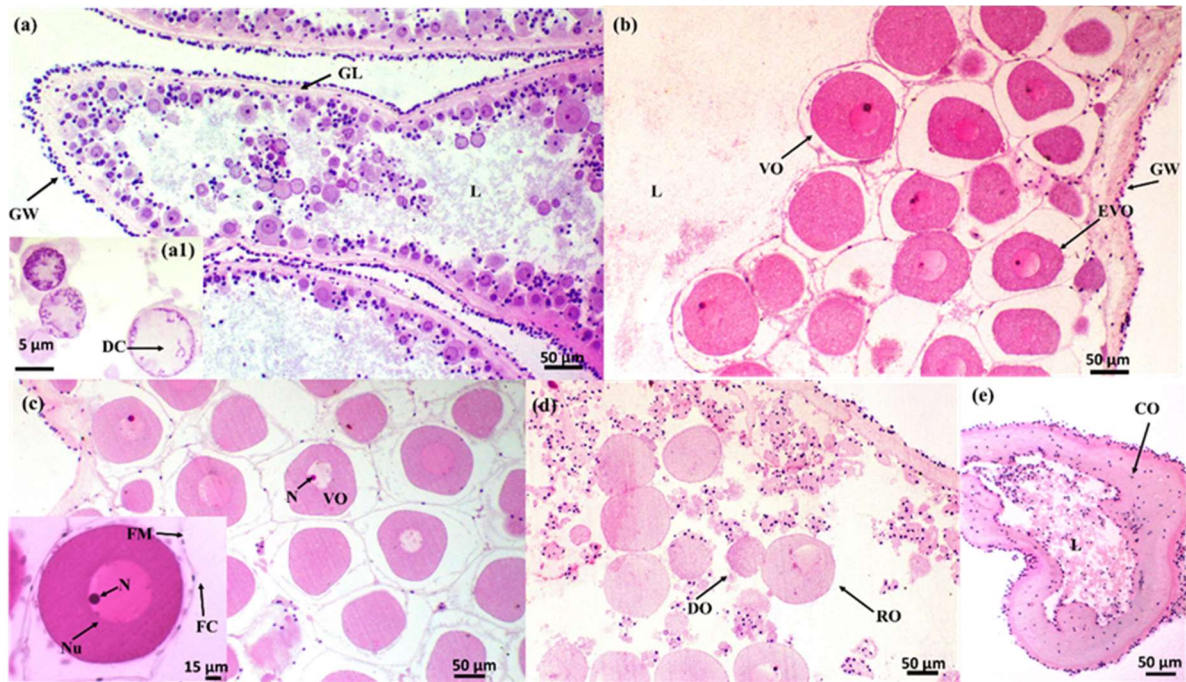


Fig. 2.17: Microscopic characteristics of *H. tubulosa* female gonads. a (Recovery Stage), b (Growing stage), c (Mature stage), d (Partly spawned stage), e (Spent stage). GW = gonad wall; EV = early vitellogenic oocytes; L = lumen of the gonad; GL = germinal layer; DC = diplotene chromosomes; EVO = early vitellogenic oocyte; VO = vitellogenic oocyte; N = nucleolus; Nu = nucleus; DO = degraded oocyte; RO = relict oocyte; CO = connective tissue.

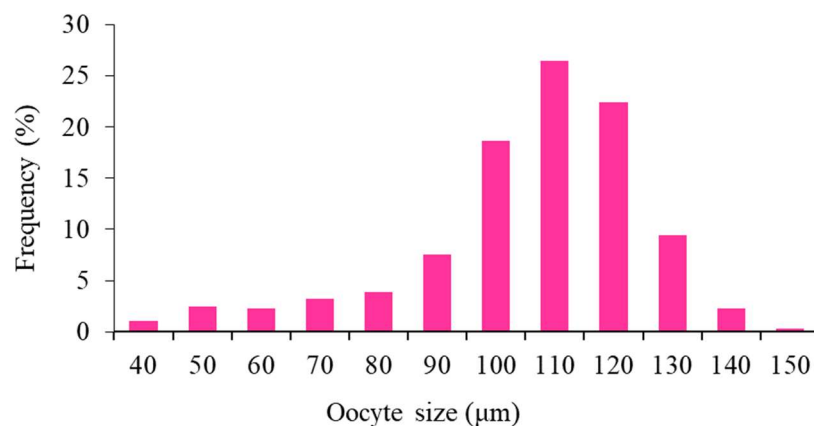


Fig. 2.18: Oocyte distribution in Mature females' gonads. The size of the oocyte presented a unimodal distribution, with a peak at 110 µm.

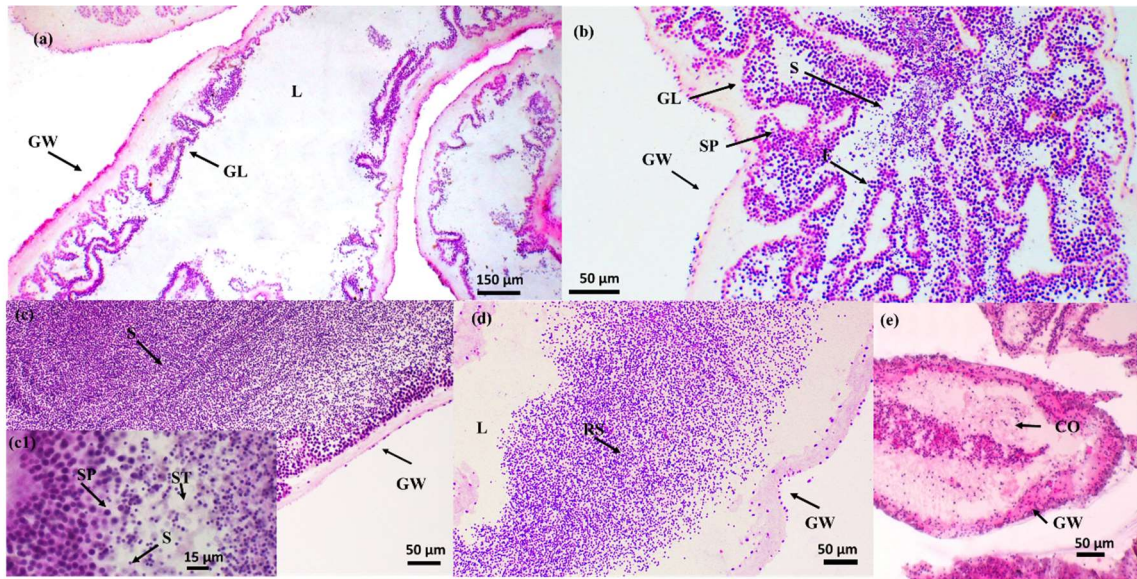


Fig. 2.19: Microscopical characteristics of *H. tubulosa* male gonads. a (Recovery Stage), b (Growing stage), c (Mature stage), d (Partly spawned stage), e (Spent stage). GW = gonad wall; GL = germinal layer; L = lumen; F = folds; S = spermatozoa; SP = spermatocytes; ST = spermatozoa tails; RS = residual spermatozoa; CO = connective tissue.

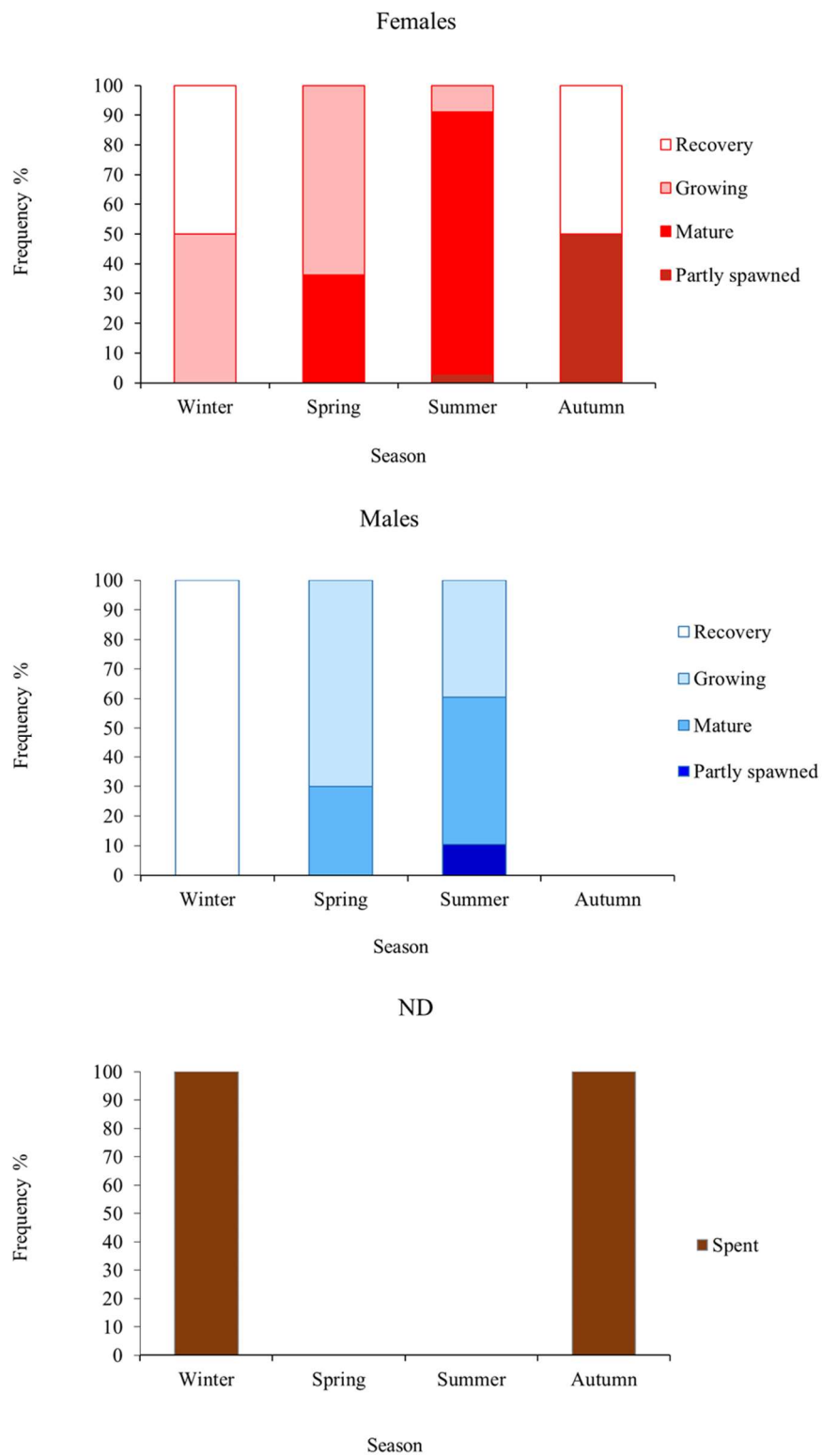


Fig. 2.20: Seasonal distribution of *H. tubulosa* females, males and unassigned specimens (ND) at each gonadal stages during the sampling seasons.

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Chapter 3

Bubbling as a trigger to spawning in *Holothuria tubulosa* (Gmelin, 1788)

3.1 Introduction

Sea cucumbers (Phylum Echinodermata) are common benthic marine invertebrates represented by more than 1500 species spread worldwide (Horton *et al.*, 2018). Deposit feeder sea cucumbers, by ingesting large amount of sediments, are one of the strongest bioturbators of marine seafloors, plying a putative key ecological role in sedimentary nutrient cycling and in benthic trophodynamics (Roberts *et al.*, 2000; Uthicke, 2001; Mangion *et al.*, 2004; Amaro *et al.*, 2010, Neofitou *et al.*, 2019).

Sea cucumbers are also an important food source, which for centuries have been harvested in Asian countries and are currently sold in the market as luxury seafood dried products, called *trepang* or *bêche-de-mer* (Conand, 2001; Ferdouse, 2004). This product can reach high price according to the sea cucumber species and quality of the processed product, for example up to 2950 US\$ dried kg⁻¹ for high-quality *Apostichopus japonicus* (Purcell *et al.*, 2012; Purcell, 2014). Moreover, due to the presence of many high-value bioactive compounds, sea cucumbers are exploited for their extracts, which are used in the pharmaceutical and cosmetics industry, as well as in Chinese traditional medicine (Fredalina *et al.*, 1999; Zhao *et al.*, 2007; Bordbar *et al.*, 2011; Purcell, 2014). The high market value and the increasing demand, have led to the overexploitation of most valuable sea cucumber species, at the point that in some cases the populations were not able to recover with self-recruitment (Hasan, 2005; Friedman *et al.*, 2011; Ramírez-González *et al.*, 2020). Consequently, the sea cucumbers fishery shifted to new target species, including those in the Mediterranean Sea, where it already provoked negative effects on wild populations (González-Wangüemert *et al.*, 2018).

The species we focused on is *Holothuria tubulosa* Gmelin 1788, one of the most commercially exploited Mediterranean sea cucumber (Sicuro and Levine, 2011; González-Wangüemert *et al.*, 2015, 2018; Dereli and Aydin, 2021). This species is a

gonochoric and broadcast spawner that lacks in sexual dimorphism (Despalatović *et al.*, 2004).

In order to meet the product demand with the reduction of the fishing pressure, the aquaculture of sea cucumbers has been recently investigated. The technologies for commercial-scale aquaculture have been provided for the most valuable historically exploited species, including *Holothuria scabra*, *A. japonicus* and *Isostichopus fuscus* (Chang *et al.*, 2004; Gamboa *et al.*, 2004; Mercier *et al.*, 2004; Agudo, 2006; Ivy and Giraspy, 2006; Raison, 2008; Purcell *et al.*, 2012; Tuwo and Tresnati, 2015). Experimental attempts to breeding Mediterranean sea cucumbers, were recently reported by different authors (Domínguez-Godino *et al.*, 2015; Domínguez-Godino and González-Wangüemert, 2018; Tolon *et al.*, 2017; Rakaj *et al.*, 2018, 2019; Laguerre *et al.*, 2020). However, the adaptation of the technologies and the breeding protocols used with tropical species to the Mediterranean ones presents some criticisms, including the low effectiveness of the stimuli used to collect the gametes. In such protocols, the trigger most used to achieve the spawning of sea cucumbers, is the thermal stress, alone or combined with mechanical stressors such as air exposure (Battaglene *et al.*, 2002; Hu *et al.*, 2010, 2013; Dabbagh *et al.*, 2011; Dominguez-Godino *et al.*, 2015, 2018; Günay *et al.*, 2018; Rakaj *et al.*, 2018, 2019; Laguerre *et al.*, 2020; Schagerström *et al.*, 2021). Other effective triggers include conspecific spermatozoa and administration of high quantity of phytoplankton or supplementary feeding powder (Agudo *et al.*, 2006; Hu *et al.*, 2013; Gianasi *et al.*, 2019). One of the aspects that can influence a hatchery production, is the densities of the eggs and the larvae in the rearing tanks. In particular, the hatching rate at different densities can affect the survival of the embryos and the final productivity of the hatchery, thus, is considered one of the most important criteria for the reproduction of sea cucumbers (Liu *et al.*, 2010; Asha and Diwakar, 2013; Ivy and Giraspy, 2006; Günay *et al.*, 2018). Despite this, such approach is poorly explored in the breeding studies of *H. tubulosa* breeding.

In order to better understand these mechanisms, the aims of this study are, firstly, to explore the effect of different triggers on the spawning of *H. tubulosa* broodstock, and secondly, to investigate the effect of different stocking densities on the hatching rate of fertilized eggs.

3.2 Material and methods

3.2.1 Collection of broodstock and acclimation

A total of 200 adult specimens (mean weight: 207.03 ± 5.95 ; mean \pm se) were collected by divers (5 – 10 depth) from the Gulf of Teulada (Italy, Sardinian Sea, $38^{\circ}55'42''$ N; $8^{\circ}43'9''$ E) in August 2020. This sampling period corresponds to the reproductive phase of *H. tubulosa* as reported in previous studies (Despalatović, 2004; Dereli *et al.*, 2016, see also Chapter 2). However, to confirm the maturity stage of the collected broodstock, 20 extra specimens were sampled and dissected in the same occasion, and the maturity stage was verified by the gonads' color, gonads' morphology and the gonado-somatic index (GSI) (Chapter 2):

$$\text{GSI} = (\text{Gonad Weight} / \text{Wet Weight}) \times 100$$

After collection, each broodstock was placed in a plastic bag containing 3 L of seawater and transported to the experimental hatchery of the University of Cagliari (Italy) in cooling boxes (at *in situ* temperature) to avoid thermal stress, delivery time to the hatchery was within 1.5 h. Once in the hatchery specimens were divided into four acclimation tanks (350L), supplied with air and running seawater, previously sand-filtered, skimmed and treated with ozone, at a reference temperature of 25°C, with natural light regime, for 48h prior to the experiment.

3.2.2 Spawning trials

Each treatment was replicated in 6 independent tanks (15-L), containing 3 sea cucumbers each. Having *H. tubulosa* no sexual dimorphism the broodstock were divided randomly in the tanks. To trigger the gametes emission from broodstock, a set of stimuli already used for *H. tubulosa* or other sea cucumbers species or marine invertebrates was considered. The stimuli used included: thermal stressors (alone or combined with air exposure), the addition of conspecific sperms or feeding excess and the bubbling (Agudo 2006; Dominguez-Godino *et al.*, 2015; Ferranti *et al.*, 2018; Rakaj *et al.*, 2018, 2019; Gianasi *et al.*, 2019). Unless otherwise specified, the water temperature in the treatment tanks was set at a reference value of 25° C, the same temperature as the acclimation tanks. In details the triggers tested were:

TS: Thermal shock. Broodstock were transferred from the acclimation tank to the treatment tank with a water temperature of 28-30°C. After 180 min of exposure, the temperature was re-established at the reference value of 25°C.

DTS: Dry + thermal shock (heat - cold). Specimens were kept out of the water and exposed to air for 30 min, then they were moved in the tanks and exposed to the water temperature of 28–30°C over 75 min. After the heat exposure, specimens were exposed to cooler water at 21°C. After 75 min of exposure to cool water, the temperature was re-established at the reference value of 25°C.

CP: Conspecific sperms. *H. tubulosa* sperms were collected after dissection of three males. The content of the gonads was cut, mixed, filtered, equally divided and introduced in the six treatment tanks.

FA: Feeding excess (*Amphora spp.*). 1L of *Amphora spp.* culture in exponent growth was added in each treatment tank.

FP: Feeding excess (feeding powder). 0.1g/L of commercial supplement feeding powder (EASY DRY SELCO®) was added to the treatment tanks.

BU: Bubbling. The water in the treatment tanks was vigorously bubbled, using standard aquarium air stones positioned on the bottom of the tanks (*sensu* Ferranti *et al.*, 2018).

CTRL: Control (no stimuli). The specimens were placed in the treatment tanks in static water at the reference temperature of 25°C and no stimuli were added.

The triggers used are summarized in Tab. 3.1. The experiments started at 9 AM, all the treatments lasted 180 minutes. At the end of the exposure, specimens were moved in the spawning tanks (one for each replica), filled with seawater (25°C). During the treatments, and the next 9 h (total of 12 h from the beginning of the trials), the behavior of sea cucumbers was observed to detect whether they assumed the pre-spawning position and/or spawned. When spawning occurred, the spawners were isolated in separate tanks.

3.2.3 Collection of gametes, fertilization and hatching rate

To perform the hatching rate trials, among the specimens triggered with the same stimulus (only the stimulus bubbling lead to the emission of enough specimens), the gametes of three males and three females were collected. Eggs were siphoned from the spawning

tanks, gently mixed and divided into three tanks containing 10 L seawater, and successively fertilized with 3 ml of the water from the males' spawning tanks. One hour later, time that is considered enough to ensure fertilization (Domínguez-Godino and González-Wangüemert, 2018), the number of fertilized eggs was estimated volumetrically by collecting 1 ml subsample (n=3). The fertilized eggs were counted under microscope and the occurrence of fertilization was confirmed by the presence of the fertilization envelope in the eggs (Rakaj *et al.*, 2018).

The hatching rate trials were conducted considering the following densities: 4, 8, 25, 50, 100, 200 eggs ml⁻¹. After count, eggs were placed in the trial tanks (2L, three replicates), according to the different final densities, and were raised to volume with seawater (previously filtered at 5µm and UV sterilized). The embryos were kept in the dark, with static water at 24°C for 24h. After 24h, the hatching rate was volumetrically estimated by collecting 1 ml subsample (n=3) from each replicate and the number of swimming larvae in the gastrula stage was counted. The hatching rate was estimated for each batch as the proportion of swimming larvae (counted volumetrically) over the number of incubated eggs expressed in percentage (Carboni *et al.*, 2013).

3.2.4 Statistical analysis

One-way analysis of variance was performed using a modelled set of data. Data of the spawning trials (average number of spawners expressed in percentage) and of the hatching rate trials (number of hatched eggs expressed in percentage) were arcsin transformed ($x' = \arcsin\sqrt{x}$). Prior to performing the ANOVA, we tested for homogeneity of variance with Cochran's test. The *post-hoc* Tukey HSD test was applied to test significant effects between each treatment and the Control and/or among treatments ($P < 0.05$). All the statistical analyses were conducted with the software STATGRAPHICS PLUS 5.1 professional edition (Statistical Graphics Corp., Rockville, MD, USA).

3.3 Results

3.3.1 Spawning trials

All the specimens used in the experiment showed no signs of stress (e.g. evisceration) and no mortality occurred. The triggered specimens showed the pre-spawning behavior,

followed by the emission of the gametes. In the present study, the achievement of such position by *H. tubulosa* was followed by the oscillation of the head, the simultaneous contraction of the specimens' body and the emission of the gametes (Fig. 3.1). Such behavior was more pronounced in females than males. During the pre-spawning, was also possible to observe the bulge of the gonopore and the changing of its color, from brown to pale orange in both males and females. Shortly after, the release of gametes occurred and took place with several vigorous jets in females with about 5-10 minutes intervals. Less vigorous and longer-lasting jets were observed in males. Statistical analysis showed significant differences among treatments (Tables 3.2; 3.3). The specimens in the Control did not spawn and did not show pre-spawning behavior, also the treatments CS and FP did not stimulate any specimens. The treatments TS and DTS triggered the spawning of the $6\% \pm 13.6$ (mean % \pm standard deviation) of the sea cucumber but were not different from the Control. The treatment FA triggered the $22\% \pm 17.2$ of specimens after 6 h from the beginning of the experiment and was different from the Control. The specimens in the treatment BU spawned during the stimulation and continued during the observing time, with $61\% \pm 25.1$ of triggered specimens. This treatment was significantly different from all the other treatments, including the Control (Tab. 3.3; Fig. 3.2) and was the only stimulus able to trigger enough specimens to perform the hatching rate trials.

3.3.2 *Hatching rate*

The eggs released in the treatment bubbling were estimated in 5.4 ± 1.5 sd million oocytes. This batch was used to assess the hatching rate of the fertilized eggs, using six different stocking densities (4, 8, 25, 50, 100, 200 eggs ml^{-1}). The ANOVA indicated significant differences among treatments ($P < 0.05$) (Tab. 3.4). The *post hoc* Tukey HSD showed that among the treatments tested, the density of 200 eggs ml^{-1} was significantly different from all the others ($P < 0.05$) (Tab. 3.5). The hatching rate decreased with the increasing of densities from 4 to 100 eggs ml^{-1} and was $44\% \pm 6.4$ (mean % \pm standard error); $38\% \pm 6.9$; $32\% \pm 7.2$; $23\% \pm 2.7$ and $28\% \pm 5.6$ respectively for 4, 8, 25, 50 and 100 eggs ml^{-1} (Fig. 3.3).

At the higher density, 200 eggs ml⁻¹, the hatching rate drastically decreased (3%±0.6) (Fig. 3.3).

3.4 Discussion and conclusions

The spawning event in sea cucumbers is generally preceded by the pre-spawning behavior, which consists in the changing of the position of reproductive specimens respect to the sea bottom. Indeed, 1/3 of the ventral part of spawners remained in contact with the bottom while the other 2/3 is upright. In this position, the gonopore, near the oral cavity is as far as possible from the bottom resulting in the most efficient position to improve the gametes' dispersion (Ocaña and Tocino 2005). The pre-spawning behavior was followed by the release of gametes that took place with several vigorous jets in females and less vigorous and longer-lasting jets in males, similarly to the observation reported by Rakaj *et al.* (2018).

The trials using stimuli as the thermal shock and dry + thermal shock were able to trigger some specimens, but they were not significantly different from the Control. This result is not in contrast with the studies that considered the thermal shocks as the most effective trigger for the spawning of *H. tubulosa* and other sea cucumbers. Indeed, the same authors reported the occasional failure of this stimuli and/or the need to collect large number of broodstock to proceed with the larval rearing (Battaglione, *et al.*, 2002; Agudo 2006; Dabbagh *et al.*, 2011; Rakaj *et al.*, 2018, 2019; Dominguez-Godino *et al.*, 2015; Domínguez- Godino and González-Wangüemert, 2018).

The other moderate (22%) effective stimulus was the addition of an excess of the diatom *Amphora spp.*, the addition of live phytoplankton has been reported to be one of the most effective triggers with the species *Cucumaria frondosa* (Gianasi *et al.*, 2019), but was not effective with *H. arguinensis* (Dominguez-Godino *et al.*, 2015). On the other hand, the addition of powdered feeding as a trigger to spawning is mostly used with other deposit feeders, including *H. scabra*, *H. fuscogilva* and *Holothuria spinifera* (Battaglione *et al.*, 2002; Agudo, 2006; Asha and Muthiah, 2007; Asha and Diwakar, 2013), but in the present study was not able to trigger any specimens.

Among the investigated treatments, bubbling was the most effective stimulus, leading to the emission of more than 60% of specimens. This stimulus, despite has never

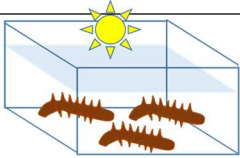
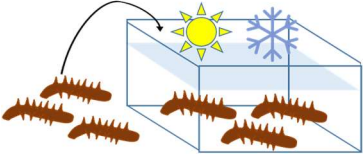
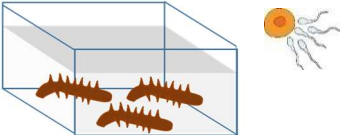
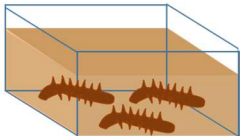
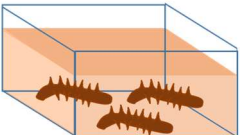
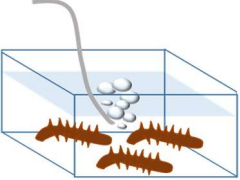
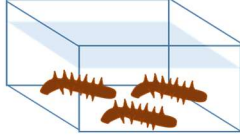
been investigated with echinoderms, is the most common method used for the reproduction of other broadcast spawners marine invertebrates as *Patella caerulea*, *Patella ferruginea* (Ferranti *et al.*, 2018, 2021), *Cellana exarata* (Corpuz, 1981), *Lottia spp.* (Kay and Emler, 2002). Despite it was not possible to establish the sex of the specimens *a priori*, since no sexual dimorphism is present in *H. tubulosa*, it is however important to stress that the bubbling was able to trigger both males and females with similar timing. This treatment acted in the increasing of the water turbulence in the tanks using vigorous air insufflations, possibly recreating the turbulence of the wave motion, whereas in all the other treatments the water in the tanks was static. Indeed, the water movement can also be the cause of the spawning of sea cucumbers during the transportation from the wild to the laboratory, which can be enough to trigger the specimens (Agudo, 2006; Rakaj *et al.*, 2018). However, field observations of *H. tubulosa* spawning mentioned the occurrence of low tide during the emission, but did not mention the water turbulence condition (i.e. wave height or current speed) (Valls, 2004; Ocaña and Tocino, 2005; Moosleitner, 2006; Bertoncini *et al.*, 2008). For other species instead, has been reported that the current speed and tidal patterns can have a role in the spawning of sea cucumbers (e.g. *Cucumaria lubrica*, *Cucumaria frondosa*) (Hamel and Mercier, 1995a,b; McEuen 1988, Mercier and Hamel 2009). The effectiveness of the increasing of the water turbulence as a trigger for the gametes' release might be an adaptive strategy of this species. In fact, a moderate water turbulence can enhance the dispersion of the gametes and increase the probability of their encounter and subsequent fertilization (Gaylord 2008), both crucial steps for the reproductive success of broadcast spawners, including *H. tubulosa*.

The gametes obtained with bubbling were able to be fertilized and to hatch into the stage of gastrula, indicating that this trigger can provide vital gametes, both oocytes and sperms. The hatching rate at the lower density was about 44% which is lower than that reported by Günay *et al.* (2018), about 80% for similar density. Such different results can be ascribed to the different hydrodynamic conditions in the water in the hatching tanks. In the present experiments we used static water, as commonly used for other echinoderms (Carboni *et al.*, 2012), whereas Günay *et al.* (2018) used continuous flow of filtered seawater and aeration. In the present study, we also explored higher densities never explored before as 50, 100, 200 eggs ml⁻¹. In particular, the density of 200 eggs ml⁻¹

showed a drastic decline of the hatching rate, indicating that high densities affect the hatching of the eggs of *H. tubulosa*.

In conclusion, this study provides new information on the effect of different stimuli on the trigger of spawning of *H. tubulosa* and on the negative effect of high stocking density on the fertilized eggs. The most effective trigger was the bubbling, a stimulus that has never been used before in sea cucumbers. Nevertheless, further studies are needed to better identify the response threshold to water turbulence in *H. tubulosa* and to evaluate the mixed effect of the bubbling coupled with other triggers, including thermal stressors and live phytoplankton supplies, in both *H. tubulosa* and other sea cucumbers species.

Tab. 3.1: Schematic representation of the treatments units, each treatment was replied in six independent tanks (15-L), each containing three sea cucumbers.

ID treatment	Treatments	
TS	Thermal shock	
DTS	Dry + thermal shock (heat - cold)	
CS	Conspecific sperms	
FA	Feeding excess (<i>Amphora spp.</i>)	
FP	Feeding excess (feeding powder)	
BU	Bubbling	
CTRL	Control	

Tab. 3.2: Results of the one-way ANOVA for the effect of different treatments on the spawning of the specimens. Df = degree of freedom; MS = mean squares; F = F-ratio; P = P-value.

Source	Df	MS	F	P
Between groups	6	2409.550	14.72	<0.05
Within groups	35	163.719		
Total	41			
Cochran test				>0.05

Tab. 3.3: Results of the Tukey's test contrasting the triggered specimens among treatments. P = P-value; ns= not significant

Contrast	Difference	P
CTRL vs TS	-5.877	ns
CTRL vs DTS	-5.877	ns
CTRL vs CS	0.000	ns
CTRL vs FA	-23.509	<0.05
CTRL vs FP	0.000	ns
CTRL vs BU	-54.122	<0.05
TS vs DTS	0.000	ns
TS vs CS	5.877	ns
TS vs FA	-17.632	ns
TS vs FP	5.877	ns
TS vs BU	-48.245	<0.05
DTS vs CS	5.877	ns
DTS vs FA	-17.632	ns
DTS vs FP	5.877	ns
DTS vs BU	-48.245	<0.05
CS vs FA	-23.509	<0.05
CS vs FP	0.000	ns
CS vs BU	-54.122	<0.05
FA vs FP	23.509	<0.05
FA vs BU	-30.613	<0.05
FP vs BU	-54.122	<0.05

Tab. 3.4: Results of the one-way ANOVA testing for the effect of different eggs densities on the hatching rate. Df = degree of freedom; MS = mean squares; F = F-ratio; P = P-value.

Source	Df	MS	F	P
Between groups	5	374.496	11.07	<0.05
Within groups	12	33.839		
Total	17			
Cochran test				>0.05

Tab. 3.5: Results of the Tukey's test for the hatching rate of the fertilized eggs at different densities. P = P-value; ns= not significant

Contrast	Difference	P
4 vs 8 eggs ml ⁻¹	3.283	ns
4 vs 25 eggs ml ⁻¹	7.570	ns
4 vs 50 eggs ml ⁻¹	12.794	ns
4 vs 100 eggs ml ⁻¹	9.865	ns
4 vs 200 eggs ml ⁻¹	31.675	<0.05
8 vs 25 eggs ml ⁻¹	4.287	ns
8 vs 50 eggs ml ⁻¹	9.510	ns
8 vs 100 eggs ml ⁻¹	6.581	ns
8 vs 200 eggs ml ⁻¹	28.392	<0.05
25 vs 50 eggs ml ⁻¹	5.223	ns
25 vs 100 eggs ml ⁻¹	2.295	ns
25 vs 200 eggs ml ⁻¹	24.105	<0.05
50 vs 100 eggs ml ⁻¹	-2.929	ns
50 vs 200 eggs ml ⁻¹	18.882	<0.05
100 vs 200 eggs ml ⁻¹	21.810	<0.05

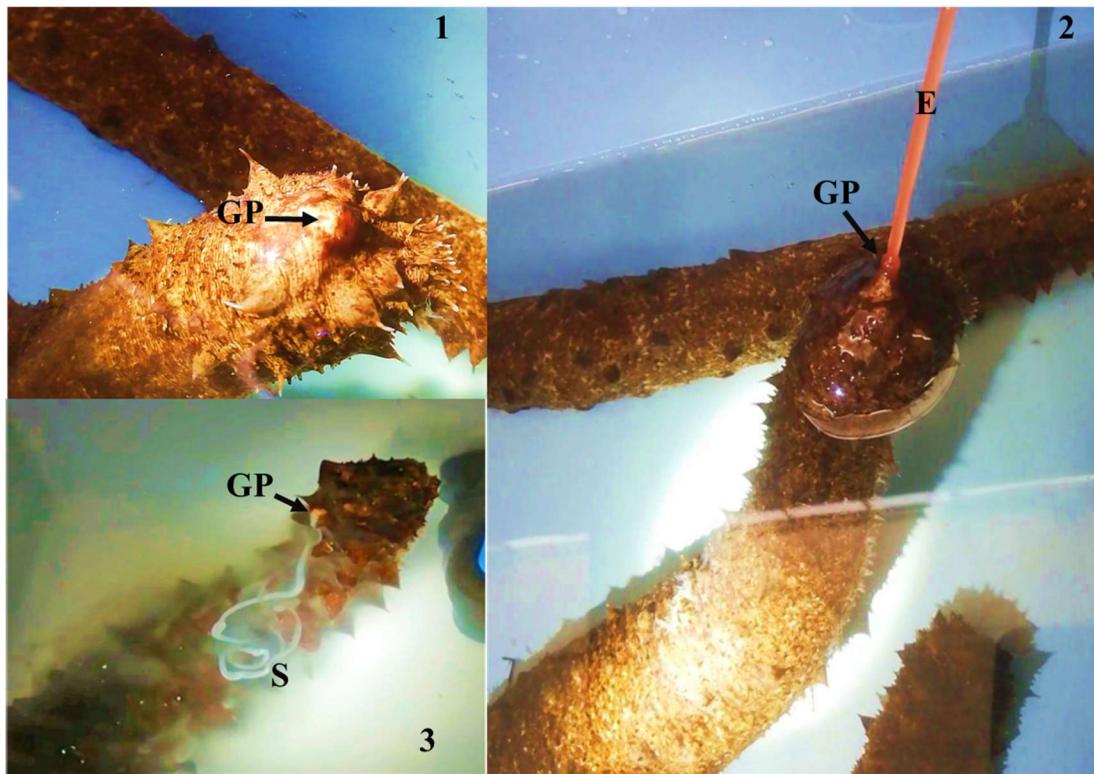


Fig. 3.1: Spawning of *H. tubulosa*. **1:** Specimen in pre-spawning position showing a clear bulge of the gonopore. **2:** Shortly after the bulge of the gonopore, gametes were vigorously ejected from the female in several jets. **3:** Less vigorous and long lasting ejection was observed for male specimens. GP, gonopore; E, eggs; S, Sperms.

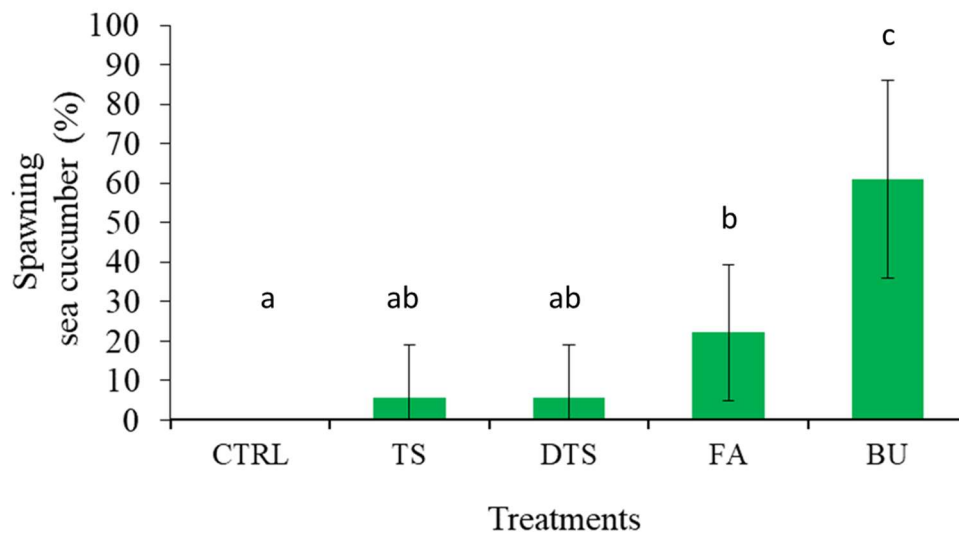


Fig. 3.2: Percentage of spawning of sea cucumber *H. tubulosa* triggered with the treatment TS: Thermal shock; DTS: Dry + thermal shock; FA: Feeding excess (*Amphora spp.*); BU: Bubbling; and the Control (CTRL). Treatments CS and FP not shown. Lowercase letters indicate the results of the post-hoc tests as from the results reported in Tab. 3.3. Error bars indicate standard deviation of the mean.

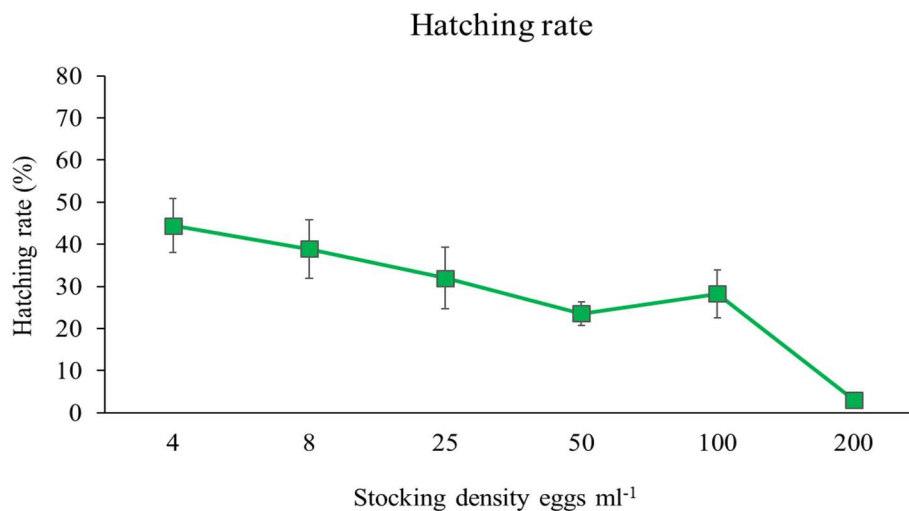


Fig. 3.3: Variation of the hatching rate at different stocking densities. Error bars indicate standard error.

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Chapter 4

Use of algal conditioned plates to foster the settlement of *Holothuria tubulosa* Gmelin 1788 competent larvae

4.1 Introduction

The sea cucumber *Holothuria tubulosa* (Gmelin, 1788) is a common echinoderm that inhabits the coastal areas of the Mediterranean Sea and the Eastern Atlantic Ocean (Tortonese, 1965). As continuous deposit-feeder, *H. tubulosa* is generally associated with rich organic bottoms and seagrass beds where it plays a key ecological role in the nutrient cycling and in the sediment bioturbation (Bulteel, Jangoux and Coulon, 1992; Coulon and Jangoux, 1992, Roberts *et al.*, 2000; Costa *et al.*, 2014). In the last decade, *H. tubulosa* has been strongly harvested and destined to Asian markets (González-Wangüemert *et al.*, 2014, 2018), where sea cucumbers are sold as delicacy seafood and used as a source of bioactive compounds in the pharmaceutical and cosmetic industries (Kinch *et al.*, 2008; Bordbar *et al.*, 2011; Purcell 2012; Purcell *et al.*, 2014; Janakiram *et al.*, 2015; Yang and Bai, 2015). The increasing demand and the high commercial value of sea cucumbers led to their overexploitation, and in extreme case to the collapse of wild populations (Purcell, 2010; Sicuro and Levine, 2011; Toral-Granda, 2008, González-Wangüemert *et al.*, 2014, 2018; Conand *et al.*, 2014, Ramírez-González *et al.*, 2020). For these reasons, in some countries (i.e. Italy, Decree n. 30-December 2020 Ministry of Agricultural, Food and Forestry Policies), the commercial and recreational fishery of sea cucumbers is forbidden.

A suitable alternative to reduce the pressure on sea cucumbers' wild stocks and to produce high-value biomass, could be represented by their breeding in captivity. Sea cucumbers' aquaculture is commonly practiced with historically exploited species as *Holothuria scabra* and *Apostichopus japonicus* (Agudo 2006; Purcell *et al.*, 2012; Shi *et al.*, 2013; Pietrak *et al.*, 2014, Purcell and Wu, 2017). More recently, also new-target species were candidates for aquaculture, including *H. tubulosa* (Rakaj *et al.* 2018). However, some critical phases are still present on the technology adaptation of the rearing

protocols from other species to the new target ones. One of the main issues is the low settlement success and the low survival rate of post-settlers (Domínguez-Godino *et al.*, 2015, 2018; Günay *et al.*, 2018; Rakaj *et al.*, 2019; Laguerre *et al.*, 2020). The procedure for sea cucumbers breeding can be divided into four main steps: 1) spawning (see Chapter 3), 2) larval rearing, 3) nursery 4) growth-out of the juveniles (Agudo 2006). In general, the larval cycle of *Holothuroidea* species is characterized by three main stages, it starts with a feeding planktonic auricularia (early, mid and late) followed by a non-feeding planktonic doliolaria, which settle into a pentactula, which feeds on the substrate (Strathmann, 1975; Smiley *et al.*, 1991; Ito and Kitamura, 1997; Yanagisawa, 1998; Domínguez-Godino *et al.*, 2015, 2018; Rakaj *et al.*, 2018, 2019; Laguerre *et al.*, 2020).

The process of settlement is one of the trickiest stages during the larval development of benthic marine invertebrates, probably, due to the complex processes of interaction between biotic and abiotic factors operating at different spatial and temporal scales (Rodríguez *et al.*, 1993). In absence of suitable or preferred substrata, competent larvae may continue to swim and return to the water column. Alternatively, larvae may respond to a surface or water-borne cues, attaching to the substrate and performing the metamorphosis, which is an irreversible process that indicates the passage from the planktonic larva to a benthic settler (Hadfield *et al.* 2001; Mos *et al.*, 2011).

Several cues have been used to stimulate the metamorphosis of marine invertebrates, in particular, for sea cucumbers, the following are commonly used: natural biofilms, addition of *Sargassum sp.* extract or other seagrasses, and settlement plates covered with diatoms biofilm (Agudo 2006; Domínguez-Godino *et al.*, 2015, 2018; Rakaj *et al.*, 2018, 2019; Laguerre *et al.*, 2020).

A substrate that has been reported to improve the settlement of veliger larvae in abalone *Haliotis discus* and *Haliotis laevigata* (Takahashi and Koganezawa 1988; Daume and Ryan, 2004) and in the echinopluteus of sea urchins *Strongylocentrotus intermedius* and *Paracentrotus lividus* (Takahashi *et al.* 2002; Hannon *et al.*, 2017), is the green algae *Ulva lens* belonging to the family Ulvaceae (Phylum: Chlorophyta). This green alga grows over surfaces by expanding the disc-shaped thallus. The cells in the central part of the thallus develop into sporangia, which at maturity release the flagellated zoospore, which in presence of substrata can germinate in a short time range (Nielsen, 1977).

In view of the fact that the process of settlement is fundamental for larval survival and subsequent stages, this study aims to investigate the effect of different biofilms on the settlement success of *H. tubulosa* competent larvae (doliolaria). The biofilms used were the following: the one made with *Amphora spp.* that belong to the family Catenulaceae (Phylum: Ochrophyta), a diatom commonly used as a potential settlement cue for invertebrate larvae (Ito and Kitamura 1997; Daume *et al.*, 2000; Hannon *et al.*, 2017), and the biofilm made with the green alga *U. lens* (Hannon *et al.* 2014). This study aims to respond to the following question 1) Does the biofilms influence the settlement of *H. tubulosa* doliolaria larvae? To answer this question, we tested the null hypothesis that different biofilms do not influence the settlement of competent larvae of *H. tubulosa*.

The practical feedback of the study is to increase the survival rate and growth of *H. tubulosa* post-larvae, a process that can affect the production yield in the aquaculture of this species.

4.2 Material and methods

The experiment was conducted at the facility of the University of Cagliari “Laboratorio Sperimentale di Acquacoltura” located on the seaside of the Santa lagoon, Sardinia, Italy in September - October 2020.

4.2.1 Algal culture and settlement substrates

U. lens and *Amphora spp.* were cultured on site in 4L f/2 medium at 1ml/L, and sodium metasilicate (Na_2SiO_3) was added 1ml/L to the culture media for *Amphora spp.* (Hannon *et al.*, 2017). The cultures were kept at 20°C under a photoperiod of 12 h light and 12 h dark.

4.2.2 Substrate conditioning

The fitness of metamorphosis of *H. tubulosa* was tested with three treatments: 1) *Amphora spp.* (Amphora); 2) *U. lens* (Ulvela) 3) no substrate as Control.

The experimental design considered multiple chamber plates (6 chambers each plate; 4 replicates each treatment) with a volume of 3 ml for each chamber, where algae were inoculated (Fig. 4.1). The treatment *Amphora spp.* was inoculated in chambers filled with

the f/2 medium with the addition of sodium metasilicate (Na_2SiO_3); the treatment *U. lens* was inoculated in chambers filled with the f/2 medium with *U. lens* zoospores obtained using the protocol described by Hannon *et al.* (2014). The plates inoculated with *U. lens* and *Amphora spp.*, were held under ambient light conditions at 20°C for 15 days prior the settlement trials, to allow the growth of the algal biofilms.

4.2.3 Larvae and competency

The gametes were obtained from broodstock collected in August from south Sardinia (Latitude: 38°55'46.1"N, Longitude: 8°43'16.1"E, Mediterranean Sea, Italy). The larvae used in this study were reared using the protocol described by Rakaj *et al.* (2018) with some adjustments. The larvae were bred in 150-L tank (1 larvae ml^{-1}) and were fed with the pabulum made a mix of the microalgae *Chaetoceros calcitrans*, *Isochrysis galbana*, and *Tetraselmis suecica*. After 25 days from the fertilization, the larvae reached the doliolaria stage ($407.7 \pm 3.2\mu\text{m}$). A total of 72 doliolaria were transferred individually in the chambers (one doliolaria per chamber), previously conditioned with the algal biofilms described above, plus the Control plates (without biofilm). Before moving the doliolaria in the settlements plates, the medium was exchanged with filtered ($5\mu\text{m}$) and UV-sterilized seawater. The plates were kept at 24°C with 12h light and dark. Every day the 50% water in the chambers was exchanged and every five days the larvae were observed to define the development of the settlement process. The settlement was considered successfully achieved when the larvae reached the pentactula stage, showing the first tube foot (ambulacral podium) and the five anterior tentacles (Fig. 4.2) (Rakaj *et al.*, 2018, 2019). When the second posterior podium appeared, the specimens were considered juveniles. The progress of the settlement was followed under stereomicroscope by observing the morphological changes every five days until the 35th day.

The settlement success was calculated as the ratio of the number of settlers (pentactula and juveniles) on the number of initial larvae, expressed in percentage.

4.2.4 Statistical analysis

Data expressed in percentage were previously arcsin transformed ($x' = \arcsin\sqrt{x}$). One-way ANOVA ($P < 0.05$) was performed to test the null-hypothesis formulated in the introductory paragraph.

The *H. tubulosa* settlement performance was tested at the end of the experiment after 35 days. Prior to the analysis, Cochran's C-test ($P > 0.05$) was used to check the assumption of the homogeneity of variances. *Post-hoc* multiple comparisons were performed using Tukey HSD test; STATGRAPHICS PLUS 5.1 professional edition (Statistical Graphics Corp., Rockville, MD, USA) was used for statistical analysis.

4.3 Results

In all the treatments, except the Control, the first pentactula was observed within five days, the number of settlers increased until the 25th day in all the treatments with biofilms. However, after the 30th day, a reduction of the settled pentactula was observed caused by the death of the post-settlers (Fig. 4.3). The ANOVA indicated that significant differences occurred among treatments (Tab. 4.1). The settlement success at the end of the experiment (35th day) was about the 21%±12.5 (mean ± se) with *Amphora spp.*, and 54%±12.5 with *U. lens*, which was the only treatment different from the Control (Tab. 4.2; Fig. 4.3).

The percentage of swimming doliolaria decreased with the progression of the settlement. In detail, doliolaria larvae disappeared in *U. lens* after 20 days and in *Amphora spp.* after 30 days (Fig. 4.4). In the Control plates, at the end of the experiment, 62% of the swimming doliolaria were still present (Fig. 4.4), and the treatment *U. lens* showed the highest post-settler survival, with 25% of the post settlers that reached the juveniles stage, while in the treatment *Amphora spp.* only 4% of the larvae reached the juvenile stage (Fig. 4.4).

4.4 Discussion and conclusion

Like other marine invertebrates, sea cucumbers' larvae seems to be sensitive to settlement cues, which can induce metamorphosis from planktonic larvae to benthic post-settlers (Morse 1985; Gosselin and Jangoux 1996; Takahashi *et al.* 2002; Mos *et al.*, 2011). The settlement of echinoderms involves the presence of a variety of cues, including

macroalgae, microalgal and bacterial biofilms and the presence of conspecific adults, which can be detected by larvae (Dworjanyn and Pirozzi, 2008; Mos *et al.*, 2011). When the larva touches the substrate, exhibits exploratory behavior, repeatedly touching the surface with the tentacles, briefly attaching and swimming away before finally settling and proceeding with the metamorphosis (Scheibling and Robinson, 2008).

Sea cucumbers larvae bred in captivity are generally induced to settle on natural biofilms obtained immersing the plate in diatoms culture for a few days, or adding an extract of *Sargassum sp.* or seagrass leaves (Agudo 2006, Rakaj *et al.*, 2018, 2019). In the current experiment, larvae of *H. tubulosa* showed exploratory behavior for several days in the treatments with algae biofilms, until larvae settled or died.

The results show that all the plates conditioned with algal biofilm allowed the settlement of the doliolaria and its metamorphosis into pentactula. In particular, the best settlement performance was achieved with *U. lens* biofilm, which allowed the metamorphosis of the 54% of the larvae into pentactula. The set of biofilms used here is quite new for the settlement induction of sea cucumbers and the results obtained are difficult to compare with reference data from literature. The only study that explored the survival of *H. tubulosa* settlers and post-settlers was conducted by Rakaj *et al.* (2018) using benthic diatoms (*Navicula spp.*, *Nitzschia spp.* and *Phaeodactylum tricorutum*) as settlement substrata, obtaining the survival of the 7% of post-settler after 30 days, however, the authors reported a possible underestimation of such survival rate.

Since the doliolaria larvae actively swim but is a non-feeding stage, as doliolaria delays the metamorphosis the larval size decreased due to the utilization of the endogenous resources, mainly stored in the hyaline sphere (Peters-Didier and Sewell, 2019). For this reason, the settlement speed is crucial to perform the metamorphosis and survive. It is particularly interesting to observe that larvae that survived in the Control plates were still in the doliolaria stage for the whole experiment. This fact indicates that the presence of some cue is a necessary condition to proceed with the metamorphosis of *H. tubulosa*, and that larvae are able to delay metamorphosis when no substrate is available. This behavior has been previously observed also in *Holothuria scabra*, which was able to delay the settlement for a relatively short period, about 96h (Mercier *et al.*, 2000). Even *Stichopus californicus* could survive for 10 days in absence of suitable

substrata (Smiley et al., 1991) and *Cucumaria frondosa* larvae for 8 days (Hamel and Mercier, 1996).

These results could be explained by the Desperate Larva Hypothesis (DLH), according to which, lecithotrophic larvae become less discriminating in their settlement requirements over time, due to depletion of energy resources (Knight-Jones, 1953). This hypothesis was then revised to be applicable to the non-feeding stage of the larval cycle (Botello and Krug, 2006), reporting that as the non-feeding stage continues, larvae became “desperate” to settle and the level of selectivity towards the substrata decrease. Hence, the larvae will take the risk to settle in non-optimal substrata and perform the metamorphosis in spite of extending the planktonic period. However, older larvae will settle, but only in response to a minimum cue, because performing metamorphosis in complete absence of substrate will be fatal for the feeding post-settler (Botello and Krug, 2006).

This hypothesis can explain both the persistence of doliolaria stage in the control plates and the different number of juveniles observed with the two biofilms. Indeed, the two biofilms did not show significant differences in settlement success, but the lower number of juveniles in the treatment *Amphora spp.* (4%) can indicate that while the cue seems to be enough to induce the settlement, *Amphora spp.* might not be able to ensure the survival of the post-settlers and their growth. Indeed, a common practice to improve the post-settlers survival and growth is to give supplementary food, such as live phytoplankton or dried algae powder, to add extra nourishment (Agudo 2006; Domínguez-Godino *et al.*, 2015; Domínguez-Godino and Gonzalez-Wangüemert, 2018; Huang *et al.*, 2018; Rakaj *et al.*, 2018, 2019). However, no information on the nutritional and caloric requirements of the sea cucumbers settler and juveniles are present in literature.

In conclusion, the biofilm that allowed the maximum settlement success was the green algae *U. lens* confirming the high settlement performance of this biofilm in the metamorphosis of echinoderms larvae, including *H. tubulosa*. The results stimulate further study at a pilot scale using *U. lens* biofilm mixed with other algal species in order to achieve the best settlement performance and survival of the post-settlers. These steps are indeed crucial to achieve the feasibility of sea cucumbers aquaculture.

Tab. 4.1: Results of the one-way ANOVA for different treatments on the settlement of *H. tubulosa* doliolaria larvae. DF = degree of freedom; MS = mean squares; F = F-ratio, P= P-value.

Source	DF	MS	F	P
Between groups	2	2303.6	8.83	<0.05
Within groups	9	260.9		
Total	11			
Cochran test				>0.05

Tab. 4.2: Results of the Tukey's test for settlement of *H. tubulosa* doliolaria larvae, contrasting the Control with the biofilms treatments in different days. P = P-value; ns= not significant.

Contrast	Difference	P
Control vs <i>Amphora spp.</i>	-20.065	ns
Control vs <i>U. lens</i>	-47.791	<0.05
<i>Amphora spp.</i> vs <i>U. lens</i>	-27.725	ns

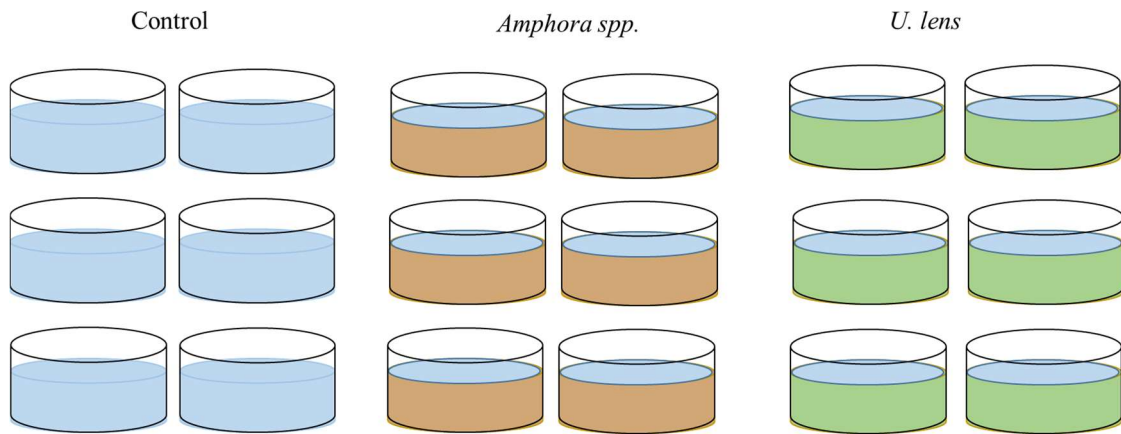


Fig. 4.1: Schematic representation of the experimental design. Each treatment consisted in six chambers in which was placed one doliolaria. Each experiment was replicated four times.

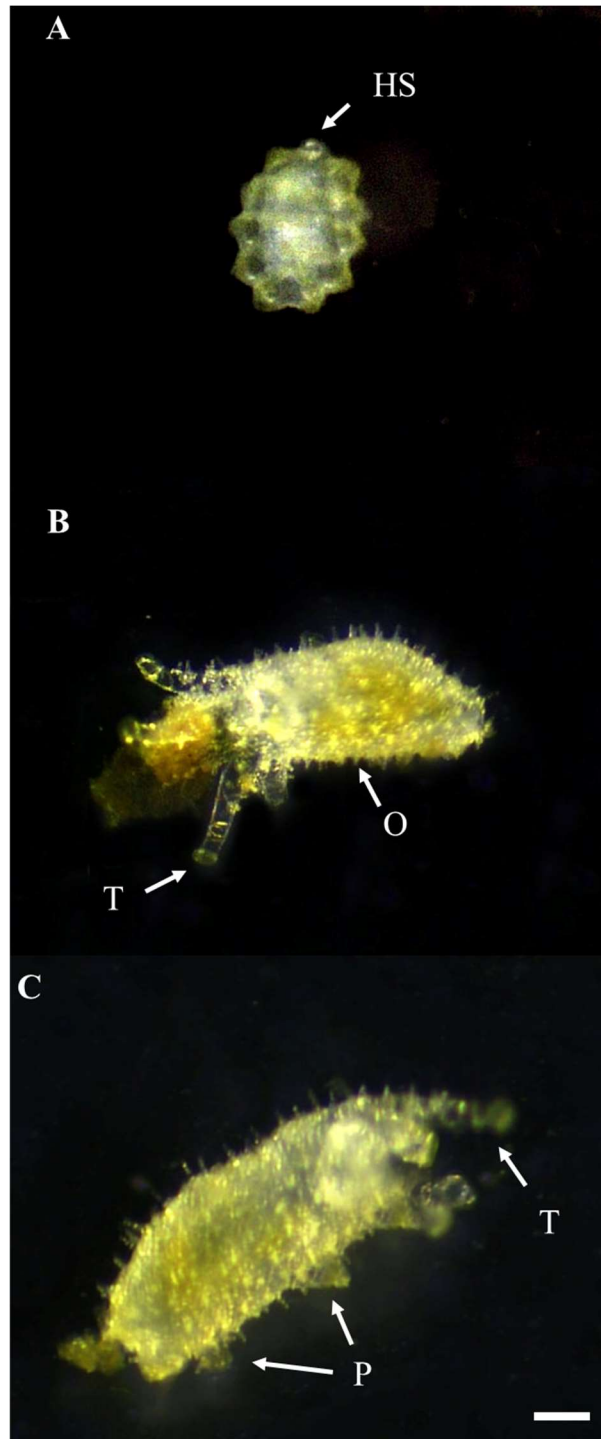


Fig. 4.2: Different larval and post-larval stages. A: swimming doliolaria that presents visible hyaline spheres (HS); B: settled pentactula with buccal tentacles (T) and derma ossicles (O), C: juvenile with two ambulacral podia (P). Scale bar =100 μ m.

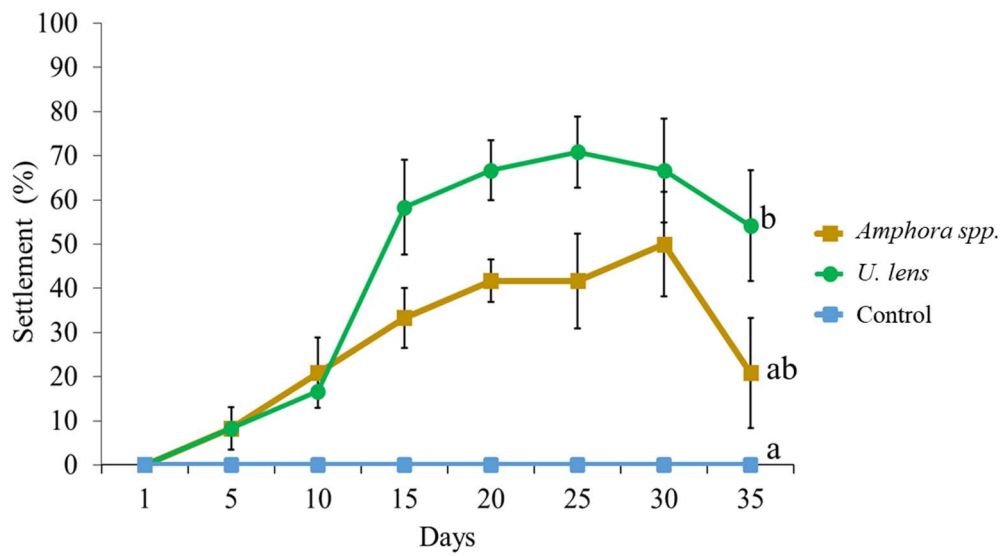


Fig. 4.3: Percentage of settlement success in the different treatments along the experiment. Lowercase letters indicate the results of the Tukey's test for the settlement of *H. tubulosa* larvae from the results reported in Table 4.2. Error bars indicate standard error.

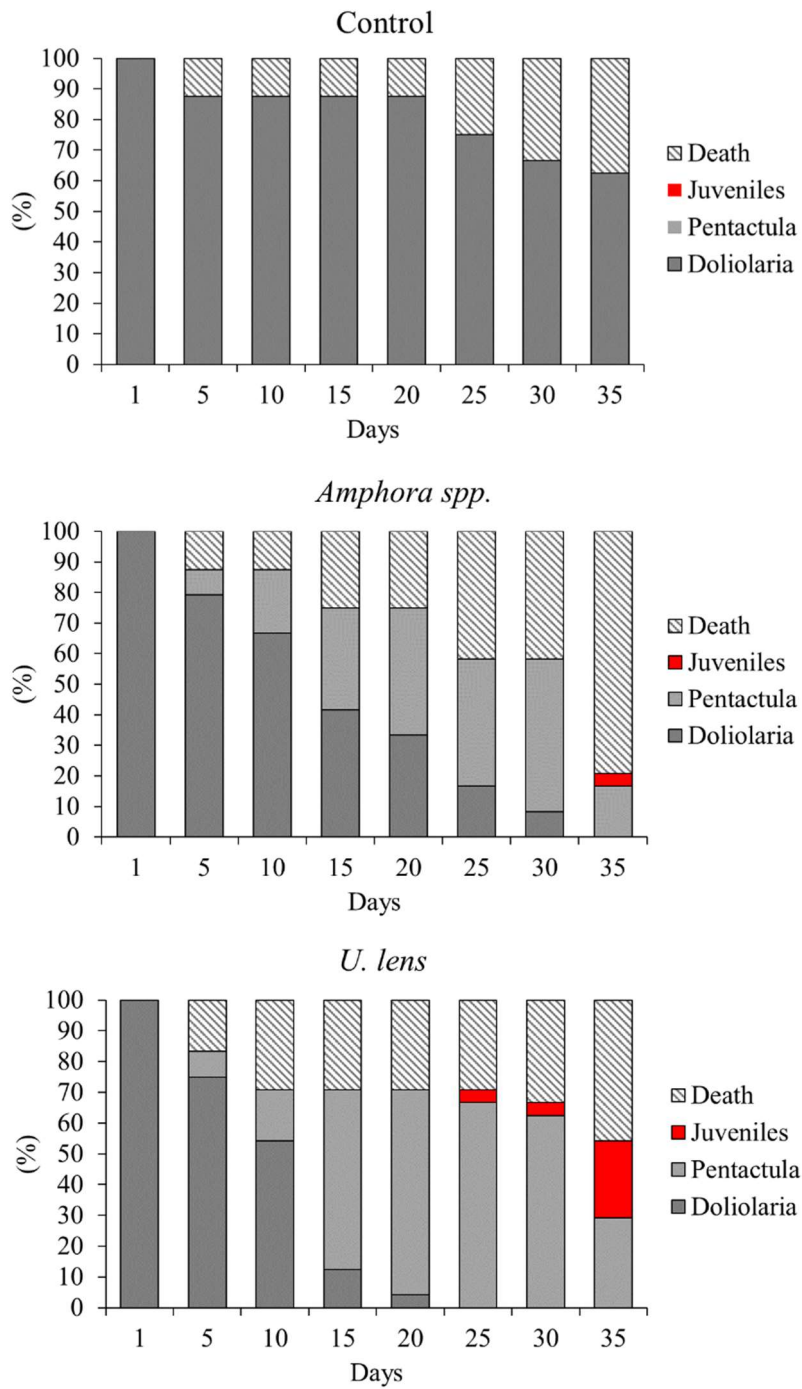


Fig. 4.4: Variation of the frequency of the different larval stages along the whole experiment in the Control and in the treatments *Amphora spp.* and *U. lens*.

4.5 References

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Chapter 5

Effects of *Holothuria tubulosa* on quantity, biochemical composition, and nutritional quality of sedimentary organic matter

5.1 Introduction

Marine depositivorous organisms play a key role in nutrient cycling by bioturbation, from coastal areas to hadal depths (Uthicke 1999; Roberts *et al.*, 2000; Slater and Cartoon, 2009; Amaro *et al.*, 2010; Slater *et al.* 2011a; Purcell *et al.*, 2016). Bioturbation can indeed influence the sediment permeability, the chemical gradients in pore water and organic matter (OM) remineralization rates (Reise 2002, Lohrer *et al.*, 2004; Solan *et al.*, 2004; Meysman *et al.*, 2006 a, b). Because of their feeding behavior, the increased (unsustainable) exploitation of low trophic level organisms is rising concern not only for the conservation of wild populations but also for the possible consequences of their decline on benthic ecosystem functioning (Andreson *et al.*, 2011a, b).

Sea cucumbers (Phylum Echinodermata) are common members of the benthic communities and occur in all world oceans and seas, counting more than 1500 species (Horton *et al.*, 2018). Deposit feeding sea cucumbers act as strong bioturbators and can rework large amounts of sediments ($5.9 - 12.9 \text{ kg dw m}^{-2} \text{ yr}^{-1}$), ingesting the whole sediment and feeding on the sedimentary OM (Coulon and Jangoux, 1993; Uthicke and Karez, 1999; Mangion *et al.*, 2004, Hartati *et al.*, 2020). By such a feeding behavior, sea cucumbers contribute to the redistribution and remineralization of sediment organic loads (MacTavish *et al.*, 2012; Purcell *et al.*, 2016, Yamazaki *et al.*, 2019), thus modifying the geochemical structure of the seafloor (Amaro *et al.*, 2010). Bioturbation by sea cucumbers can be limited to the upper layer of the seafloor or reach several centimeters depth in the sediment, depending on the ability of the different species to dig into the sediment (Mercier *et al.*, 1999; Roberts *et al.*, 2000; Amaro *et al.*, 2010; Ramón *et al.*, 2019). During the feeding activity, sediment particles are captured by the sea cucumber with the tentacles and released into the pharynx; the particles are then mixed with the digestive

enzymes and compressed into a plug, which moves along the simple digestive system that ends in a cloaca in the posterior part of the animal (Zamora and Jeffs, 2011).

Previous studies reported the ability of deposit-feeding sea cucumbers to reduce the sedimentary OM loads with their feeding behavior (Slater and Carton, 2009; MacDonald *et al.*, 2013; Neofitou *et al.*, 2019). For this reason and because of their high commercial value and the overexploited status of many populations spread worldwide (Conand, 2006; Bordbar *et al.*, 2011 Purcell *et al.*, 2011; Conand *et al.*, 2014; González-Wangüemert *et al.*, 2018), sea cucumbers are considered one of the best candidates for Integrated Multi-Trophic Aquaculture (IMTA) practices (MacDonald *et al.*, 2013;; Cubillo *et al.*, 2016; Grosso *et al.*, 2021). IMTA practices aim to mitigate the environmental impacts of mariculture (Pusceddu *et al.*, 2007; Holmer *et al.*, 2008), by transforming the wastes (uneaten food and fish feces) from higher trophic levels into food sources for reared species at lower trophic levels, at the same time producing extra valuable biomass (Zhou *et al.*, 2006; Slater and Carton, 2007, 2009; Slater *et al.*, 2009; Zamora and Jeffs, 2011, 2012; Yuan *et al.*, 2013; Lamprianidou *et al.*, 2015; Shpigel *et al.*, 2018). Sea cucumbers used so far in IMTA and co-culture include high commercially valuable species like *Apostichopus japonicus* (Zhou *et al.*, 2006; Yuan *et al.*, 2013; Kim *et al.*, 2015), *Australostichopus mollis* (Slater and Carton, 2007, 2009; Slater *et al.*, 2009; Zamora and Jeffs, 2011, 2012), *Holothuria scabra* (Mathieu-Resuge *et al.*, 2020), and *Parastichopus californicus* (Paltzat *et al.*, 2008).

Recent studies conducted on the Mediterranean sea cucumber *Holothuria tubulosa* Gmelin 1788 report that this species can reduce biodeposits and feces produced by sea urchins in laboratory trials (Grosso *et al.* 2021). Moreover, *H. tubulosa* grows more beneath finfish cages than in mariculture-free areas Tolon *et al.* (2017). The reduction rate of OM and organic carbon (OC) carried out by *H. tubulosa* (ca. 10 ind m⁻²) in the field in sediments beneath sea bream and sea bass cages were estimated to be about 31% and 62%, respectively (Neofitou *et al.*, 2019).

With an eye to the potential use of *H. tubulosa* for abating, in an IMTA perspective, the consequences of benthic eutrophication caused by mariculture, we investigated: *i*) differences in quantity and biochemical composition of sediment OM and *H. tubulosa* feces; *ii*) the digestive process of sedimentary protein loads in the digestive tracts of *H.*

tubulosa. More specifically, we posed the following questions: 1) Does *H. tubulosa* influence benthic trophic status? 2) Does *H. tubulosa* influence the availability of labile components (i.e., proteins) of sedimentary OM?

To answer these questions, we tested the following null hypotheses: 1) the biochemical composition of sediment OM is not different from that of sea cucumbers' feces; 2) the protein content does not vary among the ingested sediment, the sediment inside the sea cucumber digestive tract and sea cucumbers feces.

5.2 Material and methods

5.2.1 Model species, study areas and sampling strategy

Our study is focused on the sea cucumber *Holothuria tubulosa*, one of the most common and most exploited native species across the whole coastal Mediterranean Sea, where it can be pre-eminently found in organically enriched bottoms and seagrass beds (Tortonese, 1965; Bulteel, Jangoux and Coulon, 1992; Koukouras *et al.*, 2007, Costa *et al.*, 2014; González-Wangüemert *et al.*, 2014, 2015, 2018; Aydin, 2019).

H. tubulosa specimens for the purposes of this study have been collected from two areas (Oristano and Teulada Gulfs). The Oristano Gulf (39°52'50''N; 8°28'54''E) is characterized by the dominance of sands (63-1000 µm; 75%), followed by mud (<63 µm; 14%) and gravel (>1000 µm; 11%) and by the presence of off-shore mariculture plants for fish farming. The Teulada Gulf (38°55'46''N; 8°43'17''E) is characterized by the dominance of sandy sediments (74%), followed by gravel (24%), and mud (1%), and by the presence of wide *Posidonia oceanica* meadows (Fig. 5.1).

Sediment and holothurians samplings were carried out in April, June and August 2019 and in January-February, May-June and July-August 2020 in both sites. Sediment and holothurians specimens were collected by scuba divers at depths comprised between 3 and 7 m.

At each sampling site and in each sampling date, sediments were obtained by scraping ca. 50 mL from the top 2-cm of the sea bottom with a Falcon-type tube from three 60x60 cm squared frames. From each frame, triplicate sediment samples were obtained from three random sub-squares (20x20 cm) (Moccia *et al.*, 2019). One of the

sediment replicates was obtained from the central sub-square of the frame, always positioned close to the sea cucumber (one per frame, subsequently sampled for the feces collection).

All sea cucumbers (one per squared frame) were placed into 3-L plastic bag filled with seawater collected *in situ* and maintained in a cooler box (at *in situ* temperature) during transportation to the laboratory (within 1.5 h from sampling). Once in the laboratory, sea cucumbers were singularly placed in a tank filled with seawater (15-L), pre-conditioned at temperature and salinity values as measured *in situ*. Every 6-8 hours the sea cucumbers' feces were collected, until the complete evacuation of the digestive system (within 3 days). Feces from each specimen were pooled together to ensure the storage of a sufficient amount of feces for the analyses. The sediments and the faeces were stored at -20°C until the analysis.

Another 2-3 sea cucumbers were collected in June 2019 at both sites and used for the analysis of the protein content in the sediment within the digestive tracts. Those sea cucumbers were weighted (g) before dissection and the sediment present in the intestine esophagus (ESO), mid gut (MID), and end gut (END) (Fig. 5.2) was carefully collected to avoid contaminations with the digestive tissue (Amaro *et al.*, 2010) and stored at -20°C until the analyses.

5.2.2 Biochemical composition of sedimentary OM and faeces

Protein, carbohydrate, and lipid contents of sediment and sea cucumbers feces and sediment in the gut (only proteins) were analysed spectrophotometrically (Danovaro 2010), and expressed as albumin, glucose and tripalmitine equivalents, respectively. For each biochemical assay on ambient sediment, blanks were obtained using pre-combusted sediments (450 °C for 4 h). All analyses have been carried out on triplicate samples. Carbohydrate, protein and lipid contents were converted into carbon equivalents using the conversion factors 0.40, 0.49, and 0.75 mgC mg⁻¹, respectively, and their sum reported as biopolymeric C (BPC; Fabiano *et al.*, 1995).

The percentage proteins potential assimilation in the digestive tracts was calculated as follow:

$$PA = (ESO\ PRT - END\ PRT) / ESO\ PRT \times 100$$

where:

PA = potential proteins' assimilation

ESO PRT= protein content in the sediment in the esophagus

END PRT= protein content in the sediment in the end gut

5.2.3 Statistical analyses

Spatial (between sites) and temporal (among sampling dates) differences in organic matter quantity and biochemical composition between sediment and feces were investigated with permutational analyses of variance (PERMANOVA), in both univariate and multivariate contexts. The fully orthogonal design used Matrix (2 fixed levels: sediment vs. feces), Site (2 fixed levels: Oristano vs. Teulada) and Month (6 fixed levels: April, June, August 2019, and January-February, May-June, July-August 2020) as sources of variance.

Differences in protein content between ambient sediment, sediment in the gut (three sectors: esophagus, mid gut and end gut) and feces were assessed by means of a univariate PERMANOVA using two orthogonal factors: Matrix (5 fixed levels: ambient sediment, esophagus, mid gut, end gut and feces) and Site (2 fixed levels: Oristano vs. Teulada).

The analyses of both experiments were based on Euclidean distances of previously normalized data, using 999 random permutations of the appropriate units. When significant differences were observed, pairwise tests were also carried out. All the statistical analyses were performed using the routines included in the PRIMER 6+ software (Clarke and Gorley, 2006).

5.3 Results and discussion

5.3.1 Effects of *H. tubulosa* on the benthic trophic status

Deposit-feeding sea cucumbers are generally associated with soft organic-rich bottoms, where they sift the upper sediment layers, feeding on the most readily utilizable organic matter (Yingst, 1982; Roberts *et al.*, 2000; Purcell *et al.*, 2016). The studies conducted so far on the feeding behavior of coastal sea cucumbers investigated the fate of total organic matter (TOM) and/or total organic carbon (TOC), without considering the biochemical

composition and the biopolymeric C content of the sediments, which are considered proxies of the availability of food more reliable than the total carbon pool alone (Danovaro *et al.*, 2001; Pusceddu *et al.*, 2009). To shed light on the potential role of *H. tubulosa* on the benthic trophic status, we investigated differences in quantity and biochemical composition of source sediments and sea cucumbers' feces in two distinct sites characterized by different background trophic status.

According to the thresholds of BPC sedimentary contents proposed by Pusceddu *et al.* (2011) sandy-muddy sediments in Oristano can be ranked as meso-eutrophic (BPC > 3 mgC g⁻¹) during the entire study period, whereas sandy sediments in Teulada range from oligotrophic (BPC <1 mgC g⁻¹) in winter and early spring, to mesotrophic (BPC 1-3 mgCg⁻¹) in summer, to eutrophic (BPC > 3 mgC g⁻¹) in late spring-early summer.

The results of the PERMANOVA tests show that the protein content in sediment and feces varied significantly among Sites but also among Months and Matrices, with no significant effect of interactions among factors (Tab. 5.1). In Oristano, feces protein content was significantly higher than that in the sediment in June 2019 and in Jul-Aug 2020, whereas in Teulada the feces protein content was significantly higher than that in the sediment in August and Jan-Feb (Tab. 5.2; Fig. 5.3). On the other hand, the carbohydrates content in sediment and feces did not show any significant difference among levels of the investigated factors (Tab. 5.1). Lipid contents varied significantly among Sites and Matrices, with significant effects of Si×MO, Mo×Ma and Si×Mo×Ma interactions (Tab. 5.1). In Oristano the lipid content of feces was significantly lower than that in the sediment only in April 2019, whereas in the other sampling dates lipid contents in the feces were significantly higher than that in the sediment (Tab. 5.2, Fig. 5.3). In Teulada, the only significant difference occurred in August 2019, when the lipid content of feces was significantly higher than that in the sediment (Tab. 5.2, Fig. 5.3).

BPC sedimentary contents varied significantly only between sites (Tab. 5.1), with values in sediment and sea cucumbers' feces in Oristano generally significantly higher than those in Teulada. Thus, we show here that, overall, BPC contents in sea cucumbers' feces are not significantly different from that in the sediment, suggesting that the total amount of organic C in sediments colonized by holothurians would not change significantly because of their feeding activity, at least in the short term. While this agrees

with the results obtained with other holothurians species, when considering the feces (Mercier *et al.*, 1999), it does not agree with the results of experiments showing that the amount of organic matter in sediments used by holothurians for their feeding (not considered here) can decrease on longer time scales (Paltzat *et al.*, 2008; Slater and Carton, 2009; MacDonald *et al.*, 2013; Neofitou *et al.*, 2019), thus providing a tool for a long-term reduction of sedimentary OM loads.

On the other hand, the results of the multivariate PERMANOVA revealed that OM biochemical composition varied significantly among Sites, Months and Matrices, with significant effects also of Si×Mo and Mo×Ma interactions (Tab. 5.3). More in detail, in Oristano significant differences in OM composition between sediment and feces occurred in April 2019 mainly due to the reduction of the lipids content in the feces, and in June 2019 and in Jul-Aug 2020 due to higher contents of both proteins and lipids in the feces (Tab. 5.4; Fig. 5.4). In Teulada significant differences in OM biochemical composition between sediment and feces occurred in August 2019 and Jan-Feb 2020, when contents of all chemical classes of organic compounds and their sum (BPC) in the feces were significantly higher than those in the sediment (Tab. 5.4; Fig. 5.4).

Since the trophic status of a benthic marine ecosystem is a combination of OM quantity and composition rather than a mere reflection of total organic C contents (Pusceddu *et al.*, 2009), our results allow us to conclude that *H. tubulosa* can modify the trophic status of the sea bottom where they feed on. This change, however, does not apparently have any influence on C contents, rather on its composition and nutritional quality, though with some differences in the two study sites. More in detail, our results show that, in both sites, feces can at times be enriched in protein and lipid contents when compared to the sediment, more pronouncedly in the most productive site (Oristano). Since both proteins and lipids are high-energy compounds used by holothurians for their somatic growth and gonad development, respectively (Yang *et al.* 2006; Slater *et al.*, 2011b), their enrichment in the feces could appear counterintuitive. Whatever the potential explanation (but see also the next paragraph) this result indicate that feces released by holothurians during their feeding enhance locally and temporarily the nutritional quality of the sediment. Since proteins are N-rich compounds, and N is the most limiting factor for heterotrophic nutrition (Dell'Anno *et al.*, 2002, Pusceddu *et al.*,

2009), the availability of protein-enriched feces could contribute to facilitate the transfer of energy towards higher trophic levels.

H. tubulosa is a continuous feeder that can ingest and rework large amounts of sediment (up to 17 kg m^{-2} dry weight⁻¹ of sediment per year) (Coulon and Jangoux, 1993) and can have a role on recycling seagrass detritus in *Posidonia oceanica* meadows (Costa *et al.*, 2014; Boncagni *et al.*, 2019). Thus, in a raw, *H. tubulosa*, by accelerating the recycling of OM detritus on the sea bottom, could be potentially used to condition the benthic trophic status for additional levels (further reared species, like small scavenging crustaceans) of Integrated Multi-Trophic Aquaculture (IMTA) practices or, even, for the restoration of the benthic trophic status of OM-enriched sea bottoms, for instance by partially reverting the potential adverse consequences of benthic eutrophication caused by mariculture (Neofitou *et al.*, 2019).

5.3.2 Fate of proteins ingested by H. tubulosa: role of food particle selection and estimate of potential assimilation of proteins

As delineated in the previous paragraph, we report here that protein content in *H. tubulosa* feces can, at times, be higher than that in the source sediment. As explained above, such protein enrichment could be counterintuitive, but could be linked to the specific feeding behavior of the sea cucumbers. To shed light on the mechanism behind such phenomenon we compared protein contents in ambient sediment, sediment within the gut and feces.

The results of the univariate PERMANOVA reveal that the protein content of the sediment varied significantly between Sites and among Matrices (Tab. 5.5). However, the results of the post-hoc tests (Tab. 5.6) reveal that, while in the Teulada Gulf (Fig.5.5) the protein content did not vary significantly among ambient sediment, gut sections, and feces, in the Oristano Gulf (Fig. 5.5), protein content in the esophagus is significantly higher than that in the source sediment, and then significantly decrease in the end gut (Tab. 5.6, Fig. 5.5).

We show here that proteins' content in the esophagus can be (though significantly only in the Oristano Gulf) from 2 to 2.7 times higher than that in the source sediment, suggesting the presence of a sort of accumulating or concentration mechanism. This result is consistent with the results reported on the protein content of the deep-sea holothurian

Molpadia musculus gut, in which the concentration factor was estimated to be 3.1 (Amaro *et al.* 2010). Other studies, based on total N or total OM contents, reported a similar pattern in *Apostichopus mollis* (Slater *et al.*, 2011; Zamora and Jeffs 2011) and *Parastichopus californicus* (Paltzat *et al.*, 2008). The increase in proteins (or total OM) contents in sediment from the esophagus can be explained by the presence of a selection mechanism of the food particles ingested by the holothurians. Several studies have indeed proven that holothurians, rather than simply picking up sediment particles with mucus and digestive fluids, can select, possibly driven by chemo-selection mechanisms, food particles with their tentacles (Uthicke and Karez, 1999; Roberts *et al.*, 2000; Dar and Ahmad, 2006; Paltzat *et al.*, 2008; Slater *et al.*, 2011; Navarro *et al.*, 2013; Ramón *et al.*, 2019; Hartati *et al.*, 2020). However, we report here that such a potential explanation appears valid only for holothurians from the more OM enriched bottoms (Oristano). Such a difference could be related to the different sediment grain size in the two sites.

In Oristano sediments comprise a conspicuous fraction of mud and fine sand, which are the target of particles selection mechanisms with buccal tentacles (Mezali and Soualili, 2013), resulting in a potential assimilation of 30% and 40% of the ingested proteins in the digestive tract, in the Oristano and Teulada site, respectively. The different protein assimilation rates in holothurians from the two sites can be related to the inverse relationship between assimilation rates and availability of organic substrates (Roberts *et al.*, 2000; Zamora *et al.*, 2011), with the latter higher in Oristano (30% assimilation) than in Teulada (40% assimilation). Overall, thus, our results strongly support the hypothesis that the sea cucumber *H. tubulosa* can select food particles from the surrounding sediment, concentrate them in the esophagus, but also that the potential assimilation is strongly dependent upon the relative amount of food available.

5.3.3 Concluding remarks

The results of this study allowed us to demonstrate that the sea cucumber *H. tubulosa* can influence the benthic trophic status, specifically modifying the biochemical composition of sedimentary organic matter rather than its content.

Our results proved also that *H. tubulosa* influences particularly the sedimentary contents of the most labile molecules (i.e., proteins and lipids) and that, by producing

protein- and lipid-enriched feces, can contribute to make more labile substrates available for higher trophic levels.

Based on the changes in protein concentration of sediment within the digestive tract we also provided elements in support of the hypothesis of an active selection of labile food particles by holothurians and that this mechanism is, however, dependent upon the actual availability of organic substrates.

Tab. 5.1: Results of univariate PERMANOVA testing for the effects of Sites (Si), Months (Mo) and Matrices (Ma) and their interactions on protein, carbohydrate, lipid and BPC contents. DF = degree of freedom; MS = mean squares; Pseudo-F = F; P(MC) = probability level after Monte Carlo simulations. (*) = P < 0.05; (**) = P < 0.01; (***) = P < 0.001; ns = P > 0.05.

Source	DF	Proteins				Carbohydrates			
		MS	Pseudo-F	P(MC)	% EV	MS	Pseudo-F	P(MC)	% EV
Site (Si)	1	28.886	53.338	***	33	0.432	0.445	ns	0
Month (Mo)	5	3.532	6.521	***	11	1.252	1.290	ns	2
Matrices (Ma)	1	6.714	12.397	***	7	0.680	0.701	ns	0
Si×Mo	5	1.202	2.220	ns	5	1.534	1.580	ns	6
Si×Ma	1	0.504	0.931	ns	0	0.762	0.786	ns	0
Mo×Ma	5	1.082	1.999	ns	4	0.331	0.341	ns	0
Si×Mo×Ma	5	1.037	1.915	ns	7	0.157	0.161	ns	0
Residual	115	0.542			33	0.970			92

Source	DF	Lipids				BPC			
		MS	Pseudo-F	P(MC)	% EV	MS	Pseudo-F	P(MC)	% EV
Site (Si)	1	25.299	57.514	***	27	7.513	8.714	**	11
Month (Mo)	5	0.537	1.220	ns	0	1.253	1.453	ns	2
Matrices (Ma)	1	2.516	5.721	*	2	2.512	2.913	ns	3
Si×Mo	5	1.368	3.111	*	6	1.417	1.643	ns	6
Si×Ma	1	0.160	0.364	ns	0	0.177	0.205	ns	0
Mo×Ma	5	2.787	6.335	***	15	0.893	1.036	ns	0
Si×Mo×Ma	5	2.235	5.080	***	23	0.450	0.521	ns	0
Residual	115	0.440			25	0.862			78

Tab. 5.2: Results of the pairwise comparisons contrasting proteins and lipid contents between sediment and feces in the two study sites in all sampling dates. $t = T$ value, $P(\text{MC}) =$ probability level after Monte Carlo simulations. (*) = $P < 0.05$; (**) = $P < 0.01$; (***) = $P < 0.001$; ns = not significant.

Site	Month	Proteins		Lipids	
		t	P(MC)	t	P(MC)
Oristano (Sandy- Mud)	Apr	1.003	ns	3.092	*
	Jun	6.783	***	3.258	*
	Aug	0.930	ns	3.282	**
	Jan - Feb	2.150	ns	0.987	ns
	May - Jun	0.178	ns	2.788	*
	Jul - Aug	4.022	**	2.490	*
Teulada (Sandy)	Apr	1.810	ns	2.231	ns
	Jun	0.039	ns	1.385	ns
	Aug	3.240	*	4.584	**
	Jan - Feb	6.508	***	0.620	ns
	May - Jun	0.225	ns	0.107	ns
	Jul - Aug	0.991	ns	1.023	ns

Tab. 5.3: Results of multivariate PERMANOVA testing for the effects of Site, Month and Matrices and their interactions on the biochemical composition of OM. DF = degree of freedom; MS = mean squares; Pseudo-F = F; P(MC) = probability level after Monte Carlo simulations. (*) = $P < 0.05$; (**) = $P < 0.01$; (***) = $P < 0.001$; ns = $P > 0.05$.

Source	DF	MS	Pseudo-F	P	%EV
Site (Si)	1	54.617	27.982	***	24
Month (Mo)	5	5.321	2.726	**	5
Matrices (Ma)	1	9.910	5.077	*	4
Si×Mo	5	4.104	2.103	*	6
Si×Ma	1	1.427	0.731	ns	0
Mo×Ma	5	4.200	2.152	*	6
Si×Mo×Ma	5	3.429	1.757	ns	8
Residual	115	1.952			47

Tab. 5.4: Results of the pairwise comparisons testing for differences in the biochemical composition between sediment and feces in the two study sites during the study period. t = T value, P(MC) = probability level after Monte Carlo simulations. (*) = $P < 0.05$; (**) = $P < 0.01$; ns = not significant.

Site	Month	t	P(MC)
Oristano	Apr	2.397	*
	Jun	2.057	*
	Aug	1.823	ns
	Jan - Feb	1.408	ns
	May - Jun	1.306	ns
	Jul - Aug	2.736	**
	Apr	1.519	ns
Teulada	Jun	0.652	ns
	Aug	3.426	**
	Jan - Feb	3.106	**
	May - Jun	0.120	ns
	Jul - Aug	1.228	ns

Tab. 5.5: Results of the univariate PERMANOVA carried out to test for differences in the protein content of the source sediment, digestive tract and feces of *H. tubulosa* in the two study sites (Oristano vs. Teulada). DF = degrees of freedom; MS = mean square; Pseudo-F = F statistic; P(MC) = probability level after Monte Carlo simulations %EV = percentage of explained variation; (*) = $P < 0.05$; ns = not significant.

Source	DF	MS	Pseudo-F	P(MC)	%EV
Site (Si)	1	5.1095	7.3881	*	24
Matrices (Ma)	4	1.9695	2.8477	*	14
Si×Ma	4	1.3179	1.9055	ns	14
Residual	26	0.6916			48

Tab. 5.6: Results of the pairwise comparison testing for difference in protein contents in the source sediment (S), digestive tracts (esophagus = ESO; mid gut = MID; end gut = END) and feces (F) in the two study sites (Oristano and Teulada). P(MC) = probability level after Monte Carlo simulations; t = t value, (***) = $P < 0.001$; (**) = $P < 0.01$, (*) = $P < 0.05$; ns = not significant.

Site	Contrast	t	P(MC)
Oristano	S, F	6.783	***
	S, ESO	6.860	***
	S, MID	0.061	ns
	S, END	3.537	*
	F, ESO	2.100	ns
	F, MID	2.862	ns
	F, END	4.063	*
	ESO, MID	2.682	ns
	ESO, END	7.475	**
	MID, END	1.369	ns
Teulada	S, F	0.039	ns
	S, ESO	0.682	ns
	S, MID	0.518	ns
	S, END	0.041	ns
	F, ESO	0.942	ns
	F, MID	0.603	ns
	F, END	0.140	ns
	ESO, MID	0.134	ns
	ESO, END	1.037	ns
	MID, END	0.615	ns

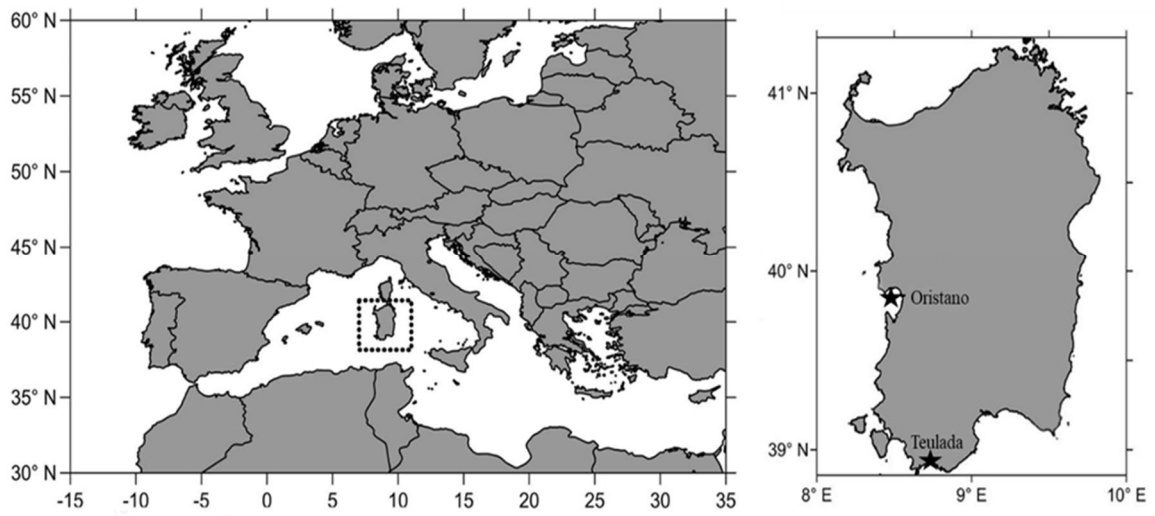


Fig. 5.1: Location of the sampling areas in the Oristano and Teulada Gulfs.

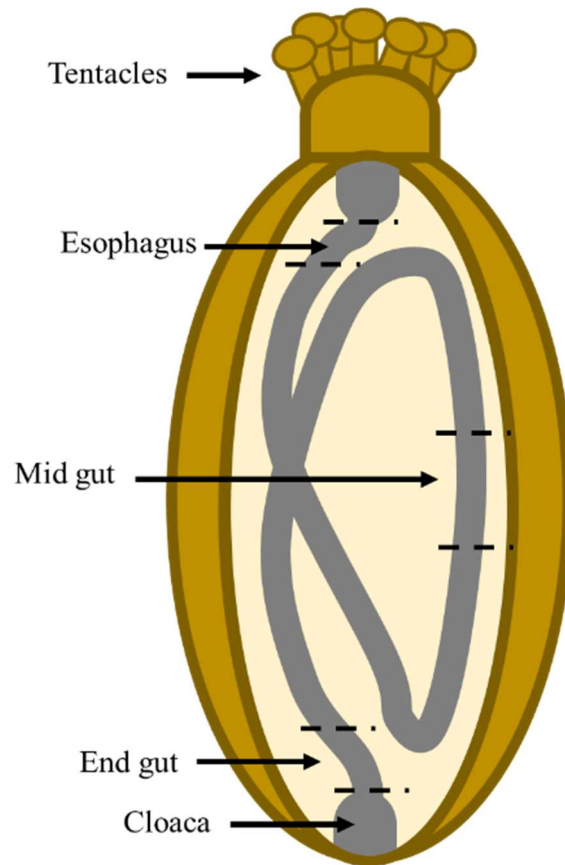


Fig. 5.2: Schematic representation of sea cucumbers' digestive tract.

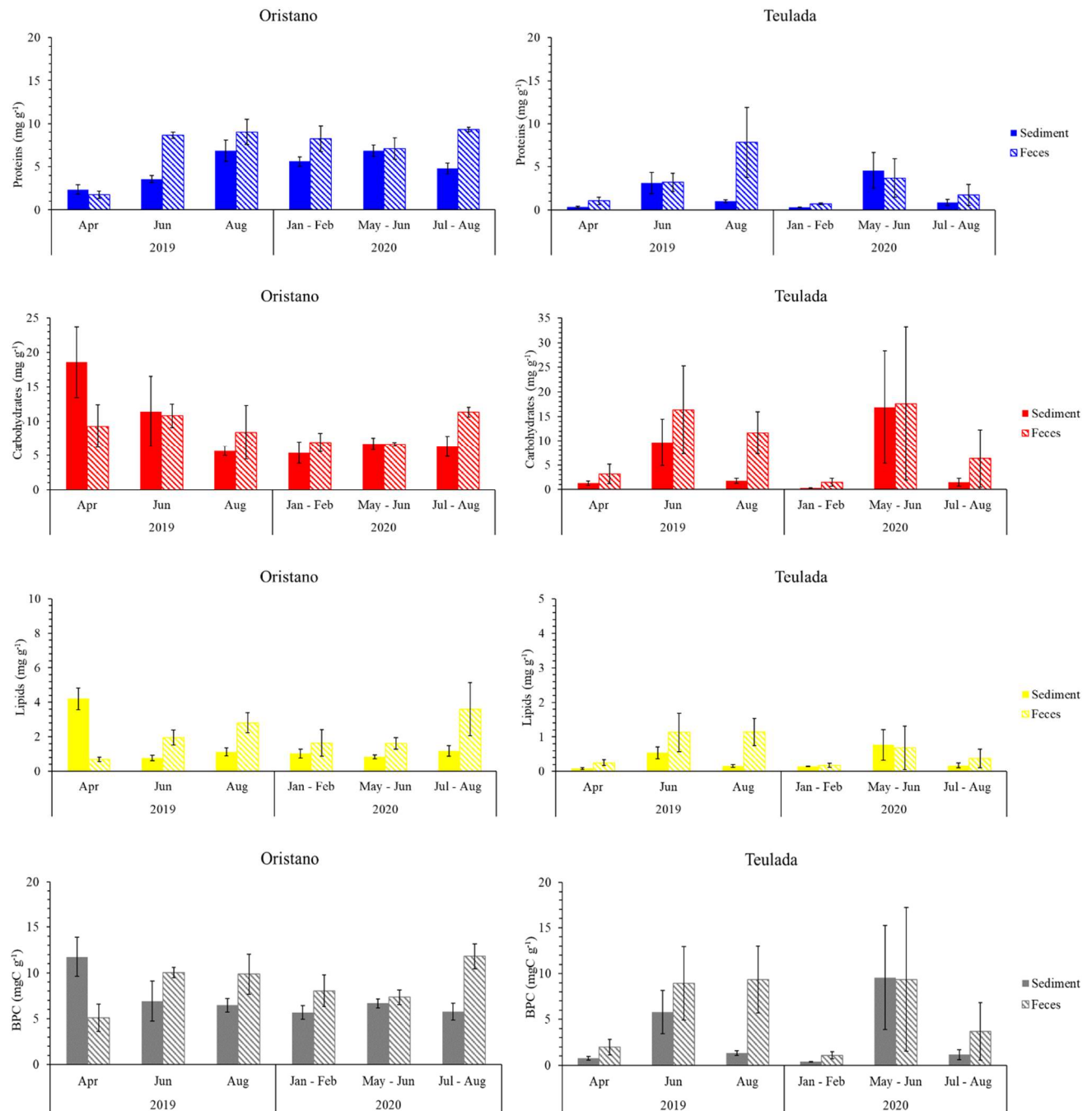


Fig. 5.3: Protein, carbohydrate, lipid, and BPC contents in sediment and feces from Oristano and Teulada during the study period. Error bars indicate standard error.

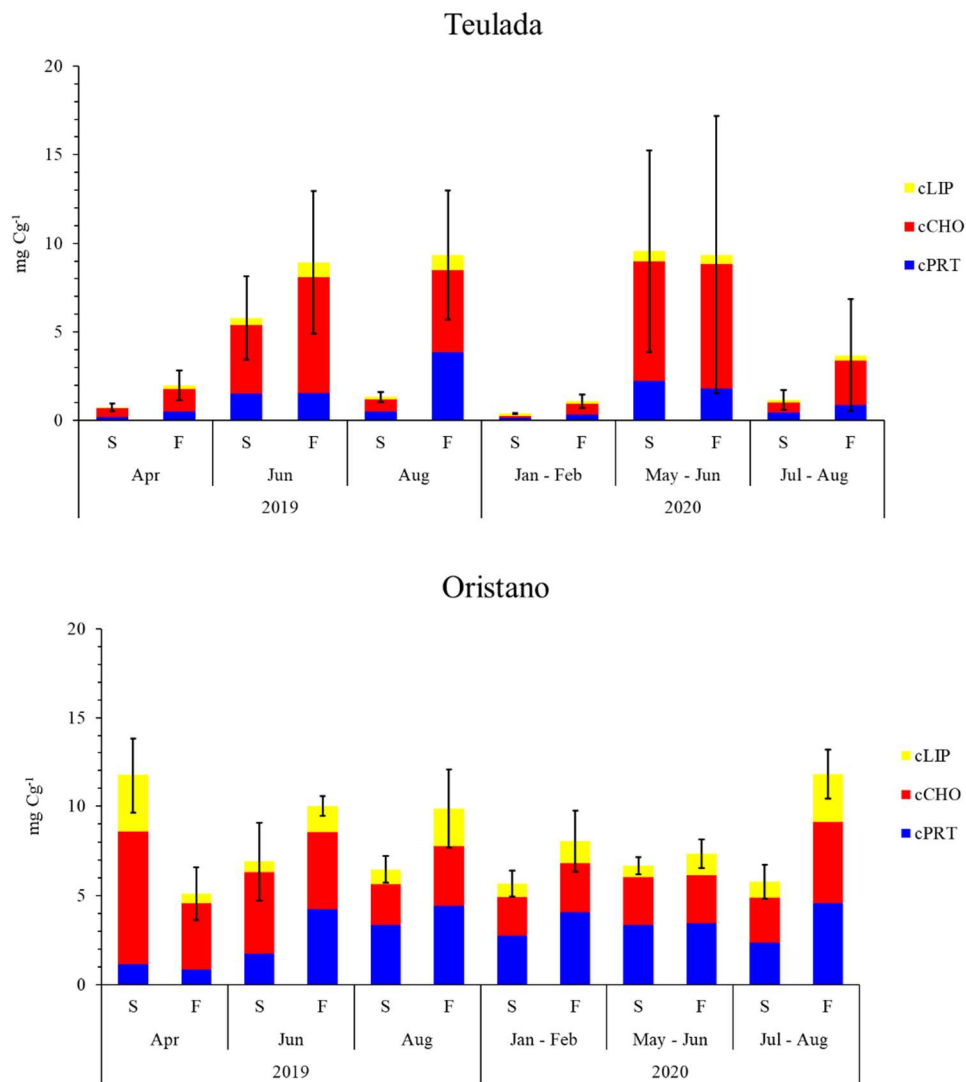


Fig. 5.4: Variations in sediment (S) and feces (F) OM contents (proteins, carbohydrates and lipids expressed in mgCg^{-1}) in Oristano (Sandy-Mud) and Teulada (Sandy). S= sediments; F=feces. Error bars indicate the standard error of the biopolymer C. cLIP = lipid; cCHO = carbohydrate; cPRT = protein.

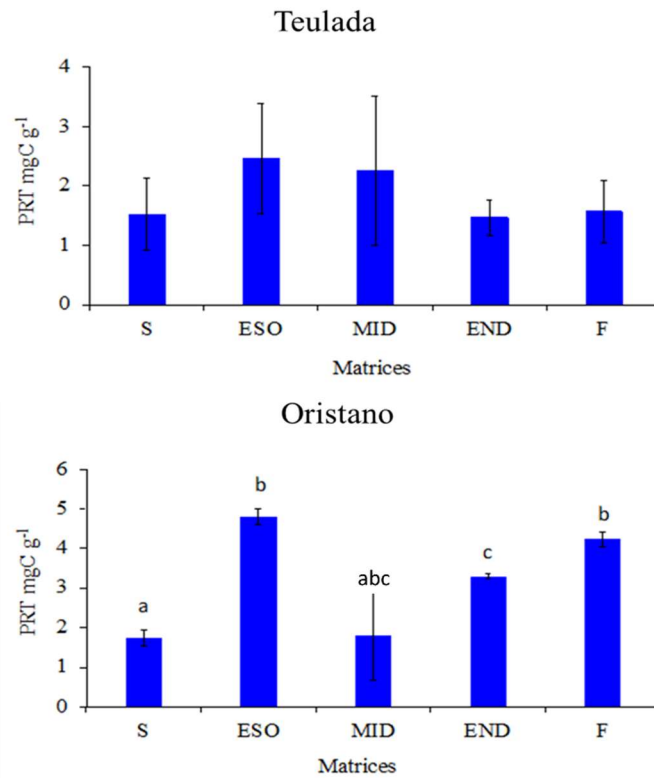


Fig. 5.5: Protein contents of source sediment (S), sediment in the esophagus (ESO), mid gut (MID), and end gut (END) and feces (F) of holothurians from the two study sites. Lowercase letters indicate the results of the post-hoc tests as from the results reported in Tab. 5.5. Error bars indicate standard error.

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Chapter 6

General conclusions and future perspectives

6.1 Recap of most relevant results

This thesis aimed at investigating biological and ecological traits, including the reproductive biology, larval development, feeding behavior and trophodynamic role of the sea cucumber *Holothuria tubulosa*.

In **Chapter 1**, I narratively reviewed the information on the holothurians' biology and exploitation and management issues, providing cues on their potential use for IMTA practices, with focus on the Mediterranean species *Holothuria tubulosa*.

In **Chapter 2**, I explored temporal patterns of the reproductive biology of *H. tubulosa* in the Central Western Mediterranean Sea, identifying the different stages of gonads development through new macroscopic approaches based on the color of gonads, confirmed by the histological analyses.

In **Chapter 3**, I explored the different stimuli triggering the spawning of *H. tubulosa*, identifying air bubbling as the most effective. In this regard, I inferred that the positive effect of water turbulence on the release of gametes might be an adaptive strategy of this species to favor fertilization and dispersal of resulting zygotes and then, larvae. I report also that eggs' hatching rate decreases with the increase of the stocking density of fertilized eggs.

In **Chapter 4**, I explored the use of different algal biofilms, commonly used for the settlement of sea cucumbers larvae and other echinoderms larvae, as a cue to induce the metamorphosis of *H. tubulosa* doliolaria. The experiments revealed that the best settlement and survival of the post-settlers occurs when the *Ulvela lens* biofilm.

In **Chapter 5**, I explored the trophodynamic of *H. tubulosa* in two sites with different grain sizes and trophic status. The results of this study reveal that: *i*) the sea cucumber *H. tubulosa* can influence the benthic trophic status, specifically modifying the biochemical composition of sedimentary organic matter rather than its content; *ii*) *H. tubulosa* influences particularly the sedimentary contents of the most labile molecules (i.e.,

proteins and lipids) and, by producing protein- and lipid-enriched feces, can contribute to make more labile substrates available for higher trophic levels; iii) *H. tubulosa* is most likely able to actively select labile food particles with the tentacles and that this mechanism is dependent upon the actual availability of organic substrates.

6.2 Future perspectives

The overexploitation of sea cucumbers claims for the urgent need of applying a management policy to regulate the sea cucumbers fishery and to fight the illegal catches. The fishing pressure reached such high levels to affect most commercial stocks, and, in certain cases, the wild stocks are already insufficient to support commercial harvests or stock recovery. The lack of documented information about these fisheries makes, to date, almost impossible predicting sea cucumbers' stocks recovery, if ever possible. On the one hand, this claims for the need of more and more data, relying on science-based assessment of stocks size, but also on the biology and reproduction potential of wild populations. Intensive rearing of new biomass after reproduction and settlement in captivity could represent a possible "exit strategy" to counteract stocks impoverishment with large-scale restocking practices. If holothurians rearing in captivity will become an easy, safe and low-cost procedure, based on the role of holothurians in regulating OM loads in marine sediments, they, on the one hand, appear to be the best candidates for restoration actions of eutrophicated sediments, but also important targets of IMTA practices, especially if accompanied by other species profiting of the enrichment in labile substrates determined by the release of feces by holothurians.

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