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Study of the reproductive biology and chemical communication of sea cucumbers (*Holothuria arguinensis* and *H. mammata*)

UNIVERSIDADE DO ALGARVE

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Doutoramento em Ciências do Mar, da Terra e do Ambiente

Ramo Ciências do Mar

Especialidade em Ecologia Marinha

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Declaração de autoria de trabalho:

Declaro ser a autora deste trabalho que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Declaration of authorship:

This work has not previously been submitted for a degree in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Nathalie Marquet

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To myself I am only a child playing on the beach, while vast oceans of truth lie undiscovered before me.

Isaac Newton

Summary

New sea cucumber fisheries are emerging in the Mediterranean Sea and Atlantic Ocean in response to a strong Chinese market demand. However, little is known about the biology of the new target species, hindering decisions on their management. The main objective of the present thesis was to study the reproductive biology and the role played by chemical communication and chemosensory systems in *Holothuria arguinensis* and *Holothuria mammata*. The different populations sampled in a narrow range along the Iberian Peninsula varied in size/weight, gonadal production, and maturity profile within each species, suggesting the influence of singular features of each location. However, they had all the same general reproductive pattern with a summer-autumn spawning. These results, essential to manage populations, were also useful to determine when to develop bioassays to test whether and how these species communicate during reproduction. Male sea cucumbers, but not females, release chemicals that attract and induce spawning in both sexes. A preliminary analysis of the male spawning water suggests a pheromone with multiple components, among them possibly phosphatidylcholine derivatives. Histology, histochemistry and immunohistochemistry of the potential chemosensory structures involved in the detection of these cues – tentacles, papillae and tube feet – show no obvious differences between them. However, the disc was the most specialized area, with a specific nerve arrangement, rich in nitric oxide synthase and containing numerous cells some of which are likely sensory neurons. The analysis of tissue transcriptomes revealed the presence of at least 591 G-protein-coupled receptors among them at least seven putative odorant receptors distributed mainly in the tentacles, oral cavity, calcareous ring and, papillae and tegument. Overall, this thesis gives valuable insights for sea cucumber fisheries management in the region and a better understanding of how sea cucumbers communicate during reproduction.

Key words: sea cucumbers, reproduction, pheromones, chemoreceptors, transcriptome

Sumário

Estão a emergir novas pescarias de pepinos do mar no mar Mediterrâneo e Oceano Atlântico em resposta à forte procura do mercado chinês. Contudo, sabe-se pouco sobre a biologia e história de vida destas novas espécies alvo, o que prejudica as decisões sobre a sua gestão. O principal objectivo desta tese é o estudo da biologia reprodutiva e o papel da comunicação química e sistema quimiosensorial em *Holothuria arguinensis* e *Holothuria mammata*. Sabe-se que a comunicação química está envolvida nos processos reprodutivos em vários invertebrados marinhos. No entanto, tem sido pouco investigada nos equinodermes e sabe-se pouco acerca da natureza química dos compostos e dos mecanismos sensoriais envolvidos, assim como dos efeitos que desencadeia. Esta tese tem como objectivos específicos: i) adquirir conhecimentos sobre as características reprodutivas das espécies de interesse, ii) determiner se e como os pepinos do mar comunicam durante a reprodução e procurar identificar os compostos envolvidos, iii) caracterizar as estruturas em contacto directo com o ambiente que potencialmente estão envolvidas na detecção de sinais químicos, e iv) caracterizar o reportório de quimioreceptores e identificar possíveis receptores de odorantes nos tecidos analisados.

As populações de *H. arguinensis* e *H. mammata* amostradas numa estreita faixa territorial da Peninsula Ibérica variaram tamanho, perfil de produção e maturação das gónadas, o que sugere a influência de características únicas de cada local. Havia maiores indivíduos de *H. arguinensis* e com gónadas mais pesadas em Sagres e na Ria Formosa comparado com Olhos de Água onde se encontraram mais indivíduos imaturos. A gametogénese e a postura era mais longa em Sagres o que pode ser devido a condições nutricionais e de maré (subtidal vs intertidal). Os indivíduos de *H. mammata* na Ria Formosa também eram maiores e com gónadas mais pesadas do que em Murcia e Olhos de Água, possivelmente ligado a diferenças n alimentação devido ao tipo de substrato (vasa/areia vs rochas). Estes resultados, incluindo o tamanho à primeira maturação e fecundidade, são importantes para a gestão das espécies nestas regiões. Além disso, sabendo quendo estas espécies se reproduzem permitiu determiner quando deviam ser feitos os bioensaios para estudar a comunicação química nos pepinos de mar.

Os bioensaios tiveram como objectivo testar a hipótese de que os pepinos de mar libertam químicos durante a agregação e postura. Os resultados mostram que a água condicionada por machos, mas não homogenatos de gónada ou fluido celómico, atrai machos e fêmeas. Também, a água condicionada de macho, com ou sem espermatozóides, induziu a postura em ambos o sexos, sendo que a água de fêmea não teve qualquer efeito. Isto mostrou

claramente que os machos atraem e induzem a postura em ambos os sexos. A água de macho foi analisada através de cromatografia líquida associada a espectrometria de massa, sendo identificada a presença de derivados de fosfatidil colina; contudo, a sua função potencial como feromonas necessita de confirmação experimental.

As estruturas corporais em contacto difecto com o ambiente – os tentáculos, as papilas e os pés ambulacrários – e potencialmente envolvidos ma detecção de estímulos químicos foram caracterizadas através de histologia, histoquímica e imunocitoquímica para identificar terminais nervosos que pudessem ser utilizados para electrofisiologia e fazer a triagem dos possíveis coompostos feromonais. Não se encontraram diferenças óbvias na organização nervosa e tecidular. Contudo, o disco na extremidade destas estruturas parece ser um candidato promissor para ensaios electrofisiológicos futuros pois confirma-se ser a área snsorial de cada estrutura.Ó disco caracteriza-s por um arranjo nervoso específico, incluindo uma placa nervosa distinta, rica em óxido nitroso e contendo abundantes células, muitas das quais positivas para sinaptotagmina e serotonina, provavelmente neurónios sensoriais anteriormemte descritos.

Finalmente, para melhor compreender como a percepção sensorial ocorre nos pepinos do mar, o reportório de receptores acoplados a proteínas G (GPCR) foram analisados em seis bibliotecas de transcriptómica de diferentes tecidos (tentáculos, ovário, testículo, cavidade oral, anel calcáreo, papila e tegumento). Foram identificados 591 GPCR através de pesquisa de sequências por semelhança; contudo não foram encontrados ortólogos de receptores de odorantes humanos. Através da análise filogenética, que incluiu sequências proteicas de humano, enguia anfioxo, ouriço marinho e anémona, pelo menos sete receptores de odorantes foram encontrados no pepino do mar. Estas sequências pertencem a dois grupos, um mais próximo do ouriço do mar e outro mais próximo da anémona, o que sugere origem de diferentes ancestrais. Foram encontrados amino ácidos conservados característicos da assinatura de receptores de odorantes de metazoários. Além disso, estes estavam presentes principalmente nos tentáculos, cavidade oral, anel calcário, papila e tegumento, em acordo com uma potencial função sensorial.

No seu conjunto, esta tese fornece nova e importante informação para a gestão dos pepinos do mar e para melhor compreender como comunicam durante a reprodução.

Palavras chave: pepinos do mar, reprodução, feromonas, receptores quimiosensoriais, transcriptoma.

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Chapter I

General introduction

Sea cucumbers are fished worldwide and mainly exported to Asian countries where they are consumed for their nutritional and medicinal properties (Chen 2003; Conand 1989). Due to uncontrolled exploitation and/or inefficient management, sea cucumber populations are suffering from overfishing, mainly in the Indo-Pacific region (Anderson et al. 2011; Purcell et al. 2013). This situation, driven by Chinese demand and reduced stocks elsewhere, has resulted in the development of new sea cucumber fisheries in the Mediterranean Sea and Atlantic Ocean (González-Wangüemert and Borrero-Pérez 2012; González-Wangüemert et al. 2013b). However, the biology of the new target species, including *Holothuria arguinensis* and *H. mammata*, is poorly understood, which hinders their management. One of the key aspects in their management is the understanding of reproductive biology to allow the development of adequate fisheries plans and breeding/restocking aquaculture. Consequently, reproductive cycle, fecundity or size at first sexual maturity parameters are needed in these new target species. Furthermore, there is little knowledge about the ecology of these animals and the factors that control the reproductive cycle, spawning aggregations and spawning itself.

Among the factors controlling reproduction, temperature and photoperiod play an important role, although their relative contribution for gonadal growth and spawning is still not completely understood (Mercier and Hamel 2009). Furthermore, photosensitivity in these animals probably plays a limited role and they may rely on chemical communication for attraction and reproductive synchrony as in other invertebrates (e.g. Hardege and Bentley 1997; Watson et al. 2003). However, studies of chemical communication in sea cucumber remain scarce and mostly speculative; as broadcast spawners, they are expected to depend on chemical exchanges during reproduction. Until now, the only clear evidence of chemical communication in sea cucumbers demonstrated that exposure to mucus produced by mature individuals accelerated the gonadal development of less mature individuals of the same sex (Hamel and Mercier 1996a, 1999). A part from that, little is known about the nature of the compounds, the sensory mechanisms involved and the effects elicited.

Thus, the present thesis will investigate the reproductive processes in sea cucumbers and the possible role of chemical communication in aggregation and spawning in sea cucumbers.

1.1. Thesis aims

The main objective of this thesis was to study the reproductive biology, including chemosensory systems and chemical communication in sea cucumbers. The focus of the study was *Holothuria arguinensis*, the most abundant species, although the reproductive cycle of *H. mammata* was also studied.

The specific objectives, reflecting each experimental chapter, were:

1) Acquire basic knowledge on the reproductive characteristics of the two species, including their reproductive cycles, reproductive structures and the possible influence of the environmental factors on their reproductive activities.

2) Determine whether and how sea cucumbers use chemical communication during their reproduction, and attempt to identify the compounds involved.

3) Characterize the sensory structures in contact with the environment, namely the tentacles, papillae and tube feet, as a preliminary step to electrophysiology.

4) Characterize the chemosensory G-protein-coupled receptors (GPCRs) in target tissues with a potential role in chemosensation, and identify putative odorant receptors (ORs) and their distribution within these tissues.

1.2. Thesis outline

This thesis is organized into six chapters, starting with a general introduction followed by four experimental chapters and a final chapter with an overall discussion and conclusions.

Chapter I explains the reasons why this work was performed and describes the thesis aims and outline. Sea cucumbers as model organisms and an exploited resource are introduced and the importance of studying their reproductive biology is highlighted. Then, the reproductive characteristics of sea cucumbers and chemical communication related to reproduction are reviewed. To conclude this chapter, the two studied species, *H. arguinensis* and *H. mammata*, are presented.

Chapter II (aim 1) describes and compares the reproductive biology of *H. arguinensis* and *H. mammata* from different locations in the Iberian Peninsula. It reveals that populations from both species living in a narrow latitudinal range display the same general reproductive pattern with a summer-autumn spawning. Differences in size/weight, gonadal production and maturity profile among the three locations within each species were seen, and suggested to be influenced by the specific features of each location. The influence of temperature and photoperiod are then discussed in relation to the reproductive cycle in *H. arguinensis*.

Chapter III (aim 2) tests the hypothesis that sea cucumbers release chemical compounds to aggregate and spawn. The main results obtained in this chapter reveals that males release chemicals that attract and induce spawning in male and female conspecifics, although females do not have any effect on aggregation and spawning in either sex. The male pheromone is probably a mixture with at least one labile compound. Preliminary evaluation of male spawning water showed the presence of a phosphatidylcholine; however, further investigation is needed to assess its role as part of the spawning pheromone.

Chapter IV (aim 3) characterizes the structures in contact with the environment, namely the tentacles, papillae and tube feet. All structures presented similar tissue and nervous composition, and were rich in nitric oxide synthase. The disc of the structures was revealed as a strong candidate to perform electrophysiology as it was the most specialized area with a specific nervous arrangement constituted of a distinct nerve plate from where nervous fibers extended in the disc and to the stem. The disc contained many cells arranged differently according to the structures, some of them might correspond to the sensory neurons described previously in ultrastructural studies.

Chapter V (aim 4) reveals the presence of at least 591 GPCRs and at least seven putative odorant receptors (ORs) from six target tissues (calcareous ring, oral cavity, testis, ovary, tentacles, papillae and tegument). The candidate odorant receptors cluster in two groups: one with the sea urchin and other with the sea anemone. Conserved amino acids characteristic of OR motifs were found in these sequences, and their distribution reveals that they are mainly found in the tissues in contact with the environment. The evolution of ORs is then discussed.

Chapter VI integrates the major finding of each separate chapter into a final discussion, and highlights the main conclusions. It also discusses the research perspectives that should be undertaken in the future.

1.3. What are sea cucumbers and why are they important?

1.3.1. The phylum Echinodermata: from a symbol of marine life to a model organism

The phylum Echinodermata contains approximately 7,000 extant species, exclusively marine, and occurring in all marine habitats from shallow intertidal areas to the deep sea, and in all climate zones. They are grouped into five well-defined classes containing some of the most charismatic marine invertebrates: Asteroidea (starfishes), Crinoidea (sea lilies and feather stars), Echinoidea (sea urchins), Holothuroidea (sea cucumbers) and Ophiuroidea (brittle stars). As such, the phylum has become a symbol of marine life (Micael et al. 2009).

Echinoderms occupy a unique phylogenetic position in the animal kingdom as they are the only major group of invertebrates within the deuterostome clade, which includes the chordates (Turbeville et al. 1994; Van Veghel 1993). They share many distinguishing features, including the presence of a water-vascular system, a calcium carbonate endoskeleton and a radial pentamerous symmetry developing from bilaterally symmetrical larvae (Fell 1972). Due to their unique characteristics and evolutionary position, echinoderms have been used for more than a century as model organisms in different research areas, including cell biology and immunology (e.g. Brown 1995; Evans et al. 1983).

Currently, research on echinoderms covers broad fields such as evolutionary developmental biology and biotechnology (Arnone et al. 2015; Matranga 2005). They are also target organisms in marine ecology studies due to their high ecological relevance, with several described keystone species (Iken et al. 2010; Navarrete et al. 2000). Additionally, they are valuable bio-indicators in ecotoxicological studies due to their sensitivity to many types of contaminants (Coteur et al. 2003; Dupont and Thorndyke 2007).

1.3.2. Sea cucumbers: weird creatures, valuable resources

Holothurians, known as sea cucumbers, are a diverse and abundant group of soft bodied and worm-like animals belonging to the class Holothuroidea which includes approximately 1,400 species divided into 6 orders: Aspidochirotida, Dendrochirotida, Malpodida, Apodida, Elasipodida and Dactylochirotida (Pawson 1982). They represent a considerable part of the benthos, up to 90% of the biomass in many areas, but with some holopelagic species (Miller and Pawson 1990; Rogacheva et al. 2012).

Sea cucumbers are the only class of the phylum Echinodermata to have an unpaired gonad, a calcareous ring, a tentacular feeding organ, and a secondary bilateral symmetry (for summary, see Pawson 2007) (Figure 1). Their endoskeleton consists of microscopic calcareous

spicules, unlike other echinoderms that possess well-developed skeletal plates (Gutschick 1979). Some species possess peculiar organs, Cuvierian tubules, which are expelled through their anus when threatened (VandenSpiegel et al. 2000). However, the most impressive characteristic of sea cucumbers is their ability to regenerate all their internal organs ejected after evisceration, a process which occurs when sea cucumbers are confronted by a chemical or physical stress (García-Arrarás and Greenberg 2001; Mashanov and J.E. 2011; Wilkie 2001; Zang et al. 2012).

Although sea cucumbers might seem unattractive at first sight, they have ecological as well as socio-economic values associated with nutritional and medicinal properties, which make them particularly important. As sea cucumbers are mostly deposit feeders and bioturbators, they are key components in maintaining healthy marine ecosystems by recycling of nutrients, stimulating algal growth, oxygenating sediment and regulating both carbonate content and water pH (Massin 1982; Purcell et al. 2013; Schneider et al. 2011; Uthicke 2001; Uthicke and Klump 1998; Wolkenhauer et al. 2010). Besides their ecological importance, sea cucumbers represent an important economic resource mainly exported to Asian countries where they are used in traditional medicine, as culinary delicacies and as aphrodisiacs (Bordbar et al. 2011; Chen 2003; Chen 2004; Conand 1989). Their trade is also important in developing countries where they represent a considerable source of income (Eriksson et al. 2011; Friedman et al. 2011; Ochiewo et al. 2010).

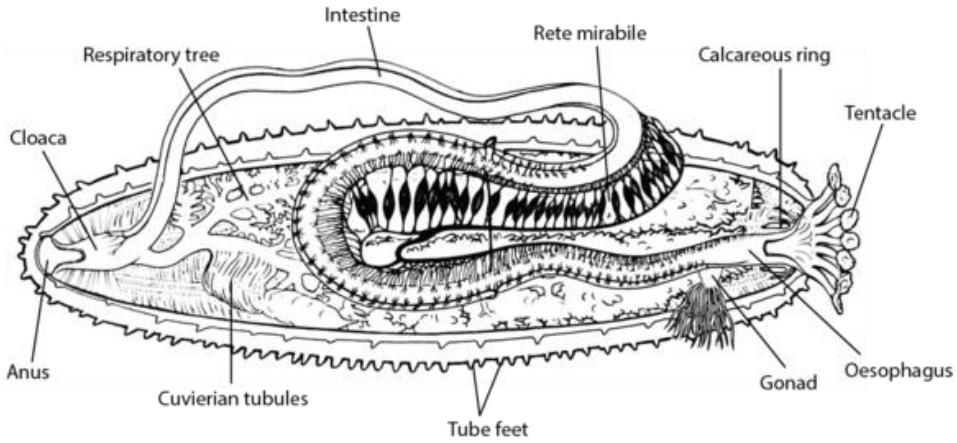

Figure 1. General anatomy of a sea cucumber (Cannon and Silver 1986).

1.4. Sea cucumber exploitation

Sea cucumbers are a highly appreciated seafood product which is exploited in wild fisheries and is commercially cultivated. The main consumed product is the dried body wall, commonly known in western Pacific islands as "bêche-de-mer" (according to the Merriam-Webster dictionary sea worm from the Portuguese "bicho-do-mar"), although the viscera and the body wall are also eaten raw or pickled (Akamine 2004; Conand 1989; Conand and Byrne 1993).

Sea cucumber fisheries have developed worldwide with the largest areas exploited in the Indo-Pacific region. The annual global catch of sea cucumbers has been estimated at 100 000 tons (Purcell 2010) with an annual global market value valued between US$ 56 and 130 million (Ferdouse 2004). Most of the commercial species belong to the orders Aspidochirotida and Dendrochirotida, with at least 66 species fished in more than 70 countries worldwide (Conand 2006b; Purcell 2010). Sea cucumber fisheries are very diverse, ranging from artisanal fisheries, where tropical species are collected by hand or free-divers in shallow waters, to industrialized fisheries with both dredging boat and SCUBA/hookah diving for temperate species (Kinch et al. 2008; Purcell et al. 2013).

Due to the ever-increasing Chinese demand, combined with ineffective fisheries management, sea cucumber populations are suffering from a dramatic decline worldwide, particularly in the Indo-Pacific region (Anderson et al. 2011; Kinch et al. 2008; Purcell 2010; Purcell et al. 2013). This is compounded by specific biological characteristics, such as slow growth rate, late maturity, low recruitment rate and density-dependent reproductive success, that make sea cucumbers particularly vulnerable to overfishing (Bruckner et al. 2003; Conand 2006a, b; Uthicke et al. 2004).

As an answer to overexploitation, sea cucumber aquaculture was considered as a solution (Conand 2008). China is the country that has developed the most extensive sea cucumber aquaculture with an annual production exceeding 80,000 tons (Chang and Yu 2004; Chen 2004). Nowadays, many countries in the Indo-Pacific are developing sea cucumber aquaculture as a priority (Jimmy et al. 2012). Fisheries plans have also been considered to react to overfishing but some populations have been so decimated that their recovery is improbable even with the best regulatory measures (Friedman et al. 2011). This has resulted in the development of new fisheries in the north-eastern Atlantic Ocean and the Mediterranean Sea targeting new species, including *Holothuria arguinensis* and *H. mammata* (see section 1.8 for descriptions of these species) (Aydin 2008; González-Wangüemert and Borrero-Pérez 2012;

González-Wangüemert et al. 2013a; González-Wangüemert et al. 2013b; Sicuro and Levine 2011). The problem with these new fisheries is the lack of biological knowledge about the new target species which will hinder decisions on population management. As a step to acquire knowledge about the new target species, the present thesis focused on the reproductive biology of *H. arguinensis* and *H. mammata*.

1.5. Why study reproductive biology ?

Reproductive biology is an important subject to study in order to support sustainable fisheries activities (Morgan 2008). Among the strategies that can be established based on reproductive information are a minimum capture size and a closed season to protect spawning individuals. Ideally, these regulatory measures should be established before a fishery is exploited; however, this is rarely the case. Another area where reproductive biology knowledge plays a critical role is in the development of breeding and aquaculture programs, useful to help in supplying the strong market demand, but also to restore and enhance wild stocks suffering from overexploitation (Mercier and Hamel 2009; Wang et al. 2015). Much progress has been made in sea cucumber aquaculture; however, several steps such as the spawning induction (see section 1.7.1), still need to be optimized to improve profitability and reliability (Purcell et al. 2012). Finally, understanding the reproductive processes can be of a great help in managing invasive species, as a result of human introduction or global warming, notably by the possible use of reproductive pheromones (e.g. using pheromone traps) (Corkum and Belanger 2007).

In the present study, the reproductive biology was investigated through the study of the reproductive characteristics and chemical communication, including the chemosensory tissues and receptors. Together, this will give valuable information to manage and cultivate the species of interest. In the following sections, general knowledge about the reproductive characteristics in sea cucumbers will be explained and the definition, the biological role(s) and the mechanisms of reproductive pheromones will be reviewed.

1.6. Reproductive characteristics of sea cucumbers

Sea cucumbers exhibit several reproductive modes and strategies according to species depending on several factors such as their specific life history, type of larval development and response to environmental cues (Mercier and Hamel 2009). In the present thesis, the main reproductive characteristics of the sea cucumbers belonging to the order Aspidochirotida will be introduced, as this order contains most commercial sea cucumber species, including those studied in this thesis.

1.6.1. Life cycle

The life cycle of most sea cucumbers consists of two phases; a sedentary, and benthic adult phase preceded by a planktotrophic, pelagic larval phase (Ruppert and Barnes 1994). With the exception of few cases of hermaphroditism, most species are gonochoric with no external morphological differences between the sexes (Hyman 1955; Smiley et al. 1991). Sea cucumbers are broadcast spawners and release their gametes into the water column, where fertilization occurs. This is followed by larval development until larvae are ready to settle in the benthos as juveniles, and subsequently mature into adults to complete the life cycle (Smiley et al. 1991) (Figure 2). Larval development includes three main successive stages, feeding auricularia, non-feeding doliolaria, and pentactula. During the pentactula stage, the larvae settle on a substrate and undergo metamorphosis into benthic juveniles. If no suitable substrate is available, the larvae are able to detach themselves from the substrate and delay metamorphosis until they find a more appropriate surface (Mercier et al. 2000). The critical stages during sea cucumber development are metamorphosis and settlement of the larvae; mortality rates can exceed 90% (Smiley et al. 1991; Yanagisawa 1998).

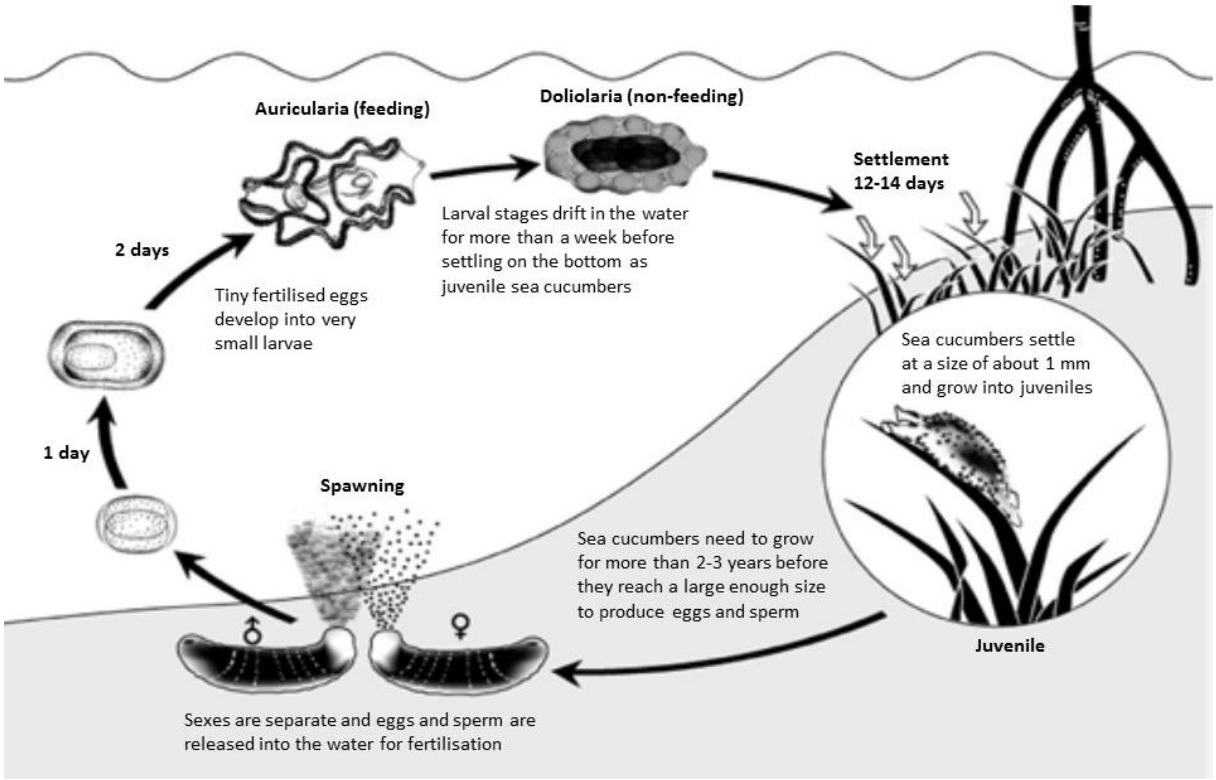

Figure 2. Life cycle of a sea cucumber based on the sandfish *Holothuria scabra* (Adapted from Battaglene (1999) and modified by Friedman et al. (2008)).

1.6.2. Anatomy of the reproductive system

The reproductive system of sea cucumbers is constituted of a single gonad located in the anterior dorsal region (Ruppert and Barnes 1994). The gonad is composed of a group of branched tubules which can be distributed either on one side of the gonad, generally the right side in the family Holothuroidea, or both sides of the gonad as observed mostly in the family Stichopidae. All tubules are joined at the base from which extends a gonoduct, running along the dorsal mesentery, and ending in a gonopore, situated close to the mouth and tentacles, from which the gametes are released to the environment (Smiley 1994). During the reproductive cycle, the gonadal tubules develop through four main stages – recovery, growth, mature, spent – in which they vary in length, width, shape, ramifications and colour (Figure 3).

Sea cucumbers show wide variability in morphology of gonad structure and development compared to the conservative morphology seen in other echinoderms (Hyman 1955; Sewell et al. 1997). Two main gonadal developmental patterns have been described: 1) a uniform development in which all tubules have the same gametogenic stage and 2) a development in cohorts, known as the tubule recruitment model (TRM), in which the tubules within a cohort are synchronous but asynchronous between cohorts (Smiley 1988). According to this model, there is a progressive recruitment of primary tubules (the smallest tubules) at the anterior part of the gonad in year N, which become secondary tubules (the intermediate-sized tubules) in year N+1, and fertile tubules (the largest tubules) at the most posterior end of the gonad in year N+2 (Figure 4). The three main assumptions of the TRM are: distinctive cohorts of gonad tubules, resorption of spent tubules, and no overlapping generations of oocytes.

This model has been criticized, and its applicability to sea cucumbers seems to be limited (Sewell et al. 1997) since it has been shown that gonad development can vary between location and geographical position within the same species. For example, in the east coast of the northern island in New Zealand, *Stichopus mollis* absorbed the spent tubules and the gonadal base, supporting the TRM, whereas it maintained spent tubules in the south of the same island, rejecting the TRM (Sewell 1992). In addition, some sea cucumbers do not fit into either of these two models, such as *Isostichopus badionotus* in Venezuela (Foglietta et al. 2004) and *Holothuria glaberrima* in Colombia (Gómez 2011). It appears thus that gonadal development in sea cucumbers is more complex than first thought and deserves further investigation.

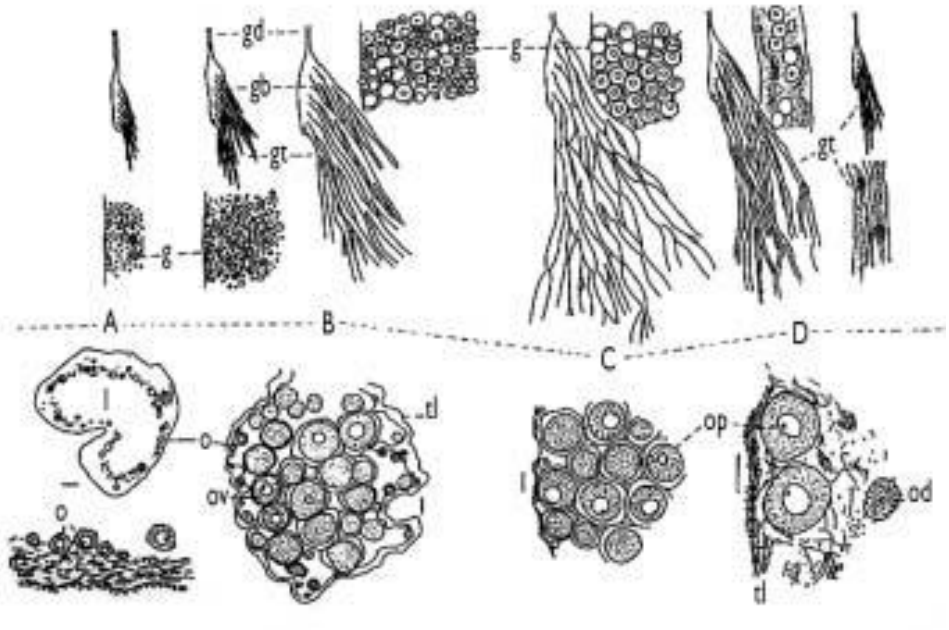

Figure 3. Main maturity stages in *Holothuria leucospilota* of ovarian tubules (above line) and microscopic representation (below line). A: recovery stage, B: growing stage, C: mature stage, D: spent. *gb*: gonadal base; *gd*: gonoduct; *gt*: gonadal tubules; *l*: lumen; o: previtellogenic oocytes; *od*: degenerated oocytes; *op*: postvitellogenic oocyte; *ov*:vitellogenic oocyte*; t*: tubule; *tl*: tubule lining. Scale bars in A, B, C, and D: 20, 40, 50 and 50 μm. (Purwati and Thinh Luong-van 2003)

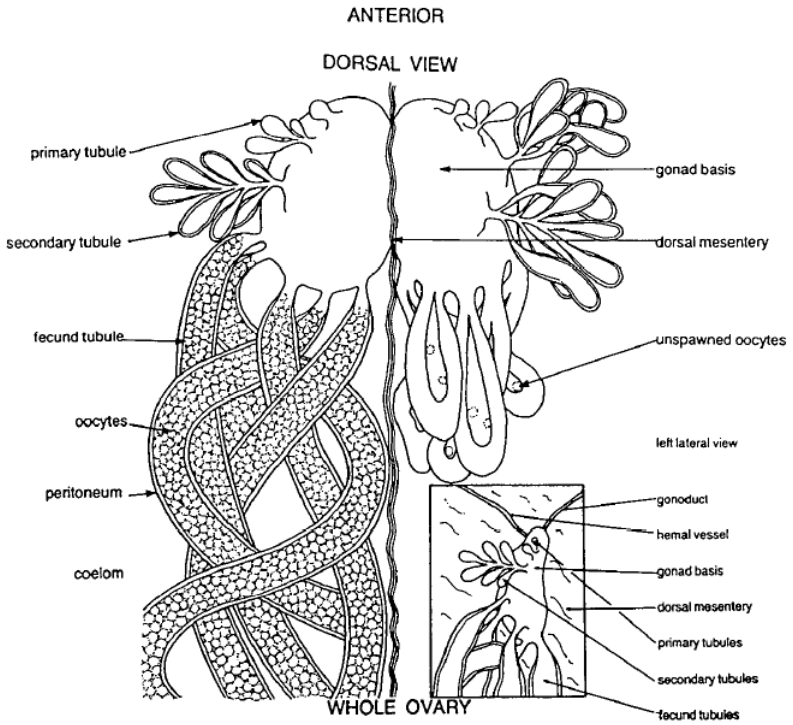

Figure 4. The tubule recruitment model (TRM) by Smiley (1988) in *Parastichopus californicus*. The left side represents the prespawning condition and the right side shows the post-spawning, or resting-phase, gonad. (Smiley and Cloney 1985).

1.6.3. Pattern of reproduction

Sea cucumbers may reproduce aperiodically, when no cyclical pattern is detectable (generally in deep-sea and equatorial species), and periodically when reproduction follows cyclical patterns (mainly found in shallow water species) (Baillon et al. 2011; Mercier and Hamel 2009; Smiley et al. 1991). In periodic patterns, sea cucumbers display mostly seasonal cycles with a short discrete spawning period in temperate species (e.g. Hamel et al. 1993; Santos et al. 2015; Tuwo and Conand 1994), and much longer in tropical species (Chao et al. 1995; Conand 1993; Guzmán et al. 2003).

Reproductive cycles, from gametogenesis to spawning, are seen as a means to synchronize individuals of a population in order to maximize fertilization success and to take advantage of optimal conditions for offspring survival (Giese and Kanatani 1987; Levitan 1995b). They are thought to be the result of complex interaction between endogenous and exogenous factors, including inter-individual communication (discussed in the section 1.7) and environmental factors (Mercier and Hamel 2009).

Among the most cited environmental factors, temperature and photoperiod have been largely correlated with the onset of gametogenesis, while changes in water temperature, food availability, light intensity, water turbulence, salinity and phytoplankton blooms have been suggested to trigger spawning (e.g. Chao et al. 1995; Conand 1981; Despalatovic et al. 2004; Drumm and Loneragan 2005; Guzmán et al. 2003; Hamel et al. 1993; Hamel and Mercier 1996b; Muthiga 2006; Ramofafia et al. 2000).

1.7. Chemical communication, pheromones and reproduction

Animals use many different signals or cues to communicate: visual, tactile, auditory, electrical and chemical (Cardé and Baker 1984). Chemical communication is the most ancient and widespread mode of communication in the biosphere; all organisms from single-celled bacteria to mammals are able to detect chemical cues (Wyatt 2003b). When chemicals are exchanged between individuals belonging to the same species, and evoke a specific innate effect, they are called pheromones. These are released in the environment by a sender and detected by a receiver in whom a specific reaction occurs such as a stereotyped behaviour or a development/physiological process (Karlson and Luscher 1959; Wyatt 2010). They do not require previous learning or experience, and are mutually beneficial to both sender and receiver. They can be composed of a single compound or a mixture of several compounds and are water-soluble and/or volatile depending on the environment (Wyatt 2003b).

1.7.1. Reproductive pheromones

In aquatic environments, chemical communication is particularly important as vision and hearing might be limited. In such environments, pheromones mediate a wide variety of behaviours such as migration, individual recognition, group cohesion, aggregation, territorial marking and reproduction (Brönmark and Hansson 2000; Sorensen and Stacey 2004). Reproductive pheromones have been attributed two main roles: 1) recognition of mating partners and formation of breeding aggregation and 2) synchronization of reproductive processes such as gametogenesis and spawning (see review by Gomez-Diaz and Benton 2013). These are particularly important for broadcast spawners in which the fertilization rates can be considerably affected if gametes are not released at the same time and place (Denny and Shibata 1989; Levitan 1995a; Pennington 1985).

In marine invertebrates, the influence of reproductive pheromones has been demonstrated in an increasing number of studies; however, few of them have identified the signal molecules (Agosta 1992; Hardege 1999). The best studied cases come from Nereid polychaetes in which it has been shown that the nuptial dance and gamete release are controlled by sex pheromones from the coelomic fluid of mature individuals (Boilly-Marer and Lassalle 1978). The nuptial dance coordinating pheromone has been identified in *Platynereis dumerilii* and *Nereis succinea* as a ketone, 5-methyl-3-heptone, and is concentration species-specific, while different substances such as uric acid, inosine, glutamic acid and small glutathione-like are associated with gamete release (Hardege et al. 2004; Zeeck et al. 1990; Zeeck et al. 1988; Zeeck et al. 1998; Zeeck et al. 1996). In the opistobranch molluscs *Aplysia* spp., a peptide pheromone, attractin, is released during egg laying and stimulates attraction while maintaining mating and egg-laying aggregations (Cummins and Bowie 2012; Cummins et al. 2005; Painter et al. 1998). In crustaceans, the first sex pheromone has recently been identified in the shore crab *Carcinus maenas* as the nucleotide uridine diphosphate (UDP) (Hardege et al. 2011). It is a metabolic by-product from chitin biosynthesis which is accumulated before and during molt, and is predominantly released in the urine. This pheromone is released by females and induces stereotyped sexual behaviours in males, including pre- and post-copulatory guarding of the female and initiation of mating.

Chemical communication in echinoderms remains poorly investigated and mostly speculative, although progress has been made in recent years to better understand the influence of chemical cues during reproduction. In echinoderms, they are thought to be involved in the fine-tuning of breeding aggregations and spawning synchrony (see review by Mercier and

Hamel 2009). Echinoderms are known to form breeding aggregations in which individuals are generally found to be more mature than solitary individuals (Run et al. 1988; Tyler et al. 1992; Unger and Lott 1994; Young et al. 1992). These aggregations are believed to increase fertilization success by increasing the probability of gamete encounter during spawning (Levitan et al. 1992) and by influencing the synchronization of gametogenesis (Leite-Castro et al. 2016). It has also been shown that mucus secreted by mature individuals in the sea cucumber *Cucumaria frondosa* contains a biologically active substance which accelerates gonadal development of less mature individuals of the same sex (Hamel and Mercier 1996a, 1999). Chemical attraction among conspecifics has been demonstrated in some laboratory studies; however, little information is found on how groups are formed during the pre-breeding period and, in particular, whether the signal comes from males or females, or both (e.g. Campbell et al. 2001; Dix 1969).

Regarding spawning, the endogenous mechanisms of final oocyte maturation and gamete release have been quite well studied, mainly in starfish, in which the gonad-stimulating hormone (i.e. the primary mediator of oocyte maturation), is one of the best known endogenous factors in echinoderms (Giese and Kanatani 1987; Kanatani 1973; Kanati and Shirai 1969). However, little is known about the identity, the origin and the mechanism of action of spawning pheromones, although their existence was suggested more than 40 years ago (Beach et al. 1975). It was thought that sperm and/or chemicals released with sperm stimulated females to spawn, as males generally start to spawn before females (e.g. Levitan 2002; McEuen 1988; Miller 1989; Smiley et al. 1991; Thorson 1950). This hypothesis has been tested in starfish (Caballes and Pratchett 2017; Hamel and Mercier 1995) and sea urchins (Reuter and Levitan 2010; Starr et al. 1990), but not in sea cucumbers. Also, the effect of spermatozoa has never been separated from chemicals released with them, and the reciprocal effects of both sperm and eggs on spawning induction were rarely tested.

The discovery of sex pheromones inducing spawning in sea cucumbers may help to improve aquaculture. Until now, aquaculture relies largely on thermal shock to induce spawning (Battaglene 1999; Hamel et al. 2001; James 1994; Smiley et al. 1991). Although this technique is simple (i.e. transfer mature animals to water 4-5°C warmer), the spawning success varies with the protocol and species used (Léonet et al. 2009; Yanagisawa 1998). Consequently, the use of sex pheromones could be a more reliable alternative to stimulate individuals to spawn than thermal shock, and thus contribute to improve the reliability and profitability of sea cucumber aquaculture.

1.7.2. Perception of chemicals signals

In all organisms, the dominant sense involved to perceive and detect chemical cues is olfaction. The mechanisms of detection, including the olfactory systems and the organization of the olfactory signaling pathways, show some remarkable similarities across species (Ache and Young 2005; Hildebrand and Shepherd 1997). Chemical detection always starts with the binding of an odorant molecule to an olfactory receptor (OR) located in the membrane of the olfactory sensory neurons (OSNs) (Wyatt 2014). This binding induces a conformational change in the ORs which activates a cascade of enzymatic reactions, involving multiple intracellular secondary messengers, that leads to the opening of ion channels, and ultimately causes a generator potential in the ORs (Lancet et al. 1988). If the generator potential is of sufficient amplitude, it triggers an action potential which will relay the information along the axon of the OSNs to the central nervous system. The axons of the OSNs that express the same ORs converge in spherical conglomerates of nerve cells, called glomeruli, which are located in the olfactory bulbs in vertebrates and the antennal lobes in insects (Hildebrand and Shepherd 1997; Touhara and Vosshall 2009). From these structures, the information is transmitted to other nerve cells, known as mitral cells in mammals and projection neurons in insects, which are connected to other regions in the brain (Wyatt 2003a). This glomerular organization, found in vertebrates and insects, has also been reported in decapod crustaceans (Schmidt and Ache 1992), molluscs (Cummins et al. 2009), and even in echinoderms (Hoekstra et al. 2012). However, it is not known if this comes from a common ancestor or results from a logical response to the demands of olfactory processing, i.e. convergent evolution (Strausfeld and Hildebrand 1999).

1.7.2.1. Olfactory receptors

Chemosensation in animals is generally mediated by receptors belonging to the large superfamily of G-protein-coupled receptors (GPCRs). This family is characterized by a highly conserved structure across animal phyla - seven transmembrane domain proteins with three intracellular loops (IL1-3) and three extracellular loops (EL1-3) (Ache and Young 2005; Krishnan et al. 2015). The GPCRs bind extracellular chemicals such as pheromones, neurotransmitters, hormones or photons, and activate the intracellular pathways that, ultimately, lead to a physiological and/or behavioural response (see review by Bockaert and Pin 1999; Lefkowitz 2007). According to the 'GRAFS' (initials of families) classification scheme, GPCRs are divided into five families based on their ligand affinity and sequence similarity: Glutamate,

Rhodopsin, Adhesion, Frizzled/Taste and Secretin (Schiöth and Fredriksson 2005). The Rhodopsin family contains the most numerous GPCRs involved in vertebrate olfaction, including the odorant receptors; the largest group with about 2,130 genes (Kaupp 2010; Mombaerts 2001). This group often contains sequences organized in tandem array resulting from gene duplication and gene loss and has led to the great diversity of receptors across phyla (Ache and Young 2005). Furthermore, GPCRs have undergone frequent lineage-specific expansion over time contributing to the low similarity between invertebrates and vertebrates (Bargmann 2006) (see chapter V for more details).

Echinoderms must rely heavily on olfaction for their daily activities since they lack other well-developed senses (except mechanoreception and light-sensing eyespots). It has been demonstrated that they are able to respond to a wide range of chemical stimuli (e.g. Burke 1984; Campbell et al. 2001; Nishizaki and Ackerman 2005). Chemosensory GPCRs have been characterized in the sea urchin *Stronylocentrotus purpuratus* from which 678 to 979 rhodopsin-like GPCRs were identified (Burke et al. 2006a; Raible et al. 2006). With an approach more oriented to the search for odorant receptors, 192 chemosensory receptors were identified in the same species of sea urchin (Burke et al. 2006a). In the starfish *Acanthaster planci*, 772 to 775 rhodopsin-like GPCRs have been identified (Hall et al. 2017) and 63 putative odorant receptors were recently discovered (Roberts et al. 2017). Knowledge of GPCRs is increasing in the echinoderm phylum, and in metazoans in general; however, there is no information about the chemosensory GPCRs in sea cucumbers. Consequently, the present thesis aimed to characterize the overall GPCRs in sea cucumbers, with special attention dedicated to the rhodopsin family to look for putative odorant receptors.

1.7.2.2. Sensory structures

Chemosensory GPCRs are generally expressed in the sensory epithelia of specialized structures - olfactory or chemosensory organs - where the OSNs are gathered. These structures play a fundamental role in setting the sensitivity of the olfactory system in response to different chemical signals (Kaissling 1990). They are found within a cavity, the nasal cavity in vertebrates (vomeronasal organ and olfactory mucosa), while they are generally spread on external appendages in invertebrates, such the anterior region in nematodes, the tentacles in gastropods, and the antennae and maxillary palps in insects (Bargmann and Horvitz 1991; Kaupp 2010; Shorey 1976; Wertz et al. 2006).

In echinoderms, the structures that have been attributed a sensory role are external prolongations of the body wall which are connected to the water-vascular system. In sea

cucumbers, these structures are the tentacles, the tube feet and the papillae. The role of these structures varies and has been associated with feeding, respiration, locomotion, substratum fixation, burrowing and mechanoreception (Bouland et al. 1982; Flammang 1996; VandenSpiegel et al. 1995). However, their role in chemical detection has rarely been investigated. One reason for this is the difficulty in performing electrophysiological assays, notably because of the small size of the neurons and the lack of knowledge about the nervous system in adult echinoderms (Cobb 1978; Díaz-Balzac et al. 2014; Pentreath and Cobb 1972).

The main nervous system in echinoderms is composed of five radial nerve cords (RNCs) which are connected to the circumoral nerve ring. Each RNC is composed of ectoneural and hyponeural subdivisions which are thought to innervate different parts. Although there are exceptions, the ectoneural component innervates the body wall and its appendages (i.e. tentacles, tube feet and papillae) while the hyponeural component innervates the muscles (Cobb 1985; Hyman 1955; Inoue et al. 2002). The RNCs have been quite well studied (see summary by Díaz-Balzac et al. 2016), in contrast with the body appendages which were until recently only investigated through ultrastructural studies and classical histology (e.g.Bouland et al. 1982; Cavey 2006; Flammang and Jangoux 1992; Pentreath and Cobb 1972; VandenSpiegel et al. 1995). Much progress has been made recently to better understand the innervation of the body structures, particularly thanks to the development of a new antibody against synaptotagmin which is specific to neurons in echinoderms (Burke et al. 2006b; Nakajima et al. 2004) and RN1 which is an antibody raised against a homogenate of radial nerve in sea cucumber (Díaz-Balzac et al. 2010) (see chapter IV for more details).

However, despite this recent progress, it is important to keep tracking the distribution and localization of neurotransmitters and other nervous signaling molecules in different species to better understand the functions of different sensory structures in sea cucumbers. In this context, and as a preliminary step to electrophysiology, the present thesis characterized the tissue organization and the innervation of the structures in contact with the environment of the studied species through classical histology, histochemistry and immunohistochemistry.

1.8. *Holothuria arguinensis* Koehler & Vaney, 1906 and *Holothuria mammata* Grube, 1840

Holothuria arguinensis and *Holothuria mammata* are both deposit feeders belonging to the order Aspidochirotida and target species of the recent developing fisheries in the north-eastern Atlantic Ocean and the Mediterranean Sea (e.g. Aydin 2008; Borrero-Pérez et al. 2011;

González-Wangüemert et al. 2013b). At the beginning of the present thesis, little was known about their biology, ecology or life-history.

1.8.1. Holothuria arguinensis Koehler & Vaney, 1906

H. arguinensis (Figure 5) is a north-eastern Atlantic species ranging from the Berlengas Islands (Portugal) to Morocco and Mauritania, including the Canary Islands (Costello 2001; Rodrigues 2012; Thandar 1988). However, this species is spreading into the Mediterranean Sea where it has been found on the eastern coast of Spain (González-Wangüemert and Borrero-Pérez 2012) and Algerian coast (Mezali and Thandar 2014). It is found from the intertidal to 52 m depth, and is generally associated with sandy substrate, macro-algal beds and seagrass meadows (González-Wangüemert and Borrero-Pérez 2012; Navarro 2012).

Recent ecological and behavioural studies on *H. arguinensis* have shown that this species moves continuously during day and night without any sheltering behaviour, although it is often covered with seagrass leaves and algae as camouflage (González-Wangüemert and Borrero-Pérez 2012; Navarro 2012; Navarro et al. 2014; Siegenthaler et al. 2015). It is also the first European sea cucumber species that has been reproduced in a hatchery, and its larval development has been estimated at 18 days (Domínguez-Godino et al. 2015).

1.8.2. Holothuria mammata Grube, 1840

H. mammata (Figure 6) is distributed widely throughout the Mediterranean Sea and north-eastern Atlantic Ocean, including the coast of Portugal and the Macaronesian Islands of the Azores, Madeira and the Canary Islands (Borrero-Pérez et al. 2009). It is found from the intertidal zone to 25 m depth, and is mainly associated with rocky shores where it is predominantly nocturnal (González-Wangüemert et al. 2013a; Navarro 2012; Navarro et al. 2013; Siegenthaler 2013). However, it can sometimes be found in habitats such as muddy/sandy substrates with sea grass meadows where animals show diurnal and nocturnal feeding (González-Wangüemert et al. 2016; Siegenthaler et al. 2017). It possesses small Cuvierian tubules, used as defensive system in sea cucumbers, but interestingly they are never expelled in this species (VandenSpiegel and Jangoux 1988). Its reproductive cycle has been studied in Peniche (Portugal) where the period of gonad maturation occurs from February to April (Santos 2013).

Figure 5. Picture of *H. arguinensis* in Olhos de Água (Portugal).

Figure 6. Picture of *H. mammata* in Olhos de Água (Portugal).

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Chapter II

Sea cucumbers, *Holothuria arguinensis* and *H. mammata*, from the southern Iberian Peninsula: variation in reproductive activity between populations from different habitats.

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Sea cucumbers, *Holothuria arguinensis* and *H. mammata*, from the southern Iberian Peninsula: variation in reproductive activity between populations from different habitats.

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2.1. Abstract

New fisheries in the western Mediterranean and north eastern Atlantic target the sea cucumbers *Holothuria arguinensis* and *H. mammata*; however, lack of biological information hinders management decisions. Here, the reproductive biology of populations of the two species was investigated in the southern Iberian Peninsula. Different populations located along a narrow latitudinal range displayed the same general reproductive pattern of summer-autumn spawning. However, significant differences in size, gonadal production and maturity profile between locations suggests the influence of site-specific factors. In Sagres and Ria Formosa *H. arguinensis* individuals were larger and had larger gonads than in Olhos de Água, which had relatively more immature animals. The spawning and active gametogenesis periods were also longer in Sagres, possibly linked to specificity of food availability and tidal conditions. Ria Formosa also had larger *H. mammata* individuals with larger gonads than in Murcia and Olhos de Água, possibly reflecting differences in feeding activity in different substrates (muddy/sandy vs rocky). Gametogenesis in *H. arguinensis* may be triggered by decreasing photoperiod and temperature, and spawning by increasing temperature. Altogether, these results, which include fecundity and size at first maturity, provide an important basis for the scientific management of sea cucumber fisheries in the region.

Key words: Holothurian, reproduction, first maturity, fecundity

2.2. Introduction

Sea cucumbers are bottom-dwelling echinoderms found in all regions of the ocean, from intertidal to deep-sea and from polar to tropical regions (Conand 1989, 2004). As sea cucumbers are mostly deposit feeders and bioturbators, they have a key role in maintaining healthy marine ecosystems by mixing of sediments, recycling of nutrients, stimulating algal growth, and regulating both carbonate content and water pH (Massin 1982; Purcell 2004; Purcell et al. 2016; Schneider et al. 2011; Uthicke 2001a, b; Wolkenhauer et al. 2010). Besides their ecological importance, sea cucumbers represent an important fishery resource mainly exported to Asian countries (Bordbar et al. 2011; Chen 2003; Chen 2004; Conand 1989). Currently, at least 66 sea cucumber species are fished worldwide in more than 70 countries (Purcell 2010; Purcell et al. 2013).

Sea cucumber populations have been subjected to increased exploitation which, in combination with ineffective fisheries management, has led to severe overfishing throughout the world, particularly in the Indo-Pacific region (Anderson et al. 2011; Kinch et al. 2008; Purcell 2010; Purcell et al. 2013). This has been compounded by the low recruitment, slow growth rate, late maturity and density-dependent reproductive success of sea cucumbers that make them especially vulnerable to overexploitation (Bruckner et al. 2003; Conand 2006a, b; Uthicke et al. 2004).

New sea cucumber fisheries are also being developed rapidly in the Northeastern Atlantic Ocean and Mediterranean Sea, in Turkey, Italy, Spain, Greece and Portugal, in response to the strong Chinese market demand. *Holothuria arguinensis* (Koelher and Vaney, 1906) and *Holothuria mammata* (Grube, 1840) are two of the target species for these fisheries with prices ranging from 70 to 350 euros per kilo and could reach 1-2 million $US of total annual revenue (González-Wangüemert and Borrero-Pérez 2012; González-Wangüemert et al. 2013b; González-Wangüemert et al. 2016). *H. arguinensis* is present from the Berlengas Islands (Portugal) to Morocco and Mauritania, including the Canary Islands (Costello 2001; Rodrigues 2012). In the Mediterranean, it has recently been registered on the eastern coast of Spain and on Algerian coast (González-Wangüemert and Borrero-Pérez 2012; Mezali and Thandar 2014). This species is found from the intertidal zone to 52 m depth, and is frequently observed on macroalgal-dominated beds and sea grass meadows of *Cymodocea nodosa* and *Zostera noltii* (González-Wangüemert and Borrero-Pérez 2012; Navarro et al. 2012; Navarro et al. 2014; Siegenthaler et al. 2015). *H. mammata* is distributed widely throughout the Mediterranean Sea and northeast Atlantic Ocean, including the coast of Portugal and the Macaronesian Islands of

the Azores, Madeira and the Canary Islands (Borrero-Pérez et al. 2011). It is found from the intertidal zone to 25 m depths, and is mainly associated with rocky shores though it can also be found on muddy/sandy substrate dominated by sea grass meadows (Borrero-Pérez et al. 2011; Borrero-Pérez et al. 2009; González-Wangüemert et al. 2013a; González-Wangüemert et al. 2016; Siegenthaler et al. 2015).

Understanding the reproductive biology of a species is central to sound fisheries management, such as the establishment of a closure season during spawning and a minimum capture size. Potential productivity of fisheries, an essential parameter to define the resilience of a population under exploitation or in the face of human activity disturbances, can also be determined by studying reproductive processes (Morgan 2008). Finally, knowledge about reproductive characteristics can help to restore and enhance wild stocks and are essential for breeding and aquaculture programs (Mercier and Hamel 2009; Wang et al. 2015).

Sea cucumbers display reproductive cycles typically associated to predictable fluctuations in environmental factors maximizing the fertilization success through synchronization between individuals (Levitan 1995; Mercier and Hamel 2009). Reproductive activity is thought to be regulated by endogenous and exogenous cues such as photoperiod, water temperature, salinity, food availability, tidal flow, light intensity and phytoplankton blooms (Conand 1981; Drumm and Loneragan 2005; Hamel et al. 1993; Hamel and Mercier 1996b; Ramofafia et al. 2000; Ramofafia et al. 2003). However, the specific influence of each of these factors has rarely been explicitly tested (Mercier and Hamel 2009).

In order to promote the scientific management of *H. arguinensis* and *H. mammata* it is essential to acquire baseline knowledge about their ecology, population dynamics, and reproduction. Therefore, the objectives of the present study are: (1) to provide a detailed description of the morphology of reproductive structures in relation to the reproductive cycle of the two species; (2) to obtain an insight of the seasonal and inter-population variability of the reproductive cycle; (3) to determine the size at first sexual maturity; and (4) to examine possible relationships between the reproductive cycle and environmental parameters.

2.3. Materials and Methods

2.3.1. Sampling locations and collection of specimens

Individuals of *H. arguinensis* were collected at three locations along the Algarve coast (southern Portugal) of similar latitude: Ria Formosa (RF; 37°00’35.02’’N; 7°59’46.10’’O), Sagres (SA; 37°00’44.78’’N; 8°55’49.51’’O), and Olhos de Água (OD; 37°05’18.76’’N;

8°11’34.86’’O; Figure 1). At each location, between 10 and 15 individuals were collected monthly from May 2013 to April 2014. The collection program was extended until December 2014 in Sagres for inter-annual variability analysis, and this location was chosen because it had a denser population of sea cucumbers in this area. Individuals of *H. mammata* were collected in Ria Formosa and in Olhos de Água in the Atlantic Ocean, and in Murcia at Los Cocedores (MU; 37°24’20.39’’N; 1°37’02.27’’O; Figure 1) in the Mediterranean Sea. Collections of *H. mammata* were more spaced in time than that of *H. arguinensis*, and around 20 individuals were collected each season, because of logistical factors and their low density in the Ria Formosa (González-Wangüemert et al. 2013a; Siegenthaler 2013).

Ria Formosa is a sheltered mesotidal coastal lagoon extending for about 55 km along the south coast of Portugal, with a mean depth of 1.5 m. Sea cucumbers were collected in the intertidal zone composed of mud and muddy-sand flats where perennial seagrasses such as *Cymodocea nodosa*, *Zoltera noltii* and *Z. marina* and green mat-forming macroalgae (Ulvales) dominate (Asmus et al. 2000). Olhos de Água is 20 km to the west of the Ria Formosa, a sheltered mesotidal coast moderately exposed to the WSW prevailing waves. The intertidal zone where the sea cucumbers were collected had rock pools and platforms alternating with sandy sediment areas (Moura et al. 2006; Rosa et al. 2013). Sagres, 70 km to the west of Olhos de Água, is a mesotidal moderately exposed coastal area (Bettencourt et al. 2004). Sea cucumbers were collected in the subtidal zone (about 2 m depth) off Praia da Baleeira, next to Sagres harbor, that is characterized by sandy and rocky areas. This area is affected by summer upwelling, due to a dominant coastal northerly wind, that supplies nutrients to the euphotic zone (Relvas and Barton 2002; Sousa and Bricaud 1992; Wooster et al. 1976). Los Cocedores is located in a sheltered bay where sea cucumbers were collected subtidally (1.5 – 2m depth) from rocky substrate close to sandy patches covered by *C. nodosa* and *Posidonia oceanica* meadows.

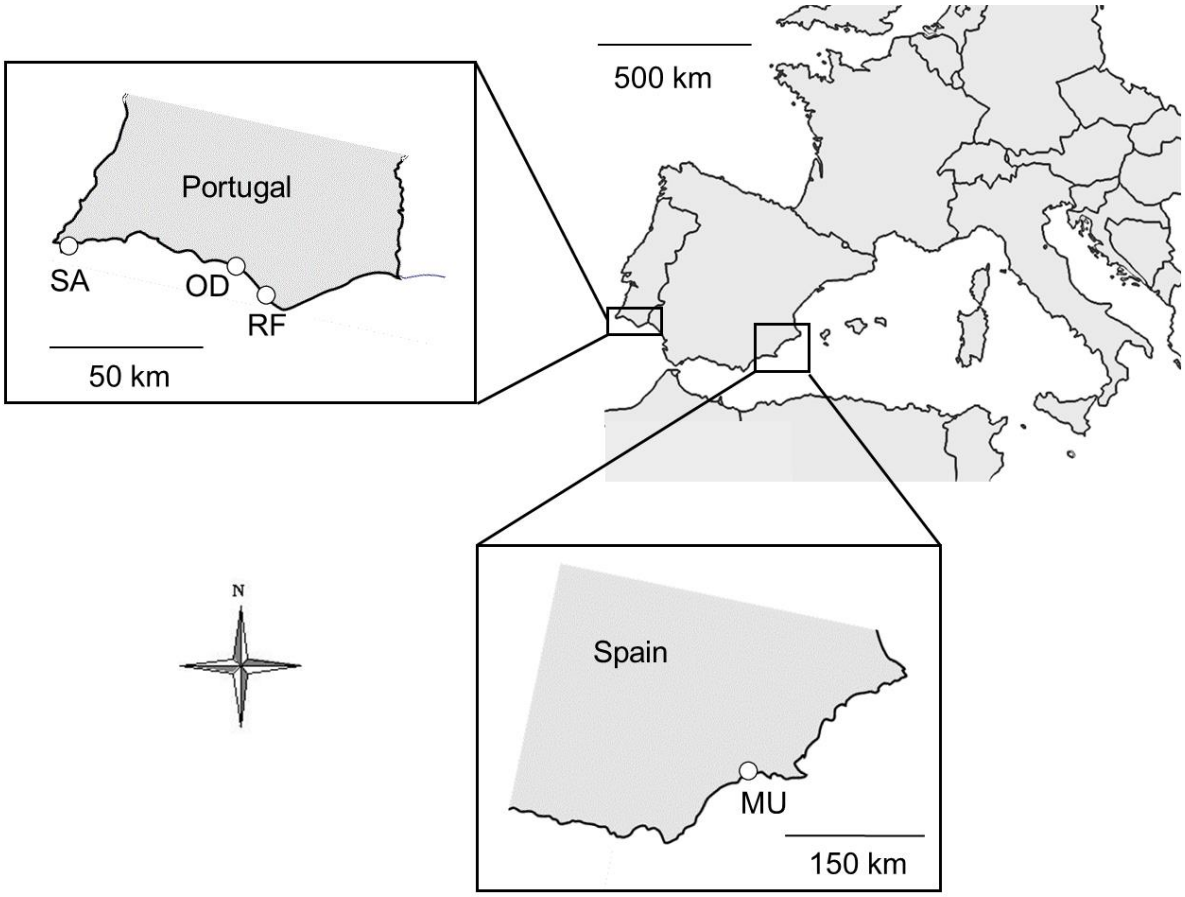


Figure 1. Sampling locations of *H. arguinensis* (SA, OD and RF) and *H. mammata* (OD, RF and MU).

2.3.2. Biometric measurements

Immediately upon arrival at the laboratory, total length (TL), total weight (TW), body wet weight after dissection and removal of internal organs and coelomic fluid (gutted body weight, GBW) and gonad weight were measured for each individual. For histological analysis, a small piece (around 1cm) in the tubule's mid region was removed and fixed in Bouin's solution for 24h and was then stored in 70% ethanol. The remaining gonad was fixed in 10% buffered formaldehyde for measurement of the gonadal tubules and to estimate fecundity (see below). The gonad index (GI) was calculated for each individual as GI / GBW * 100 (Conand 1981). Length-weight relationships (LWR) were inferred for each population according to $GBW = a\,TL^{b}$ (Keys 1928), where GBW is the gutted weight in g, TL is the total length in mm, a is the regression intercept on the Y-axis and b is the regression slope.

2.3.3. Maturity stages of gonads

A scale of gonadal maturity was established based on the morphology and histological analysis following the criteria of Conand (1981) modified by Ramofafia et al. (2000): I.

Immature, II. Recovery, III. Growing, IV. Mature, V. Partly-spawned, VI. Spent. The length and diameter of 10 to 15 tubules, taken randomly or from the most representative cohorts, were measured under the microscope using a ruler and a microscope eyepiece graticule. For histology, paraffin sections of the testes were stained with Masson's trichrome (Humason 1972) and the ovaries with V.O.F. (brilliant yellow-green, Orange G and acid fuchsin) (Gutiérrez 1967).

2.3.4. Fecundity and sexual maturity

Absolute fecundity was determined for 63 *H. arguinensis* and 27 *H. mammata* individuals from a small piece of gonad fixed in 10% formalin as described by Muthiga et al. (2009). The size/weight at first sexual maturity, defined as the size/weight when gonads of 50% of individuals were undergoing gametogenesis (stage 2, 3, 4, 5 and 6) (Conand 1981), was determined from 92 *H. arguinensis* (49 from 2013 and 43 from 2014) collected in SA between May and June. This was not estimated for *H. mammata* due to lack of small sized individuals.

2.3.5. Environmental factors

Monthly averaged sea surface temperature (SST) and chlorophyll a concentration with a 4 km resolution were retrieved from the Moderate Imaging Spectroradiometer-Aqua (MODIS-Aqua) dataset available from the National Aeronautics and Space Administration (NASA) Goddard Earth Sciences Data and Information Services Center (GESDISC) between May 2013 and April 2014 for all studied locations, and until December 2014 for SA. Visualization was performed using Giovanni, a web-based application developed by the GESDISC (Acker and Leptoukh 2007). As complementary information, the upper layer of the surface sediment where sea cucumbers feed was sampled in March 2014 at each location to determine (i) the organic matter content and (ii) carbonate content (see Supplementary material 1).

2.3.6. Statistical analyses

Data are presented as mean ± standard error of the mean (SEM), unless otherwise stated (see Supplementary material 2 for more detailed information about the statistical analyses). PERMANOVA (Anderson 2001) was used to test differences in: 1) TL, GBW, length and diameter of gonadal tubules between location with as fixed factors location (3 levels) and sex (2 levels); 2) GI between location with as fixed factors location (3 levels) and month (12 levels

for *H. arguinensis* and 4 levels for *H. mammata)*; 3) GI between years in SA for *H. arguinensis* with as fixed factors year (2 levels) and month (8 levels). A theoretical 1:1 sex ratio of each population was tested using a chi-square test (χ^2). Sexual dimorphism in each location for each species was tested with the Mann-Whitney test.

Length-weight relationships were used to determine whether the growth of each sea cucumber population was isometric or allometric (Ricker 1973). The significance of the regression was assessed with the F-statistic, and the slope b for each population was tested with a Students t-test for deviations from the isometric coefficient b=3 (Sokal and Rohlf 1995). Pearson's correlation was used to determine relationships between GW and GBW before spawning in all locations.

Absolute fecundity was compared between locations for each species using analysis of covariance (ANCOVA) with absolute fecundity as a dependent variable and gutted body weight as a covariate followed by the Tukey HSD post-hoc test for pairwise comparisons and unequal sample size. Spearman's correlation was used to estimate the relationship between fecundity and GI in each species. To estimate the size and weight at first sexual maturity, data were fitted to a logistic curve, using the Levenberg-Marquardt algorithm (Marquardt 1963).

H. arguinensis monthly mean GI was cross-correlated (1 month lag interval) with monthly mean sea surface temperature, chlorophyll *a* concentration and length of photophase over 12 months in 2013 at the three locations and during the 8 months in common between 2013 and 2014 (from May to December) in SA, using GI as the lagged variable. Organic and carbonate content were analyzed using one-way ANOVA followed by Tamahane and SNK post-hoc tests for pairwise comparisons.

2.4. Results

2.4.1. Population characteristics

There were significant differences in TL among locations (PERMANOVA, df = 2, n = 552 for *H. arguinensis*, n = 165 for *H. mammata*, p(perm)<0.001 for each species) and in GBW (PERMANOVA, df = 2, p(perm)<0.001 for each species) (Figure 2). For *H. arguinensis*, TL and the GBW varied significantly and had the following order: SA > RF > OD (Post-hoc pair-wise tests, p(perm) < 0.001 for all combinations; Figures 2a, b). *H. mammata* had similar TL (Post-hoc pair-wise tests, p(perm) = 0.97) and GBW (p(perm) = 0.84) at MU and OD, but at RF they were longer and heavier (p(perm) < 0.001 for all combinations; Figures 2c, d). There was no sexual dimorphism in TL (*H. arguinensis* PERMANOVA, df = 2, interaction location

x sex, p(perm) = 0.37 and *H. mammata* p(perm) = 0.08) or GBW (*H. arguinensis* PERMANOVA, df = 2, interaction location x sex, p(perm) = 0.25 and *H. mammata* p(perm) = 0.08). The sex ratio in each population did not differ significantly from 1:1 (Chi-squared tests, *H. arguinensis*, df = 1, SA: $\chi^2 = 0.002$, p = 0.97; OD: $\chi^2 = 0.136$, p = 0.71; RF: $\chi^2 = 0.501$, p = 0.48; *H. mammata*, RF: $\chi^2 = 1.976$, p = 0.16; OD: $\chi^2 = 0.134$, p = 0.71; MU: $\chi^2 = 0.439$, p = 0.51).

Log(TL) was positively correlated to Log(GBW) with correlation coefficients of 0.529, 0.753, 0.483 at SA, OD and RF, respectively, for *H. arguinensis* (ANOVA, $p < 0.001$ for each population; Table 1) and 0.717, 0.679 and 0.623 at MU, OD and RF, respectively, for *H. mammata* ($p<0.001$; Table 1). The slope *b* varied from 0.980 at RF to 1.437 at OD for *H. arguinensis* and from 0.924 at MU to 1.175 at OD for *H. mammata*. The slope *b* was significantly lower than 3 (Students t-test, $p < 0.001$ in each case), indicating negative allometry.

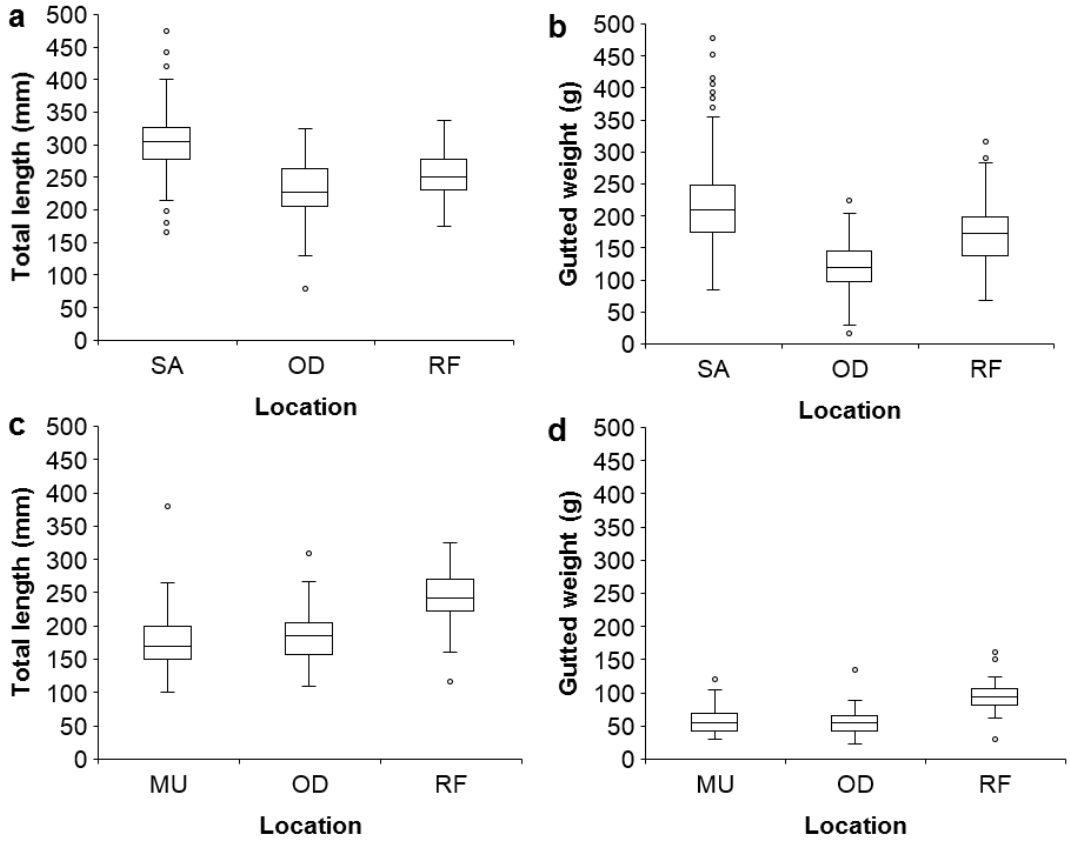


Figure 2. Box-whisker plots of morphometric characteristics of the studied populations: (**a, c**) mean total length and (**b, d**) gutted weight of (**a, b**) *H. arguinensis* (SA: n = 288; OD: n = 165; RF: n = 178) and (**c, d**) *H. mammata* (MU: n = 80; OD: n = 100; RF: n = 45).

Table 1. Characteristics and parameters of the length - gutted body weight relationships in each population for *H. arguinensis* (1) and *H. mammata* (2). Note: a and b are the parameters from equation GBW = a TL^b in which GBW is gutted body weight and TL is total length

Species	Location	N	Length range (mm)		Gutted weight range (g)		a	b	S.E. (b)	r	t-value	p-value
			Min	Max	Min	Max						
1	SA	288	165	474	84.21	478.44	0.565	1.038	0.098	0.529	10.565	p<0.0001
1	OD	178	80	325	17.47	223.68	0.047	1.437	0.094	0.753	15.177	p<0.0001
1	RF	165	175	337	68.77	323.61	0.737	0.980	0.139	0.483	7.034	p<0.0001
2	MU	80	100	380	33.00	104.00	0.474	0.924	0.102	0.717	9.098	p<0.0001
2	OD	100	109	310	38.11	134.58	0.119	1.175	0.128	0.679	9.162	p<0.0001
2	RF	45	117	325	29.57	162.07	0.373	1.002	0.192	0.623	5.227	p<0.0001

2.4.2. Gonad morphology

The gonad in both *H. arguinensis* and *H. mammata* contains a multitude of tubules joined at their base and attached to a dorsal mesentery leading to a gonopore from which the gametes are released. The development of the tubules during gametogenesis follows the same pattern in the different locations. The length and the diameter of tubules increased until reaching a maximum when individuals were fully mature, and became shorter and thinner after the spawning period. The six maturity stages based on the morphology and the histological characteristics of the gonadal tubules are detailed in Supplementary materials 3, 4 and 5.

In *H. arguinensis*, cohorts of tubules at different stages were observed in a single gonad, varying in relative abundance and type between individuals and, in some cases, all tubules were at the same stage. Pre-vitellogenic oocytes lining the germinal epithelium were also observed side by side with mature oocytes (Supplementary material 4). After spawning, the spent tubules were completely resorbed while other categories of tubules persisted in the gonad. The length and the diameter of the most represented category of tubules varied significantly between geographical locations according to the following order: SA > RF > OD (PERMANOVA, df = 2, p(perm) < 0.05 for each case; Post-hoc pair-wise tests, p(perm) < 0.001 for each combination; Supplementary material 6). No sexual dimorphism was observed in tubule length (PERMANOVA, df = 2, location and sex interaction, p(perm) = 0.65) or diameter (p(perm) = 0.15).

In *H. mammata*, in each individual all tubules were at the same stage in all gonads analyzed. However, as in *H. arguinensis*, smaller oocytes were observed lining the tubular epithelium side by side with mature oocytes (Supplementary material 4). All tubules regressed after spawning. Tubule length and diameter of individuals from RF was significantly larger

than those from MU and OD (PERMANOVA, df = 2, p(perm) < 0.001 in each case; Post-hoc pair-wise tests, p(perm) < 0.01 in each case; Supplementary material 7) but there were no significant differences in tubule length (p(perm) = 0.12) and diameter (p(perm) = 0.96) between individuals from the two latter locations. No sexual dimorphism was observed in tubule diameter (PERMANOVA, df = 2, location and sex interaction, p(perm) = 0.92). However, males had significantly longer tubules than females in RF and MU (PERMANOVA, df = 2, location and sex interaction, p(perm) < 0.05; Post-hoc pair-wise tests, p(perm) < 0.05 in both cases).

2.4.3. GI and gametogenesis

H. arguinensis - The reproductive pattern of *H. arguinensis* was similar in the three sampled locations. Four main phases were distinguished: (1) a growth phase characterized by a gradual increase of GI (2) a final maturation phase ending by the peak of GI, (3) a spawning phase characterized by a drastic decrease of the GI, and (4) a post-spawning resting phase characterized by a low and stable GI. Within these broad phases, differences in GI and gametogenic state were observed between SA, OD and RF (Figures 3a-c). While the GI was not statistically different between SA and RF (PERMANOVA, df =2, n = 506, location, p(perm) < 0.001; Post-hoc pair-wise tests, p(perm) = 0.14), both differed from OD (p(perm) < 0.001 for RF and SA). No significant differences in GI was found between males and females at the three locations (Mann-Whitney U-tests, SA: U = 8588, p = 0.13; OD: U = 2028, p = 0.51; RF: U = 2025, p = 0.06).

At SA, mature individuals were already present in January 2014, but in OD and RF were only observed in March and April, respectively. The GI peak at SA (24.53 ± 3.88%) and RF (32.16 ± 5.53%) was reached in June 2013 at similar levels (PERMANOVA, df = 22, interaction location x month, p(perm) < 0.01; Post-hoc pair-wise tests, p(perm) = 0.26), but it was a month earlier and significantly smaller at OD (8.63 ± 2.00%: p(perm) < 0.001 for all combinations). However, almost 30% of OD individuals were immature in June 2013.

During the spawning phase there was a decrease of more than 85% in mean GI at the three locations. This phase lasted over 6 months at SA, although it was particularly intense from July to October 2013 with a peak in August 2013 (68.75% of spent individuals). At OD and RF, the spawning phase extended over 4 months from July to October 2013, and was less intense than at SA and the highest percentage of spent individuals (46.67%) occurred in September 2013.

After release of the gametes, individuals entered a resting phase, the duration of which was considerably different between SA and RF. At SA, the individuals were predominantly in resting phase (60%) only in October 2013, and from then on most individuals (> 60%) were already at the growing stage (GI ca. 4%). In contrast, at RF most individuals (> 50%) were in the resting phase for four months, with a mean GI around 1%, from October 2013 to January 2014. At OD, the GI stayed low, at around 1%, from October to December 2013 with around 20% of individuals at the resting stage and more than 60% immature.

Gametogenesis started earlier at SA (October 2013) than at OD (December 2013) and RF (November 2013). Individuals at the gonad growth stage were predominant (> 50%) for longer at SA (5 months) compared to OD (3 months) and RF (2 months). During gonad growth the GI increased by 342%, 388% and 227% at SA, OD and RF, respectively.

Comparison of the breeding season at SA between 2013 and 2014 over the 8-month overlap between May and December (Figure 3a) revealed the monthly GI was significantly different between the two years (PERMANOVA, df = 1, n = 228, interaction year x month $p(perm) < 0.001$). In particular, post-hoc PERMANOVA tests revealed that the GI in May, July, August and September was significantly different between the two years ($p < 0.05$ for all combinations). The GI peak in 2013 was reached a month earlier (June) than in 2014 (July). However, the spawning phase started two months later in 2014 and a similar decrease of GI occurred in the two years (88.38% in 2013 and 86.27% in 2014). The spawning period extended from August to December in 2014, with 45.33% of spent individuals detected during that period compared to 41.38% in 2013.

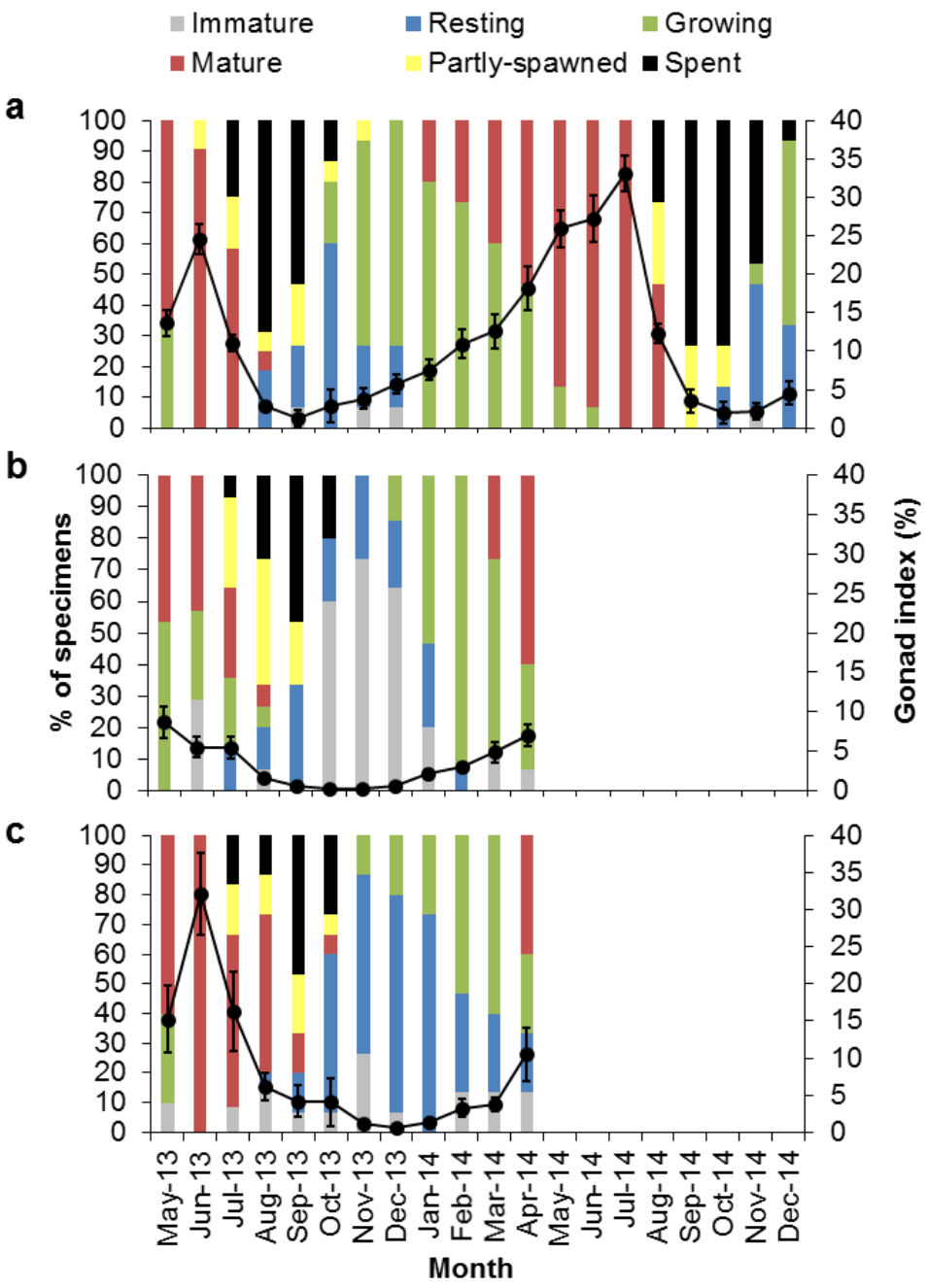


Figure 3. Gametogenic cycle and mean gonad index ± SEM (line, right Y-axis) of *H. arguinensis* at (**a**) SA, (**b**) OD and (**c**) RF. Histograms show relative frequencies of gonad stages per month from May 2013 to April 2014 for OD and RF, and to December 2014 for SA (n = 10 to 15 per month).

H. mammata - The same four main reproductive phases were found for *H. mammata* as for *H. arguinensis* (Figures 4a-c): (1) an increase of GI from spring to summer, (2) a drastic decrease of GI from summer to autumn, (3) a stabilization of GI from autumn to winter, and (4) a gradual increase of GI from winter to spring. The GI was significantly different between locations (PERMANOVA, df = 2, n = 225, p(perm) < 0.001). Individuals from OD and MU had the same GI (Post-hoc pair-wise tests, p(perm) = 0.77) which was significantly smaller than at RF (p(perm) < 0.001 for all combination). The GI peak in summer was significantly higher at RF (18.93 ± 4.15 %) than at OD (6.23 ± 1.71 %) and MU (3.54 ± 0.72 %) (PERMANOVA, df = 6, interaction location x season, p(perm) < 0.01; Post-hoc pair-wise tests, p < 0.01 for all combination) with no differences between the latter two locations (Post-hoc pair-wise tests, p = 0.65). No differences in GI between males and females was detected at any of the three locations (Mann-Whitney U-tests, MU: U = 395.50, p = 0.90; OD: U = 414, p = 0.07; RF: U = 182, p = 0.63).

From spring to summer, the mean GI increased by 30%, 78% and 51% in RF, OD and MU, respectively, (Figures 4a-c) and most individuals (> 60%) became mature. From summer to autumn, the GI decreased 74%, 75%, and 88% at RF, OD and MU, respectively. At RF and OD, more than 15% released their gametes in summer while at MU spawning occurred in the autumn. At RF spawning individuals (including partly-spawned and spent) were found in summer and winter, at OD in summer and autumn and at MU in autumn and winter. The highest percentage of spent individuals was found in autumn at all locations: 50%, 26% and 60% at RF, OD and MU, respectively. From autumn to winter, the mean GI stabilized at around 1% at OD and MU, while it was still decreasing from 5% to 2% at RF. Most individuals were at the recovery stage (> 55%) in winter with some at the growing stage at OD (5%) and MU (20%). Interestingly, at MU only 5 individuals out of 20 had visible gonads in winter. Also at MU, the gonad growth stage was predominant in spring (65%) while at OD and RF during the same period was around 37% and mature were 45%.

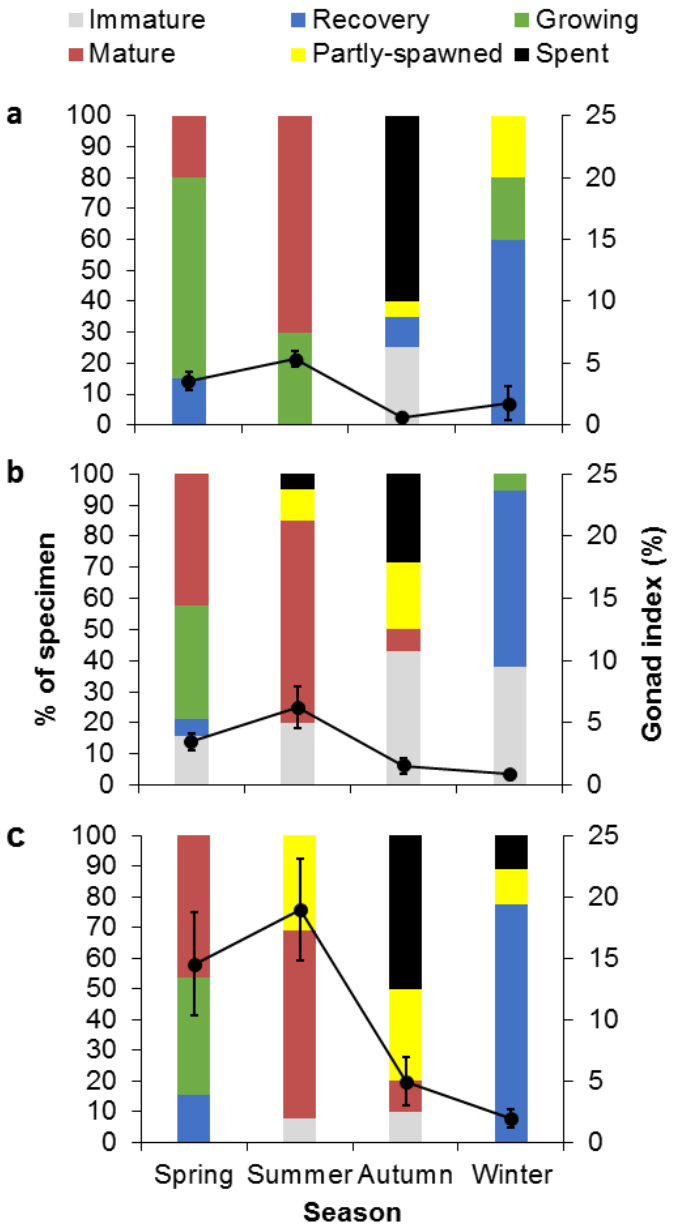


Figure 4. Gametogenic cycle and mean gonad index ± SEM (line, right Y-axis) of *H. mammata* at (**a**) MU (n = 65), (**b**) OD (n = 90), RF (**c**, n = 45). Histograms show relative frequencies of gonad stages per season from Spring 2013 to Summer 2014.

2.4.4. Soma-gonad relationships

For both species, there was a positive correlation between log(GW) and log(GBW) before spawning ($p < 0.05$ in each case, Pearson's correlation; Figures 5a-b). In *H. arguinensis*, the correlation coefficients were 0.50, 0.67 and 0.52 for SA (n = 77), OD (n = 37) and RF (n = 27) respectively. In *H. mammata*, the correlation coefficients were 0.55 for MU (n = 37), 0.59 for OD (n = 31) and 0.52 for RF (n = 23).

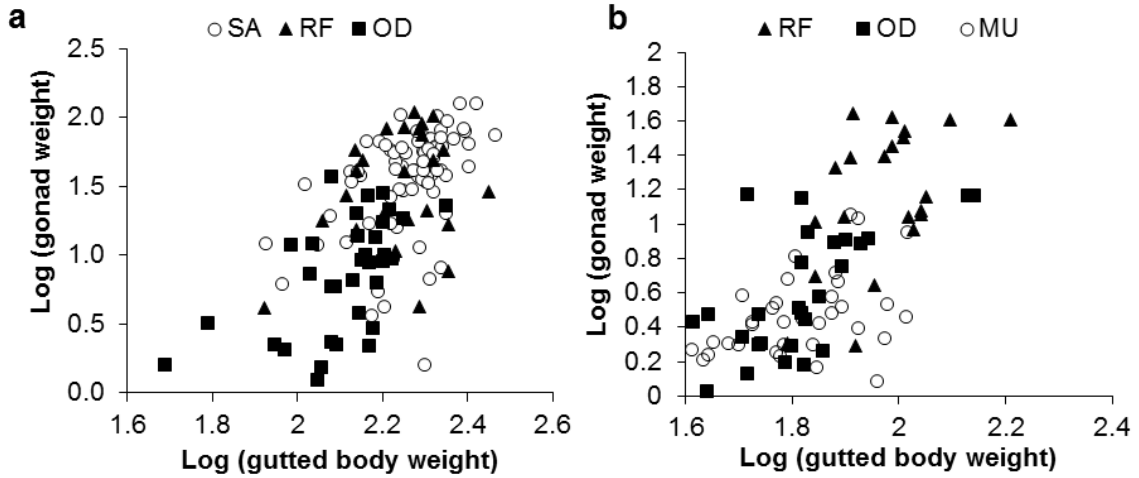


Figure 5. Relationship between gonad weight and gutted body weight before spawning in (**a**) *H. arguinensis* in SA (n = 77, r = 0.50), OD (n = 37, r = 0.67), and RF (n = 27, r = 0.52), and (**b**) *H. mammata* in MU (n = 37, r = 0.56), OD (n = 31, r = 0.55) and RF (n = 23, r = 0.52). All Pearson's correlations were significant at the $p < 0.05$ level.

2.4.5. Fecundity and sexual maturity

The absolute fecundity in *H. arguinensis* varied from 0.27 to 12.77 x 10^6 oocytes/female with a mean of 5.09 ± 0.42 x 10^6 oocytes/female (Figure 6a) while in *H. mammata* fecundity ranged from 0.10 – 6.21 x 10^6 oocytes/female with a mean of 1.32 ± 0.30 x 10^6 oocytes/female (Figure 6b). For *H. arguinensis*, individuals from RF and SA has similar fecundities (ANCOVA, square root transformation, df = 2, n = 63, $p < 0.01$; Unequal Tukey HSD, p = 0.99), but both had a higher absolute fecundity than those from OD (ANCOVA, square root transformation, df = 2, n = 63, $p < 0.01$; Unequal Tukey HSD, $p<0.01$). In *H. mammata*, individuals from RF had a higher absolute fecundity than those from OD and MU (ANCOVA, log transformation, df = 2, n = 27, $p < 0.05$; Unequal Tukey HSD, $p < 0.01$) with no differences between the latter two (p = 0.94). Absolute fecundity was positively correlated (Spearman

correlation; $p < 0.05$) to GI both in *H. arguinensis* (SA: r = 0.81, RF: r = 0.88, OD: r = 0.80) and *H. mammata* (RF: r = 0.84, OD: r = 0.87).

The length at first sexual maturity (TL_{50}) for *H. arguinensis* was estimated to be between 210 and 230 mm, while the GBW at first maturity (GW_{50}) was between 110 and 130 g. The TW at first sexual maturity (TW_{50}) was between 220 and 260 g (Supplementary material 8).

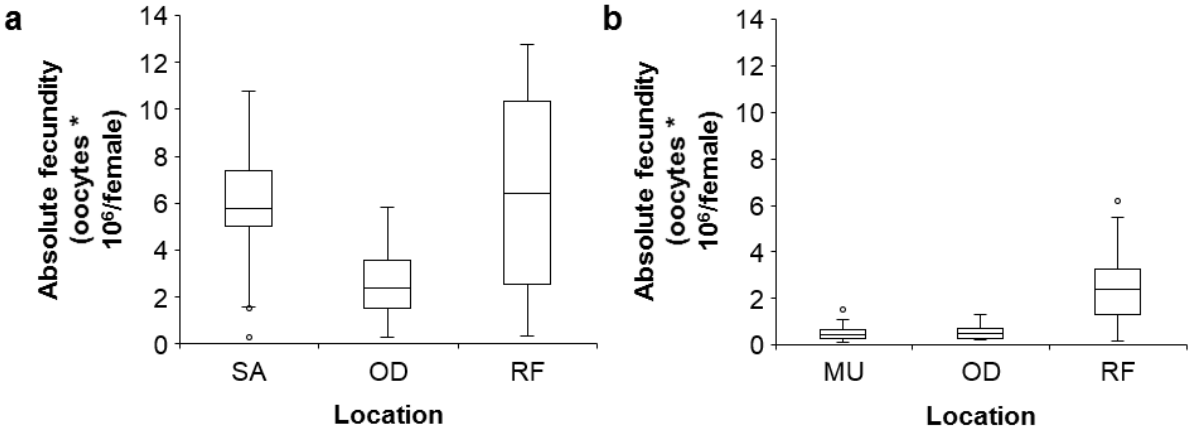


Figure 6. Box-whisker plots of the absolute fecundity of mature females from (**a**) *H. arguinensis* collected at SA (n = 23), RF (n = 20) and OD (n = 20) and from (**b**) *H. mammata* collected in MU (n = 8), OD (n = 10) and RF (n = 9).

2.4.6. Reproductive activity and environmental parameters

The mean monthly sea surface temperatures showed a clear seasonal cycle at the three locations with a maximum in September and a minimum in February (Figure 7a). The mean sea surface temperature was slightly higher at RF (17.8 ± 0.95 °C) than at OD (17.6 ± 0.97 °C) or SA (16.7 ± 0.66 °C). Significant cross-correlations were found for *H. arguinensis* in 2013 between the mean GI and mean sea surface temperature in the three sites (Figure 8a). The largest positive correlations were detected between lags +1 and +4, meaning that maximal GI values were reached from 1 to 4 months before the temperature peaked, while minimal GI values were reached from 1 to 4 months before the temperature minima. For the common 8-month sampling period, from May to December, in 2013 and 2014, at SA, significant cross-correlation between these two parameters was observed in 2013 at lag -1 while no correlation was found in 2014. The latter may reflect the difference in temperature between years with a decrease of temperature from June to July in 2014 when it increased gradually in 2013. In all locations gametogenesis started when temperature was decreasing (October-November), with the SA individuals started earlier. In contrast, gonad maturation was observed when temperature was increasing at OD and RF (March-April) whereas it started earlier at SA (January) when the

temperature was still decreasing. Spawning started when temperature increased rapidly (June-July), with the highest percentage of spent individuals identified when the temperature was maximal at RF and OD (September). At SA, the highest percentage of spent individuals was found a month before the peak temperature (August). The difference in water temperature a month before and a month after the GI peak was 4.67 ºC, 5.94 ºC and 6.05 ºC at SA, OD and RF, respectively. Although the peak of water temperature at SA was in September for both years, the GI peak and the onset of spawning differed between years, possibly related to differences in summer water temperatures (Figure 7a).

The longest photophase in June was 14.66 ± 0.03 h and the lowest in December was 9.66 ± 0.01 h (Figure 7b). In 2013, significant positive correlations were observed between the mean GI and mean photophase at 0 and +2 months lag including all locations, meaning that GI correlated with the photophase or preceded the photophase by 1 to 2 months (Figure 8b). The peak GI coincided with the summer solstice. At the three locations, gametogenesis initiated after the autumnal equinox, during short days (October-December) whereas spawning occurred after the summer solstice (June-July). At SA, from May to December, significant positive correlations were found between the mean GI and the mean monthly photophase 0 and +1 month later in 2013 and +1 month later in 2014.

The pattern of chlorophyll *a* did not show clear seasonal variation at the three locations (Figure 7c). The mean concentration of chlorophyll *a* was similar at RF (1.78 ± 0.31 mg/m^3) and OD (1.70 ± 0.35 mg/m^3), but both had a higher mean value than SA (0.95 ± 0.13 mg/m^3). All three locations had a peak of chlorophyll *a* in November 2013, corresponding to four to five months after the beginning of spawning in all locations. No significant cross-correlations were found between mean monthly GI and chlorophyll *a* concentration at any of the locations analyzed (Figure 8c) or during the 8 months in common between both sampling years (2013 and 2014) at SA. However, the largest mean chlorophyll peak was in August 2014 (4.76 ± 0.01 mg/m^3) coinciding with the beginning of the spawning (Figure 7c).

The complementary information on sediment analysis at each location showed that the organic matter was significantly lower at OD (0.63 ± 0.03 %) relative to RF (1.35 ± 0.18 %), SA (1.07 ± 0.04 %) and MU (1.73 ± 0.03 %) (ANOVA, df = 3, n = 48; Tamhane test: 35.72, $p < 0.001$ for all combinations), with no differences among the latter. Mean values in carbonate content varied significantly between all four locations according to the following order: SA (52.46 ± 1.98 %) > OD (27.46 ± 1.77 %) > MU (17.95 ± 0.47 %) > RF (8.90 ± 0.98 %; ANOVA, arcsin transformation, df = 3, n = 48; SNK test, $p < 0.01$ in each case, Supplementary material 9).

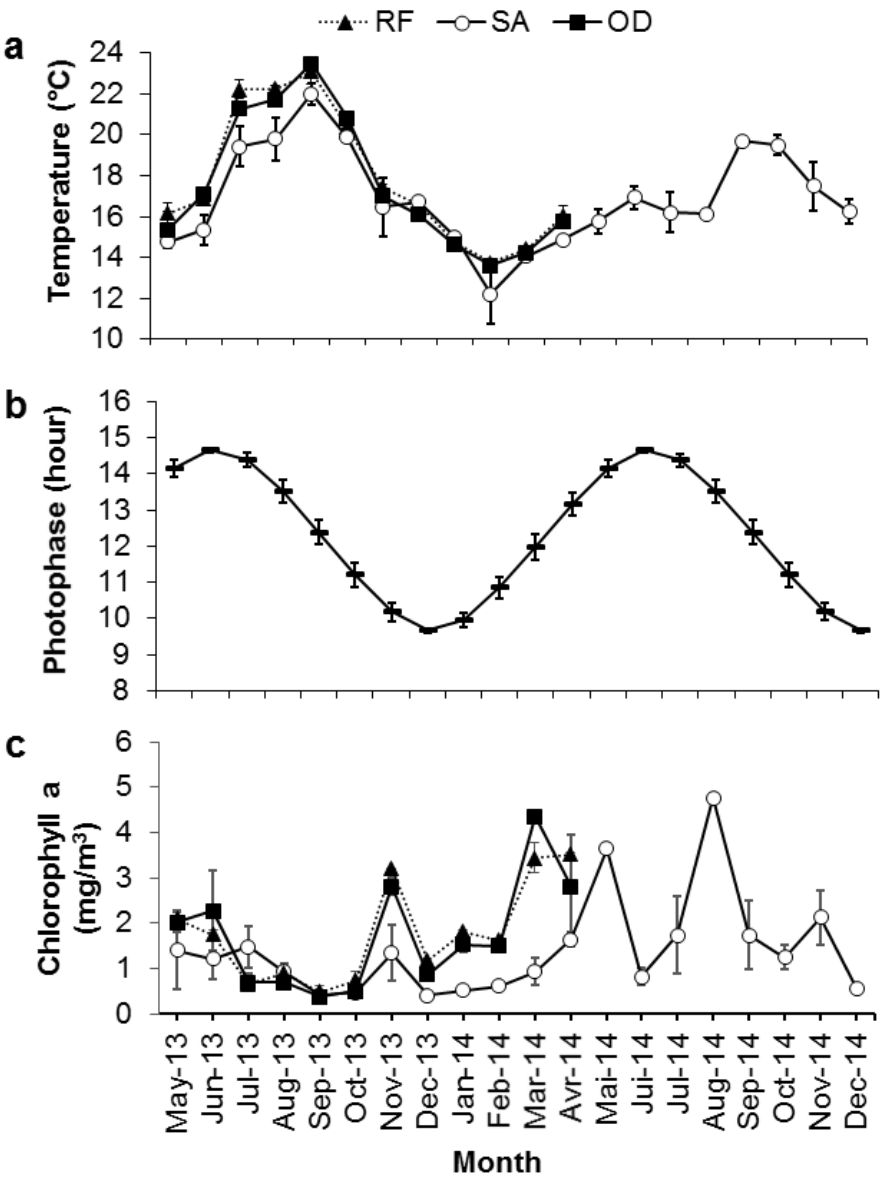


Figure 7. Seasonal variations of environmental parameters: (**a**) sea surface temperature (°C), (**b**) photophase duration (h) and (**c**) chlorophyll *a* (mg/m^3) at SA, RF and OD. See Materials and Methods for details.

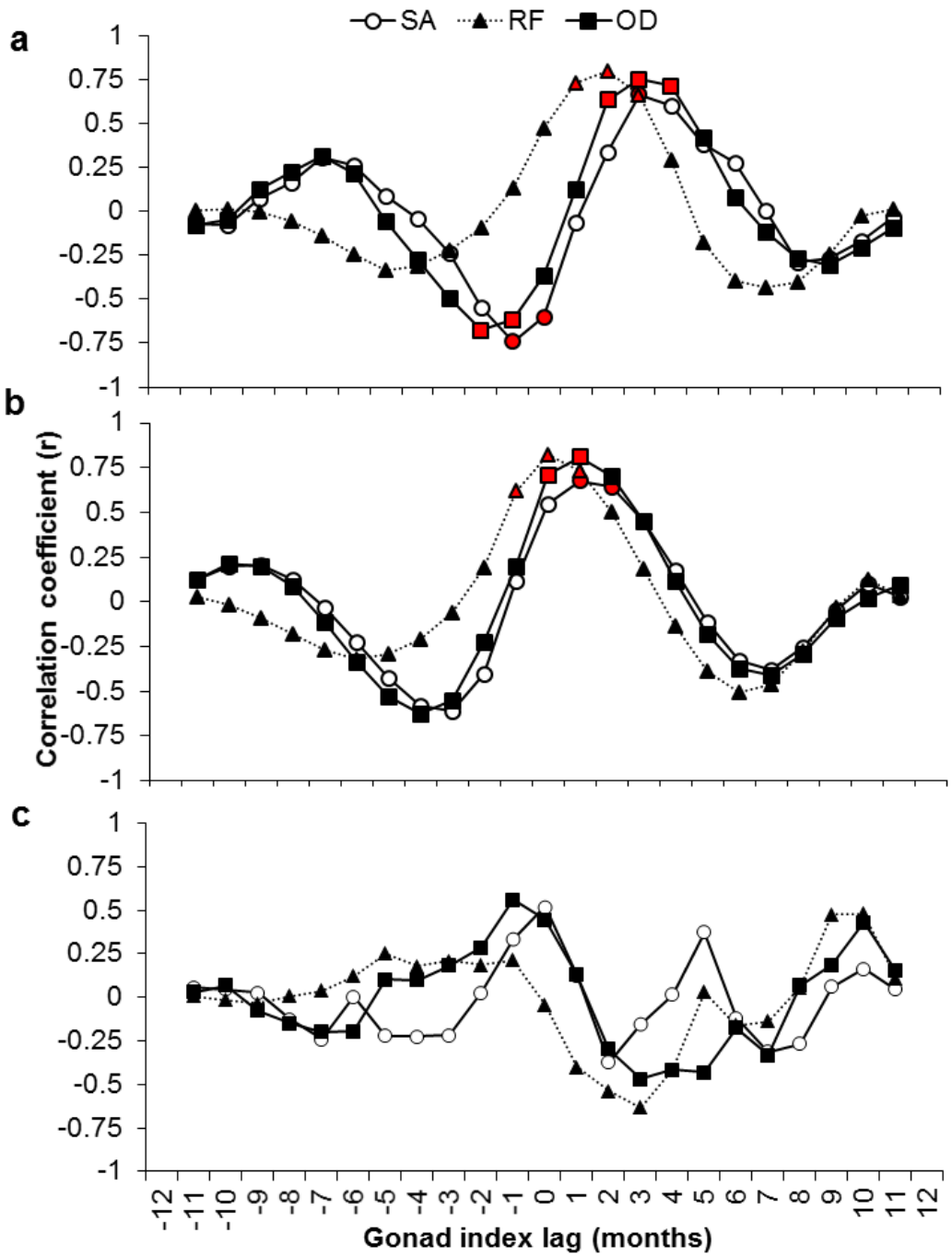


Figure 8. Cross-correlation function analysis (CCF) between gonad index and (**a**) temperature, (**b**) photophase duration, and (**c**) chlorophyll *a* for *H. arguinensis* at each studied site. Red symbols indicate significant correlations at p = 0.05.

2.5. Discussion

Separate sea cucumbers populations of *H. arguinensis* and *H. mammata* have similar seasonal reproductive patterns largely correlated to temperature and photoperiod. However, significant differences in average size/weight, fecundity and timing of reproductive activity for the sea cucumber populations studied was reflected in the size and maturity of the gonad, and this was most likely linked to local conditions.

The two species of sea cucumber studied are gonochoric, without sexual dimorphism, exhibiting a balanced sex ratio as commonly observed in many Aspidochirote holothurians (e.g. Asha and Muthiah 2007; Conand 1981; Despalatovic et al. 2004; Kazanidis et al. 2014; Mezali et al. 2014; Navarro et al. 2012). The studied populations had negative allometric growth, with the strongest positive correlations between TL and GBW generally observed in populations of *H. mammata*, reflecting a difference in body shape between the two species (Cone 1989; Herrero-Pérezrul and Reyes-Bonilla 2008). Such a pattern has been already described for most of holothurians (e.g. Bulteel et al. 1992; Conand 1993; Herrero-Pérezrul et al. 1999; Kazanidis et al. 2010; Poot-Salazar et al. 2014) where negative allometry was explained by the cylindrical shape of the body (Conand 1989) and the fact that the thickness of some part of the body wall was independent the size of the individuals (Ramón et al. 2010).

The size distribution of individuals varied between locations suggesting the importance of local environmental conditions. However, population structure may also reflect differing recruitment dynamics between locations, particularly at OD where the proportion of immature individuals was highest for both species. The individuals of both species at this location, where the organic matter was the lowest, were also smaller. Intertidal conditions at RF may also be potentially more stressful (air exposure, variable temperature) compared to the more stable conditions in the subtidal SA where the individuals can spend more energy on growth rather than on physiological changes linked to survival during air exposure. For *H. mammata*, however, tidal constraints and food availability may not be the main factors affecting the inter-population size differences, since small individuals could be found in the subtidal zone at MU where the organic matter is high. Conversely, the smaller sizes at MU and OD could be a consequence of preference of this species for rough substrates where individuals adopt nocturnal feeding behavior (Aydin and Erkan 2015; Navarro 2012; Navarro et al. 2013a; Navarro et al. 2013b) in contrast to the soft substrate at RF where feeding activity may occur day and night. Although carbonate content has in some cases been associated with organic carbon productivity, the amount of carbonate in the sediments at the experimental locations

does not seem to influence the size/weight of the sea cucumbers since it was the lowest at RF where larger individuals for both species were found.

Not only were there significant differences in size distribution between sea cucumber populations but their condition and fecundity varied. GW was positively correlated to GBW before spawning (when GIs were larger) which indicates that larger individuals have proportionally larger gonads and are potentially more fecund. *H. arguinensis* individuals at SA and RF not only were larger but also had larger absolute fecundity and GI than those at OD. Similarly, for *H. mammata*, individuals were larger and had larger absolute fecundity and GI at RF compared to OD or MU. This might be indicative of more favorable feeding and environmental conditions for both species at SA and RF allowing diversion of a higher proportion of energy to reproduction thus explaining their higher reproductive output (larger fecundity and GI) (Thompson 1983).

First sexual maturity occurs later in *H. arguinensis* (for *H. mammata* it was not estimated) from SA (TL: 210-230mm; EW:110-130g; TW:220-260g) than in *H. sanctori* from Gran Canaria (TL:201-210mm; EW:101-110g; TW:176-200g (Navarro et al. 2012). *H. arguinensis* had on average 5-fold higher absolute fecundity than *H. mammata*, but both had lower fecundity compared to larger tropical species at about 9 to 17.10^6 oocytes/female according to Conand (1993). At equivalent sizes, our results obtained for *H. arguinensis* are similar to those estimated by Domínguez-Godino et al. (2015) for the same species, ranging from 1.5 to 9.6 x 10^6 released eggs per female, and also those of the temperate *H. forskali* that varied between 2 and 7 x 10^6 oocytes per female (Tuwo and Conand 1994).

The difference in gonad morphology between *H. arguinensis* and *H. mammata* highlights the diversity of gametogenic processes that exists in the Holothuroidea. The gonads of *H. mammata* followed a uniform development, similar to what has been described in species such as *H. scabra* (Demeuldre and Eeckhaut 2012), *H. fuscogilva* (Ramofafia and Byrne 2001) and *H. spinifera* (Asha and Muthiah 2007). The gonad development pattern in *H. arguinensis* generally followed the tubule recruitment model (TRM), with tubules organized in distinct cohorts, representing different maturity stages with a single generation of gametes within each tubule, and incomplete resorption of the gonad after spawning (Smiley 1988). However, some specificities were observed: 1) some individuals had only tubules at one stage, 2) some tubules appeared to have more than one generation of oocytes. Exceptions to the TRM have been also described for other species (Foglietta et al. 2004; Gómez 2011) and included variations in gonad structure and development which were not only found between species but also between locations and seasons for the same species (Sewell et al. 1997). Possible effects of local

environmental conditions on tubular development have been suggested (Hamel and Mercier 1996b) but require systematic investigation.

The general pattern of reproduction was similar between the two species and populations in line with previous studies of sea cucumbers at narrow latitudinal ranges (Brewin et al. 2000; Byrne 1990; Byrne et al. 1998; Kazanidis et al. 2014). The reproductive cycle was seasonal with spawning during the warmer period (summer-autumn) as typically described for temperate sea cucumber species (Costelloe 1988; Despalatovic et al. 2004; Kazanidis et al. 2014; Mezali et al. 2014; Navarro et al. 2012; Sewell 1992; Sewell and Bergquist 1990; Tuwo and Conand 1992). Temperature and photoperiod correlated positively to GI, with some lag in *H. arguinensis,* indicating their potential role in the regulation of the reproductive cycle, as previously found for other sea cucumbers (e.g. Conand 1993; Hamel and Mercier 1996b; Muthiga 2006; Navarro et al. 2012; Ramofafia et al. 2000; Santos et al. 2015; Shiell and Uthicke 2006; Tuwo and Conand 1992). In the locations studied, gametogenesis initiated after the autumnal equinox, under short days (< 12h) and decreasing temperature (below 20ºC) whereas spawning started after the summer solstice when the photophase started to decrease and under increasing temperatures (above 20ºC). The importance of temperature is highlighted by the variability in the timing of gametogenesis and spawning between 2013 and 2014 and between locations. Furthermore, temperature shocks have been used by the aquaculture industry and in the laboratory to induce spawning in sea cucumbers (e.g. Domínguez-Godino et al. 2015; Mercier and Hamel 2009; Smiley et al. 1991). In addition, factors such as the lunar cycle, phytoplankton blooms, tidal flux, light intensity, and social cues (e.g. aggregative behavior, diffusible chemical signals) have also been suggested to trigger spawning (e.g. Giese and Kanatani 1987; Hamel and Mercier 1996a, 1999; Leite-Castro et al. 2016; Mercier and Hamel 2009).

It is tempting to suggest that the photoperiod is a permissive factor for both gametogenesis initiation and spawning and that temperatures below 20ºC are required for gametogenesis to develop and above 20ºC for spawning in the species studied. Furthermore, environmental factors such as food abundance associated to the more stable conditions of the subtidal habitat may facilitate faster replenishment of gonads after the spawning and ensure an extended release of gametes owing to storage of more nutrients (Bourgoin and Guillou 1990; Byrne 1990; Byrne et al. 1998). The prolonged spawning at SA could also be size-related, as larger animals are more fecund and generally known to spawn earlier and over a longer period (Scott et al. 2006; Secor 2000). In contrast, at RF and OD the sea cucumbers were intertidal and subjected to repeated temperature and exposure to solar radiation that may have contributed to

reduce the reproductive period and extended the recovery phase. Clearly, this is an area that requires investigation to pinpoint the specific contribution of the aforementioned environmental factors in the reproductive process.

Breeding periods coincide usually with optimal environmental conditions to maximize fertilization success and ensure offspring survival (Mercier and Hamel 2009). Our results did not detect a relationship between the maturity stages or GI and chlorophyll *a* concentration. A sea surface chlorophyll *a* peak was only detected in November, 5 to 6 months after the beginning of the spawning, and was unlikely to influence larval development which in *H. arguinensis* has been estimated to be 18 days (Domínguez-Godino et al. 2015). Navarro et al. (2012) obtained a similar result for *H. sanctori* in the Canary Islands and suggested the larvae were able to develop in low-food environment. However, the satellite measurements used in the present study may not reflect local conditions and therefore more focused studies on the early life stages would be required to determine the proximate factors critical for larval survival and growth.

2.6. Conclusion

Our study showed that populations in *H. arguinensis* and *H. mammata* living in a narrow latitudinal range have the same general reproductive pattern with spawning during summer-autumn and a recovery phase in winter. This pattern was correlated to temperature and photoperiod in *H. arguinensis* which with small deviations fluctuated similarly in the studied locations. The differences in size/weight, gonadal production and maturity stages between locations most likely were influenced by the particular features of each location such as the food availability and tidal stress. Populations of *H. arguinensis* and *H. mammata* are not at present under official exploitation in Portugal, although unregulated fishing may be putting pressure on stocks. The reproductive parameters obtained in the present work will provide an important basis for establishing regulatory measures for the management of sea cucumbers and preserve biodiversity.

2.7. Supplementary materials

Supplementary material 1. Methodology used to characterize the sediment at the studied locations.

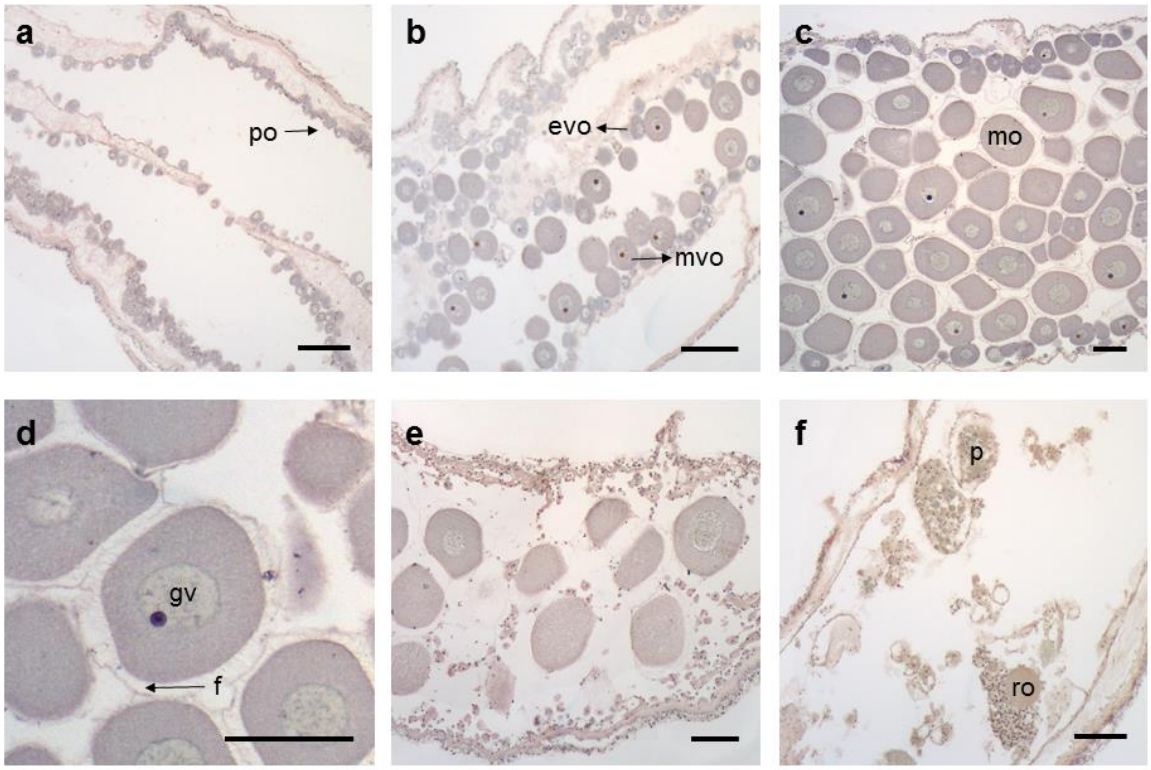
To characterize the sediment of each studied location, the upper layer of the surface sediment where sea cucumbers feed was sampled in March 2014. Twelve samples per location were dried in a drying oven at 60°C for 48 hours and processed to determine (i) organic matter content and (ii) carbonate content. The organic matter content was determined by carbonization at 450°C for 4 h. The organic matter content is the weight lost during carbonization (Gillan et al. 2005). Since the skeleton of sea cucumbers is composed of magnesium calcite, carbonates could be important for their development. To determine the carbonate content, dried sediments were decarbonated by dissolution with 37% HCl until effervescence disappeared. The decarbonated sediments were rinsed with distilled water, centrifuged and dried at 60°C during 48 hours. The carbonate content is the weight lost during the acid dissolution (Dean 1974).

Supplementary material 2. Statistical analyses.

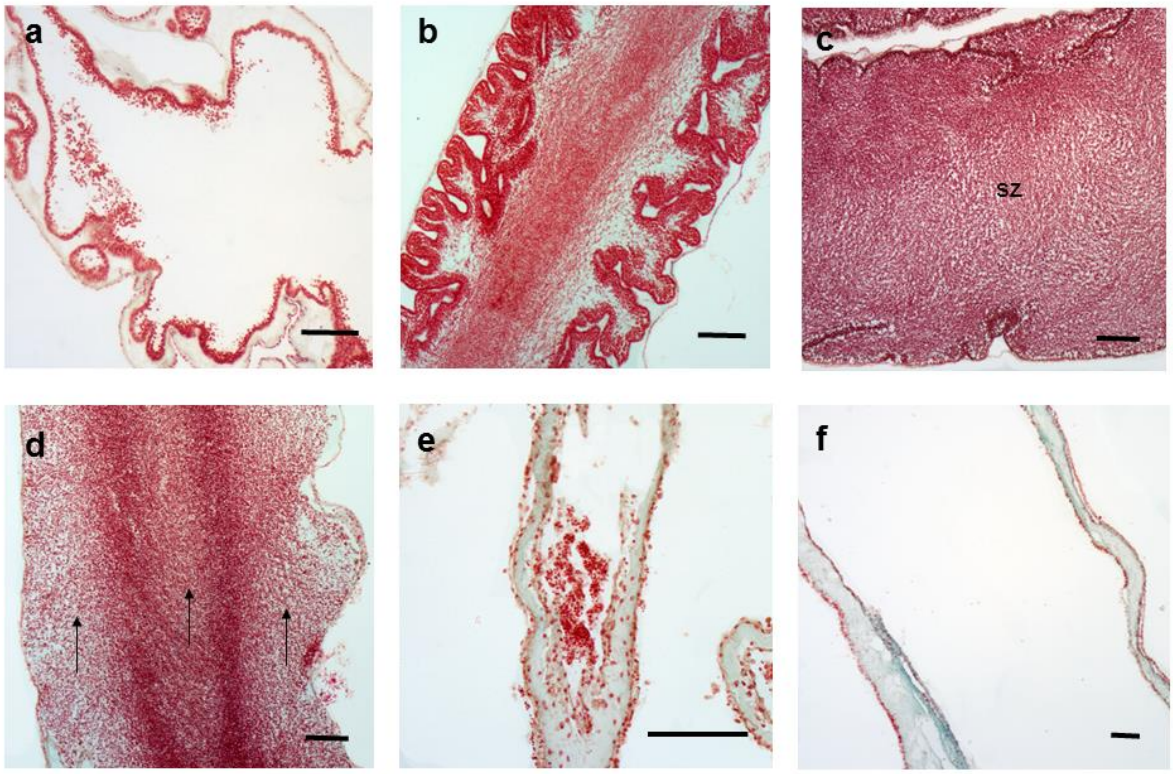
The protocol of Zuur et al. (2010) was followed to check for outliers, variance homogeneity, normality and relationships between variables before statistical analysis. Data were transformed when necessary to fulfill the prerequisites for parametric analysis (Shapiro-Wilk's and Cochran's tests) and were analyzed using SPSS version 17.0. If the prerequisites were not met even after data transformation, non-parametric tests were used. The significance level for all analyses was set at $p<0.05$. When the design was unbalanced, non-parametric permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001) with a type III (partial) sum of squares was applied as recommended by Anderson et al. (2008). Analyses were based on Euclidean distance and on 9999 permutations of residuals under a reduced model. When a factor was identified as statistically significant, post-hoc PERMANOVA pair-wise tests were conducted using 9999 permutations. Analyses were performed using Primer 6 with the add-on package PERMANOVA+ (Primer Ltd, Plymouth, UK).

Supplementary material 3. Macroscopic and microscopic description of the gonadal tubules in *H. arguinensis* and *H. mammata* at the different stages of the gametogenesis.

Maturity stage	Sex	Description
I. Immature	F/M	Short and thin tubules ending by a round shape. Distribution of the germinal cells along the tubule wall with a lumen completely empty.
II. Recovery	F	Extension and thickening of the tubules with previtellogenic oocytes (<20 µm) lining the germinal epithelium and an empty lumen. Degenerating oocytes and nutritive phagocytes can still be observed.
	M	Extension and thickening of the tubules with the beginning of invagination of germinal epithelium along which occurs layers of spermatogonia. Few spermatozoa can be observed in the lumen.
III. Growing	F	Thick and long tubules with still previtellogenic oocytes lining the germinal epithelium. Early (21-40 µm) and mid (41-80 µm) vitellogenic oocytes invade progressively the lumen of the tubule. No residual oocytes from the previous spawning.
	M	Thick and long tubules with abundant spermatogonia lining the numerous invaginations of the germinal epithelium and increasing of spermatozoa in the lumen.
IV. Mature	F	Maximal length and width of tubules with a thin wall and a lumen densely packed with mature oocytes (120-180 µm). Each oocyte is characterized by a well-defined germinal vesicle and germinal epithelium.
	M	Maximal length and width of tubules with a smooth and thin tubule wall and a lumen completely filled with mature spermatozoa. No early stages of spermatogenesis.
V. Partly-spawned	F	Shrinkage and wrinkling of tubules with loosely patches of mature oocytes and few disintegrated oocytes.
	M	Beaded appearance of shrunken tubules filled by patchy and low density of spermatozoa.
VI. Spent	F	Thin and wrinkled tubules with a lumen characterized by empty space, unspawned oocytes and some nutritive phagocytes.
	M	Thin and wrinkled tubules with empty area and relict spermatozoa in the lumen.



Supplementary material 4. Histological characterization of maturity stages in females *H. arguinensis* and *H. mammata* with VOF stain (**a**) Recovering ovary with pre-vitellogenic oocytes (*po*); (**b**) Growing ovary with active vitellogenesis with early (*evo*) and mid (*mvo*) vitellogenic oocytes; (**c, d**) Mature ovary with large mature vitellogenic oocytes (*mo*) within follicles (*f*) with a large germinal vesicle (*gv*); (**e**) Partly-spawned ovary with loosely packed of unspawned oocytes; (**f**) Spent ovary with relict oocytes (*ro*) and phagocytes (*p*) degrading oocytes. Scale bars represent 100 µm.



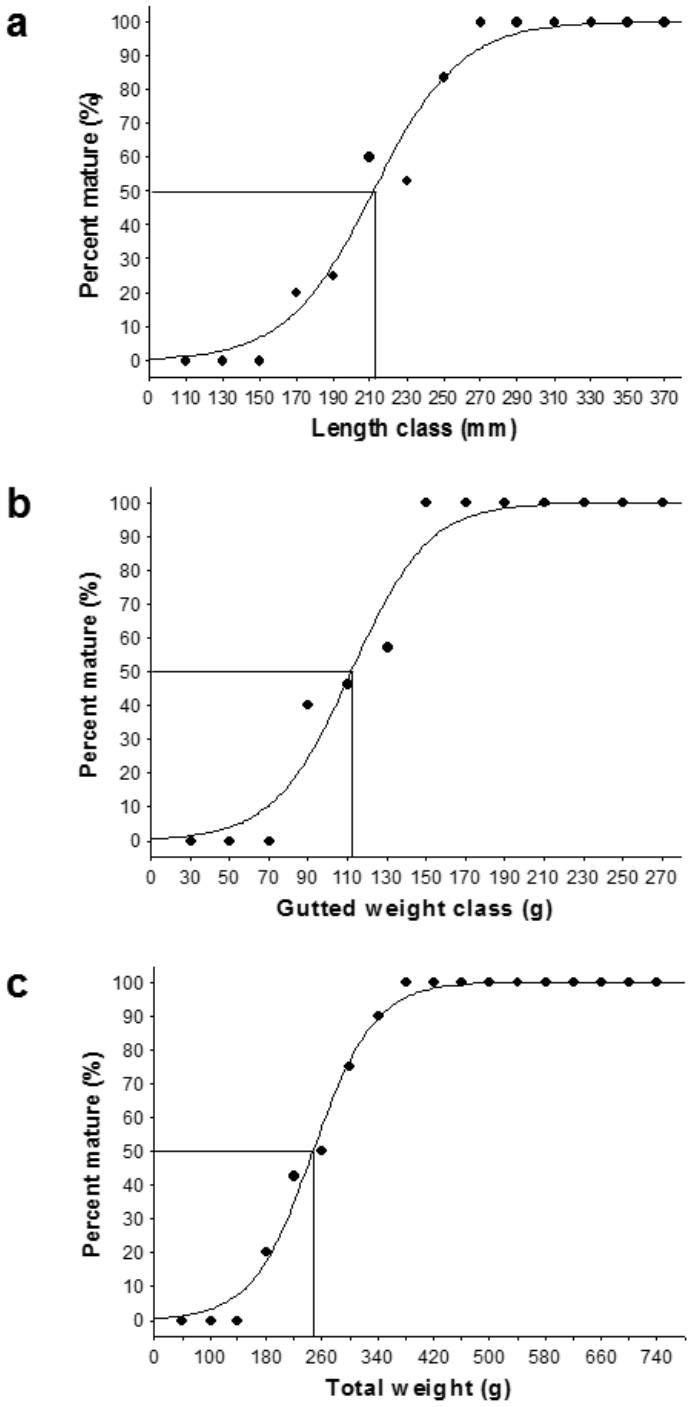
Supplementary material 5. Histological characterization using Masson's trichrome stain of the main maturity stages detected in males *H. arguinensis* and *H. mammata* gonads. (**a**) Recovering testis with developing spermatocytes lining the germinal epithelium; (**b**) Growing testis with invaginations of the germinal epithelium and increasing abundance of spermatozoa in the lumen; (**c**) Mature testis with the lumen completely filled with spermatozoa (sz); (**d**) Partly-spawned testis with area less dense areas of spermatozoa (arrow); (**e, f**) Spent testis with unspawned spermatozoa (**e**) and an empty lumen (**f**). Scale bars represents 100 µm.

Supplementary material 6. Morphometry of the gonadal tubules in the three populations of *H. arguinensis.*

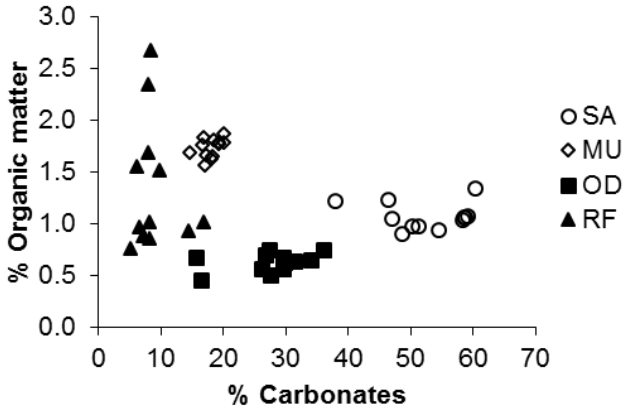
Maturity stage	Sex	Site	N	Tubules characteristics		
				Lenght (mm)	**Diameter (mm)**	**Colour**
I. Immature	F/M	SA	60	9.90 ± 5.73	0.24 ± 0.11	Translucent
		OD	615	10.41 ± 3.02	0.34 ± 0.09	
		RF	240	16.02 ± 78.10	0.31 ± 0.12	
II. Recovery	F	SA	285	53.00 ± 25.46	0.75 ± 0.23	Translucent to whitish
		OD	195	20.70 ± 4.98	0.55 ± 0.10	
		RF	450	28.21 ± 36.22	0.63 ± 0.36	
	M	SA	225	45.95 ± 25.28	0.60 ± 0.29	Translucent to whitish
		OD	165	21.53 ± 6.32	0.57 ± 0.13	
		RF	360	30.18 ± 46.31	0.59 ± 0.58	
III. Growing	F	SA	465	77.34 ± 41.32	1.15 ± 0.34	Light pink to
		OD	165	39.22 ± 6.35	1.06 ± 0.33	to light orange
		RF	300	54.84 ± 56.46	1.31 ± 1.24	
	M	SA	540	102.31 ± 51.67	0.89 ± 0.34	White to cream
		OD	210	47.87 ± 13.34	0.80 ± 0.18	
		RF	195	66.23 ± 91.58	0.74 ± 0.47	
IV. Mature	F	SA	750	111.27 ± 39.20	1.57 ± 0.51	Light orange to
		OD	165	67.78 ± 21.36	1.85 ± 0.36	to reddish orange
		RF	300	101.99 ± 38.05	1.87 ± 0.44	
	M	SA	660	124.24 ± 59.18	1.37 ± 0.58	Cream to beige
		OD	60	100.32 ± 8.97	1.43 ± 0.17	
		RF	285	119.56 ± 38.05	1.45 ± 0.42	
V. Partly-spawned	F	SA	75	47.00 ± 28.84	0.95 ± 0.11	Pale orange
		OD	92	29.55 ± 6.51	0.76 ± 0.08	
		RF	60	62.27 ± 22.93	1.09 ± 0.19	
	M	SA	210	67.08 ± 29.96	0.89 ± 0.54	Beige with
		OD	107	46.39 ± 5.60	0,67 ± 0.08	brown patches
		RF	60	71.28 ± 67.52	0.88 ± 0.23	
VI. Spent	F	SA	345	60.17 ± 34.25	0.54 ± 0.19	Brownish to translucent
		OD	75	36.34 ± 4.45	0.44 ± 0.05	with rust blotches.
		RF	45	20.71 ± 5.33	0.41 ± 0.07	
	M	SA	435	50.94 ± 20.89	0.41± 0.12	Brownish to translucent
		OD	75	21.73 ± 6.35	0.47 ± 0.08	with rust blotches.
		RF	105	17.36 ± 10.27	0.43 ± 0.17	

Supplementary material 7. Morphometry of the gonadal tubules in the three populations of *H. mammata*.

Maturity stage	Sex	Site	N	Tubules characteristics		
				Lenght (mm)	**Diameter (mm)**	**Colour**
I. Immature	F/M	MU	67	6.10 ± 2.80	0.38 ± 0.08	Translucent
		OD	136	6.48 ± 6.13	0.37 ± 0.08	
		RF	30	7.30 ± 1.39	0.40 ± 0.08	
II. Recovery	F	MU	44	6.63 ± 1.78	0.86 ± 0.96	Translucent to whitish
		OD	191	10.17 ± 9.91	0.83 ± 0.62	
		RF	75	27.34 ± 10.11	0.78 ± 0.11	
	M	MU	75	13.88 ± 5.30	0.78 ± 0.25	Translucent to whitish
		OD	135	12.57 ± 12.93	0.85 ± 0.29	
		RF	30	33.17 ± 7.05	0.89 ± 0.13	
III. Growing	F	MU	95	18.76 ± 6.46	1.14 ± 0.25	Light orange to
		OD	89	16.74 ± 8.62	1.03 ± 0.36	to intense orange
		RF	61	40.06 ± 16.16	1.18 ± 0.12	
	M	MU	167	21.59 ± 6.60	0.89 ± 0.23	White to cream
		OD	86	19.05 ± 4.92	0.88 ± 0.23	
		RF	15	36.13 ± 2.75	0.84 ± 0.05	
IV. Mature	F	MU	112	25.30 ± 5.96	1.42 ± 0.37	Intense red
		OD	97	25.29 ± 10.93	1.42 ± 0.35	
		RF	137	47.60 ± 22.67	2.39 ± 0.51	
	M	MU	126	30.80 ± 14.32	1.21 ± 0.43	Cream to beige
		OD	134	28.21 ± 7.17	1.22 ± 0.42	
		RF	90	61.54 ± 10.25	2.35 ± 0.31	
V. Partly-spawned	F	MU	15	17.73 ± 9.03	1.42 ± 0.19	Pale orange to yellow
		OD	11	16.91 ± 9.00	1.43 ± 0.20	
		RF	60	26.52 ± 4.98	1.95 ± 0.18	
	M	MU	15	12.93 ± 4.92	0.79 ± 0.06	Beige with
		OD	35	12.20 ± 6.36	0.89 ± 0.15	brown patches
		RF	60	49.03 ± 52.72	1.51 ± 0.30	
VI. Spent	F	MU	91	7.22 ± 1.92	0.45 ± 0.06	Brownish to
		OD	10	5.20 ± 2.53	0.39 ± 0.14	translucent
		RF	45	12.00 ± 1.11	0.34 ± 0.06	with rust blotches
	M	MU	30	12.33 ± 1.51	0.45 ± 0.06	Brownish to
		OD	6	6.33 ± 1.21	0.40 ± 0.04	translucent
		RF	15	23.73 ± 1.03	0.25 ± 0.06	with rust blotches



Supplementary material 8. Size (**a**), gutted weight (**b**) and total weight (**c**) at first sexual maturity in *H. arguinensis.*



Supplementary material 9. Percentage of organic matter *versus* percentage of carbonates in each of the studied location.

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Chapter III

Chemicals released by male sea cucumber mediate aggregation and spawning behaviour

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Chemicals released by male sea cucumber mediate aggregation and spawning behaviours

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3.1. Abstract

The importance of chemical communication in reproduction has been demonstrated in many marine broadcast spawners. However, little is known about its use in echinoderms, the nature of the compounds involved or their mechanism(s) of action. Here, the hypothesis that the sea cucumber *Holothuria arguinensis* uses chemical communication for aggregation and spawning was tested. Water conditioned by males, but not females, attracted both males and females; gonad homogenates and coelomic fluid had no effect on attraction. Male spawning water, but not female spawning water, stimulated males and females to release their gametes; the spermatozoa alone did not induce spawning. *H. arguinensis* male spawning water also induced spawning in the related *H. mammata.* This indicates that males release chemicals - pheromones - together with the gametes that induce spawning in conspecifics and possibly sympatric species. Finally, the male pheromone is likely a mixture with at least one labile compound (biological activity is lost after four hours at ambient temperature) and may include phosphatidylcholine derivatives. The identification of pheromones in sea cucumbers offers a new ecological perspective and may have practical applications for their aquaculture.

Key words: Echinoderm, spawning, aggregation, pheromone, sea cucumber, behaviour

3.2. Introduction

Broadcast spawning is considered as the most ancient and widespread mode of reproduction in marine invertebrates (Giese and Kanatani 1987; Wray 1995). The main disadvantage of this reproductive mode is the rapid dispersal of gametes in the environment, which can reduce fertilization rates and subsequent larval production (Denny and Shibata 1989; Pennington 1985). To counteract this, organisms have adopted different behavioural strategies including breeding aggregation and synchronization of reproductive activities (e.g. Babcock 1995; Giese 1959; Levitan and Petersen 1995; Yund 2000).

Aggregative behaviours among echinoderms are believed to facilitate gametogenesis and spawning through inter-individual chemical exchange and by increasing the probability of gamete encounter (Levitan 1991; Levitan et al. 1992; Mercier and Hamel 2008; Tominaga et al. 2004). Also, field observations show that grouped animals, irrespective of the sex ratio, are riper than solitary individuals (Young et al. 1992). Although aggregations are regularly found in echinoderms, what brings individuals group together is still poorly understood.

Aggregation and spawning in echinoderms are thought to be largely influenced by environmental factors such as temperature, photoperiod, lunar periodicity and tidal cycles (reviewed by Giese and Kanatani 1987; Giese et al. 1991). However, chemical communication plays a determinant role in the fine tuning of these processes in several marine broadcasters such as polychaetes, crustaceans, molluscs and echinoderms (e.g. Bamber and Naylor 1996; Hamel and Mercier 1996; Hardege and Bentley 1997; Painter et al. 1998; Soong et al. 2005; Watson et al. 2003).

In echinoderms, the existence of pheromones synchronizing gamete release among individuals has been suggested by Beach et al. (1975). However, evidence is largely circumstantial and little is known about the nature, origin and mechanisms of action of the putative pheromones (Mercier and Hamel 2009). Kato et al. (2009) induced spawning of mature sea cucumbers by injection of a gonadatropic neural peptide (NGIWYamide), extracted from buccal ring nerves, into the coelomic cavity. This technique, originally developed in starfish (Chaet and McConnaughy 1959), proved ineffective when the radial nerve extract was simply added to the water, rejecting any pheromonal role (Kanatani 1973). In contrast, intra-coelomic injections of perivisceral coelomic fluid (PCF) from spawning individuals successfully induced spawning directly and when added to the water, suggesting that the coelomic fluid contains a pheromone (Mercier and Hamel 2002).

Generally, males start to spawn before females, suggesting that spermatozoa and/or chemicals released with the sperm induce spawning in females (e.g. Himmelman et al. 2008; Levitan 2002; McEuen 1988; Miller 1989; Smiley et al. 1991; Thorson 1950). Sperm suspensions have been experimentally tested on the spawning behaviour in starfish (Caballes and Pratchett 2017; Hamel and Mercier 1995) and sea urchins (Reuter and Levitan 2010; Starr et al. 1990). However, the reciprocal effects of male and female gametes have rarely been experimented, and the origin and identity of the chemicals involved have never been established in echinoderms.

Sea cucumber aquaculture programs are being developed to sustain the Chinese demand and to enhance wild populations severely weakened or disappeared due to worldwide over-exploitation (Anderson et al. 2011; Lovatelli et al. 2004). Thermal shock remains the most common used method to stimulate spawning in their aquaculture (Battaglene 1999; Hamel et al. 2001; James 1994; Smiley et al. 1991). However, this method gives inconsistent and variable results according to the protocol and species used (Léonet et al. 2009; Yanagisawa 1998); therefore, the identification of spawning pheromones may provide an interesting alternative.

The sea cucumber *Holothuria arguinensis* is a recent fisheries target and the first sea cucumber species to be reared in captivity in Europe (Domínguez-Godino et al. 2015). A better understanding of the chemical factors influencing the reproductive biology of this broadcast summer-autumn spawner (Marquet et al. 2017) could give valuable insights to improve the management of species reared in captivity (Hamel and Mercier 2004) and could help to control invasive species (Witzgall et al. 2010), which represent a major threat to biodiversity and cause significant damage to worldwide economy (Pimentel et al. 2001). A typical case is found in the Mediterranean Sea where many Indo-Pacific species, including sea cucumbers (Antoniadou C. and Vafidis 2009), have invaded the area through the Suez Canal (Galil 2009). Here, the hypothesis that *H. arguinensis* release chemical signals for aggregation and spawning was tested and a preliminary chemical evaluation of the released substances during spawning was carried out.

3.3. Material and Methods

3.3.1. Ethics statement

Sea cucumbers, *H. arguinensis,* were collected and handled in agreement with the license issued by ICNF, The Institute for Nature and Forest Conservancy of Portugal (License N° 635/2015/CAPT, N°95/2016/CAPT, N° 490/2016/CAPT). The species is not endangered or protected.

3.3.2. Collection, gonadal biopsy and maintenance of specimens

Adult *H. arguinensis* longer than 210 mm (Marquet et al. 2017) were collected in Portugal from the Ria Formosa (37°00'35.02''N; 7°59'46.10''O) for the aggregation behaviour assay during late spring 2015 and from Sagres (37°00'44.78''N; 8°55'49.51''O) for the spawning experiment during summers of 2015 and 2016. The sex and the maturity stage of the sea cucumbers were determined by observation under a light microscope (Leica DM2000) of a gonadal biopsy taken from a small incision on the dorsal side of the animal previously anesthetized in 5% $MgCl_2$ (Hamel and Mercier 1996). All experiments were performed at least one week after the biopsy to allow recovery. Females and males were kept in separate tanks (1.2 x 1.0 x 0.6 m) and fed four times a week with sediment collected from their natural environment.

3.3.3. Y-maze tests of attraction

To test the capacity of water-borne stimuli to attract conspecifics, a glass Y-maze (30 cm height, 3 mm thick) was used with a stem of 55 cm long and 25 cm wide separating in two arms of 40 cm long and 12.5 cm wide (Figure 1a) at the end of which stimuli were added. Water inflow was 0.70 l / min in each arm and was drained out of the maze through two holes (2 cm diameter) connected to a standpipe which maintained the water height (10 cm). To assess the plume dynamics within the maze, dyes were delivered to both arms. The tests revealed small-scale turbulence within the arms after the injection of the dyes, but little mixing between water of the two arms in the stem section. The Y-maze was surmounted at 2 m height by an infrared video camera equipped with infrared filter (ICD-49E, Ikegami Tsushinki, Japan) and at 1.2 m height by two automated infrared light sources (IR-56, Microlight, Russia) oriented diagonally with respect to the bottom of the Y-maze (Figure 1b). The videos were stored in AVI files in a hard drive and displayed with everfocus Player Application (EFPlayer v 1.0.6.4.).

Based on preliminary tests, experiments were carried out over four hours at night, when this species is more active. All animals used in this experiment were early mature adults. Test animals were placed at the entry area of the Y-maze (A; Figure 1a) and given the choice of control seawater and seawater containing the stimulus. The stimuli tested were: (1) conspecific-conditioned water (CCW), (2) gonad homogenates (ovary and testis), and (3) coelomic fluid (CF). To produce conspecific-conditioned water, two individuals of known sex were placed in an aquarium (30 x 20 x 20 cm) from which water flowed by gravity to one of the Y-maze arms at a rate of 0.7 l / min. Control seawater was also provided at 0.7 l / min to the other arm. Separate pools of five testes (120 g in total) and five ovaries (120 g in total) were homogenised from fresh gonad with a mortar and a pestle and filtered (100 µm pore size) to remove large particles. Coelomic fluid (10 ml) was collected from separate pools of five males and five females using a sterile needle inserted in the body wall of the animals and withdrawn by gravity. Gonad homogenates and coelomic fluids were frozen after collection. The day of the experiment they were thawed and diluted in 1200 ml of seawater and injected in the maze with a peristaltic pump at a rate of 10 ml / min during the first two hours of the experiments, with the seawater inflow of 0.7 l / min.

At least 10 males (M) and 10 females (F) were tested for each combination (receiver vs donor: M vs. M, M vs. F, F vs. F., F vs. M) and stimulus. Each animal was taken from a pool of 20 males and 20 females kept in separate tanks (1.2 x 1.0 x 0.6 m) and was used only once for each stimulus. Between trials, the Y-maze was rinsed and cleaned of debris, and clean seawater was allowed to flow through the entire maze for 15 min to remove any residual stimulus. The stimulus side was alternated (B-C; Figure 1a) between each successive test to eliminate any arm preference.

The behaviours registered in each test were (1) first choice of arm and (2) percentage of time spent in each arm (in each case when the full body entered one of the two arms). The effect of each stimulus on the first choice of arm (stimulus or control) was evaluated by a chi-square test to determine if the observed frequency was different from a random choice (50/50). The nonparametric Wilcoxon signed-ranks test was used to compare the percentage of time spent in each arm (stimulus or control).

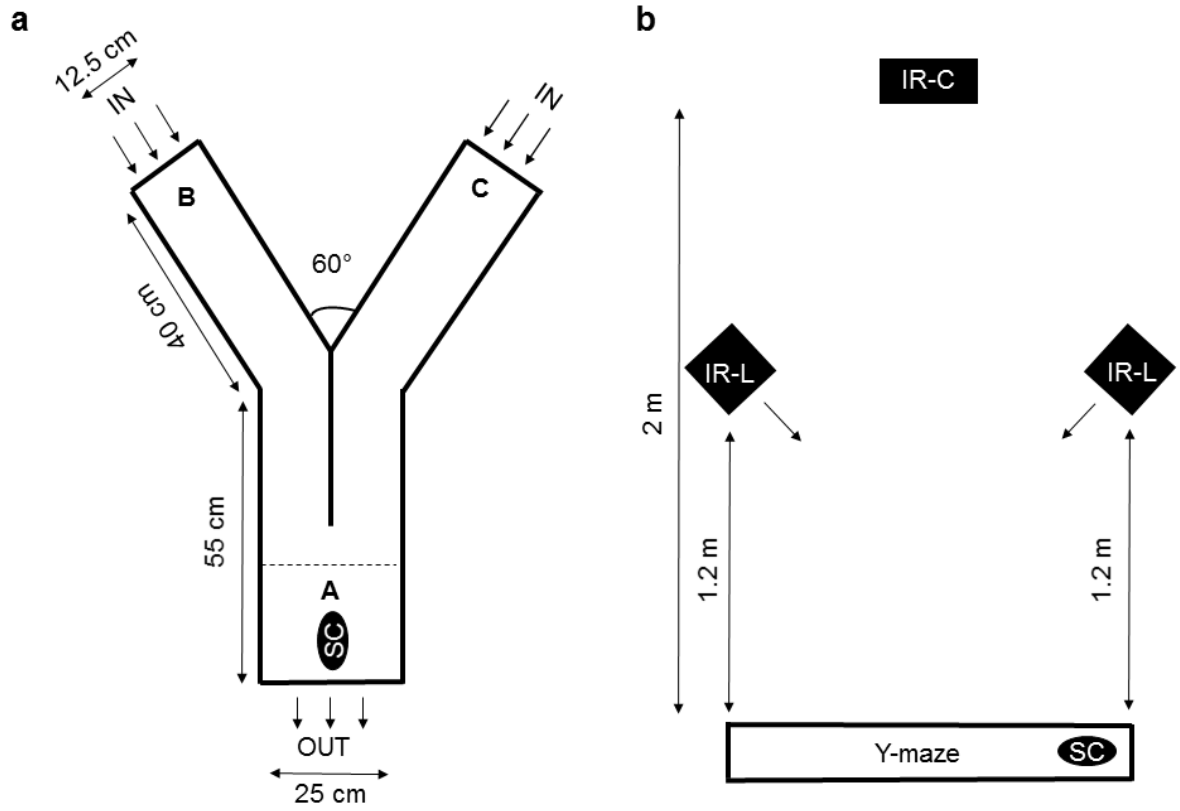


Figure 1. Schematic representation of the experimental setup used in the attraction experiment. (**a**) Overhead view showing the entry area A, the stimulus and control sides B or C. (**b**) Side view showing the position of the camera IR-C and light source IR-L. SC indicates the initial positioning of the test sea cucumber.

3.3.4. Spawning water tests

Two sets of experiments were designed to test the effect of spawning water on spawning of sea cucumbers. The first tested if male or female spawning water could induce spawning in conspecifics and the second tested for heterospecific responses in the closely related and sympatric species *H. mammata*. All aquaria were filled with seawater coming from the same source and with the same physicochemical properties (22-25°C, 35 ppt salinity). All experiments were performed at night before or on the full or new moon, and only reproductively mature sea cucumbers, previously selected through a gonadal biopsy, were used.

For each trial, sea cucumbers used to obtain the spawning water (the donors) were placed in a larger aquarium (40 x 40 x 40 cm) and induced to spawn by thermal shock (TS). For the TS, the donors were placed in an aquarium with 5 to 6°C cooler water for 10 minutes before being returned to their original aquarium. Spawning occurred within 1 hr for males and 2 hr for females. The test sea cucumbers were placed individually in a series of smaller experimental aquaria (26 x 16 x 16 cm) in the morning or the day before the experiment in order to ensure spawning was not induced by a change of environment, e.g. from the transfer from

the larger to the smaller aquarium (in which case they were not used in the experiment). The tests consisted of addition of 250 ml of female or male fresh spawning water, with or without spermatozoa, or spermatozoa in seawater, always to the same corner of the small aquarium containing either a male or a female. To test for interspecific spawning activity, 250 ml of male spawning from *H. arguinensis* was added to a small aquarium containing an isolated male or female *H. mammata*. Control aquaria received 250 ml of seawater added the same way as the test seawater. Spermatozoa were filtered from spawning water (0.7 µm pore size; Whatman, GF/F).

The results were scored (spawning or not spawning) after one or two hours for males and females, respectively. Statistical significance of percent spawning of stimuli versus the seawater control was evaluated by a Fisher's exact test (two-tailed). Difference of time in spawning response was compared between males and females using the Mann-Whitney test.

3.3.5. Stability and fractions tests

In order to characterize the active substance(s) in spawning water, tests were designed to determine whether the biological activity 1) was extractable by HLB+ universal cartridges (reverse-phase sorbent, Waters corporation, Millipore, Milford, Mass., USA), and 2) was stable. HLB+ cartridge extracts (fraction retained in cartridge and eluted in 5 ml of methanol) and filtrates (the flow-through fraction) of 1 l fresh male spawning water from which sperm and particles had been filtered, as indicated above, were obtained following the generic protocol in the manufacturer's manual. For the spawning tests, the same experimental setup as above was used with the following stimuli: HLB cartridge extract (E), HLB cartridge filtrate (F), E and F together (E + F), fresh spawning water (FSW), spawning water aged 2 hr (2h FSW) and spawning water aged 4 hr (4h FSW). The solution containing extract was prepared by adding 1.25 ml of methanol extract to 250 ml of sea water (E) or 1.25 ml of methanol extract to the 250 ml filtrate (E + F). Each extract (E) and filtrate (F) was used in 4 tests. Two control aquaria were used, sea water only and methanol (1.25 ml diluted in 250 ml sea water). If sea cucumbers spawned within the expected period, the test was stopped. If they did not respond, the complementary stimulus was added (E or F) or FSW. Finally, if they did not respond to FSW, a TS was provided. FSW and TS were used as positive controls to determine if unsuccessful spawning was due to the sea cucumber not being ready to spawn. Those that did not respond to any stimuli were not considered in the analysis.

Statistical significance of percent spawning of stimuli versus the seawater only control was evaluated by a Fisher's exact test (two-tailed).

3.3.6. Preliminary chemical characterization spawning water

HLB+ extracts of filtered seawater taken from the same aquarium before and after spawning (males: n = 5; females: n = 2) were used for subsequent analyses by mass spectrometry. The mass spectrometer was a Bruker Esquire HCT *ultra* ion trap, equipped with an electrospray ionization source (ESI) (Agilent), operating in the negative and positive polarities. For ESI-MS^n studies (direct injection) the typical spray and ion optics conditions were the following: capillary voltage, 4.0 kV; nebulizer gas pressure, 30 psi; drying gas, 300°C; drying gas flow, 6 l/min; capillary exit voltage, 208 V; skimmer voltage, 15 V. The solutions were infused into the ESI source using a syringe pump (model 781100, KDScientific, USA), at a rate of 4 µl/ min. Infusion was performed using samples extracted with methanol, after washing the HLB+ cartridges with ultra-pure water. This washing step removes excess salts, which quench the formation of ions under ESI. Direct injection allowed us to obtain fragmentation spectra of order higher than 2 (MS^n, n > 2).

The samples were also analyzed by liquid chromatography (LC, Agilent Technologies 1200 Series) coupled to the above described mass spectrometer (LC-MS), under Auto-MS mode in both, positive and negative polarity. Under LC-MS operation the spray and ion optics conditions were the following: capillary voltage, 3.5 kV; drying gas (nitrogen), 330ºC at 7 l/min; nebulizer gas pressure, 35 psi; capillary exit voltage, 104 V; skimmer voltage, 32 V.

A Hamilton PRP-1 reversed phase LC column (15.0 cm length, 2.1 mm internal diameter, 5 µm average particle diameter), stabilized at 25°C was used for chromatographic separation. The eluent system was ultra-pure water (A) and acetonitrile (B), both with 0.1 % formic acid, and ethyl acetate (C). The gradient started with 52% A, 38% B and 10% C. After 5 min an increase of B and C up to 73% and 25%, respectively, took place over 8 min. The eluent was then allowed to recover to the initial conditions (52% of A, 38% of B and 10% of D) in 1 min and then stabilize for additional 5 min before the next run. The flow was 0.35 ml/ min. Full-scan mass spectra were generated in the range of 100.00-1500.00 *m/z*, both under negative and positive ESI. The data were analysed using the software Data Analysis software v 3.4 (Bruker Daltonics esquire 6.1).

Under LC-MS a separation by LC took place before ESI-MS analysis. This separation allowed for the removal of excess salts in the injected sample. As salts came out from the column in the first 1-2 minutes, the flow was sent to waste instead of to the ion source. LC

separation also allowed for the observation of less complex full scan spectra and for detection of compounds less prone to ionize and therefore not visible under direct injection. The AutoMS mode allowed for fragmentation (MS^2) of compounds detected after LC separation. This process was done for both polarities in a single run.

Compound assignment was based on the *m/z* values, isotope distributions and fragmentation patterns. The presence of compounds possessing a phosphatidylcholine moiety was confirmed by injection of a phospholipid authentic sample, specifically compound 1,2-stearoyl phosphatidylcholine present in the standard Sigma P5394.

3.4. Results

3.4.1. Y-maze tests of attraction

For each stimulus, at least 85% of the sea cucumbers chose to move from the entry area to one of the arms. After spending some time in one of the arms, about half of the sea cucumbers went back to the entry area and re-entered the same or the other arm of the Y-maze, and eventually repeated this behaviour.

Males (M) more often chose first the male-conditioned water arm than the control (Figure 2a, $p < 0.05$, $\chi^2 = 5.24$), whereas for females (F) there was no apparent preference ($p = 0.17$, $\chi^2 = 1.87$). Males and females showed no preference in their first decision when confronted with female-conditioned water (Figure 2b, M: $p = 0.72$, $\chi^2 = 0.13$; F: $p = 0.74$, $\chi^2 = 0.11$). However, both males and females stayed significantly longer in the arm with the male-conditioned water (Figure 2c, M: $p < 0.01$, $Z = 3.21$; F: $p < 0.05$, $Z = 2.50$). In contrast, males and females spent the same percentage of time in water conditioned by females and control water (Figure 2d, M: $p = 0.10$, $Z = 1.63$; F: $p = 0.90$, $Z = 0.13$).

CF from males or females did not induce first choice preference for any arm in males (Figure 3a, M-CF: $p = 1.00$, $\chi^2 = 0.00$; Figure 3b, F-CF: $p = 0.65$, $\chi^2 = 0.20$) or females (Figure 3a, M-CF: $p = 0.65$, $\chi^2 = 0.20$; Figure 3b, F-CF: $p = 0.36$, $\chi^2 = 0.83$). Similarly, males and females spent similar time in the two arms when the stimulus was CF from male (Figure 3c, M: $p = 0.96$, $Z = 0.06$; F: $p = 0.15$, $Z = 1.43$) or female (Figure 3d, M: $p = 0.39$, $Z = 0.87$; F: $p = 0.20$, $Z = 1.27$).

As with the CF, ovary (O) and testis (T) homogenates failed to induce a preference for one arm either as first choice (Figure 4a, T vs. M: $p = 0.65$, $\chi^2 = 0.20$; T vs. F: $p = 0.61$, $\chi^2 = 0.25$; Figure 4b, O vs. F: $p = 1.00$, $\chi^2 = 0.00$; O vs. M: $p = 0.65$, $\chi^2 = 0.20$) or in the time spent in one arm (Figure 4c, T vs. M: $p = 0.28$, $Z = 1.07$; T vs. F: $p = 0.14$, $Z = 1.48$; Figure 4d, O vs. F: $p = 0.14$, $Z = 1.48$; O vs. M: $p = 0.21$, $Z = 1.26$).

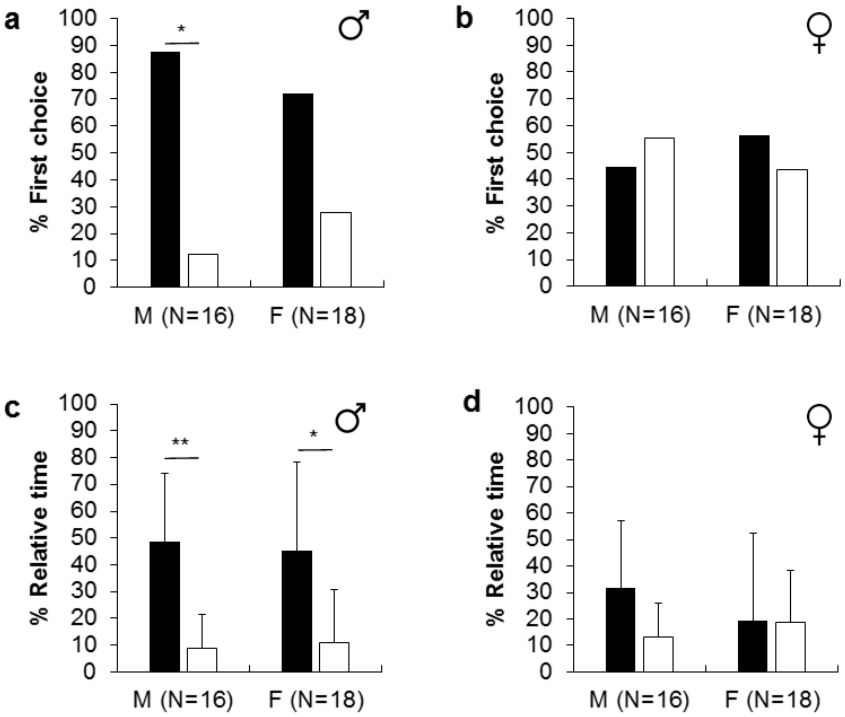


Figure 2. Percentage of first choice (**a, b**) and relative time (**c, d**) spent in the conditioned-conspecific water (stimulus, black) and in the control arm (sea water, white). Error bars are one standard deviation. * $p < 0.05$, ** $p < 0.01$.

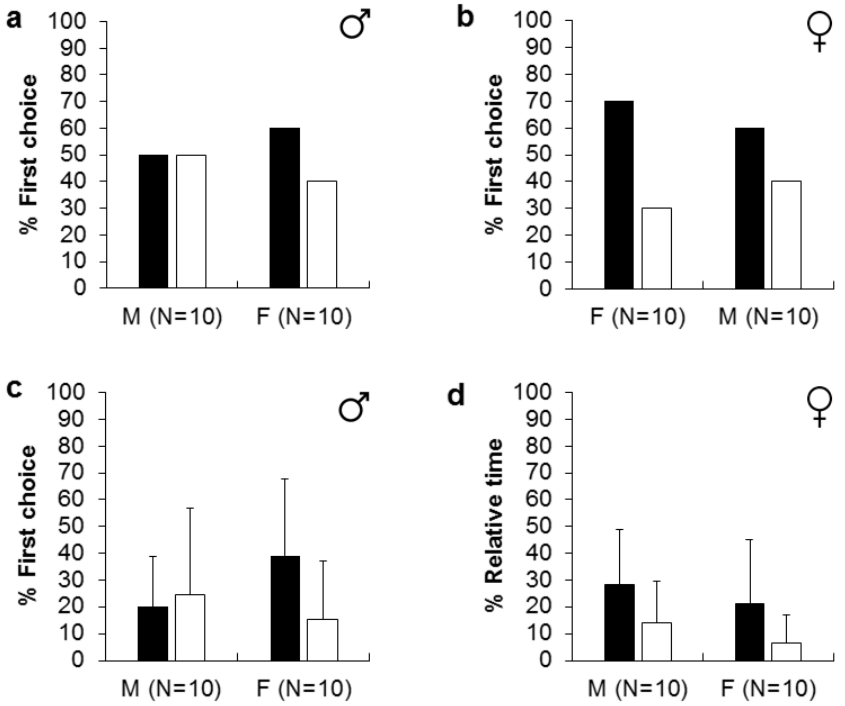


Figure 3. Percentage of first choice (**a, b**) and relative time (**c, d**) spent in the coelomic fluid (stimulus, black) and in the control arm (sea water, white). Error bars are one standard deviation.

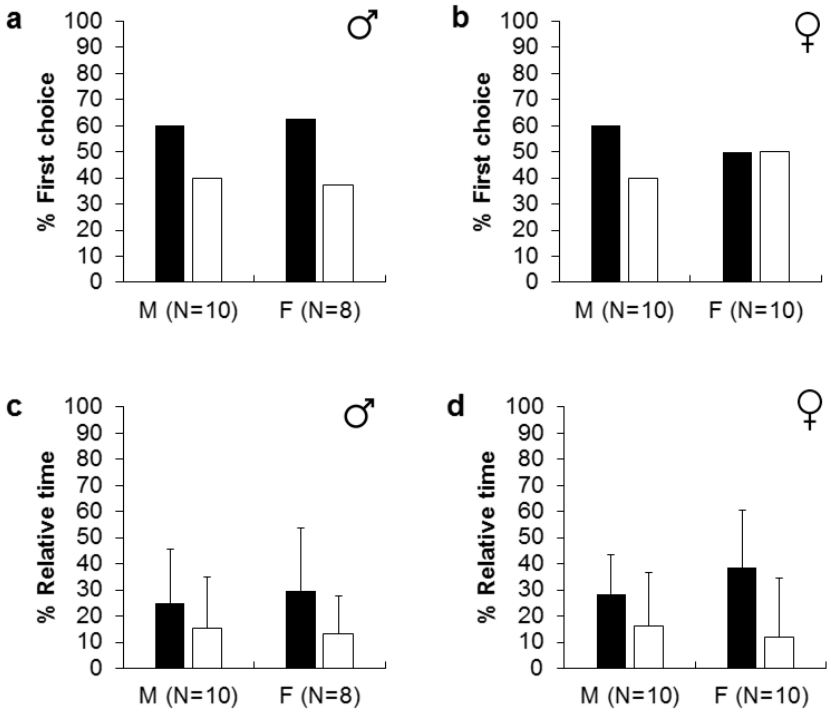


Figure 4. Percentage of first choice (**a, b**) and relative time (**c, d**) spent in the gonad (ovary and testis) homogenate (stimulus, black) and in the control arm (sea water, white). Error bars are one standard deviation.

3.4.2. Spawning behaviour

Thermally or chemically stimulated male and female *H. arguinensis* adopted a pre-spawning behaviour in which their anterior body region swayed from one side to another, with tentacles extended outside of the oral cavity (Figure 5a), while the posterior body region rested against the bottom or the side of the glass aquarium (Figures 5c-d). Both males and females released gametes from a gonopore (Figure 5b), located at the dorsal side of the anterior part at the opposite side of the oral cavity and was clearly visible only during spawning.

Males started to release gametes between 40 min and 1 hr after stimulation (mean: 49.70 ± 8.17 min, N = 10) and continued to slowly release a continuous flow of sperm for at least one hour (Figure 6b), with some individuals still spawning after 3h. The latency of response of females was longer than that of males (Mann-Whitney *U* test, U = 16.50, N = 10, $p < 0.01$), varying between 50 and 100 min (mean: 70.30 ± 18.41 min, N = 10). However, in contrast to the continuous slow release of males, females released their gametes quickly and briefly in 3 to 5 pulsatile jets (Figure 6a). Similarly, when males and females received the stimulus at the same time, males also spawned longer than females and generally continued to spawn at least until females stopped releasing gametes.

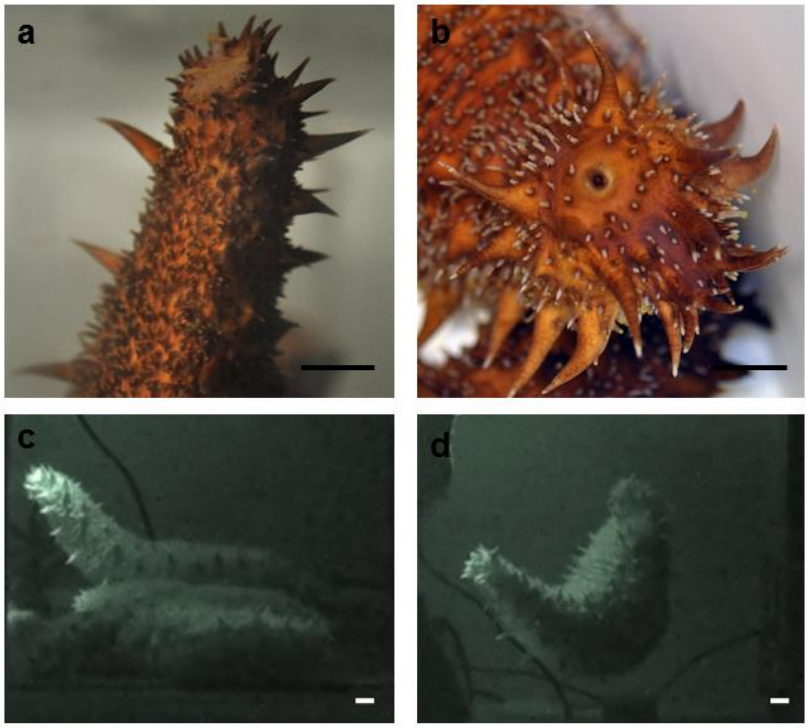


Figure 5. Spawning behaviour in *H. arguinensis* showing the tentacles outside of the oral cavity (**a**), the gonopore from where the gametes are released (**b**) and the posture of the complete body during spawning (**c, d**) in the dark. Scale bars: 1 cm.

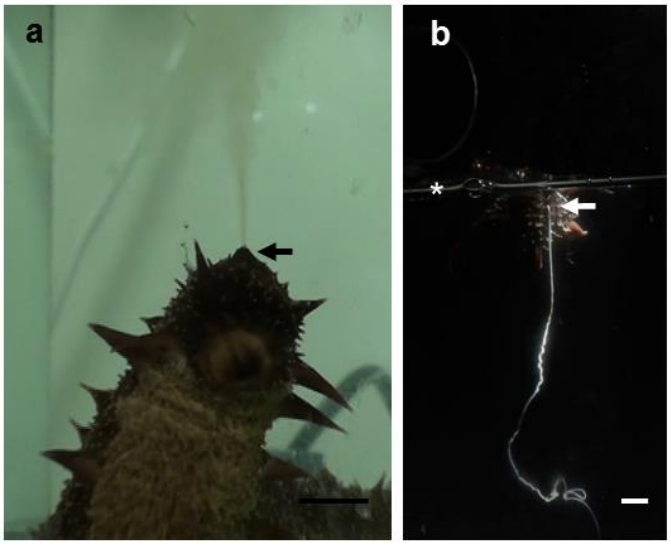


Figure 6. Gamete release by a female (**a**) and a male (**b**) in *H. arguinensis*. Arrows indicates the gonopore from where gametes are released. The female is on the bottom of the aquarium while the male has his posterior part attached to one side glass of the aquarium and releases the sperm from the water surface (asterisk) to the bottom of the aquarium. Scale bars: 1 cm.

3.4.3. Spawning water tests

All males and nearly all females spawned when male spawning water was added to their aquaria (Fisher's exact tests, males or females, $p < 0.0001$), unlike with female spawning which had no effect on spawning of either males or females (Table 1). If sperm was removed from the spawning water, more than three quarters of males and females still released their gametes (males or females, $p < 0.001$), but sperm itself had no effect on spawning of either sex ($p > 0.05$ for both).

Interspecific spawning was observed between *H. arguinensis* and *H. mammata*. All males and 5/6 female *H. mammata* spawned when they received male spawning water from *H. arguinensis* in their aquarium (Fisher's exact tests, males, $p < 0.01$; females, $p < 0.05$, Table 1).

Table 1. Results of the spawning experiment. Number of males and females responding to the different tested stimulus. Fisher's exact tests (two-tailed) were performed by comparing the percentage of individuals that answered to the different tested stimuli with the percentage of individuals answering in the control test. N.a.: not applicable (i.e. zero against zero).

Test	**Spawning frequency**					
H. arguinensis	**Male**			**Female**		
	Yes	**No**	**p**	**Yes**	**No**	**p**
Female spawning water	0	11	n.a.	3	8	0.27
Male spawning water	12	0	<0.0001	11	1	<0.0001
Male spawning water without spermatozoa	9	1	<0.0001	8	2	<0.001
Spermatozoa with sea water	2	8	0.16	1	9	1.00
Control	0	14		1	15	
H. arguinensis* vs. *H. mammata	**Male**			**Female**		
	Yes	**No**	**p**	**Yes**	**No**	**p**
H. arguinensis male spawning water	6	0	<0.01	5	1	<0.05
Control	0	6		0	6	

3.4.4. Stability and fractions tests

All sea cucumbers spawned when they received FSW and 6/7 spawned with 2h FSW (Fisher's exact tests, $p < 0.01$ in both cases; Figure 7; Table 2). However, no sea cucumber released gametes with 4h FSW ($p = 1.00$). When fractions E or F were added individually to the experimental tank, less than a quarter of sea cucumbers started to spawn ($p > 0.05$ in both cases). After the addition of the complementary stimulus, only 4/7 and 1/10 spawned with E and F, respectively ($p > 0.05$ in both cases). However, more than 50% of sea cucumbers spawned directly after E and F were added together (close to statistical significance, $p = 0.06$).

The sea cucumbers that did not spawn with extracts, or aged spawning water, were induced to spawn by FSW or TS. If they failed to respond to any stimuli they were not considered in the analysis.

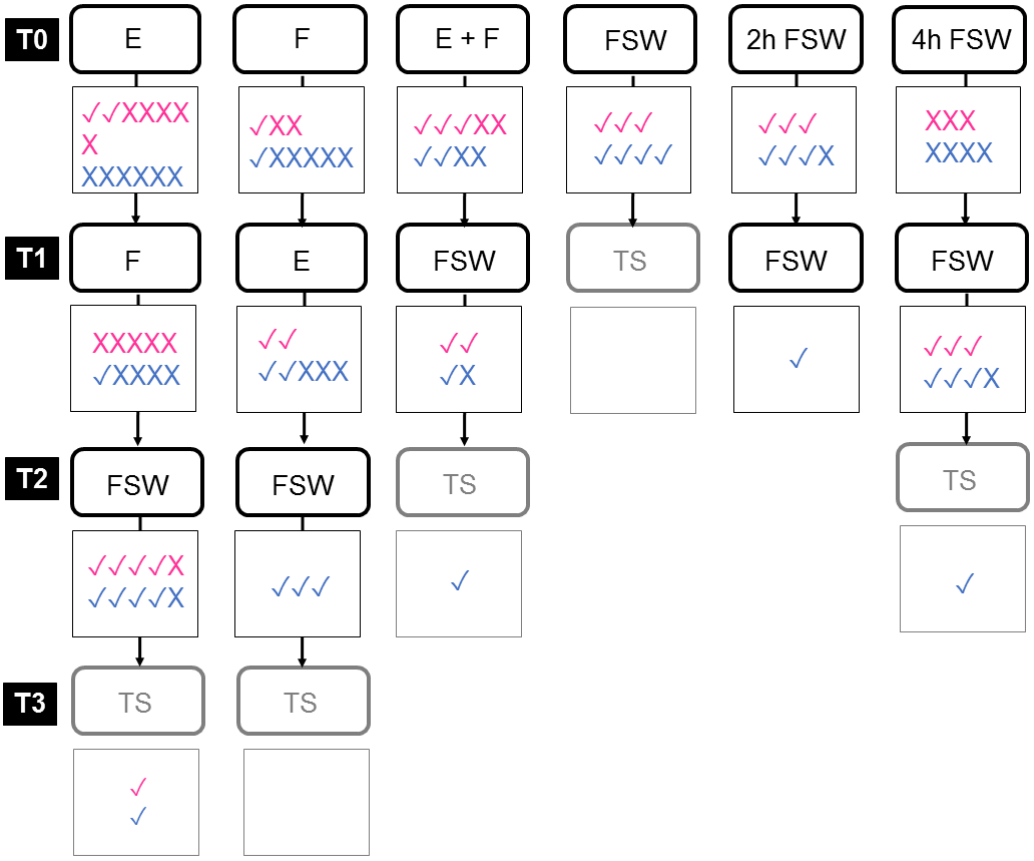


Figure 7. Results obtained in the extract vs. filtrate experiment. E: extract, F: filtrate, FSW: fresh spawning water, 2h FSW: spawning from 2hours, 4h FSW: spawning water from 4 hours, TS: thermal shock. ✓: spawning and X: no spawning with pink for female and blue for male.

Table 2. Results of the stability and fraction tests experiment. Number of sea cucumbers (males and females pooled) responding to the different tested stimulus. Fisher's exact tests (two-tailed) were performed by comparing the percentage of individuals that answered to the different tested stimuli with the percentage of individuals answering in the control test.

Test	Spawning frequency		
	Yes	No	p
E (T0)	2	11	1.00
F (T0)	2	7	0.58
F (T1)	1	9	1.00
E (T1)	4	3	0.10
E + F	5	4	0.06
FSW	7	0	<0.001
2h FSW	6	1	<0.01
4h FSW	0	7	1.00
Control	1	9	

3.4.5. Chemical characterization of spawning water

Full scan LC-MS profiles of water extracted before and after spawning of males and females were clearly different under both ionization polarities (Supplementary material 1). Since only male spawning water was active, only these LC-MS profiles were analysed further. Major differences between samples obtained before and after spawning could be seen between 8 and 11 min. Among those compounds detected by MS, the most intense was found at *m/z* 808.7 (positive polarity) in the water after spawning, and was absent before spawning (Figure 8a). A much smaller peak of this compound was also seen in female spawning water (Figure 8b). Under positive polarity, Auto MS gave a fragmentation spectrum showing a major signal at *m/z* 184.1 (Figure 8c). This result was confirmed by direct injection of the sample into the mass spectrometer (ESI-MS). The *m/z* value and the daughter ion at *m/z* 184.1 under positive polarity are common in phospholipids possessing a phosphatidylcholine moiety (Hsu and Turk 2009). To confirm the presence of a phosphatidylcholine moiety, the phosphatidylcholine phospholipid standard 1,2-stearoyl phosphatidylcholine was selected and studied by ESI-MS^n. This compound is readily seen at *m/z* 790.5 under positive polarity as it contains a net positive charge. The fragmentation led to a major signal at *m/z* 184.1 (Figure 8d), which is the same daughter ion observed for the fragmentation of *m/z* 808.7. This result indicated that the unknown compound possesses a phosphatidylcholine moiety.

Another signal detected by LC-MS that could be associated with male spawning was seen at *m/z* 287 under positive polarity (Figure 9a). This compound was detected in male water prior to spawning but increased greatly after spawning. A much smaller peak was seen in female water which did not increase after spawning (Figure 9b). The fragmentation spectra are shown in Figures 9c-e.

The signal intensity of the two compounds increased with time with a maximal intensity after 30 min and 90 min after the beginning of the spawning process for the *m/z* 808.7 and *m/z* 287, respectively. Their signal intensity then decreased progressively, even though the sea cucumber continued to release sperm. This is consistent with the reduction of spawning activity seen in the bioassay (Figures 10a, b).

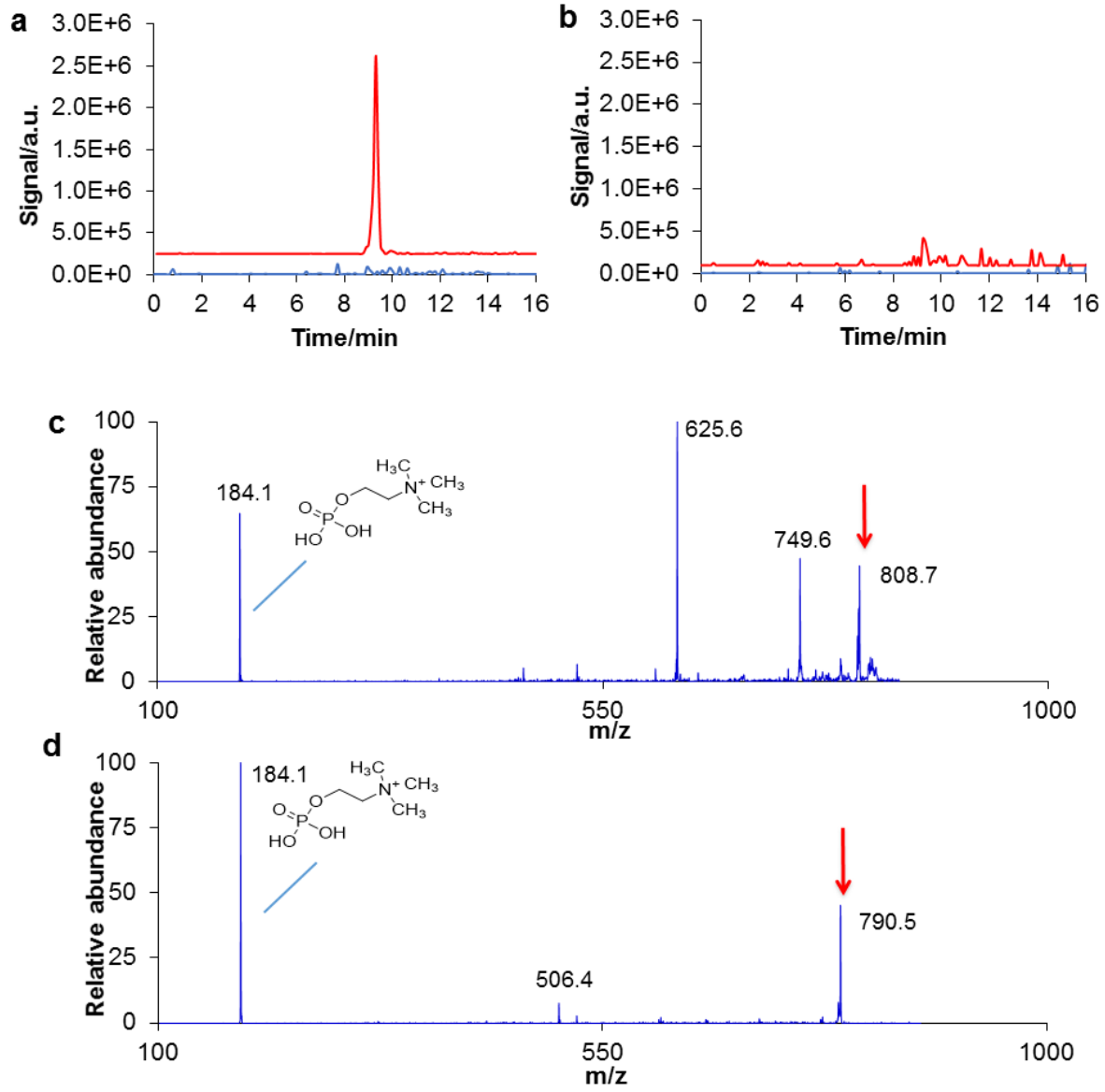


Figure 8. a – b LC-MS single ion traces of methanol extracts at *m/z* 808.7 under positive polarity: (**a**) male before (blue) and after (red) spawning; (**b**) female before (blue) and after (red) spawning. **c – d** Fragmentation spectra of (**c**) MS^2(808.7) and (**d**) MS^2(790.5), the standard 1,2-stearoyl phosphatidylcholine. The inserted structures were assigned to ion with m/z 184.1. The arrows indicate the fragmented peaks.

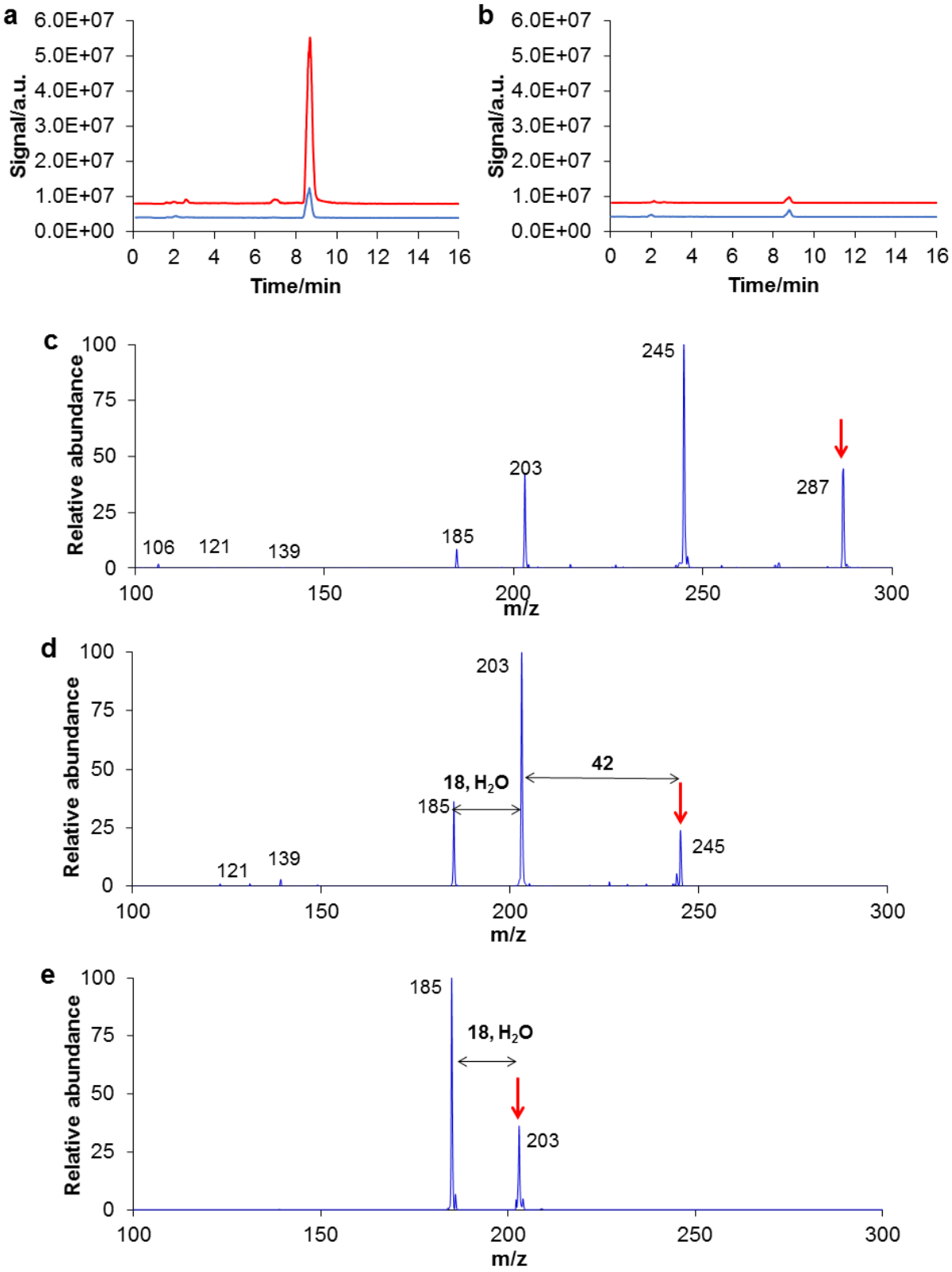


Figure 9. a - b LC-MS single ion traces of methanol extracts at *m/z* 287 under positive polarity: (**a**) male before (blue) and after (red) spawning; (**b**) female before (blue) and after (red) spawning**. c, d, e** Fragmentation spectra of m/z 287, (**c**) MS^2(287), (**d**) MS^3(287→245) and (**e**) MS^4(287→245→203). The arrows indicate the fragmented peaks.

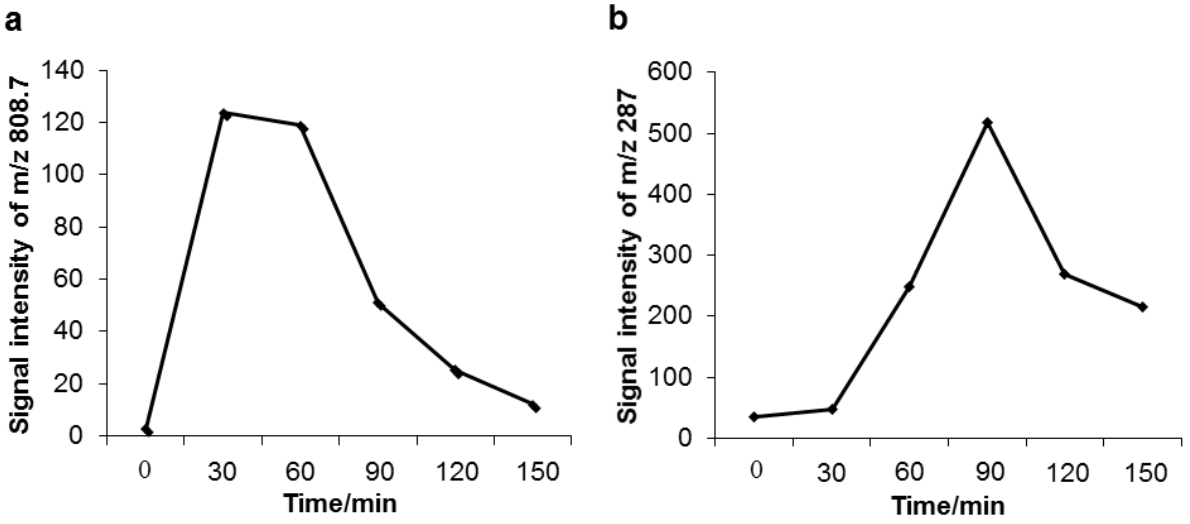


Figure 10. Signal intensity of (**a**) *m/z* 808.7 and (**b**) *m/z* 287 obtained in consecutive extractions of water after spawning.

3.5. Discussion

The present study demonstrates that chemical cues produced by male sea cucumbers attract conspecifics and trigger spawning in both sexes, indicating an important role of the chemosensory system in the coordination of their natural aggregation and spawning behaviours.

Sea cucumber aggregation substance(s)

Pre-spawning males and females spent more time in the arm of the Y-maze with male-conditioned water. This indicates that males release (a) chemical(s) to the water attractive to both sexes. The coelomic fluid and gonad homogenates (ovary and testis) did not attract either sex and are therefore unlikely sources of aggregation odorants. This contrasts with the avoidance reaction of sea urchins, when confronted with conspecific coelomic fluid or gonad extract (Campbell et al. 2001; Mann et al. 1984). Another possible source of odorants is the mucus which, in *Cucumaria frondosa*, has been shown to accelerate gonadal development of less mature individuals through chemical exchange with more mature individuals (Hamel and Mercier 1999). However, we have no indication that more mucus is released during spawning than at any other times.

That only the males produce/release the aggregation pheromone could be a strategy to draw sea cucumbers to the same place to spawn, while limiting sperm dispersion through male-male groups and maximizing fertilization success through male-female groups (Levitan and Petersen 1995; Levitan et al. 1992). Specific male behaviours have also been reported such as male aggregation in brittle stars (Hagman and Vize 2003) and sex recognition by mechano-

reception in male starfishes (Run et al. 1988). As described briefly here, *H. arguinensis* perform a "nuptial" sequence before spawning, which culminates in gamete release and a sperm mass that slowly disperses. Whether, in the wild, this happens in pairs or in a promiscuous mating mode is not known. Further investigations are needed to better understand the triggers and benefits of sea cucumber breeding aggregations and to determine if male attraction is also present outside the pre-spawning period.

Sea cucumber spawning pheromone(s)

Male spawning water, even without spermatozoa, induced spawning in males and females, whereas female spawning water had no effect. This suggests that males release (a) chemical(s) during spawning which stimulate(s) both sexes to release gametes. To our knowledge, it is the first time this has been shown in sea cucumbers, and is consistent with sea urchins (Reuter and Levitan 2010) and starfish (Caballes and Pratchett 2017), although these studies did not remove the spermatozoa first. The fact that spawning occurred without the need for heat shock, the most common method currently used in aquaculture, emphasizes how powerful the signal is in triggering the reproductive axis.

Consistent with previous studies on marine broadcast spawners, males were quicker to release their gametes than females, a feature that has been suggested to be favoured by sexual selection when males are competing to fertilize the ova, to enable fertilization of more eggs over larger areas (Levitan 1998, 2005; Thorson 1950). In the present study, males also spawned longer than females; they continued to spawn at least until females stopped. This behaviour has also been reported in other Holothuroidea (Hamel and Mercier 1995; McEuen 1988) and in other marine broadcast spawners such as Ophiuroidea (Selvakumaraswamy and Byrne 2000), Echinoidea (Levitan 2002), Polychaeta (Hardege and Bartels-Hardege 1995; Hardege and Bentley 1997) and Appendicularia (Miller 2005). Releasing sperm more slowly than eggs was shown to be a good strategy to avoid sperm attaching uselessly to fertilized eggs, since the permanent block preventing subsequent sperm attachment to the eggs takes longer time to form than the first block preventing polyspermy (Gould and Stephano 2003; Marshall and Bolton 2007).

Interestingly, as with *H. arguinensis*, and with similar efficacy, male and female *H. mammata* released their gametes in response to *H. arguinensis* male spawning water. This suggests that the two species use the same or similar chemical signals. Heterospecific spawning inducing activity has been documented in other Holothurians and in other invertebrates (Babcock et al. 1992; McEuen 1988; Sewell and Levitan 1992; Van Veghel 1993; Watson et al. 2003). Heterospecific spawning activity has been suggested to result from the coevolution

of pheromones in response to the reduced predation risks through predator swamping (Harrison et al. 1984).

Spawning was rarely induced when male spawning water was 4 hr old, indicating degradation or evaporation of spawning substance(s). Similarly, separate addition of the eluate or filtrate had no activity, which could be at least partly restored if the complementary fraction was added subsequently. This suggests that the spawning pheromone consists of more than one compound. However, solid-phase extraction of water could take between 1 and 3 hours; some loss of activity of the extracts could therefore be explained by degradation of the active compound(s) during this time.

Chemical characterization of potential pheromones

In this study, two compounds were found in male spawning water under positive polarity, at *m/z* 808.1 and *m/z* 287, while they were absent or present at much lower concentrations in female water and before male spawning. Both showed degradation within two hours, consistent with the loss of biological activity of male spawning water. They are thus good candidates to be involved in sea cucumber spawning. The compound *m/z* 808.1 was identified as a phospholipid with a phosphatidylcholine moiety. Phosphatidylcholine derivatives have been characterized as key substances governing group recognition in catfish (Matsumura et al. 2007) and as phagostimulants in the nuptial secretion of a species of cockroach (Kugimiya et al. 2002). However, to our knowledge, they have never been associated with spawning activity in any animal taxon.

Pheromones are highly diverse across different animal taxa, and are composed either of a mixture of different chemical compounds or a single compound (Wyatt 2010). Some fish use steroids and/or prostaglandins as sex pheromones (Stacey 2015) while others use amino acids (Yambe et al. 2006) or bile acids (Li et al. 2002). In marine invertebrates, for example, peptide pheromones have been identified in the sea-slug *Aplysia* and nereid worms (Cummins et al. 2005; Hardege et al. 2004; Zeeck et al. 1988) and nucleotide pheromones in crustaceans (Hardege et al. 2011).

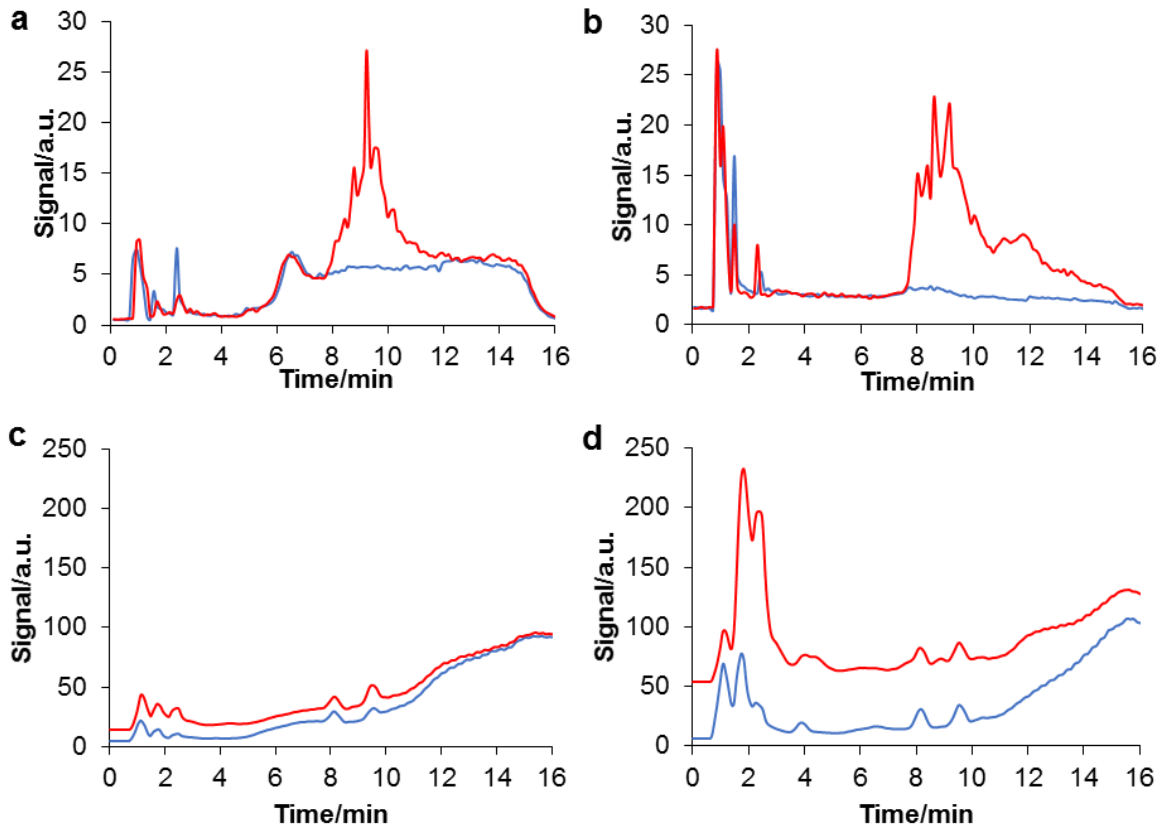
The chemical identification of pheromones is challenging due to the relatively small amount of pheromone secreted, to the large variety of substances usually present in natural waters, and to their possible lability (Shimizu 1985). Here, the compounds correlated with spawning need to be isolated, purified and submitted to further analysis, namely nuclear magnetic resonance (NMR) and high resolution mass spectrometry to assign a final structure. The identified compounds would need then to be tested on the animal to confirm their biological activity (Wyatt 2014).

3.6. Conclusion and perspectives

Our study contributes to the better understanding of chemical communication involved in reproduction in sea cucumbers, and particularly the role played by males. However, reproductive success in sea cucumbers results most likely from a combination of chemical cues and one or more exogenous factors. For example, in the sea urchin *Lytechinus variegatus*, the presence of phytoplankton, the phase of the moon and obscurity were suggested to increase the sensitivity to sperm suspension in spawning trials (McCarthy and Young 2002; Reuter and Levitan 2010). In the present study, experiments were carried out around the new and full moon and at night as they have been shown to be linked to spawning success in sea cucumbers (Mercier and Hamel 2009).

The identification of pheromones in the aggregation and spawning events have ecological significance and may have important practical applications in the management of sea cucumbers in aquaculture and in the control of invasive species. The use of pheromones could be potentially used to attract and capture invasive sea cucumbers, as they can have a significant negative impact on benthic ecosystems. Further investigations are needed to identify the chemical compounds produced by males in aggregation and spawning, including the use of high resolution mass spectrometry and NMR.

3.7. Supplementary materials



Supplementary material 1. Full scan LC-MS profiles of water extracts in males in (**a**) positive polarity and (**b**) negative polarity, and in females in (**c**) positive polarity and (**d**) negative polarity before (blue) and after (red) spawning.

3.8. References

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Chapter IV

Characterization of sensory structures in the sea cucumber *Holothuria arguinensis*

Manuscript in preparation

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Characterization of sensory structures in the sea cucumber *Holothuria arguinensis*

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4.1. Abstract

Sea cucumbers lack well-developed senses except mechanoreception and are expected to rely on chemical communication to survive and reproduce. Accordingly, they need sensory structures to detect and convert the extracellular chemical signals into an intracellular message that leads to a behavioral or physiological response. In the present study, the main structures in contact with the environment – the tentacles, tube feet and papillae – were characterized in the sea cucumber *Holothuria arguinensis* using three methods: 1) classical histology, 2) NADPH-diaphorase histochemistry and 3) immunohistochemistry. All the structures of interest had the same general tissue organisation: an inner mesothelium, a connective tissue layer, a nerve plexus and an outer epidermis layer covered by a cuticle. The main nervous structures in all appendages consisted of a longitudinal nerve and a nerve plexus surrounding the stem, from which nervous fibers extended to the mesothelium and innervated the disc. Nitric oxide synthase was abundant in all appendages, suggesting an important role for nitric oxide in the biology of sea cucumbers. The three methods used did not indicate any specific appendage as a strong candidate to perform electrophysiology. However, the specific nervous arrangement of the appendages' disc, consisting of a distinct nerve plate, rich in cells and fibers containing potential sensory cells which stained positively for neuronal markers makes this region a strong candidate as a target for future electrophysiology studies.

Key words: sensory, nervous system, histology, immunohistochemistry, NADPH-diaphorase, sea cucumber

4.2. Introduction

The ability to detect chemical signals in the environment is essential for reproduction, food detection and social interactions in all organisms (Bargmann 2006; Kaupp 2010). In multicellular animals, the detection of these chemicals takes place through specialized sensory structures, chemosensory or olfactory organs, which discriminate between different signals with appropriate selectivity and sensitivity (Hildebrand 1995; Spehr and Munger 2009). The structures of the olfactory pathway share the same general organization across phyla such as the presence of olfactory receptor cells (ORCs), perireceptor processes and organization of the central nervous system (Ache and Young 2005; Eisthen 2002). However they differ anatomically depending on the complexity of the organism and its environment (Firestein 2001; Holley et al. 2012).

The olfactory structures in vertebrates (olfactory mucosa and vomeronasal organ) are located within a chamber, the nasal cavity. In contrast, in invertebrates, they are located on various external appendages (Shorey 1976) such as the anterior region in nematodes (Bargmann 2006), the tentacles in gastropods (Wertz et al. 2006), and the antenna and maxillary palp in insects (Kaupp 2010). In all cases, these structures are adapted to collect and guide the odorant molecules to the ORCs (Hildebrand and Shepherd 1997), making them particularly important in setting the sensitivity of the olfactory systems (Kaissling 1990).

Echinoderms are slow-moving and broadcast spawning marine invertebrates which are expected to communicate using chemical stimuli (Sloan and Campbell 1982), as it is typical in organisms that lack or have only rudimentary vision and hearing (Hildebrand 1995). Numerous studies have shown that they are able to perceive and react to waterborne stimuli from predators, damaged conspecifics, potential food sources and conspecific mates (e.g. Campbell et al. 2001; Cyrus et al. 2015; Dix 1969; Hamel and Mercier 1996; Mann et al. 1984; Soong et al. 2005; Unger and Lott 1994). In chapter III, the release of chemicals by male sea cucumbers that attract conspecifics and induce spawning in males and females was demonstrated. However, the sensory structures involved in the detection of these cues have not yet been identified. This is partly due to the difficulty in performing electrophysiological assays as a consequence of: 1) the small size of nerve cells, relative to other invertebrates, and their hard protective calcareous endoskeleton (Cobb 1978), and 2) the lack of knowledge about the nervous system of adult echinoderms mostly because of the shortage of molecular markers to clearly identify neurons (Díaz-Balzac et al. 2014).

In sea cucumbers, the main structures which are in direct contact with the environment, and are most likely to perceive environmental stimuli and convert them to a neuronal signal, are the tube feet, papillae and tentacles; all of which are connected to the water-vascular system and the integument. These structures are thought to have a sensory role (Bouland et al. 1982; Hyman 1955; Pentreath and Cobb 1972; VandenSpiegel et al. 1995); however, their functional nervous organization has been less studied than the radial nerve cords (RNCs) (for summary, see Díaz-Balzac et al. 2016), the principal structures of the echinoderm nervous system (Cobb 1987; Hyman 1955). Until recently almost all knowledge on the nervous system of echinoderms was based on classical histology carried out early during the last century and, more recently, on electron microscopy studies (e.g. Bouland et al. 1982; Cavey 2006; Flammang and Jangoux 1992; Hyman 1955; McKenzie 1987; Pentreath and Cobb 1972; VandenSpiegel et al. 1995). With the development of new neural markers, neuronal and fiber populations expressing different neurotransmitters, such as catecholamines and neuropeptides, have been identified, contributing to a broader view of the echinoderm nervous system (Díaz-Balzac et al. 2010a; Díaz-Balzac et al. 2010b; Díaz-Balzac et al. 2007; Díaz-Balzac et al. 2014; Díaz-Miranda et al. 1995; Hoekstra et al. 2012; Inoue et al. 2002).

In sea cucumbers, cells with morphological characteristics typical of sensory cells have been described in tentacles, tube feet and papillae (Bouland et al. 1982; Flammang and Jangoux 1992; VandenSpiegel et al. 1995). They were characterized as ciliated cells bearing a short and non-motile cilium and ending within the nerve plexus, which is considered a diagnostic feature of sensory cells (Holland 1984). More recently, sensory-like cells were described using immunohistochemistry. They were characterized by their distinct bipolar organization and apical processes which extended towards the epithelial surface. In some cases, glomeruli-like structures are formed below the cell bodies; these are potential sites of dense synaptic connection (Díaz-Balzac et al. 2010a; Hoekstra et al. 2012).

Despite recent progress in mapping the nervous system of sea cucumbers, the diversity of species, their enigmatic behaviour and the absence of clearly identified sensory cells and neural circuits, make more studies essential. In particular, characterization of the distribution of neurotransmitters and other nervous signaling pathways will be of great help to improve understanding of the function of different sensory structures in sea cucumbers. The present study was performed to characterize the morphology of the main tissues in contact with the environment, namely the tentacles, tube feet, papillae and integument, in the sea cucumber *Holothuria arguinensis*, in order to identify candidate structures to which electrophysiology could be applied to screen candidate pheromones. The tissues of interest were studied through,

1) classical histological methods, 2) NADPH-diaphorase staining to localize the nitric oxide synthase (NOS, the enzyme synthetizing the neuronal messenger nitric oxide) and 3) immunohistochemistry using antibodies against tubulin (a neuronal marker), serotonin (a neurotransmitter) and synaptotagmin (a specific marker of echinoderm neurons) (Nakajima et al. 2004).

4.3. Materials and methods

4.3.1. Ethics statement

Sea cucumbers, *Holothuria arguinensis*, were collected, handled and euthanized in agreement with the license N° 635/2015/CAPT and N°95/2016/CAPT of the ICNF - Instituto da Conservação da Natureza e das Florestas, Portugal. The species is not endangered or protected.

4.3.2. Animals

Adult *H. arguinensis* (>210 mm length) (Koehler and Vaney, 1906: Holothuroidea, Aspidochirotida) were collected in the intertidal zone of the Ria Formosa (37°00'35.02''N) in Faro (Portugal). They were kept at Ramalhete Marine Station and fed with sediment collected from their natural environment.

4.3.3. Morphology

The entire animal and the structures of interest (Figures 1a, b) were initially observed using an Olympus SZ-PT binocular microscope coupled to an Olympus SC35 camera. The external anatomy of *H. arguinensis* was photographed and studied in detail, and a diagram was rendered in Adobe Illustrator by Scigrades (https://scigrades.be).

4.3.4. Tissue sampling

Sea cucumbers were anesthetized with 5% $MgCl_2$ (Sigma, St. Louis, MO, USA) before tissue collection. From the anterior, middle and posterior regions, samples of the body wall (dorsal and ventral) including tube feet, tentacles and papillae were dissected out and fixed in 4% paraformaldehyde at 4°C for 1 to 24 h depending on the subsequent procedure. After fixation, samples were rinsed three times in 0.1 M phosphate-buffered saline (PBS) for 15 min and stored either in 70% ethanol for histology or sucrose for histochemistry and immunohistochemistry until further processing.

4.3.5. Histology

For standard histology, tissue samples were dehydrated in a graded sequence of ethanol (from 70% to 100%), saturated in xylene and embedded in paraffin wax (Merck, Germany). Serial sections (8 µm) were cut with a sliding microtome (Leica RM2135) and mounted on poly-L-lysine coated slides. Sections were cleared in xylene and then hydrated in a decreasing alcohol series (100 to 70%). To characterize the general tissue organization several stains were used: Alizarine Red S (Sigma-Aldrich, St. Louis, MO, USA) to stain calcium deposits, Masson's (MT) or Milligan's trichrome (MiT) to distinguish muscle and collagen, and Palmgren's silver stain to localize nerve fibers (Humason 1972). After staining, sections were rapidly dehydrated through an alcohol series (70% to 100%), cleared in xylene and mounted in DPX mountant (BioChemika, Sigma-Aldrich, Madrid, Spain).

4.3.6. Histochemistry

For NADPH diaphorase (NADPH-d) histochemistry, the tissue samples were cryoprotected with a graded series of sucrose (10 to 30%) and frozen into Tissue-Tek (Sakura Finetek, Torrance, CA) using a dry-ice and ethanol mixture. Serial sections of 20 µm were prepared with a cryostat (NX50 cryostat, Thermo Scientific, Waltham, MA, USA), mounted on 3-aminopropyltriethoxysilane (APES; Sigma-Aldrich, Madrid, Spain) coated glass slides and stored at -25°C. When needed, the slides were brought to room temperature and washed three times for 15 min in 0.2 M Tris-HCl before NADPH-d staining as described by Elphick (1997). Slides were immersed in the staining solution and kept in the dark for at least three hours: 1 mM NADPH (TetraTris salt), 0.5 mM nitroblue tetrazolium (NBT), and 0.5 % Triton X-100 in 0.2 M Tris-HCl at pH 8.0. Post-staining preparations were washed three times for 10 min in Tris-HCl before mounting in glycerol gel (Sigma-Aldrich, Madrid, Spain). The intensity of the staining is correlated to the biochemical activity of NOS (Elofsson et al. 1993). Histochemical controls omitted the NADPH from the staining solution; in this case no specific stain was observed.

4.3.7. Immunohistochemistry

Frozen sections were prepared as described above. The slides were washed three times for 15 min in PBS and blocked for 3 hours with sheep serum 3% (Sigma-Aldrich, Madrid, Spain) in Tris-carrageenan Triton X-100 (TCT, Tris buffer containing 0.7% carrageenan and

0.5% Triton X-100, pH 7.6). The primary antibodies used (Table 1) were α-tubulin (Sigma, T-9026) at a dilution of 1:250, α-synaptotagmin B (DSHB, 1E11) (Nakajima et al. 2004) neat serum and α-serotonin (Sigma, S-5545) at a dilution of 1:400 and they were incubated with tissue sections overnight at 4°C. Antibodies were optimized by testing serial dilutions on tissue sections and selecting those that gave the best signal to noise ratio. The three antibodies have previously been used in other sea cucumbers and their specificity demonstrated (Díaz-Balzac et al. 2010a; Díaz-Balzac et al. 2016; Díaz-Balzac et al. 2007; Inoue et al. 2002; Nakajima et al. 2004; Nakano et al. 2006; Tamori et al. 2007). After rinsing twice for 5 min in PBS, the slides were incubated with the secondary antibodies diluted in PBS for two hours at room temperature. The secondary antibodies used were Alexa Fluor 488 conjugated anti-mouse IgG (Molecular Probes, Eugen, Oregon) at a dilution of 1:200 for the anti-tubulin and anti-synaptotagmin antibodies and Alexa Fluor 488 conjugated anti-rabbit IgG (Molecular Probes, Eugen, Oregon) at a dilution of 1:300 for the anti-serotonin antibody. Cell nuclei were stained with DAPI (Sigma, St. Louis, MO, USA) at a dilution of 1:20000 for 5 min. After washing in PBS, the sections were mounted in glycerol gelatin (Sigma-Aldrich, Madrid, Spain). Negative control slides included omission of the primary antibodies, omission of both primary and secondary antibodies and showed little or no labelling of the tissue sections.

Table 1. Antibodies used in this study, their origin and working conditions.

Antigen	**Raised in**	**Immunogen**	**Source**	**Dilution**
α-tubulin	Mouse (monoclonal)	Microtubules from chicken embryo brain	Sigma (T-9026)	1:250
α-synaptotagmin B	Mouse (monoclonal)	Radial nerve lysate from a sea star	DSHB (1E11 deposited by Dr. Burke lab (Nakajima et al. 2004)	neat
α-serotonin	Rabbit (polyclonal)	Serotonin creatinine sulfate complex conjugated with formaldehyde to BSA	Sigma (S-5545)	1:400

4.3.8. Imaging

Stained sections from histology were analyzed under a light and a fluorescent microscope (Leica DM2000) coupled to a digital camera (Leica DFC480) linked to a computer for digital image analysis. Image processing, including brightness/contrast adjustments, were performed within ImageJ Fiji (http://imagej.net/Fiji).

4.4. Results

4.4.1. Morphology

H. arguinensis has, in common with other sea cucumbers, an elongated cylindrical shaped body, with a mouth (anterior part, A) and an anus (posterior part, P) at the opposite extremities of the central body axis (Figure 1a). The body is covered by tube feet, or locomotor podia, on both ventral (V) and dorsal (D) sides, although they are more abundant on the ventral side. The dorsal side is darker and harder than the ventral side (Figures 2a, b), and possesses papillae, or non-locomotor podia, arranged in four rows. An additional two rows of papillae are located laterally between the ventral and dorsal sides. The oral cavity contains the mouth surrounded by 20 peltate tentacles (cauliflower-like in appearance), although 19 and 21 tentacles were counted in some individuals (Figure 1b).

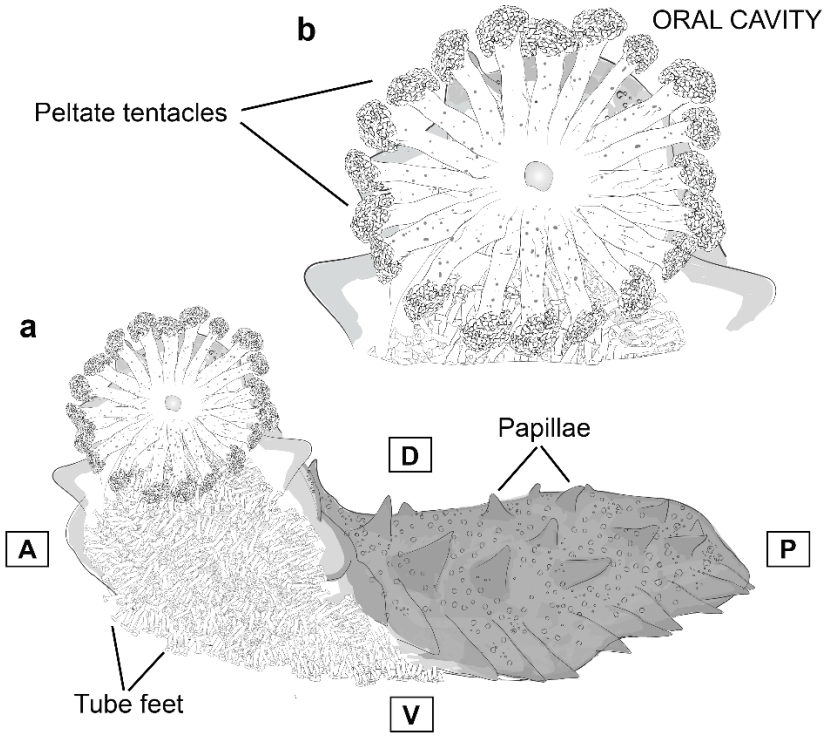

Figure 1. Schematic representation of *H. arguinensis* revealing the salient external morphological features. (**a**) General view of *H. arguinensis*. (**b**) Enlargement of the oral cavity highlighting the peltate tentacles. A: anterior, P: posterior, D: dorsal and V: ventral. Not to scale.

4.4.2. Body wall

4.4.2.1. Histology and histochemistry

MT staining revealed that the different elements constituting the body wall are the same along the animal both ventrally and dorsally: the cuticle, epidermis, loose and dense connective tissue with water-vascular canals, and a RNC accompanied by circular and longitudinal muscles (Figure 2c). The surface of the body wall is rich in calcareous spicules, mainly of the button type, as revealed by the Alizarin red staining (Figure 2d). However, no nervous elements are detected using either Palmgren's or NADPH-d staining in this region (Figures 2e, f).

The deeper part of the integument was characterized by the RNC which is composed of two neuroepithelia (hyponeural and ectoneural) separated by a thin connective tissue layer, as clearly evident in MiT stained sections (Figure 2g). Since both the ectoneural and hyponeural parts of the nervous system are hollow tubular structures, different cavities were found including the haemal lacuna and both hyponeural and epineural sinuses. Although the RNC is the main component of the sea cucumber nervous system, no obvious staining was identified with Palmgren's method (Figure 2h). However, in the same section as the RNC, many black fibers were stained by this method in the longitudinal muscle. In contrast to Palmgren's method, strong reactivity to NADPH-d was seen in both parts of the RNC, particularly at the periphery, and in the longitudinal muscle where the staining was less intense (Figure 2i).

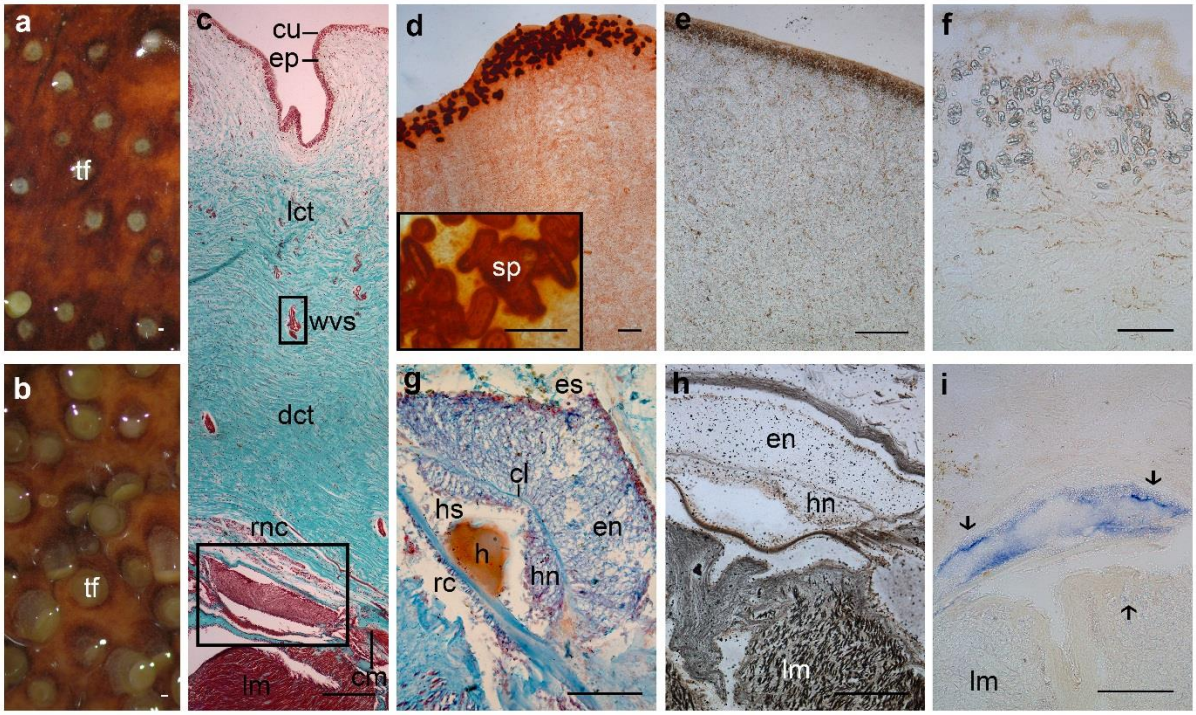

Figure 2. Histology and histochemistry of the body wall. **a – b** Macroscopic photograph of (**a**) the dorsal side and (**b**) the ventral side showing the repartition of the tube feet (*tf*) on both sides and highlighting the difference of coloration between the two sides. (**c**) Transverse section through the body wall stained with Masson's Trichrome showing the loose (*lct*) and dense (*dct*) connective tissue in *green*, the radial nerve cord (*rnc*) in *dark pink*, the circular (*cm*) and longitudinal (*lm*) muscles in *red*. **d – f** Surface part of the body wall showing (**d**) the distribution of the spicules and (insert) typical spicules (*sp*) stained with Alizarin red and the absence of (**e**) nervous elements stained with Palmgren's method or with (**f**) NADPH-d reaction in this area. **g – h** Deeper part of the body wall highlighting the radial nerve cord stained with **(g)** Milligan's Trichrome and (**h**) Palmgren where no obvious staining was seen although the longitudinal muscle (*lm*) in the same section clearly has a positive signal. (**i**) A positive histochemical reaction for NADPH-d was evident in the radial nerve cord, indicative of nitric oxide rich tissue. *cu*: cuticle, *cl*: connective layer, *es*: epineural sinus, *en*: ectoneural neuroepithelium, *ep*: epidermis, *h*: haemal lacuna, *hs*: hyponeural sinus, *hn*: hyponeural neuroepithelium, *rnc:* radial water-vascular canal, *wvs*: water vascular canal. Scale bars: 100 µm.

4.4.2.2. Immunohistochemistry

The RNCs are the main components of the holothurian nervous system and are subdivided into two parts: a large ectoneural part and a small hyponeural part, as seen in the histology section (Figure 2g). In each of the RNCs, DAPI-stained sections revealed that most of the cell nuclei are located at the periphery of these two structures, with some scattered cell nuclei seen in the interior (Figure 3b). Serotonin immunoreactivity was seen in both components of the RNC where the staining was either diffuse, or more intensely reactive in the ectoneural part (Figure 3c). Tubulin fibers were extensively labelled inside the RNCs, in both the ectoneural and hyponeural parts (Figure 3d). However, synaptotagmin labelling was mostly seen in the cells at the periphery of the two components of the RNC, where some immunopositive cells in the ectoneural part were unipolar (Figure 3e). The distribution just described of the three markers used in this work was maintained in the ventral and dorsal RNCs along the body of the animal.

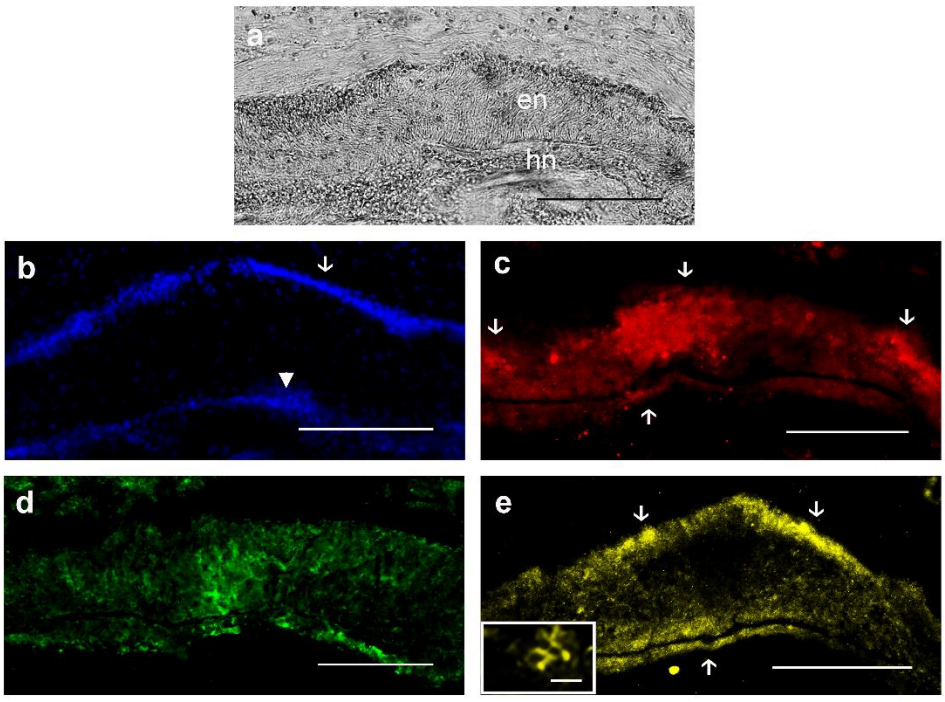

Figure 3. Immunohistochemistry of a transverse section of the radial nerve cord. (**a**) Bright light field section showing the ectoneural *(en)* and hyponeural *(hn)* parts of the RNC. (**b**) DAPI-stained section showing the abundance of cell nuclei lining the ectoneural (arrow) and hyponeural (arrowhead) components of the RNC, and the weakest abundance of cell nuclei within the RCN. (**c**) Serotonin immunolabeling is mainly distributed in the central and lateral region of the ectoneural part of the RNC and in the central region of the hyponeural part of the RNC. (**d**) Tubulin immunoreactivity is distributed extensively in both parts of the RNC. (**e**) Synaptotagmin immunoreactivity is strong in the cells lining the both part of the RNC (arrows) – insert: cell from the ectoneural part of the RNC. Scale bars: 100 µm and in the insert of e: 10 µm.

4.4.3. Water-vascular appendages

The tentacles, tube feet and papillae are protrusions of the body wall and are associated with the water-vascular system. Each of these appendages is composed of a proximal stem and a distal disc; however, the tissue organization of each appendage has distinguishing characteristics (see below).

4.4.3.1. Histology and histochemistry

4.4.3.1.1. Tentacles

In *H. arguinensis*, the stem of the tentacles is cylindrical and topped by a disc of a cauliflower shape (Figure 4a). The stem was subdivided into 5-10 secondary branches from which 3-5 tertiary branches extended and ended in a rounded papilla. Each papilla was itself composed of 2 to 5 buds (Figure 4b).

The MT and Palmgren positive tissues in the stem and the bud of the tentacles were the cuticle, epidermis, inner connective tissue, buccal or tentacular nerve, outer connective tissue (including central and external connective tissue) and mesothelium (Figures 4b, c). At the center of each tentacle, the ambulacral lumen is a continuation of the water-vascular canal system. Calcareous spicules, identified as black deposits with Palmgren staining and Alizarine red positive (not shown), were seen in the central connective tissue of the stem (Figure 4c). However, they were never seen in the bud of the tentacles. Palmgren's staining revealed the proximity of the buccal nerve to the connective tissue layer in both parts of the tentacle (Figure 4c). Peripheral nerves were clearly seen in the mesothelium within the stem (Figure 4d) and in the bud where they formed apical nerves which connected the buccal nerve to the nerve plates in the epidermal cells (Figure 4e). MiT revealed the buccal nerve plexus which formed a cylindrical meshwork and which was asymmetrically thickened to form a longitudinal buccal nerve (Figure 4f). The latter runs longitudinally on one side of the cylinder. The NADPH-d staining was similar to that seen with Palmgren's staining and showed different nitrergic fibers and cell bodies along the buccal nerve, both in the stem and in the bud. The mesothelium was also positive for NADPH-d in the mesothelium although the stain was lighter (Figures 4g-h).

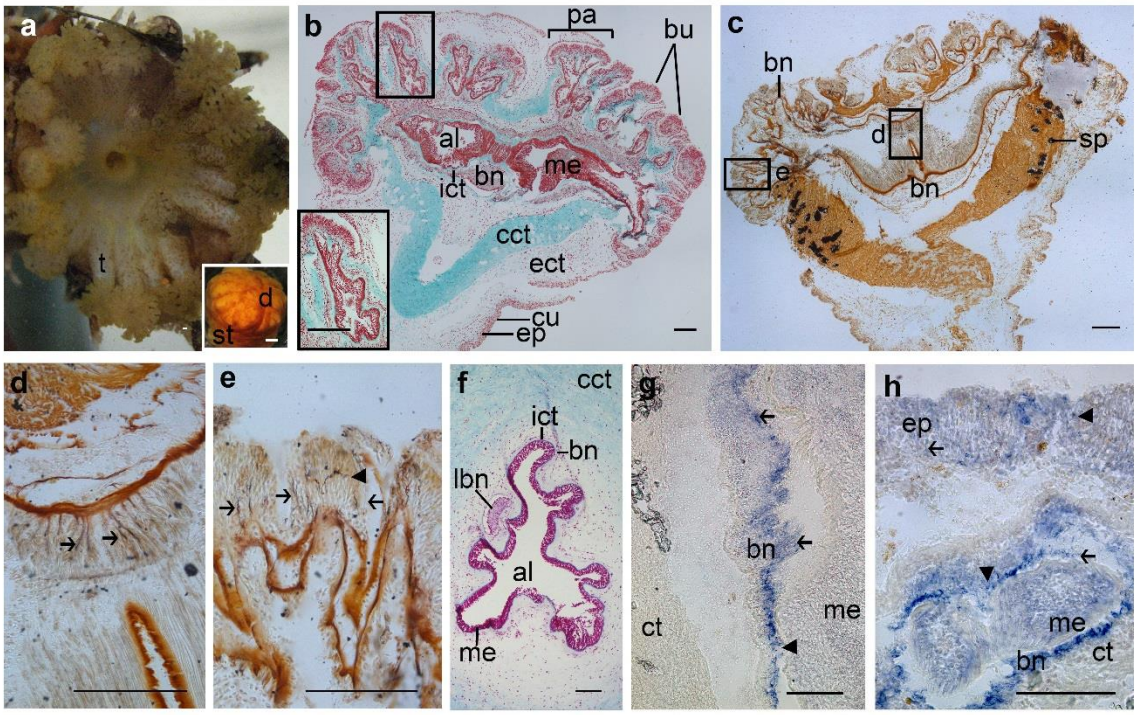

Figure 4. Histology and histochemistry of the tentacle. (**a**) Macroscopic photograph of the oral cavity showing the crown of tentacles (*t*) surrounding the mouth. **Insert** Each tentacle was composed of a stem (*st*) and a distal disc (*d*). **b-c** Longitudinal section through the tentacle stained with (**b**) Masson's trichrome showing the external (*ect*), central (*cct*) and inner (*ict*) connective tissue in *green*, the mesothelium (*me*) in *red*, and the buccal nerve (*bn*) in *dark pink*; and stained with **(c)** Palmgren's staining showing the buccal nerve (*bn*) in *dark brown* and the spicules (*sp*) in *black*. **d - e** Higher magnification (**d**) in the center of the stem where nerves are seen in the mesothelium (arrows) and, (**e**) in the bud where apical nerves (arrows) from the nerve plate (arrowhead) in the epidermis. (**f**) Transversal section in the stem stained with Miligan's Trichrome showing the longitudinal buccal nerve (lbn) and the buccal nerve plexus (*bn*) in *dark pink*, the mesothelium (*me*) in *red* and the connective tissue (*ict*: internal connective tissue, *cct*: central connective tissue) in *blue.* **g - h** Positive histochemical reaction for NADPH diaphorase in the buccal nerve and mesothelium (**g**) of the stem and (**h**) in the bud where fibers (arrows) and cell bodies (arrowheads) could be seen. *al*: ambulacral lumen, *bu*: bud, *cu*: cuticle, *pa*: papilla. Scale bars: 100 µm.

4.4.3.1.2. Tube feet

The tube feet in *H. arguinensis* had the same external morphology regardless of their position in the body and belonged to the disc-ending group of podia which is characterized by a basal cylindrical stem topped by a flattened disc (Figure 5a).

MiT staining revealed no differences in the tissue layer between the dorsal and ventral tube feet. They were all composed of the cuticle, epidermis, connective tissue (external and central), podial nerve, connective tissue (internal) and mesothelium (Figure 5b). As in the tentacle, the nervous tissue consisted of a podial nerve plexus enlarged asymmetrically to form the longitudinal podial nerve. The tube feet had a terminal disc in which two clusters of cells could be seen: one composed of densely packed epidermal cells at the tip of the disc (outer cell cluster) and another composed of loosely packed cells at the bottom of the disc (inner cell cluster). Connective tissue layers were seen between the two groups of cells and above the inner cell cluster which had the stronger staining (Figure 5c). In contrast to the tentacles, alizarine red staining showed that the calcareous spicules were only present in the terminal disc of the tube feet, below the inner cell cluster, as a support to the disc (Figure 5d). Nerve fibers were identified within the terminal disc and in the podial nerve plexus in the stem with Palmgren's staining, although the staining was masked, to some extent, by the general brown coloration of the tube feet and the black deposits in the calcium rich area (Figure 5e). The NADPH-d staining was positive in the podial nerve plexus, in the mesothelium and connective tissue (Figure 5f). In the terminal disc, the NADPH-d reaction was more diffuse than in the stem and a slight stain could be distinguished separating the two cell clusters, indicating most likely the localization of the nerve plate (Figure 5g).

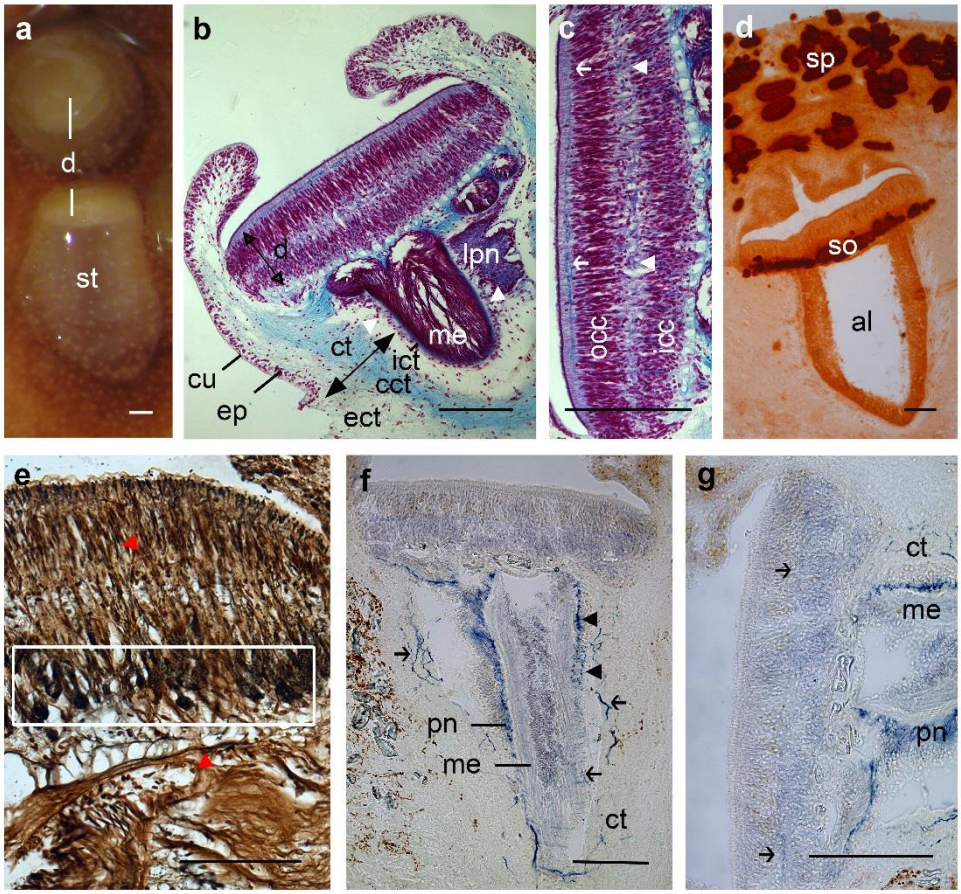

Figure 5. Histology and histochemistry of the tube feet. (**a**) Macroscopic photograph of the podia showing the stem (*st*) and the distal disc (*d*) from the ventral side. (**b**) Longitudinal section of the podia stained with Milligan's Trichrome showing the tissue layer subdivision: cuticle (*cu*), epidermis (*ep*), connective tissue (*ct*) in *blue* (external connective tissue, *ect*; central connective tissue, *cct;* internal connective tissue, *ict*), longitudinal podial nerve (*lpn*) and podial nerve plexus (arrowheads) in *light purple*, and mesothelium (*me*) in *magenta*. (**c**) Higher magnification of the terminal disc on the tube feet showing the two cell groups, the inner (*icc*) and outer (*occ*) clusters of cells, surrounded by the connective tissue above the occ (arrows) and between them (arrowheads). (**d**) Alizarine red staining showing the spicules (*sp*) and the supporting ossicles (*so*). (**e**) Higher magnification of the upper part of the tube feet stained with Palmgren's staining showing the heavily stained nerve fibers (red arrows). The white rectangle indicates the black deposits corresponding to the calcified spicules. (**f**) Positive histochemical reaction to NADPH diaphorase in the stem highlighting the podial nerve plexus (*pn*) extending in the mesothelium and in the connective tissue (fibers: arrows, cell bodies: arrowheads), and (**g**) in the nerve plate in the distal disc (arrows). Scale bars: 100 µm.

4.4.3.1.3. Papillae

Papillae are conical appendages that have a cylndrical pigmented stem surmonted by a whitish disc seen as a pointed structure (Figure 6a). Papillae contain calcareous spicules in the upper part, just below the disc, similar to the tube feet, as shown by the Alizarine red staining (Figure 6b). MiT staining showed that the stem was mainly composed of a dense connective tissue in which an ambulacral lumen communicated with the water-vascular system. From the inside to the outide, four tissue layers could be seen: the mesothelium, a thin layer of connective tissue and the podial nerve plexus (confirmed with Palmgren's staining) and an outer layer of connective tissue (Figure 6c). As for the tentacle and the tube feet, the papillae nervous tissue contained a longitudinal papillae nerve that runs on one side of the papillae and a nervous plexus which surrounds it. The NADPH-d staining showed strong reactivity in the papillae nerve, the mesothelium and the connective tissue in the stem where fibers and cell bodies could be seen (Figure 6d). The disc of the papillae was dense in cells and was supported by ossicles (calcareous spicules) as shown by the H&E staining (Figure 6e). Palmgren's staining showed numerous nerve fibers in the disc of the papillae (Figure 6f). A diffuse reactivity to NADPH-d could be seen in the disc highlighting the nerve plate, where some fibers and cell bodies could be clearly identified (Figure 6g).

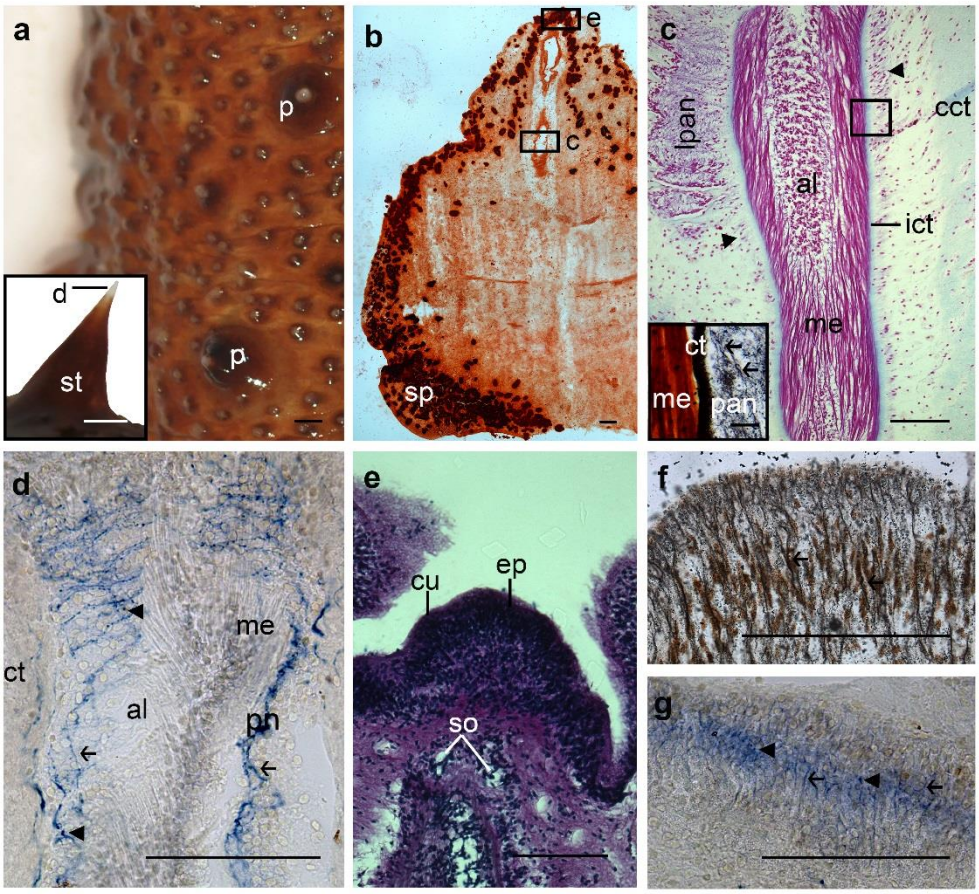

Figure 6. Histology and histochemistry of the papillae. (**a**) Macroscopic photograph of the dorsal side showing two papillae and (*insert*) a higher magnification of a papillae showing the stem (*st*) and the white distal disc (*d*). (**b**) Distribution of the spicules (*sp*) in the papillae. **c-d** Higher magnification image of the stem stained with (**c**) Milligan's trichrome clearly showing the connective tissue (*ict:* internal and *cct:* central) in *blue*, the longitudinal papillae nerve (*lpan*) and papillae nerve plexus (arrowheads) in *light purple*, the mesothelium (*me*) in *magenta* and the ambulacral lumen (*al*); with (*insert*) Palmgren's staining showing nervous fibers in the papillae nerve plexus (*pan*) in *black* (arrows); and (**d**) positive histochemical reaction with NADPH-diaphorase in the podial nerve plexus, mesothelium and connective tissue areas. **e-g** Higher magnification in the distal disc stained with (**e**) H&E, with (**f**) Palmgren's staining showing the nerve fibers (arrows) in *black* and (**g**) presence of histochemical reaction with NADPH diaphorase in some fibers (arrows) and cell bodies (arrowheads). Scale bars: a 500 µm, c in insert 25 µm, b, d e f g 100 µm.

4.4.3.2. Immunohistochemistry

In this section, the disc of the three water-vascular appendages was analyzed separately while the stem was described in one section as no major differences were seen between the three structures with the antibodies used in this study.

4.4.3.2.1. Tentacles – disc

The tentacles did not have ossicles underlying the bud, as shown in the histology section and confirmed here under bright field light (Figure 7a). DAPI staining revealed an abundance of cell nuclei in the epidermal cells of the bud (Figure 7b). The three immune markers labelled the interior of the main nervous structures in the bud of the tentacle (Figures 7c, d, e). The disc of the bud contained serotonin positive cell bodies in both clusters of cells, and a weak signal was detected in the nerve plate (Figure 7c). Bipolar cells measuring approximatively 10 µm in length were clearly distinguishable in the lower part of the bud, most likely indicative of the nerve plate (Figure 7c). Fibers within the disc and in the nerve plate were positive to anti-tubulin (Figure 7d). The synaptotagmin positive signal confirmed the presence of these elements, and highlighted the apical nerves extending from the buccal nerve plexus to the nerve plate (Figure 7e). Some cell bodies immunopositive for synaptotagmin in the epidermal layer of the bud were in close proximity to serotonergic cells, possibly the same cells (Figures 7c, e).

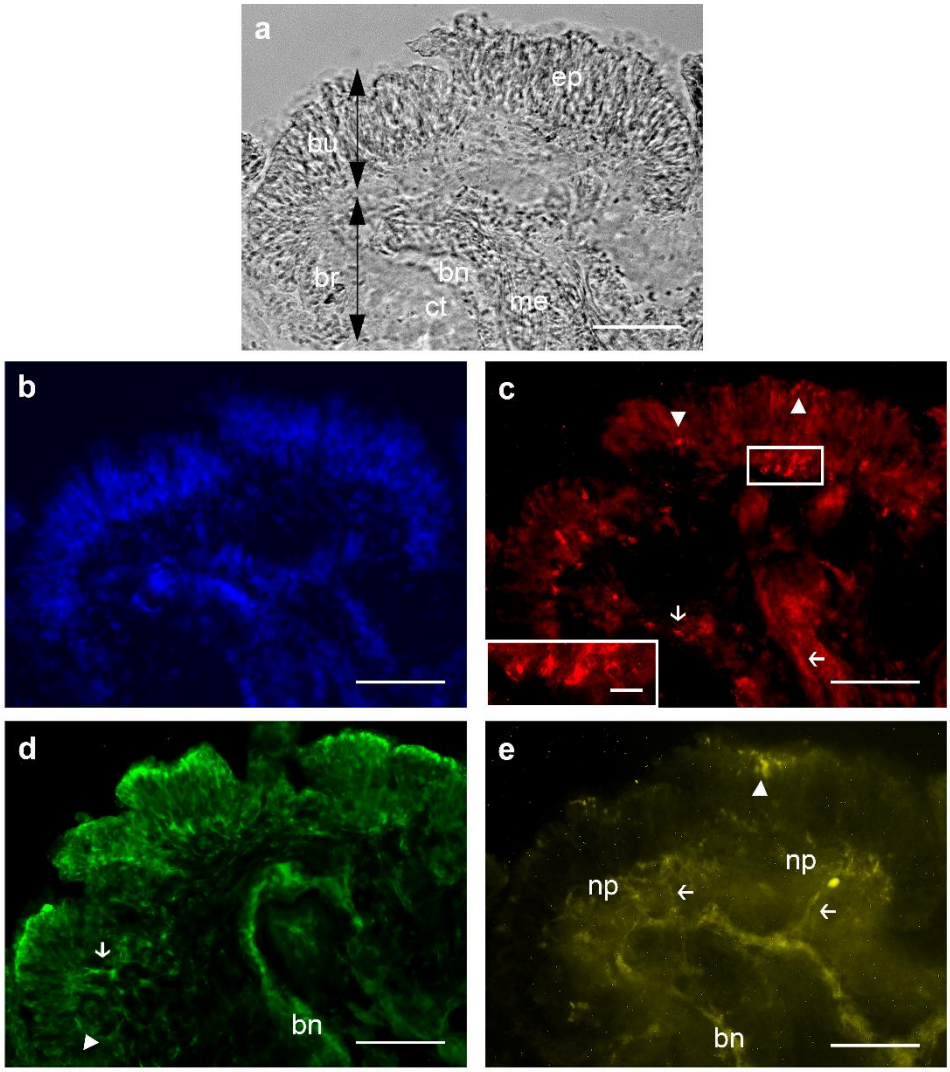

Figure 7. Immunohistochemistry of a longitudinal section of a two-bud papilla. (**a**) Section under bright field light showing the absence of supporting ossicles and the position of the different elements composing the papillae of the tentacle: buccal nerve *(bn)*, branch (*br*), bud (*bu*), epidermis (*ep*), connective tissue (*ct*) and mesothelium (*me*). (**b**) DAPI-stained section showing the densely packed outer clusters of cells (*occ*) and the loosely packed inner cluster of cells (*icc*) in the bud. (**c**) Cell bodies immunoreactivity with anti-serotonin serum where cell bodies can be seen in the two cluster of cells forming the disc of the tentacles. Bipolar cells were seen in the inferior region of the bud (insert). Anti-serotonin positive cell bodies were also seen in the nerve plate of the bud (double arrowheads) and anti-serotonin immunoreactive fibers were revealed in the buccal nerve (arrows). (**d**) Immunofluorescence against tubulin showing the buccal nerve plexus (*bn*), some fibers in the nerve plate (arrowhead) and within the clusters of cells (arrow). (**e**) Anti-synaptotagmin (1E11) immunoreactivity showing the buccal nerve plexus (*bn*), the apical nerves joining the buccal nerve plexus to the nerve plates (arrows), in which a dense group of fibers were seen. Some cell bodies were also revealed in the epidermal cells of the bud (arrowheads). Scale bars: 100 µm except in the insert of c where the scale bar corresponds to 20 µm.

4.4.3.2.2. Tube feet – disc

Bright field sections confirmed the presence of supporting ossicles only in the proximal part of the terminal disc (Figure 8a). The disc was highly cellular as shown by DAPI staining where the two clusters of cells, already seen in the histology work (inner and outer cluster of cells; Figure 5b) were distinguishable (Figure 8b). The three antibodies highlighted the nerve plate of the disc, which was located between the two group of cells composing the disc (Figures 8c, d, e). Serotonin-positive cell bodies were seen within the two clusters of cells and, with weaker staining, within the nerve plate (Figure 8c). Tubulinergic fibers were also seen in the nerve plate and in the two clusters of cells, showing the communication between these two elements (Figure 8d). The anti-synaptotagmin labelling confirmed the presence of a distinct nerve plate where cell bodies and fibers were seen (Figure 8e).

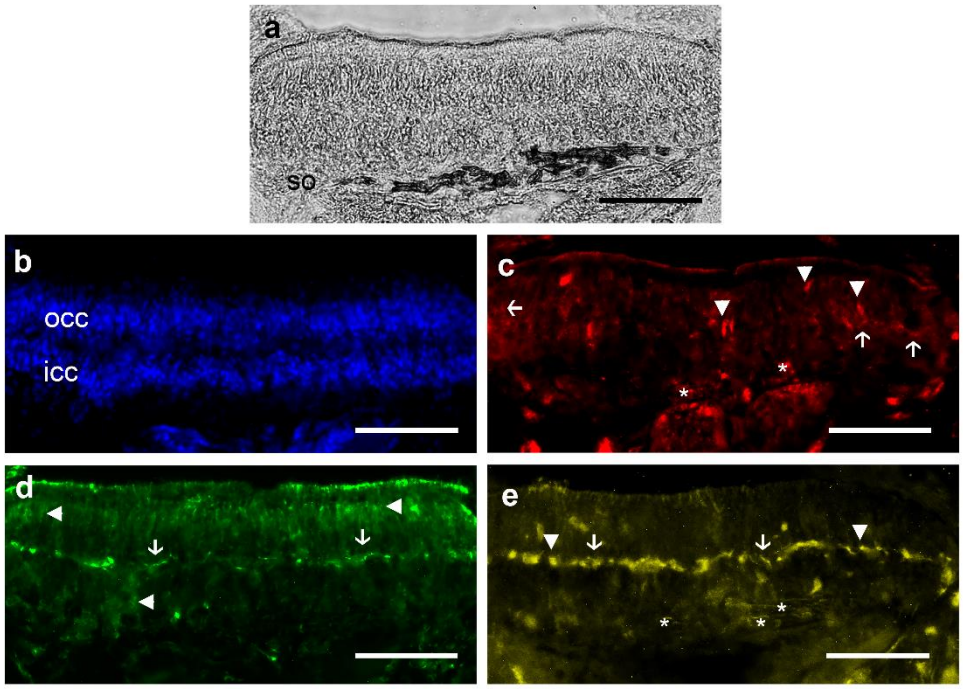

Figure 8. Immunohistochemistry of a longitudinal section through the disc of the tube feet. (**a**) Position of the supporting ossicles (*so*). (**b**) DAPI-stained section showing the nuclei of the densely packed outer clusters of cells (*occ*) and the loosely packed inner cluster of cells (*icc*). (**c**) The anti-serotonin antibody was immunoreactive with fibers (vertical arrows) stained along the nerve plate and in the disc (horizontal arrows), and within the disc (nerve plate and the two clusters of cells; arrowheads). (**d**) Tubulinergic fibers stained in the nerve plate (arrows) and between the nerve plate and the occ, and icc (arrowheads). (**e**) Synaptotagmin immunoreactivity (1E11) in cell bodies (arrowheads) and fibers (arrows) of the nerve plate. *: False positive signals from the supporting ossicles. Scale bars: 100 µm.

4.4.3.2.3. Papillae – disc

Bright field sections showed that the calcareous ossicles were not directly located below the bud but deeper within the connective tissue (Figure 9a). The DAPI-stained section revealed a highly cellular disc in the papillae (Figure 9b). However, in contrast to the tube feet, the disc of the papillae was composed of only one group of cells, as observed in the tentacles. Serotonin immunoreactive cell bodies were identified in low abundance within the nerve plate and in the epidermal cells in close contact with the environment (Figure 9c). As for the tentacles and tube feet, nerve fibers were clearly distinguishable in the nerve plate and between the epidermal cells with the anti-tubulin (Figure 9d) and anti-synaptotagmin antibodies (Figure 9e).

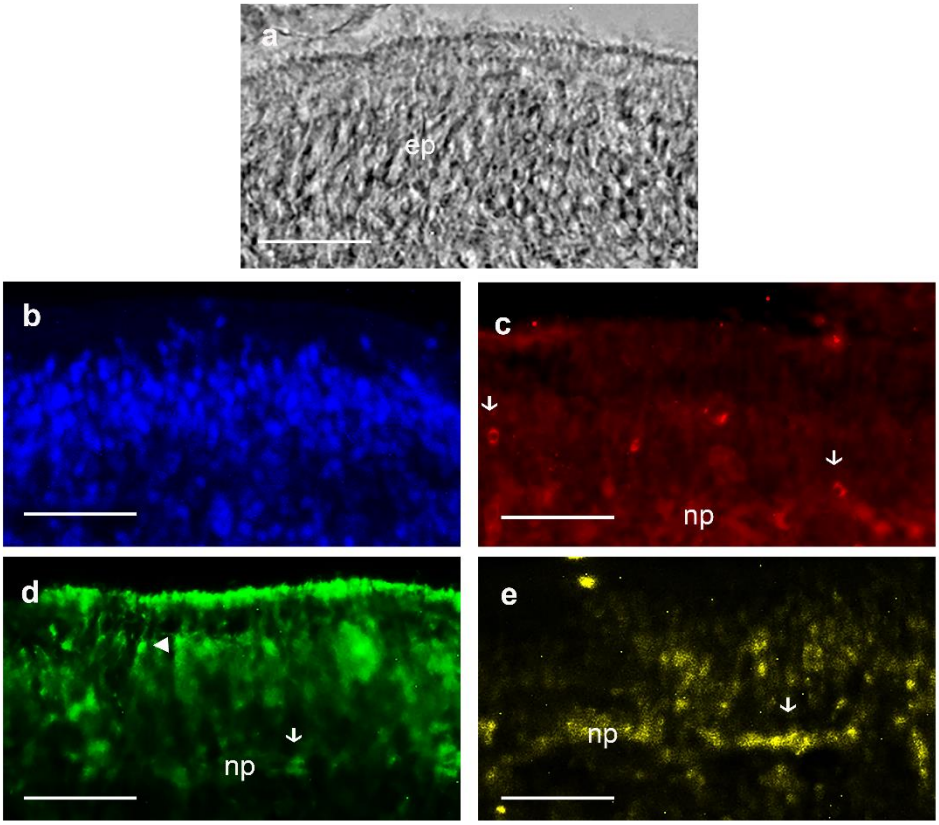

Figure 9. Immunohistochemistry of a longitudinal section through the disc of the papillae. (**a**) Section under bright field light. (**b**) DAPI-stained sections showing an abundance of nuclei in the terminal disc of the papillae. (**c**) Anti-serotonin immunoreactive cell bodies were visible in the nerve plate and in the epidermal cells (arrows). (**d**) Immunofluorescence to tubulin in fibers within the nerve plate (arrow) and between the epidermal cells (arrowhead). (**e**) Anti-synaptotagmin positive fibers in the nerve plate (arrow). Scale bars: 50 µm.

4.4.3.2.4. All appendages - stem

DAPI-stained sections showed an abundance of cell nuclei in the mesothelium and podial nerve plexus while there were fewer nuclei in the connective tissue (Figure 10b). All the nervous elements in the stem of the three different appendages (tube feet, papillae, tentacles) were stained by the three antibodies. Serotonin- (Figure 10c) and synaptotagmin- positive (Figure 10e) cell bodies were seen in the podial nerve plexus, while nerve fibers were stained with the anti-tubulin (Figure 10d) and anti-synaptotagmin antibodies (Figure 10e). These two antibodies also revealed nervous fibers in the connective tissue (Figures 10d, e) unlike the results of Palmgren histology.

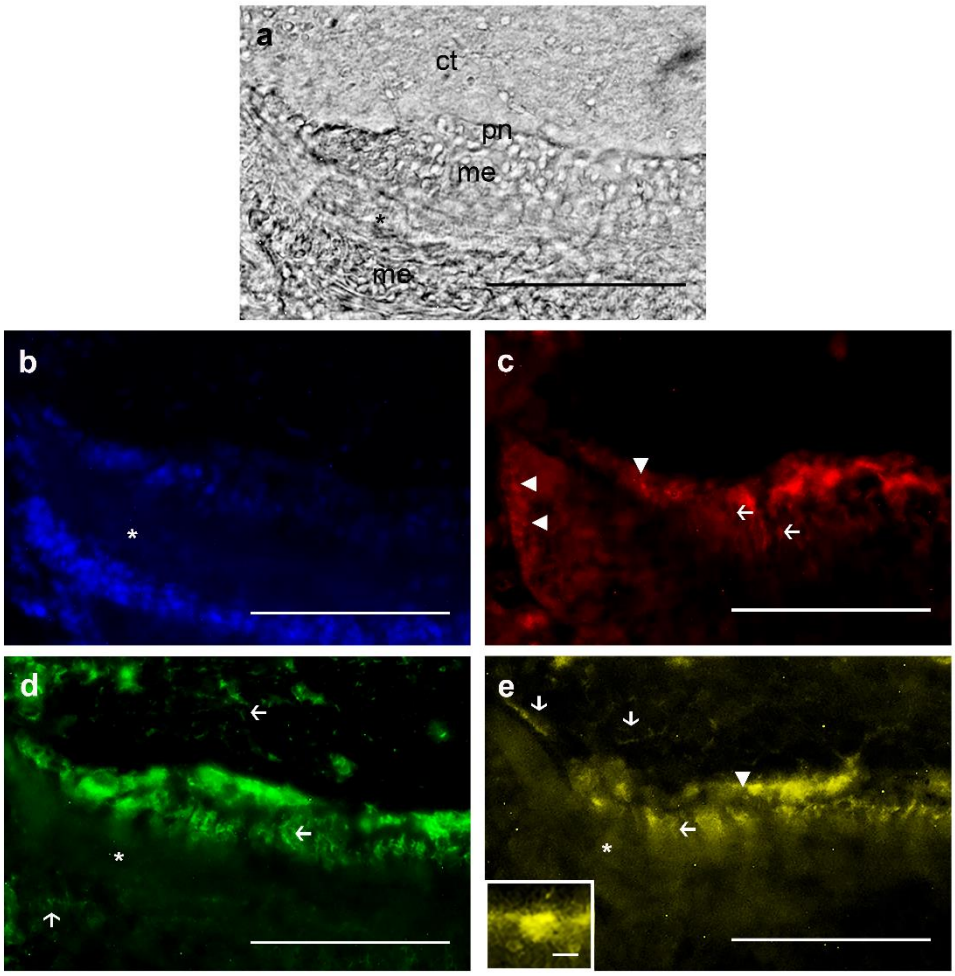

Figure 10. Immunohistochemistry of a longitudinal section through the stem of the tube feet. The same structure was observed in the stem of the papillae and the tentacle. (**a**) Section under bright field light (**b**) DAPI-stained section indicating the main structure: connective tissue (*ct*), podial nerve plexus (*pn*) and mesothelium (*me*). (**c**) Serotonin immunopositive cell bodies in the podial nerve plexus (vertical arrowheads), and in the mesothelium (horizontal arrowheads) and some fibers extending from the podial nerve plexus to the mesothelium (arrows). (**d**) Strong immunoreactivity to tubulin in the podial nerve plexus with some immunopositive tubulin fibers in the mesothelium and connective tissue. (**e**) Anti-synaptotagmin (1E11) immunoreactivity in fibers and few cell bodies (see insert) in the podial nerve plexus and in the mesothelium. * false positive resulting from the sectioning of the tissue. Scale bars: 100 µm except in the f insert where the scale bar corresponds to 10 µm.

4.5. Discussion

In this study the main morphological structures in contact with the water were analysed using histology, histochemistry and immunohistochemistry for possible clusters of nerve terminals that could suggest a chemosensory organ *H. arguinensis*. Although such a structure was not clearly identified, the discs of the three body appendages seems a promising candidate as 1) they contain groups of cells arranged differently between structures, with some stained with the neuronal markers suggesting they could be sensory neurons, 2) they possess a specific nervous arrangement with a distinct nerve place from which nerve fibers extend in the disc, and 3) they are rich in NO, and this molecule has been previously described as involved in chemosensation in invertebrates (e.g. Clifford et al. 2003; Gelperin 1994; Moroz 2006).

General tissue organization

The three body appendages were composed of a stem and a disc, forming a functional unit, and had the same general organization consisting of four tissue layers as previously reported for echinoderm appendages: an inner mesothelium, a connective tissue layer, a nerve plexus and an outer epidermis covered by a well-defined cuticle (e.g. Cavey 2006; Flammang et al. 1991; Santos et al. 2005; VandenSpiegel et al. 1995). However, the tissue layer organization and the cell and fiber composition differed depending on whether they belonged to the stem or the disc (see below). They all shared the cuticle, epidermis and connective tissue with the body wall, which is consistent with their denomination as protrusions of the body wall (Ruppert and Barnes 1994).

Localization of the nervous system and its link to the RNCs

The main nervous system of the body wall appendages consisted of a nerve plexus surrounding the stem, referred to as the fenestrated sheath, which is asymmetrically thickened to form the longitudinal nerve. As in other Holothuroidea, it is located in the deeper part of the connective tissue close to the mesothelium, although there is a thin layer of connective tissue between the two (Díaz-Balzac et al. 2010a; Flammang and Jangoux 1992). This contrasts with that seen in other echinoderms in which the nerve plexus lies just beneath the epidermis (Florey and Cahill 1977; Moore and Thorndyke 1993). This difference is proposed to be related to the thickness of the respective connective tissue (Díaz-Balzac et al. 2010a).

The innervation of the appendages is thought to come directly from the RNCs. The two components of the RNCs have been suggested to innervate different elements: the hyponeural part innervates the muscles, and the ectoneural part innervates the body wall including the appendages (Cobb 1985; Hyman 1955; Inoue et al. 2002). Díaz-Balzac et al. (2010a) supported this idea by showing unbranched lateral nerves from the ectoneural part of the RNCs to the

appendages. This proposal is also supported by the positive labelling in both RNCs and the nervous system of the appendages by several antibodies, such as RN1, anti-galanin, or anti-GFSKLYamide and those used in the present study (anti-synaptotagmin, anti-serotonin and anti-tubulin) (Díaz-Balzac et al. 2007; Inoue et al. 1999; Tamori et al. 2007).

Stem innervation

The stems of the three appendages presented the same nerve arrangement and were all immunopositive to the three markers used. Few immunoreactive cells were seen in the appendage stem, which agrees with previous ultrastructural (Cavey 2006; Flammang and Jangoux 1992) and immunohistochemical studies (Díaz-Balzac et al. 2010a; Inoue et al. 1999). In the mesothelium, immunoreactive nervous fibers stained by the three markers and Palmgren's staining were clearly seen, and extended into, or came from, the stem nerve plexus, consistent with the results obtained in *H. glaberrima* (Díaz-Balzac et al. 2010a). The present study suggests that nerve fibers cross the thin layer of connective tissue separating the mesothelium and the stem nervous plexus. This contradicts the indirect innervation proposed by previous studies in which transmitters were suggested to diffuse through the connective tissue layer (Bouland et al. 1982; Cavey 2006; Flammang and Jangoux 1992).

Interestingly, some serotonin-positive cell bodies were found in the part of the mesothelium lining the ambulacral cavity. The tissue surrounding this cavity is characterized by adluminal cells, whose function is unknown (Holland 1984). Recently, in the starfish *Acanthaster planci*, putative olfactory receptors in the tube feet and tentacles were found to be expressed in this region of the mesothelium (Roberts et al. 2017). It is therefore tempting to suggest that the cells found in the present study may detect chemicals transported into the water vascular lumen of the appendages through the madreporite or through the water vascular system.

Disc innervation

The disc was clearly different from the stem and was the most differentiated part in all appendages. It was highly cellular compared to the stem and the cellular organization differed according to the type of appendage. The disc of the tube feet and papillae consisted of a single surface; this was separated into two cell groups in the tube feet, while only one group was seen in the papillae. In contrast, the tentacles presented several buds containing one group. However, the nerve elements revealed by the neuronal markers had a specific arrangement in all appendages that consisted of a distinct nerve plate rich in cells and fibers, and with fibers joining the disc to the stem.

Based on ultrastructural studies which identified numerous ciliated cells within the disc, this structure is generally reported as the sensory part of the appendages (Bouland et al. 1982; Flammang and Jangoux 1992; VandenSpiegel et al. 1995). Here, cell bodies immunopositive with anti-synaptotagmin and anti-serotonin sera were found in the disc, at the level of the nerve plate and within the cell groups, and it seems likely that they correspond to the sensory neurons found in previous studies. It is notable that fewer cells were stained with our markers in comparison with the density of cell nuclei seen in the disc. This was also noted by Díaz-Balzac et al. (2010a) in the tentacles and tube feet of *H. glaberrima* with the RN1 antibody. Further studies are thus needed to characterize these cells.

Interestingly, no glomeruli-like structures were found in *H. arguinensis* in contrast with the papillae and tentacles of the sea cucumber *Leptosynapta clarki* (Hoekstra et al. 2012). This difference might be linked to the distinguishing characteristics of the order Apodida (to which *L. clarki* belongs) compared to order Aspidochirotida (to which *H. arguinensis* belongs). Apodid holothuroids lack tube feet, retractor muscles in the pharynx, respiratory trees, and they possess pinnate tentacle instead of the peltate tentacles seen in Aspidochirotida species (Ruppert and Barnes 1994).

Nitric oxide synthase in sea cucumbers

The NADPH-d reactivity, indicative of nitric oxide synthase (NOS), was widespread in the RNCs and in the three water-vascular appendages, which underlines the importance of nitric oxide (NO) in the biology of sea cucumbers. NO, synthesised from L-arginine by NOS, is a major signaling molecule in different phyla, and is involved in a variety of processes including immune defense, vascular regulation, muscle relaxation and, not least, neuromodulation and neurotransmission (Colasanti and Venturini 1998; Moncada et al. 1991; Palumbo 2005; Snyder and Bredt 1991).

In the present study, NOS was most abundant at the periphery of the RNCs and in the stem nervous system. This suggests an important role for NO in the nervous system of sea cucumbers. It is particularly tempting to suggest that NO might be involved in the motor activity of the appendages, taking into consideration the large extension of NO positive fibers in the nerve plexus and the mesothelium within the stem, as has been described in stomach relaxation of the starfish *Marthasterias glacialis* (Martínez 1995; Martínez et al. 1994). A subtler modulatory or sensory function, nevertheless, should not be excluded.

In addition to its presence in the stem of the appendages, NOS was also detected in scattered cell bodies and abundant fibers in the disc, where the NADPH-d staining was lighter and more diffuse than in the stem. As the disc has characteristics of a sensory structure (Bouland

et al. 1982; Flammang and Jangoux 1992; VandenSpiegel et al. 1995), one of the possible roles of NO in this area could be sensory perception. NO has furthermore been described as a modulator of chemosensory processing in several molluscs and insects, in which NOS was seen to be highly selective and abundant in chemosensory areas (e.g. Elphick et al. 1994; Gelperin 1994; Moroz 2006). Further investigations are needed to confirm the role of NO in sensory perception and particularly in chemosensation in sea cucumbers.

4.6. Conclusion

One of the main objectives of the present study was to characterize the structures in contact with the environment in sea cucumbers to establish what structures would be good candidate(s) to perform electrophysiology for screening for pheromones, an essential step to advance in the study of chemical communication in these organisms. The water vascular appendages have been attributed specific roles in feeding (tentacles); respiration, gas exchange, substratum attachment (tube feet); and mechanoreception and/or chemoreception (papillae) (Bouland et al. 1982; Flammang and Jangoux 1992; VandenSpiegel et al. 1995). However, they are also potential candidates for chemosensory reception. Tentacles seem to be an interesting organ in relation to reproduction as they are always seen outside of the oral cavity when the sea cucumber adopts its pre-spawning posture (see Chapter III). However, it is not known if this is the result of a hydrostatic pressure from the erect posture of the animal or a mechanism to improve chemical detection.

The present study did not identify one particular structure as a strong candidate for chemoreception; all had a similar tissue and nervous organization. However, it did confirm that the disc is the most specialized area within each appendage with a specific nervous arrangement, composed of a distinct nerve plate, rich in NOS, and containing numerous cells, some of which were serotonin and synaptotagmin positive. In summary, the present study indicates that the disc on the appendages could be a prime target for electrophysiological assays. If experiments targeting the disc fail for this purpose, it might be worth testing dissected RNCs, as has previously been done in starfish and brittle star (e.g. Binyon and Hasler 1970; Brehm 1977). In contrast, the body surface is rich in calcareous material and poorly innervated making it thus a poor candidate site for chemosensing. As a complementary step to electrophysiological assays, the chemoreceptors, which belong to the G-coupled-protein receptors (GPCRs) superfamily, need to be characterized in the three body appendages in order to identity putative olfactory receptors (see chapter V).

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Chapter V

Candidate odorant receptors in the sea cucumber *Holothuria arguinensis*

Manuscript in preparation

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Candidate odorant receptors in the sea cucumber *Holothuria arguinensis*

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5.1. Abstract

Sea cucumber (Holothuroidea: Echinodermata) males release chemical cues that attract and induce spawning in ripe conspecifics. However, our knowledge of how they recognize and respond to environmental signals is limited. In vertebrates, there are five different G-protein-coupled receptor (GPCR) families involved in olfaction including the odorant receptors (ORs) which are the most diverse. Here, we characterized chemosensory GPCRs in the sea cucumber *Holothuria arguinensis*. Six transcriptome libraries from tissues involved in sensing (tentacles, oral cavity, calcareous ring, and papillae and tegument) plus ovary and testis were obtained and combined to generate a single *de novo* transcriptome that was screened for putative GPCRs. Sequence similarity searches revealed the presence of at least 591 distinct GPCRs; however, no orthologs of vertebrates ORs were found. Phylogenetic analysis including human, a teleost fish (eel), two early deuterostomes (amphioxus and sea urchin) and cnidarian resulted in at least seven potential sea cucumber ORs which clustered in two groups: one closer to sea urchins ORs and the other closer to sea anemone ORs, suggesting that sea cucumber ORs may emerge from two distinct ancestors. The putative sea cucumber ORs possessed conserved amino acid sequences characteristic of OR signature motifs in metazoans and they were mostly abundant in tentacles, oral cavity, calcareous ring, and papillae and tegument, consistent with involvement in chemical sensing. Our study provides for the first time a preliminary analysis of the candidate odorant receptors in the sea cucumber *Holothuria arguinensis*.

Key words: GPCRs, chemosensory, sea cucumbers, transcriptome, odorant receptors

5.2. Introduction

All living organisms, from bacteria to vertebrates, perceive and respond to chemical cues from their environment (Bargmann 2006; Kaupp 2010). Chemical cues are essential for survival and reproduction, and mediate a variety of activities such as feeding, predator avoidance, mating and social behaviour. To detect and discriminate the vast array of chemical stimuli, animals have developed complex olfactory systems with a large repertoire of chemosensory receptors (Kaupp 2010; Touhara and Vosshall 2009). Chemosensory receptors still remain to be characterised in most metazoan lineages although they are known to be highly diverse in those already described (Churcher 2011; Spehr and Munger 2009).

A large group of chemosensory receptors belong to the G-protein-coupled receptors (GPCRs), which is one of the largest superfamilies of transmembrane receptors found in metazoan cells. GPCRs have a highly conserved structure of seven transmembrane domains (Ache and Young 2005) and convert extracellular stimuli, including odorants, photons, amino acids and peptides, into intracellular biochemical signals through a diversity of signaling cascades (mostly cAMP and calcium secondary messengers) (Rosenbaum et al. 2009). Based on sequence similarity and ligand affinity, GPCRs are classified into five main families: Glutamate (G), Rhodopsin (R), Adhesion (A), Frizzled (F) and Secretin (S) (Schiöth and Fredriksson 2005). So far, GPCRs with chemosensory functions have been found in two families, the glutamate-receptor family and the rhodopsin-type family (Bjarnadóttir et al. 2005; Liberles and Buck 2006). The latter family contains the largest number and the most diverse repertoire of GPCRs involved in vertebrate olfaction (Fredriksson et al. 2003).

Rhodopsin family members involved in vertebrate olfaction include, i) the odorant receptors (ORs) (Buck and Axel 1991), ii) trace amine-associated receptors (TAARs) (Liberles and Buck 2006) and iii) formyl peptide receptor-like proteins (FRPs) (Riviere et al. 2009). Vomeronasal receptors (type 1 and 2) are also GPCRs that are relatively well characterized in vertebrate olfaction but they do not belong to the rhodopsin family (Bargmann 2006). ORs are the largest GPCR subfamily with about 2,130 genes discovered while the four other subfamilies are significantly smaller, and contain less than 100 genes (Kaupp 2010; Mombaerts 2001). The uniqueness of this subfamily resides in its rapid evolution and the great diversity of receptor sequences across species as a consequence of gene duplication and gene loss in the vertebrate lineages, with some teleost fishes possessing fewer than 100 OR genes whereas some mammals can have more than 1000 OR genes (Niimura and Nei 2005).

In invertebrates, olfaction appears to be mediated by receptor families evolutionarily distinct from those of vertebrates (Bargmann 2006). In insects, ORs are not GPCRs and do not share sequence similarity with vertebrate ORs (Bargmann 2006; Benton et al. 2009; Kaupp 2010; Sato et al. 2008). In the nematode *Caenorhabditis elegans* and in the marine mollusc *Aplysia californica* GPCR chemoreceptors have been identified; however, they do not seem to be related to vertebrate ORs (Ache and Young 2005; Bargmann 2006; Cummins et al. 2009; Kaupp 2010). Within the phylum Chordata, analysis of urochordate genomes failed to identify potential OR-like genes in *Ciona intestinalis*, *C. savignyi*, *Oikopleura doica* suggesting that OR genes have been lost in this lineage (Churcher and Taylor 2009; Niimura 2009b). However, more than 30 vertebrate-type OR genes were identified in the cephalochordate amphioxus *Branchiostoma floridae* (Churcher and Taylor 2009; Niimura 2009a, b) and, 678 to 979 putative chemoreceptor genes were reported in the echinoderm *Strongylocentrotus purpuratus* (Burke et al. 2006; Raible et al. 2006). ORs sharing a similar structure to chordate ORs were recently described in the genome of the cnidarian *Nematostella vectensis* (Churcher and Taylor 2011). Recent phylogenetic studies have also shown that ORs of cephalochordates, cnidarians, and vertebrates, and a subset of OR genes from the echinoderm *S. purpuratus* form a monophyletic clade. Based on the taxonomic distribution of these genes, this clade would have been formed before the divergence of cnidarians and bilaterians occurring at least 700 million years ago (Churcher 2011; Churcher and Taylor 2011). The diversity of the chemoreceptor gene repertoire among nematodes, molluscs, insects, cnidarians, echinoderms and chordates reflects the dynamic odour landscape to which organisms have adapted during evolution (Bargmann 2006).

Echinoderms have the ability to perceive and react to waterborne chemical stimuli (e.g. Campbell et al. 2001; Dix 1969; Mann et al. 1984; Unger and Lott 1994). Since they are phylogenetically closer to vertebrates than to nematodes and insects, they are expected to possess ORs characteristic of the rhodopsin-GPCR like family. In the sea urchin *S. purpuratus*, 192 candidate chemosensory receptor genes, including 177 rhodopsin-like members, were identified with an approach more oriented to the search for odorant receptors (Burke et al. 2006). Evidence of chemical communication has been demonstrated in sea cucumbers (Holothuroidea: Echinodermata) during gametogenesis (Hamel and Mercier 1996, 1999) and we have established that male *Holothuria arguinensis* release chemical cues that attract and induce spawning in ripe conspecifics (Marquet et al., in preparation). However, our knowledge of how they recognize and respond to environmental signals is limited, and no study has yet been published on chemosensory GPCRs genes in sea cucumbers.

The present study aimed to characterize the chemosensory GPCR complement and their tissue distribution in the sea cucumber *Holothuria arguinensis* to gain insight into the enigmatic process of chemosensing in these organisms. Six transcriptome libraries from target tissues with a potential role in chemosensing (oral cavity, calcareous ring, tentacles and, papillae and tegument) plus ovary and testis were produced and the candidate chemosensory GPCR complement was retrieved by comparison with other metazoans. Putative chemosensory receptors were mapped to the different tissue libraries to infer location of candidate chemosensory tissue in sea cucumber. The identification of candidate chemosensory receptors will contribute to elucidate the tissues and organs where sensory perception occurs, will lead to better characterization of this process, and will permit OR deorphanisation using cell engineering techniques (Behrens et al. 2014).

5.3. Materials and Methods

5.3.1. Ethics statement

The specimens of the sea cucumber, *Holothuria arguinensis*, were collected and handled in agreement with the license of the ICNF, Instituto da Conservaçao da Natureza e das Florestas, Portugal (License N° 635/2015/CAPT). The species is not endangered or protected.

5.3.2. Collection of animals and tissues

Adult *H. arguinensis* Koehler and Vaney, 1906 (Holothuroidea, Aspidochirotida) (>210 mm length) were collected by hand in the intertidal zone of the Ria Formosa (37°00'35.02''N) in Faro (Portugal) during summer 2015 and transported live to the Center of Marine Science (CCMAR), University of Algarve. Animals were anesthetized in $MgCl_2$ (5%) before dissection to collect the tissue samples. Six tissue samples were collected and pooled from four individuals (2 males and 2 females except for gonads in which 4 individuals of each sex were pooled): tentacles, testis and ovary, papillae and tegument, oral cavity and calcareous ring. All samples were frozen on dry ice and kept at -80°C until RNA extraction.

5.3.3. RNA extraction and library preparation

Total RNA (tRNA) was extracted using the Maxwell 16 total RNA purification kit (Promega, Madrid, Spain) according to the manufacturer's instructions. Samples were homogenized using an Ultra-Turrax homogenizer (IKA T25, Staufen, Germany) and tRNA was precipitated with ethanol and quantified using a Nanodrop (1000 Spectrophotometer, Thermo Fisher Scientific, USA). Agarose gel electrophoresis (0.8% / 1 x TAE: Tris-acetate-EDTA) was

used to assess the RNA quality and integrity. RNA samples were subsequently treated with DNAse to remove any remaining genomic DNA using the Turbo DNA-free kit (Ambion, London, UK).

Library preparation and sequencing was conducted by Genenergy (Shanghai) using Illumina TrueSeq mRNA-seq library Prep kit (RNA input 2 µg, insert size of 300-400 bps) and Illumina Hi-Seq 1500 to generate 100 base paired-end reads.

5.3.4. Sequence assembly

Quality control of raw reads and their respective editing was performed with Trimgalore wrapper script version 0.3.3 (bioinformatics.babraham.ac.uk/ projects/trim_galore/) producing simple descriptive statistics and edited reads, before assembly. Tissue specific *de novo* assemblies were obtained using Trinity v. 2.0.6 (trinityrnaseq_r2012-05-18; http:// trinityrnaseq.sourceforge.net/) with the default parameters. A whole tissues transcriptome was also obtained using all tissues RNAseq samples and Trinity with "--normalize_reads" and "--min_kmer_cov 2" options defined. This program has been developed for transcriptome assembly of short reads using the de Bruijn graph algorithm (Grabherr et al. 2011).

5.3.5. Characterisation of GPCRs

The full transcriptomes of the six target tissues were translated into protein using the TransDecoder program (http://transdecoder.sf.net) and the predicted proteins were annotated using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) to generate a sub-library containing only the predicted protein sequences with TM domains. Blast searches (tBLASTn) were performed against human, sea urchin and amphioxus GPCR datasets to retrieve GPCRs using an e-value cut-off of 1e-5 (Table 1). A total of 1,330 putative holothurian GPCRs (full-length or incomplete) were obtained and were subsequently searched against the NCBI protein database using default parameters to confirm their identity. Based on sequence similarity, they were classified into the five main GPCR families as described by Fredriksson et al. (2003) . A total of 591 unique GPCR sequences of variable length were obtained and the percentage distributed between the five families was compared to that described in human (Bjarnadóttir et al. 2006), amphioxus (Nordström et al. 2008), cnidaria (Krishnan et al. 2014), and two other echinoderms (starfish and sea urchin) (Hall et al. 2017).

Table 1. Datasets used as reference to retrieve sea cucumber GPCRs.

Organism	Type of GPCRs	Reference	Extracted from:
Human	Not olfactory	Fredriksson et al. (2003)	NCBI protein database https://www.ncbi.nlm.nih.gov
Human	Olfactory	Zozulya et al. (2001)	Supplementary material Zozulya et al. (2001)
Sea urchin	Chemosensory genes identified by 7TM/PFAM/C.elegans search method	Burke et al. (2006)	Ensembl Metazoa http://metazoa.ensembl.org
Sea urchin	Chemosensory genes identified by OR Motif/HMM search method	Burke et al. (2006)	Ensembl Metazoa http://metazoa.ensembl.org
Amphioxus	Olfactory	Niimura (2009b)	Joint Genome Institute http://genome.jgi-psf.org/euk_home.html

5.3.6. Phylogenetic analyses

Due to the large number of predicted sequences and their variable length, which could negatively interfere with the analysis by generating many potential false results, only GPCRs containing more than 4 transmembrane domains (123 sequences) were used. These sequences were aligned in AliView (Larsson 2014) with odorant receptor sequences from the cnidarian *Nematostella vectensis* (Churcher and Taylor 2011), amphioxus *Branchiostoma floridae* (Churcher and Taylor 2009), sea urchin *Strongylocentrotus purpuratus* (Burke et al. 2006), eel *Anguilla anguilla* (Churcher et al. 2015), human (Zozulya et al. 2001) and with human non-olfactory GPCRs (Fredriksson et al. 2003). A total of 250 sequences of 237 amino acids in length were used to construct a maximum-likelihood tree using PhyML (PHYlogenetic inferences using Maximum Likelihood) software available in the Montpellier Bioinformatics Platform (http://www.atgc-montpellier.fr/). Before tree construction, sequence alignment was manually edited to remove poorly aligned regions and to correct imprecisions. The edited sequence alignment was then submitted to ProtTest (2.4) according to the Akaike Information Criterion (AIC) (Abascal et al. 2005) to select the statistical model which would explain the best the evolution of the receptors in the phylogeny. Maximum likelihood (ML) trees were constructed with the PhyML program (v3.0) using a Blosum62 substitution model with a gamma shape (4 rate categories) of G = 1.89 and 100 bootstrap replicates. The tree was edited in Figtree software (http://www.umiacs.umd.edu/~morariu/figtree/) and the putative sea cucumber non-OR cluster was used to root the tree. Branch support was estimated using three methods: two parametric methods, aLRT (approximate likelihood ratio test) and aBayes (approximate transformation Bayes test) and the non-parametric Bootstrap method, (BS, 100 replicates). The nodes were reported when at least one of the methods showed significant

branch supporting values, defined as, aBAYES > 0.95, SH-aLRT > 0.85 and BS > 75% by Maronna et al. (2016).

5.3.7. Multiple sequence comparisons and signature OR motif identification

Amino acid sequence motifs that are considered as common signature OR motifs in amphioxus and vertebrate ORs were searched for in the sea cucumber OR candidates. Sequences were used and aligned in ClustalW (www.ebi.ac.uk/clustalw) with the closest phylogenetic references obtained from the tree. Among the motifs of interest were, LxxxxxxRxxAlxxPL (x represents any amino acid), commonly found in zebrafish and mouse ORs (Alioto and Ngai 2005), and the motifs LxxPxYxxxxxLxxxDxxxxxxxxP (Intracellular loop 1, IL1- Transmembrane 2, TM2), MxxxxYxxxCxPLxY (TM3-IL2) and KAxxTxxxH (IL3-TM6) and NPxxYxxR (TM7), which have been identified to discriminate ORs and non-ORs in the Rhodopsin family (Churcher and Taylor 2009). The percentage of amino acid sequence similarity and identity was determined between the potential sea cucumber OR candidates and their closest receptors identified using the phylogenetic tree, and with the eel and human OR sequences using GeneDoc software (Nicholas and Nicholas 1997). The presence and localization of the TM domains was assessed using TMHMM (http://www.cbs.dtu.dk/services/TMHMM).

3.5.8. Expression of sea cucumber ORs

To characterize the tissue distribution of the OR candidates identified in the sea cucumber, the presence of the transcripts was determined in the six target tissues. A blast query (tBLASTn) looked for transcript presence/absence in each of the six assemblies with an e-value cut-off of < 1e-20 and a maximum of five sequences were retrieved. The OR candidates were estimated as present in a specific tissue when the identity was equal or more than 99% and when the sequence coverage was equal or more than 150 nucleotides between the query and the subject. The relative number of ORs in each tissue was estimated by dividing the number of OR sequences by the total number of Trinity transcripts found in each tissue.

5.4. Results

5.4.1. Transcriptome assembly and annotation

The pair-end reads from all six tissue libraries were pooled and used to assemble a single *de novo* transcriptome. This strategy was used instead of the individual tissue assemblies as this would increase the probability to obtain low expressed transcripts which may be underrepresented in each tissue transcriptome. The raw reads were assembled into 900,603 Trinity transcripts with an N50 value of 758 bp. Tissue specific *de novo* assemblies were also performed and the highest and lowest number of Trinity transcripts were found in the oral cavity (OC; 353,921 transcripts) and the ovary (GF; 86,417 transcripts), respectively (Table 2). To facilitate the identification of putative GPCRs, all transcripts were translated to 37,711 predicted proteins and then searched for the presence of TM domains.

Table 2. Descriptive statistics of the individual tissue assemblies.

	Te	**T**	**O**	**OC**	**CR**	**P+T**	**All**
Total Trinity transcripts	153,723	200,418	86,417	353,921	257,323	327,647	900,603
N50 value (bp)	656	726	678	741	656	691	758
Average length (bp)	570.86	599.89	586.91	590.40	551.68	565.13	586.79

Te: testis, T: tentacle, O: ovary, OC: oral cavity, CR: calcareous ring, P+T: papillae and tegument and All: all tissues.

5.4.2. General characterization of the sea cucumber GPCR repertoire

Of the 37,711 predicted proteins from the *H. arguinensis* transcriptome, 591 unique GPCRs were annotated. The majority of the best sequence matches against the NCBI protein database identified an ortholog in the sea urchin *S. purpuratus.* The GPCR families identified were Rhodopsin (398 members), Adhesion (126 members), Glutamate (51 members) and Secretin (15 members), with the Rhodopsin family containing the most diverse array of receptors (Figure 1a, Table 3). Although different members of the Rhodopsin family cluster were identified, putative orthologs of vertebrate ORs were absent. No members of the Frizzled family were found, and one sequence categorized as “Other GPCRs” could not be assigned to any of the above groups.

5.4.2.1. The Rhodopsin GPCRs

Members of the Rhodopsin receptor family represented approximately 67% of the total GPCRs represented in the transcriptome (Figure 1a). Within this family, homologues for the four main human Rhodopsin subgroups, alpha, beta, gamma and theta, were found (Fredriksson et al. 2003) (Figure 1b, Table 3). The alpha group contained the largest number of sequences (160) which represented 40% of Rhodopsin GPCRs. Members of the five main subfamilies of the alpha group receptor cluster were found: amine (84%), prostaglandin (2%), opsin (4%), melatonin (4%) and MECA (melanocortin, endothelia, cannabinoid and adenosine) (9%). Among the amine subfamily, two neurotransmitter receptors that are typical of invertebrates, tyramine and octopamine, were also identified.

The beta group was the second largest and constituted 23% of the Rhodopsin family. Within this group, GPCRs predicted to be activated by peptides such as gastrin, neuromedin B and orexin were identified. Around 18% of the sea cucumber Rhodopsin GPCRs was found to be part of the gamma group. Most of these receptors belonged to the SOG (somatostatin, opioids, galanin) receptor cluster (67 members) with the somatostatin receptors (39 GPCRs) being the most numerous. Only four receptors were similar to the chemokine receptor cluster, and no members of the melanin-concentrating hormone (MCH) receptor cluster were found.

The theta group contained the lowest number of representatives of the Rhodopsin family and constituted only 7% of the total GPCRs of this family. Twenty-seven receptors, most likely members of the glycoprotein receptor cluster but no members of the MAS-related receptor cluster, purine receptor cluster and olfactory receptor cluster were found. It was not possible to assign 47 of the GPCRs (12% of the Rhodopsin family) to a family and these were designated "Unclassified Rhodopsins".

5.4.2.2. Other GPCR families

Adhesion (21%) and Glutamate (9%) were the second and the third largest GPCR families identified in the sea cucumber transcriptome (Figure 1a). Metabotropic glutamate, GABA (gamma-amino-butyric acid) and calcium-sensing receptors were found in the Glutamate family (Table 3). Members of the Secretin family were the least represented (3%) with few receptor sequences found (15) (Figure 1a). The latter were putative homologues of the chordate calcitonin, corticotrophin-releasing hormone, parathyroid hormone, Pigment Dispersing Factor (PDF) and of vasoactive intestinal peptide receptors (Table 3).

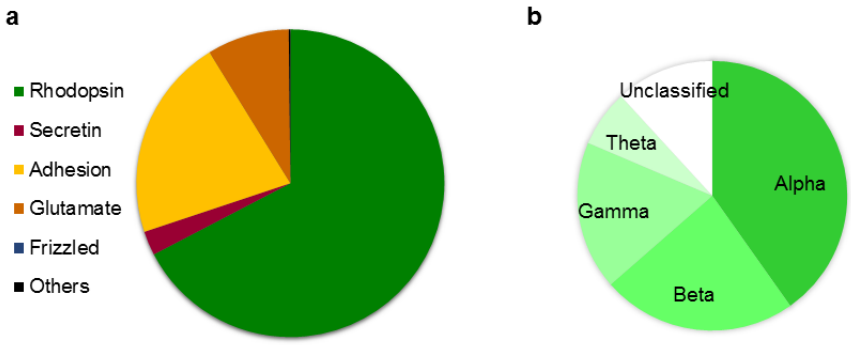


Figure 1. Proportion of GPCRs represented in the sea cucumber transcriptome. (**a)** GPCR family and (**b)** Rhodopsin group members.

Table 3. Overall identity of the sea cucumber GPCRs and number of representatives within each family.

RHODOPSIN	**398**				
ALPHA	**160**	**BETA**	**93**	**GAMMA**	**71**
Amine	**134**	Gastrin	11	**SOG**	**67**
Alpha-adrenergic	65	Neuromedin B	11	Somatostatin	39
Muscarinic	19	Orexin	11	Galanin	18
Serotonin	19	Cholecystokinin	9	Opiod receptors	5
Histamine	10	Bombesin	7	Allatostatin	4
Beta-adrenergic	8	Substance P	7	Nociceptin	1
Octopamine	5	Neuropetitde cappa	5	**Chemokine**	**4**
Dopamine	3	Neuropeptide Y	5	Cysteinyl leukotriene	4
Tyramine	3	Cardioacceletory peptide	4	**THETA**	**27**
Trace amine	2	GHSR	4	**Glycoprotein**	**27**
MECA	**9**	Prokineticin	4	TSH/LHCG/FSH	27
Adenosin binding	8	Tachykinin	3	**RHODOPSIN-LIKE**	**47**
High-affinity lysophatidic	1	Anti-diuretic	2	GPCR moody	15
acid receptor		Gonadotropin releasing	2	GPCR 54	9
Opsin	**7**	Relaxin	2	GPCR 52	6
Opsins	3	Insulin-like peptide	1	GPCR 84	5
Melanopsin	3	Neuromedin U	1	GPCR12	4
Peropsin	1	Neuropeptide A15	1	GPCR 85	3
Melatonin	**7**	Neuropeptide CCHamide	1	GPCR 173	2
Melatonin	7	Neuropeptide FF	1	GPCR 161	1
Prostaglandin	**3**	THR	1	GPCR 19	1
Prostaglandin	3			Rhodopsin GC	1
SECRETIN	**15**	**ADHESION**	**126**	**GLUTAMATE**	**51**
CRH	6	Adhesion	94	Metabrotopic glutamate	29
Calcitonin receptor	4	EGF-like mucin	14	GABA receptors	19
PTH	2	latrophilin	14	Calcium sensing-receptors	3
PDF receptors	2	Cadherins	3	**OTHER GPCRs**	**1**
Vasoactive intestinal peptide	1	BAI	1	Uncharacterized 160863	1

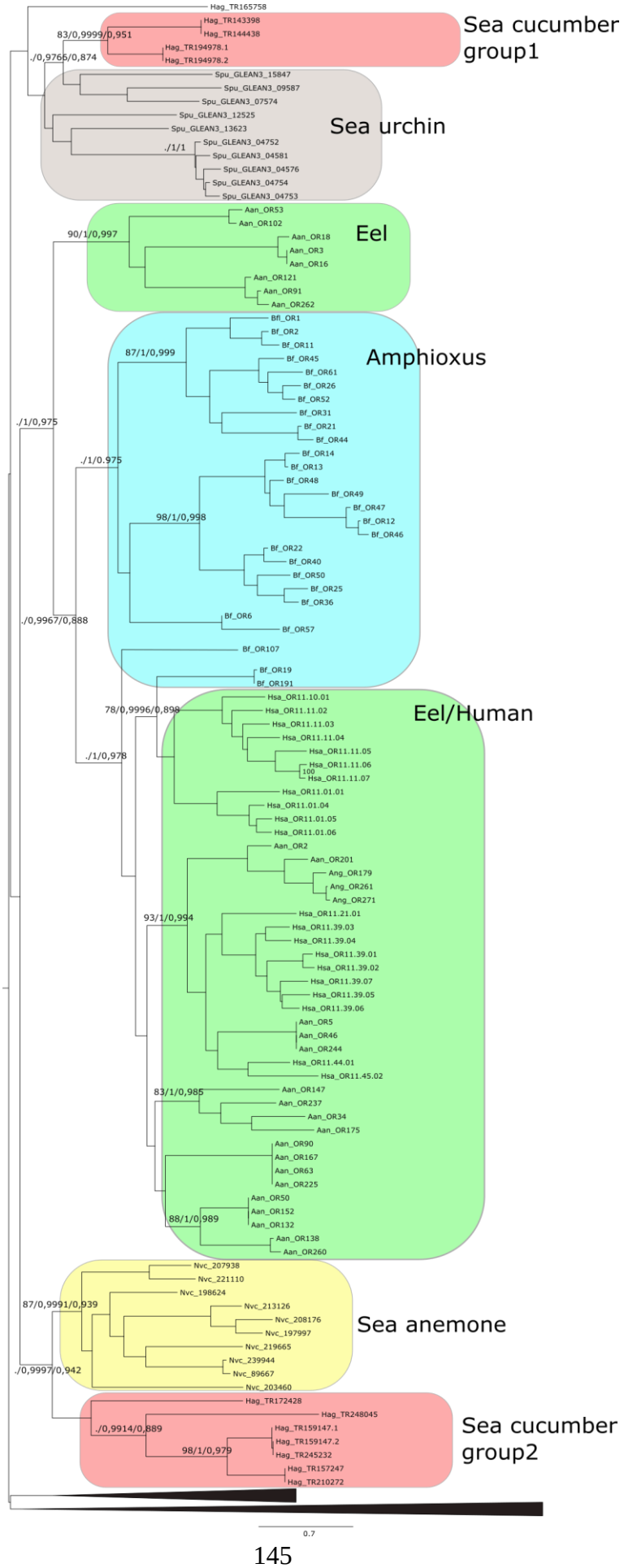
BAI: Brain-specific angiogenesis inhibitor, CRH: corticotropin-releasing hormone, EGF: epidermal growth factor, GABA: gamma-amino-butyric acid, GHSR: growth hormone secretagogue receptor, MECA: melanocortin, endothelia, cannabinoid and adenosine, PDF: pigment dispersing factor, PTH: parathyroid hormone, THR**:** thyrotropin-releasing hormone.

5.4.3. Phylogenetic analyses to identify putative ORs in sea cucumber

As no putative ORs were identified using standard database annotation, a more focused approach was taken. This consisted of comparing all putative Rhodopsin sea cucumber GPCRs with the OR repertoire from other metazoans (human, eel, amphioxus and sea urchin) using phylogeny (Figure 2). A maximum-likelihood tree was constructed using holothurian GPCRs (123 GPCRs) that contained more than 4 TMs and the OR repertoire from other metazoans, and putative OR were identified based on sequence clustering.

The tree topology suggested that sea cucumber sequences shared common ancestry with the metazoan orthologs and that twelve sea cucumber sequences could be considered as OR candidates. The clustering of these OR sequences suggested that at least two major OR groups evolved: one grouped with the sea urchin ORs (sea cucumber OR group 1, scOR1; four sequences) and another with the sea anemone ORs (sea cucumber group 2, scOR2; seven sequences). The receptor sequence Hag_TR165758 did not cluster within these two groups and thus its position as a potential OR was not clear. None of the putative sea cucumber OR sequences clustered with amphioxus or vertebrate ORs.

Clustering of the sea cucumber sequences revealed that replicates were present within the assembly. Sequences were confirmed as replicates when they shared more than 95% of amino acid identity. Of the scOR1, tree clustering and sequence identity suggests that Hag_TR143398 was identical to Hag_TR144438 and Hag_TR194978.1 to Hag_TR194978.2. Of the scOR2, Hag_TR245232 was identical to Hag_TR159147.1 and Hag_TR159147.2, and Hag_TR157247 was identical to Hag_TR210272. After removing replicate sequences, seven unique sea cucumber potential OR sequences were obtained with two from scOR1, four from scOR2 and the sequence Hag_TR165758 which was not related to either of the two main groups identified. The different approaches used for tree construction and validation supported the clustering of the holothurian sequences and overall aBayes and SH-aLRT methods gave better branching support values than the bootstrap method.


Hag_TR165758
Hag_TR143398
Hag_TR144438
83/0,9999/0,951
Hag_TR194978.1
Hag_TR194978.2
./0,9766/0,874
Sea cucumber group1
Spu_GLEAN3_15847
Spu_GLEAN3_09587
Spu_GLEAN3_07574
Spu_GLEAN3_12525
Spu_GLEAN3_13623
./1/1
Spu_GLEAN3_04752
Spu_GLEAN3_04581
Spu_GLEAN3_04576
Spu_GLEAN3_04754
Spu_GLEAN3_04753
Sea urchin
Aan_OR53
Aan_OR102
90/1/0,997
Aan_OR18
Aan_OR3
Aan_OR16
Aan_OR121
Aan_OR91
Aan_OR262
Eel
Bfl_OR1
Bf_OR2
Bf_OR11
87/1/0,999
Bf_OR45
Bf_OR61
Bf_OR26
Bf_OR52
Bf_OR31
Bf_OR21
Bf_OR44
Amphioxus
./1/0,975
Bf_OR14
Bf_OR13
./1/0.975
Bf_OR48
Bf_OR49
Bf_OR47
Bf_OR12
98/1/0,998
Bf_OR46
Bf_OR22
Bf_OR40
Bf_OR50
Bf_OR25
Bf_OR36
Bf_OR6
./0,9967/0,888
Bf_OR57
Bf_OR107
Bf_OR19
Bf_OR191
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Hsa_OR11.01.06
Aan_OR2
Aan_OR201
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Ang_OR261
Ang_OR271
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Hsa_OR11.39.02
Hsa_OR11.39.07
Hsa_OR11.39.05
Hsa_OR11.39.06
Aan_OR5
Aan_OR46
Aan_OR244
Hsa_OR11.44.01
Hsa_OR11.45.02
Eel/Human
83/1/0,985
Aan_OR147
Aan_OR237
Aan_OR34
Aan_OR175
Aan_OR90
Aan_OR167
Aan_OR63
Aan_OR225
Aan_OR50
Aan_OR152
88/1/0,989
Aan_OR132
Aan_OR138
Aan_OR260
Nvc_207938
Nvc_221110
Nvc_198624
87/0,9991/0,939
Nvc_213126
Nvc_208176
Nvc_197997
Nvc_219665
Nvc_239944
./0,9997/0,942
Nvc_89667
Nvc_203460
Sea anemone
Hag_TR172428
Hag_TR248045
./0,9914/0,889
Hag_TR159147.1
Hag_TR159147.2
98/1/0,979
Hag_TR245232
Hag_TR157247
Hag_TR210272
Sea cucumber group2
0.7

Figure 2. Phylogenetic tree of the sea cucumber GPCRs with the eel, amphioxus, human, sea anemone and sea urchin ORs. The sea cucumber groups (1, scOR1 and 2, scOR2) are circled by a red box, the vertebrate (eel and human) are in green, amphioxus in blue and sea anemone in yellow. Supporting values were represented in the following order: BS / ABayes / SH-aLRT. The non-OR sequences were collapsed (black triangle) to highlight the OR branches. The tree was rooted with sea cucumber non-OR clade.

5.4.4. Multiple sequence alignments and identification of OR signature motifs

Sequence comparisons between the full-length receptors, scOR1 and scOR2 showed a sequence similarity of 30% and a sequence identity of 10-11% (see Supplementary materials 1 and 2). The sequence comparison of scOR1 with sea urchin ORs revealed that they share low amino acid sequence identity (26-27%) and similarity (46-49%) (Table 4). The amino acid sequence identity and similarity between the full-length receptors of scOR2 and the sea anemone ORs were 13-18% and 29-38%, respectively (Table 5). Receptor sequences from both groups (1 and 2) were only 10-20% identical and 28-40% similar with the vertebrate homologues (see Supplementary materials 1 and 2). This shows that sequence conservation of the ORs was generally poor.

Amino acids that are characteristic of OR signature motifs (Alioto and Ngai 2005; Churcher and Taylor 2009) were identified in scOR1 and scOR2. Within scOR1, three OR motifs were found (Figure 3). The LxxPxYxxxxxLxxxDxxxxxxxxP motif, seen at border of IL1 and TM2, was not 100% conserved since an asparagine (N) was replaced by tyrosine (Y) in Hag_TR194978.1 and was not seen in Hag_TR144438 as this sequence was incomplete. The second motif, MxxxxYxxxCxPLxY, found at the junction between TM3 and IL2, had the first amino acid, methionine (M), replaced by an isoleucine (I) in Hag_TR194978.1 and it was not seen in Hag_TR144438 as this sequence was incomplete. The NPxxYxxR motif found within TM7 was 100% conserved in Hag_TR144438 and Hag_TR194978.1. The motif KAxxTxxxH, normally found between IL3 and TM6, was not identified in scOR1s.

Within scOR2 (Figure 4), the motif LxxPxYxxxxxLxxxDxxxxxx was poorly conserved with only leucine (L) and in some cases tyrosine (Y) sharing conserved positions relative to the consensus reference motif. The second motif, MxxxxYxxxCxPLxY, was also poorly conserved and only the tyrosine was found in some sequences. The two previous motifs were not identified in the Hag_TR172428 as this sequence was incomplete and lacked the TM domain where they are normally found. The motif KAxxTxxxH (IL3-TM6) was found in the sequence alignment but was also poorly conserved with only the lysine (K) and threonine (T) sometimes seen. Finally, the motif NPxxYxxR was only fully conserved in one sequence (Hag_TR172428) while only proline (P) was conserved in other scOR2 sequences.

In Hag_TR165758, the LxxPxYxxxxxLxxxDxxxxxx motif was poorly conserved but the amino acids proline (P) and leucine (L) were present (see Supplementary material 3). Only one amino acid was found in the other motifs: leucine (L) in MxxxxYxxxCxPLxY, threonine (T) in KAxxTxxxH and proline (P) in NPxxYxxR.

Table 4. Percentage of similarity and identity between the full-length sequences of scOR1 and the reference sequences of the sea urchins.

	Spu_09587	Spu_07574	Spu_15847	Hag_TR194978.1
Spu_09587	-	58	50	48
Spu_07574	35	-	47	49
Spu_15847	28	24	-	46
Hag_TR194978.1	27	26	27	-

% Similarity

% Identity

Table 5. Percentage of similarity and identity between the full-length sequences of scOR2 and the reference sequences of the sea anemone.

	Nvc_ 89667	Nvc_ 239944	Nvc_ 198624	Nvc_ 219665	Nvc_ 208176	Nvc_ 203460	Hag_ TR157247	Hag_ TR159147.1
Nvc_89667	-	89	37	54	49	46	35	38
Nvc_239944	87	-	54	57	50	44	34	37
Nvc_198624	33	34	-	50	52	34	36	36
Nvc_219665	33	34	30	-	46	44	29	32
Nvc_208176	30	30	30	26	-	38	32	31
Nvc_203460	26	24	14	26	21	-	33	34
Hag_TR157247	17	17	17	13	16	14	-	78
Hag_TR159147.1	15	16	18	14	13	14	57	-

% Similarity

% Identity

Figure 3. Multiple sequence alignment of scOR1 with the sea urchin ORs. The sea urchin sequences were selected based on their proximity to scOR1 given by the phylogenetic tree. The OR motifs are highlighted in red. The sea cucumber transmembrane domains were predicted on TMHMM and the consensus region is indicated by the black box and they are numbered. The intracellular (IL) and extracellular loops (EL) are numbered and labelled. The sequence in italic was incomplete.

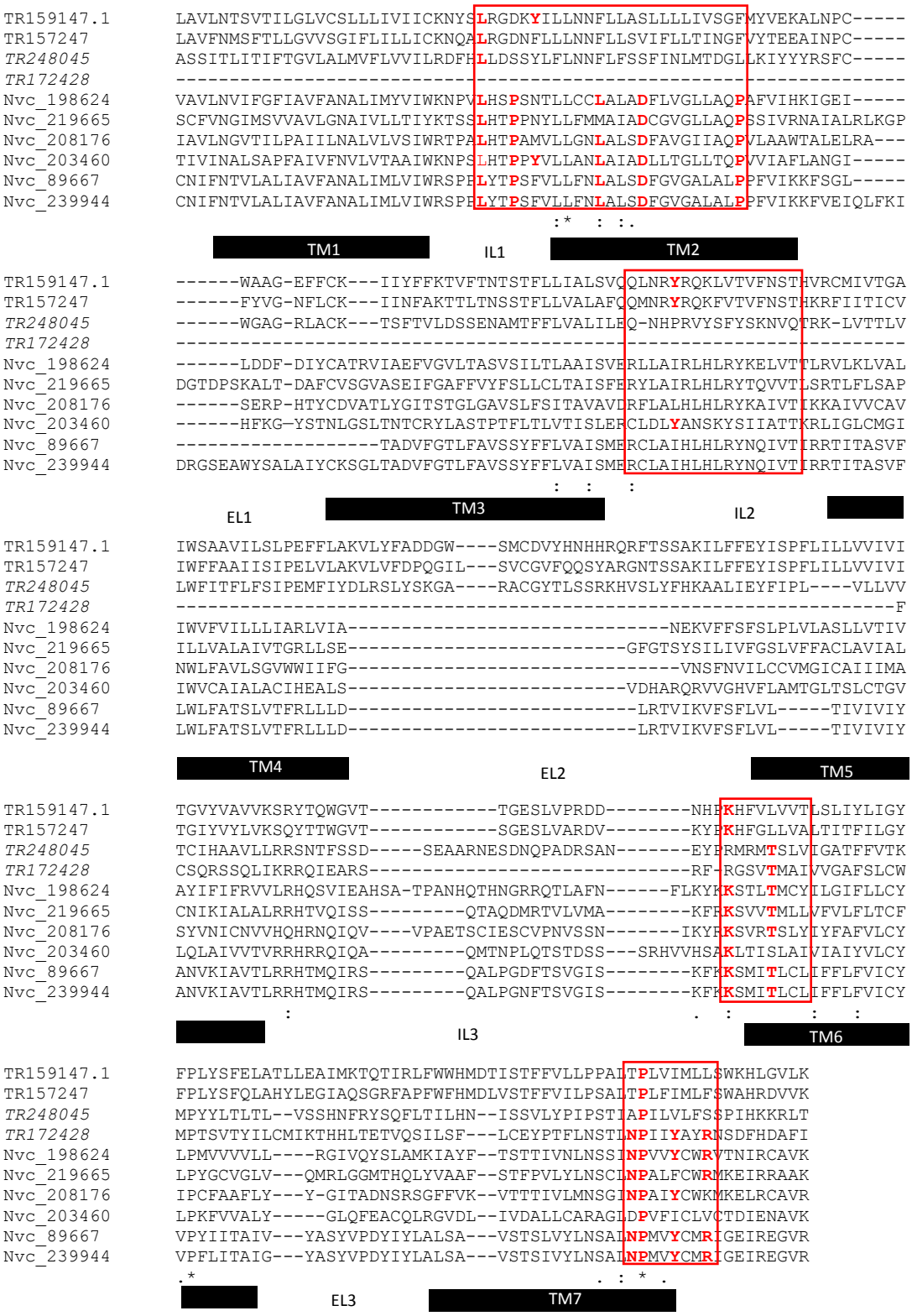


Figure 4. Multiple sequence alignment of the scOR2 and sea anemone ORs. The sea anemone sequences were selected based on their proximity to scOR2 given by the phylogenetic tree. The OR motifs are highlighted in red. Transmembrane domains of the sea cucumber receptors were predicted on TMHMM and the consensus region is represented in the black box and are numbered. The intracellular (IL) and extracellular (EL) loops are labelled. The sequences in italic were incomplete.

5.4.5. Distribution of sea cucumber ORs in the six tissue transcriptomes

The scOR1 and scOR2 members were mapped to the individual tissue libraries (Figure 5). Members of both scOR1 and scOR2 were represented in all the main tissue transcriptomes. The scOR1s and scOR2s were most abundant in the oral cavity, papillae and tegument, followed by the calcareous ring and tentacle transcriptomes. A single transcript was found in the gonads: Hag_TR165758 was found in testis, and one sequence from scOR2 (Hag_TR172478) was found in the ovary.

The relative abundance of OR candidates in each tissue transcriptome was lower in the testis and ovary (6.50 per 10^6 and 11.50 per 10^6, respectively) than in the tentacles (19.96 per 10^6), calcareous ring (19.43 per 10^6), oral cavity (19.78 per 10^6) and, papillae and tegument (18.31 per 10^6).

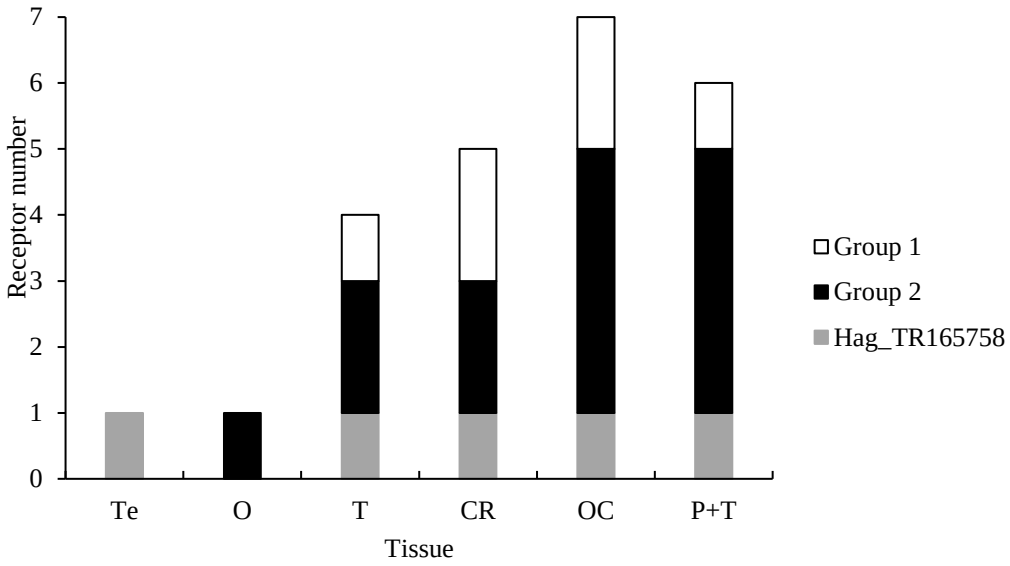


Figure 5. Distribution of OR transcripts in the six sea cucumber tissue transcriptomes. Te: testis, O: ovary, T: tentacle, CR: calcareous ring, OC: oral cavity and P+T: papillae and tegument.

5.5. Discussion

The GPCR repertoire of the sea cucumber *H. arguinensis* consists of at least 591 unique receptors, of which more than 60% were classified within the Rhodopsin family, and at least seven potential OR-like transcripts were identified. Of special interest was the finding of two clusters of odorant receptors one closer to sea urchins ORs and the other closer to sea anemone ORs, suggesting that sea cucumber ORs emerged from two distinct ancestors. The putative sea cucumber ORs possessed some conserved amino acid characteristics of signature motifs in

metazoan ORs and they were mostly found in tentacles, oral cavity, calcareous ring and papillae, and tegument, consistent with their involvement in chemical sensing.

Sea cucumber GPCRs

The number of GPCRs found in this study (591) is of the same order of magnitude, albeit smaller, than that found in other echinoderms such as the starfish *Acanthaster planci* (988) and sea urchin *S. purpuratus* (805) genomes (Hall et al. 2017), and similar to the cephalochordate amphioxus *B. floridae* genome (664) (Nordström et al. 2008). Nevertheless, more GPCR receptors are expected to be found in the sea cucumber as the data analysed in this study come from a transcriptome of six tissues at a given state and genome sequencing is necessary to identify the full complement of GPCR genes.

The largest and the most diverse GPCR present in metazoans are the Rhodopsin-GPCRs (Fredriksson et al. 2003), which corresponds to more than 50% of all the GPCRs identified in humans (Bjarnadóttir et al. 2006), amphioxus (Nordström et al. 2008), starfish and sea urchin (Hall et al. 2017) and cnidaria (Krishnan et al. 2014) (Figure 6). Most of the GPCRs in the sea cucumber transcriptome (67%) were assigned to the Rhodopsin family; this is consistent with what is encoded in the genomes of invertebrates and vertebrates (Fredriksson et al. 2003).

The second most abundant GPCR family in the sea cucumber transcriptome was the Adhesion members (21%). This family of receptors, also has a large representation in cnidaria (18%) (Krishnan et al. 2014) but is poorly represented in humans and amphioxus (around 5%) (Bjarnadóttir et al. 2006; Nordström et al. 2008). In the sea urchin (*S. purpuratus*), at least 90 adhesion-GPCRs genes were described, the majority with no mammalian homologues, suggesting either they were deleted in mammals or specifically expanded in the echinoderm genome (Whittaker et al. 2006). The number of Adhesion-like sequences found in sea cucumber (n = 126) is comparable to that found in sea urchin. Members of the Adhesion-like family are known to be involved in cellular adhesion and signaling, although almost all members are still considered to be orphan receptors of unknown function (Paavola and Hall 2012). Therefore, to explain the considerable expansion of this family within echinoderms compared to the vertebrates (around 30 members), efforts should be focused on identifying the ligands of these receptors.

The Glutamate family contains many receptors which are crucial modulators involved in neurotransmission (Hermans and Challiss 2001). In this study, the percentage of Glutamate family genes in sea cucumber was equivalent to that in starfish (around 8%), but both had more members than in the sea urchin (around 4%), human and amphioxus (around 2% for both) (Bjarnadóttir et al. 2006; Hall et al. 2017; Nordström et al. 2008). The Secretin family, known

to include receptors binding to hormones and neuropeptides, was equally represented in human, amphioxus and sea cucumber (around 2%) (Bjarnadóttir et al. 2006; Nordström et al. 2008). This family was absent in cnidaria (Krishnan et al. 2014), probably due to the lack of peptide hormones in this group (Srivastava et al. 2010).

No members of the Frizzled family were found in the sea cucumber while they represented 1% of total GPCRs in the reference organisms (Figure 6). Since these receptors are known to play a crucial role in tissue polarity and cell signaling (Huang and Klein 2004), their absence is surprising and may be an artifact resulting for their low abundance in the tissues analysed. Further studies are needed, particularly since the genome of the sea cucumber has still not been sequenced, and the transcriptomes only can provide a relative quantitative analysis as many transcripts may be the result of alternative splicing events.

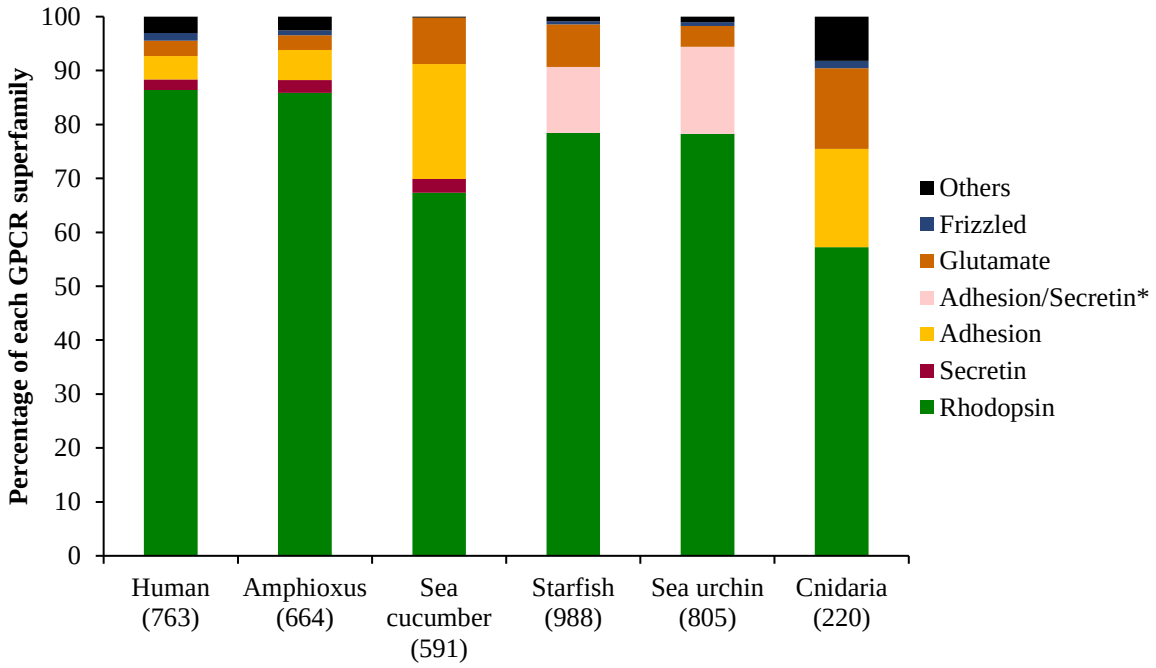


Figure 6. Percentage of GPCR families in human (V1R and T2R were not included) (Bjarnardóttir et al. 2006), amphioxus (Nordström et al. 2008), starfish and sea urchin (Hall et al. 2017), cnidaria (Krishnan et al. 2014) and sea cucumber. The total number of GPCR found in each organism is indicated within the brackets. *In some studies, secretin and adhesion receptors were not separated.

No orthologs of the receptors belonging to the ORs, TAARs, FPRs and vomeronasal receptor 1 and 2 (VR1 and VR2) were found in the sea cucumber transcriptome based on sequence similarity. This could be due to the fact that the best matches against the NCBI database were almost exclusively with the sea urchin *S. purpuratus* in which no ORs *sensus stricto* have been classified. However, phylogenetic analysis, including the sea urchin and the sea anemone, identified seven OR-like transcripts. Unsurprisingly, taking into consideration their evolutionary position, the sequences of sea cucumber ORs formed a separate clade from human, eel and amphioxus ORs and this also explains the difficulty experienced in identifying ORs in the sea cucumber.

Putative sea cucumber odorant receptors

The putative sea cucumber ORs were more similar to the sea urchin ORs than to the sea anemone, eel and human, as expected since they belong to the same phylum. Of all the species analyzed, only the sea cucumber has two distinct groups of OR transcripts. The reason for this remains at present unclear and requires further study. Grouping of the sea cucumber OR with the putative orthologues in sea urchin and sea anemone ORs may suggest that two putative types of OR ancestral genes existed but that only one group persisted in most groups, with the exception of the sea cucumber where two persisted. It remains to be established if the two independent groups of ORs in sea cucumber are functionally divergent and detect different kinds of molecules.

Of the seven OR-like identified in the sea cucumber transcriptome, some sequences shared the highest amino acid sequence identity with Octopamine and Tyramine receptors. These receptors have been attributed roles in modulating olfactory perception in certain species such as flies, bees and moths (Farooqui 2007). This raises the exciting possibility that remains to be demonstrated that these receptors may also be involved in olfaction in sea cucumbers, or in echinoderms in general.

Conserved amino acid characteristics of signature OR motifs, found in human, mouse, zebrafish (Alioto and Ngai 2005; Liu et al. 2003), amphioxus (Churcher and Taylor 2009) and sea anemone (Churcher and Taylor 2011), were also present in the sea cucumber receptor sequences. From a general point of view, the OR motifs were more conserved in the scOR1 sequences than the scOR2. Amino acids from the motif, LxxPxYxxxxxLxxxDxxxxxxxxP were found in IL1-TM2 in all sea cucumber groups. A similar motif containing a leucine (L) residue followed by downstream proline (P) and tyrosine (Y) residues is commonly found in mammalian ORs (Liu et al. 2003). The second motif, MxxxxYxxxCxPLxY (TM3-IL2), was more conserved in the sea cucumber group scOR1 than in scOR2 and the sequence

Hag_TR165758. In human, mouse and zebrafish, this motif is typically found as MAYDRYVAIC (Alioto and Ngai 2005; Zhang and Firestein 2002; Zozulya et al. 2001). The comparison between the two motifs suggested that the methionine (M), tyrosine (Y) and the cysteine (C) are the most conserved residues and may have OR-specific functions (Churcher and Taylor 2009). Conserved amino acids from the motif NPxxYxxR, which is described as a good marker for ORs (Churcher and Taylor 2009), were also found in the sea cucumber sequences.

Putative OR receptors were found in three tissues in direct contact with the environment: the tentacles, oral cavity and, papillae and tegument. This is consistent with previous studies in echinoderms that found chemoreceptors in the tube feet in the starfish *A. planci* (Roberts et al. 2017) and in the tube feet, pedicellariae and spines of the sea urchin *S. purpuratus* (Churcher 2011; Raible et al. 2006). However, potential ORs were also found in internal tissues; the calcareous ring, which is connected to the radial nerve cord, and in much less abundance in the ovary and testis. The presence of candidate OR has already been reported in other tissues such as the testis and sperm of sea urchin (Churcher 2011), in mammalian and avian testes (Parmentier et al. 1992; Steiger et al. 2008) as well as other non-olfactory tissue (Feldmesser et al. 2006). However, the roles of OR in non-olfactory tissues are not well understood but include cell-cell communication and chemotaxis (Abaffy 2015).

Evolution of OR genes

The OR repertoire is considered to reflect the sensing ability of a species to detect chemicals in its environment (Niimura and Nei 2007). Although these receptors share conserved origin across animals, they have expanded specifically within each species and thus sequence similarity is generally low. It has been shown that terrestrial vertebrates have generally more OR genes than aquatic vertebrates; some have interpreted this as inferring that olfaction is more important on land than in water (Niimura and Nei 2005, 2007). Within vertebrates, the human OR repertoire contains 388 functional OR genes (Niimura and Nei 2003), equivalent to 49% of the total GPCRs, while fishes such as zebrafish *D. rerio*, the tetraodon *T. nigroviridis* and the eel *A. anguilla* have only 62, 22 and 112 OR genes respectively (Alioto and Ngai 2005; Churcher et al. 2015; Kolmakov et al. 2008; Metpally and Sowdhamini 2005) (Figure 7). However, the OR repertoire of fish is more diverse than that of terrestrial mammals and birds (Niimura and Nei 2005); and receptor number does not seem to be a predictor of sensitivity or selectivity.

The OR repertoire of the amphioxus *B. floridae* (31) (Niimura 2009b), cnidaria *N. vectensis* (35) (Churcher and Taylor 2011), sea urchin *S. purpuratus* (192) (Burke et al. 2006)

and sea cucumber *H. arguinensis* (7) (present study), all of which are marine invertebrates, is smaller than that in humans (Figure 7). The importance of the OR gene repertoire among the total GPCRs varied from 2% in the sea cucumber to 23% in the sea urchin. Although they belong to the same phylum, the sea urchin has 16 times more OR-like genes than those found in the sea cucumber. Also, a recent study of GPCRs in the starfish *Acanthaster planci* identified 63 putative ORs (Roberts et al. 2017). The small number of OR-like genes found in the sea cucumber may be due to the restrictive approach to assign identification used this study, due to the overall small length of the sequences obtained from the transcriptome assembly. The average size of the transcripts (758 bp) obtained from the single *de novo* transcriptome assembly was smaller than the expected full-length for a GPCR (>1000 bp within the coding domain). As GPCRs are constituted by seven highly conserved sequence blocks, combination of the pooled raw reads from the distinct tissue transcriptome may have led to chimeras and fragmented assembly. Given the higher similarity of the GPCRs within the TM domains, longer sequences are needed to correctly discriminate the different families and receptors within the same family in the absence of an assembly genome. To reduce bias from the use of small sequences, only receptors with more than 4TM were considered in the analysis; this likely underestimates the number of GPCRs.

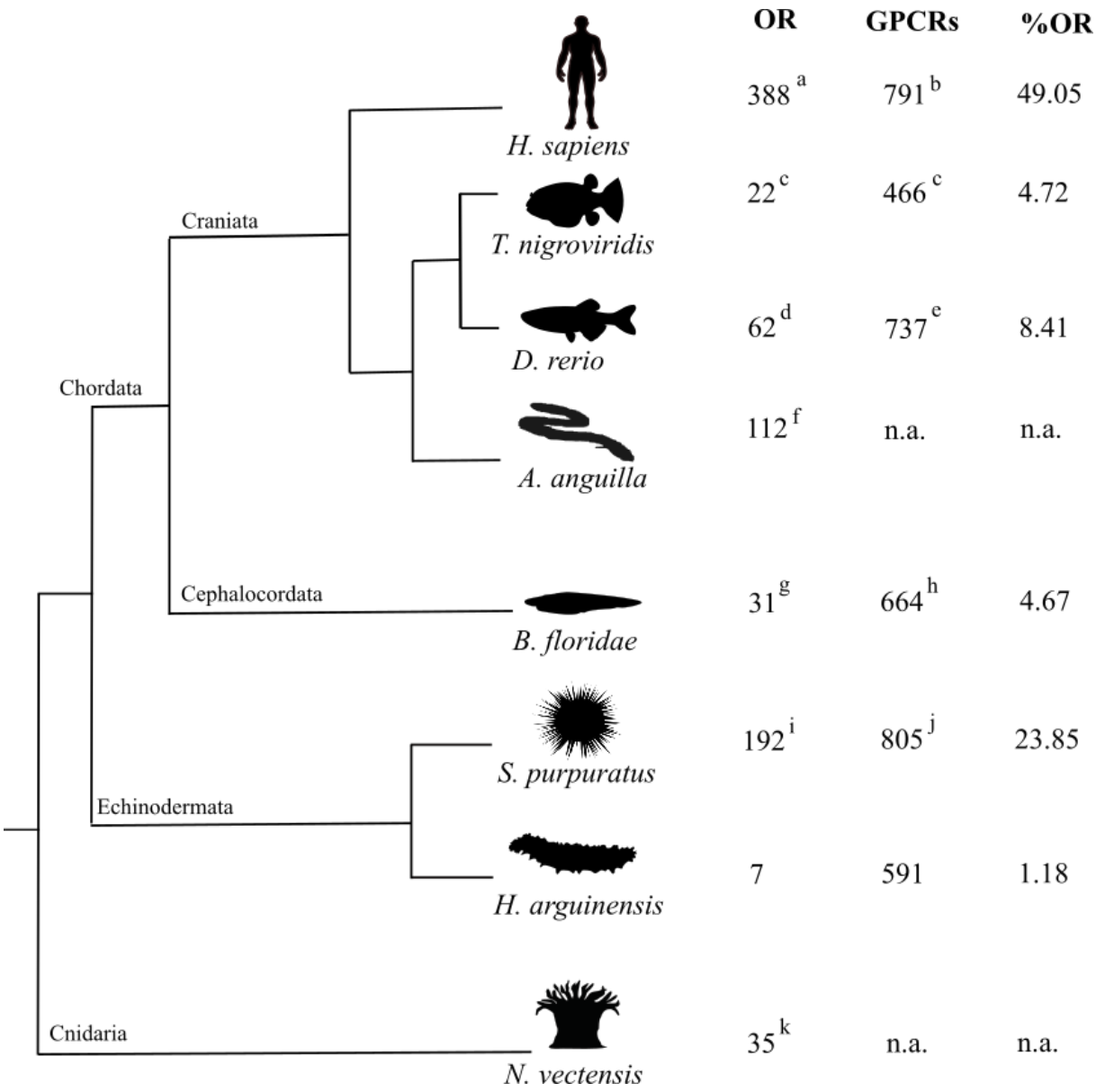


Figure 7. Dendrogram showing the number of total GPCRs, olfactory GPCRs and percentage of odorant GPCRs found in each organism. [a] Nimura and Nei 2003, [b] Bjarnadóttir *et al.* 2006, [c] Metpally and Sowdhamini 2005, [d] Alioto and Ngai 2006, [e] Fredriksson and Schiött 2005, [f] Churcher *et al.* 2015, [g] Nimura 2009, [h] Nordström *et al.* 2008, [i] Burke *et al*. 2006, [j] Hall *et al.* 2017, [k] Churcher *et al.* 2010, [l] Krishnan *et al.* 2014, n.a.: not available in the literature. The evolutionary relationship between the species is in agreement with the Tree of Life (http://tolweb.org/tree/).

5.6. Conclusion

As sea cucumbers have limited visual abilities and lack of hearing, they are expected to depend largely on chemosensation for detecting biotic and abiotic factors in their environment. Here, the family containing the most numerous receptors were the Rhodopsin, as in other metazoans, and reveal a high versatility of the cell types that can be found in sea cucumbers, although they are less complex than vertebrates. Among this family, at least seven sea cucumber candidate ORs were identified; however, chemosensory receptor transcripts other than OR may also exist. Two distinct groups of ORs were identified, and their presence in the sea cucumber transcriptome suggests that they may be functionally divergent, with scOR1 closer to sea urchin ORs and scOR2 more similar with the sea anemone ORs. This may suggest that the sea cucumber ORs contain representatives of two distinct ancestral ORs. Future work should focus on deorphanizing the identified ORs and determine their function through *in vitro* bioassays. Moreover, differences in male and female receptors, and between reproductive and non-reproductive season should also be investigated.

5.7. Supplementary materials

Supplementary material 1. Percentage of similarity and identity between the full-length sequences of sea cucumber groups (1 and 2) and the reference sequences of the eel.

	Eel_ 179	Eel_ 2	Eel_ 46	Eel_ 50	Eel_ 191	Eel_ 237	Eel_ 90	Eel_ 107	Hag_ TR194978.1	Hag_ TR157247	Hag_ TR159147.1
Eel_179	-	73	55	50	47	49	48	50	30	28	30
Eel_2	61	-	60	58	53	55	53	52	32	32	33
Eel_46	35	39	-	58	50	49	50	48	34	32	30
Eel_50	29	36	36	-	54	58	57	52	38	35	36
Eel_191	25	29	30	33	-	49	48	52	31	34	32
Eel_237	26	31	26	33	29	-	55	50	33	34	35
Eel_90	25	28	30	32	26	33	-	54	30	29	31
Eel_107	24	28	28	32	30	26	25	-	35	30	33
Hag_TR194978.1	14	17	14	18	17	18	15	15	-	30	30
Hag_TR157247	10	13	15	12	12	12	12	11	11	-	78
Hag_TR159147.1	14	15	13	14	14	14	11	14	10	57	-

% Similarity

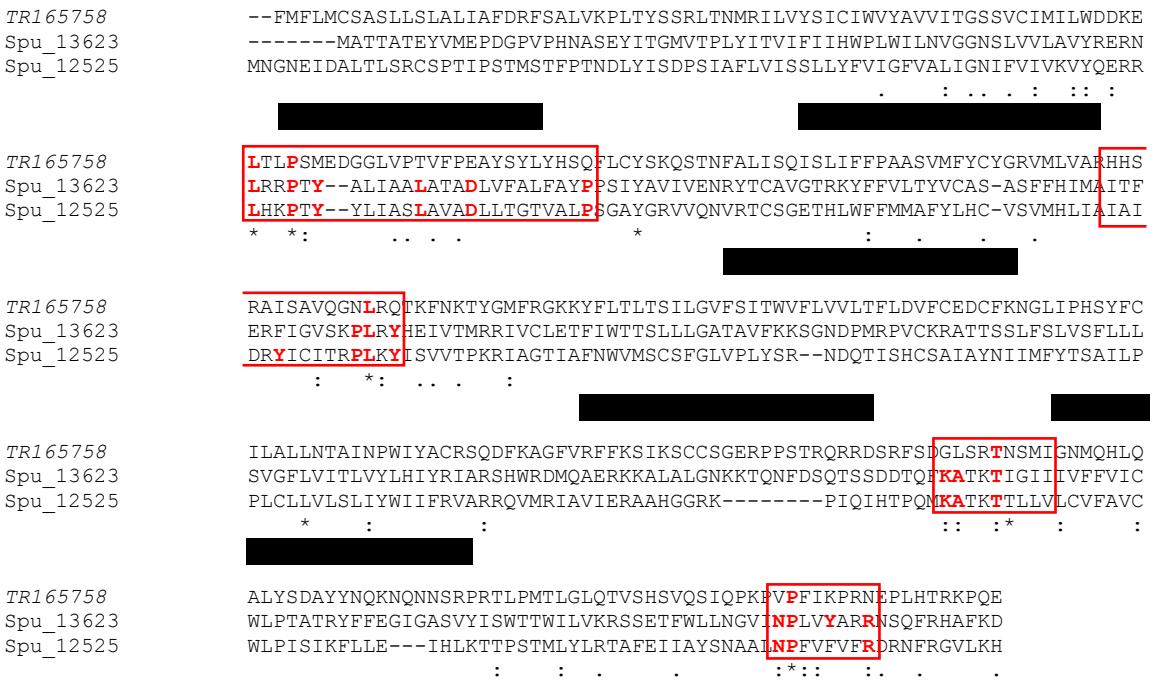
% Identity

Supplementary material 2. Percentage of similarity and identity between the full-length sequences of sea cucumber groups (1 and 2) and the reference sequences of the human.

	Hs_ 111505	Hs_ 111506	Hs_ 111504	Hs_ 111502	Hs_ 111503	Hs_ 111501	Hs_ 111401	Hs_ 111304	Hag_ TR194978.1	Hag_ TR157247	Hag_ TR159147.1
Hs_111505	-	83	82	83	82	75	73	69	39	32	31
Hs_111506	67	-	84	85	84	78	74	68	36	35	33
Hs_111504	68	69	-	86	84	81	76	70	39	36	34
Hs_111502	67	67	70	-	95	81	77	72	40	36	33
Hs_111503	65	65	68	91	-	81	76	69	38	35	32
Hs_111501	56	57	58	59	57	-	76	71	40	36	34
Hs_111401	53	52	55	60	58	57	-	72	37	35	30
Hs_111304	50	47	50	53	51	48	52	-	34	33	31
Hag_ TR194978.1	18	18	19	20	18	19	18	17	-	30	30
Hag_ TR157247	13	14	14	15	14	14	13	13	11	-	78
Hag_ TR159147.1	12	13	15	15	13	13	13	15	10	57	-

% Similarity

% Identity



Supplementary material 3. Alignment of the sequence that does not belong to any of the group with the refence sequences of sea urchin. Transmembrane domains (predicted on TMHMM) are represented in the black box based on the first sequence and OR motifs are highlighted in red.

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Chapter VI

General discussion, conclusions and future perspectives

6.1. General discussion and conclusions

This thesis reports research that I carried out that led to novel discoveries of the biology of sea cucumbers with broader implications for science and society.

Firstly, I described and compared the reproductive and biometric characteristics of *H. arguinensis* and *H. mammata* and found that the reproductive cycle in *H. arguinensis* is closely linked to photoperiod and the timing of temperature rise, and that local environmental conditions most likely dictate their size distribution.

Secondly, I discovered the decisive role played by males to form breeding aggregations and synchronize spawning. I showed that males, but not females, release powerful substances to the water that attract and induce spawning in both males and females. Furthermore, it was possible to identify phosphatidylcholine derivatives as a possible component of the sea cucumber pheromone mixture.

Thirdly, using histology, histochemistry and immunohistochemistry, I characterized the sea cucumber nervous system in tissues at the water interface, as part of a search for chemosensory structures or organs, and found that the appendages disc rich in cells and nerve fibers contained what could be sensory neurons.

Finally, using a transcriptomics approach, I found that, although sea cucumbers do not have clearly defined sensory organs, they possess a large repertoire of GPCRs, including at least seven putative odorant receptors. The latter are mainly found in the tentacles, calcareous ring, papillae and tegument, and oral cavity, supporting the morphological observations and their potential role as chemosensory structures.

The following section highlights the role of chemicals combined with environment factors in sea cucumber reproduction and discusses the sensory perception in these organisms, and finally proposes practical applications of the results obtained in this thesis for sea cucumber management.

6.1.1. Reproductive cues: combination of environmental and chemical factors

Populations of *H. arguinensis* and *H. mammata* located in a narrow latitudinal range, differ in size/weight, gonad production and maturity stages; however, they have the same seasonal reproductive pattern with a summer-autumn spawning. In line with previous studies that showed similar breeding periods between populations at similar latitudes, these results suggest that seasonal changes in environmental parameters control reproduction in these two species of sea cucumber (e.g. Conand 1981; Conand et al. 2002; Kazanidis et al. 2014; Mercier and Hamel 2009; Shiell and Uthicke 2006). Temperature and photoperiod, in particular, which

were correlated with the reproductive cycle in *H. arguinensis*, are amongst the most cited factors influencing sea cucumber reproduction (e.g. Chao et al. 1995; Gaudron et al. 2008; Guzmán et al. 2003; Muthiga 2006; Navarro et al. 2012).

Photoperiod is regularly seen as a general synchronizer as it does not vary between years, while temperature is more often attributed a role in the triggering of spawning (Giese and Pearse 1974; Himmelman 1999; Mercier and Hamel 2009). However, convincing evidence on the role of temperature as a proximal factor in spawning in nature is largely unclear (Mercier and Hamel 2009). Interestingly, as explained in this thesis, the most commonly used method in aquaculture to induce spawning in sea cucumber is the thermal shock, although its results may be inconsistent (e.g. James 1994; Smiley et al. 1991; Yanagisawa 1998). However, such abrupt temperature changes are not that common in the wild, with the exception of certain shallow waters or after summer upwelling (Berkelmans et al. 2010; McCabe et al. 2010). The mechanism of temperature shock in the discharge of gametes in sea cucumbers remains unknown.

In contrast, the effect of chemical communication on reproduction in *H. arguinensis* was clearly demonstrated in this thesis. In particular, males release chemicals that attract and induce spawning in both sexes, while females do not. To my knowledge, it is the first time that these effects have been experimentally demonstrated in sea cucumbers. Previous studies showed evidence for a role of chemical communication during gametogenesis in the sea cucumber *C. frondosa* (Hamel and Mercier 1996), while my results are strongly indicative that sea cucumbers release chemicals to facilitate aggregation and synchronize spawning. These results are also consistent with the spawning induction assays performed in other echinoderms, such as starfish and sea urchins (Caballes and Pratchett 2017; Hamel and Mercier 1995; Reuter and Levitan 2010; Starr et al. 1990).

Nevertheless, it remains to be seen what stimulates the first males to spawn. It has been suggested in starfish and sea cucumbers that females release a pheromone during or after oocyte maturation which induces males to spawn, and in turn, stimulates female spawning (Miller 1989; Smiley et al. 1991). However, we do not have evidence for a female pheromone in *H. arguinensis.* Another explanation is that spawning may be initially triggered in males that are sensitive to certain environmental variables (e.g. temperature, moon cycle, phytoplankton blooms), as males were more easily induced to spawn than females (Caballes and Pratchett 2017). It is then these males that stimulate spawning in conspecifics. Furthermore, it has been shown that the likelihood of spawning was higher in certain conditions such as the presence of phytoplankton, darkness and lunar phase (Reuter and Levitan 2010; Starr et al. 1990). Overall,

it seems that sea cucumber reproduction results from a combination of environmental and chemical factors, and that the latter act as a proximal trigger for simultaneous spawning.

6.1.2. Sensory perception

Echinoderms are able to detect and integrate a variety of external stimuli through their sensory systems. Although their sensory behaviour has been less studied than in sea urchins and starfish, it has been shown that sea cucumbers select food particles from the sediment (Plotieau 2012; Slater et al. 2011), react to touch (Bouland et al. 1982; VandenSpiegel et al. 1995) and light (Dong et al. 2010; Mercier et al. 2000), and detect chemical stimuli from conspecifics (Hamel and Mercier 1996, 1999) (chapter III). However, no olfactory organs have been identified with certainty. In this thesis, I revealed that the main structures in contact with the environment – tentacles, papillae and tube feet – were rich in cells and highly innervated at the level of their disc, suggesting a sensory role. To complement these data, the GPCR repertoire was characterized and shown to be composed of at least 591 GPCRs, with more than 60 percent belonging to the Rhodopsin family, and at least seven putative olfactory receptors. Among the structures in contact with the environment, the putative olfactory receptors were found in the tentacles, oral cavity and, papillae and tegument, supporting their role in chemosensory processes. This follows the discovery of candidate olfactory receptors in the tube feet and tentacles in the starfish (Roberts et al. 2017) and in pedicellariae, tube feet and spines in the sea urchin (Churcher 2011). Thus, sea cucumbers, and perhaps echinoderms in general, do not have a single chemosensory organ but, instead, several structures spread over their body that can detect odorant molecules. Whether these different structures detect the same, or different, molecules remains to be determined.

6.1.3. Implications for sea cucumber management

6.1.3.1. Fisheries implications

H. arguinensis and *H. mammata* are collected illegally along the Iberian Peninsula, sometimes in large amounts; it is thus urgent to implement management measures to regulate these captures before their populations become endangered.

One important regulation is establishment of a closed season to allow mature individuals to reproduce. According to the present thesis, this seasonal ban for should be established in summer-august when spawning occurred in both species. More precisely, based on monthly sampling in *H. arguinensis*, it should be ideally implemented from July to October when spawning was the most intense.

Another important parameter to take into account when establishing regulations is the minimum capture size, which is determined from estimates of the size at first sexual maturity (Conand 2006, 2008). This will allow individuals to mature and spawn before harvesting. Based on my results, only sea cucumbers longer than 210 mm and heavier than 220 g in total weight or 110 g in eviscerated weight should be authorized to be captured. I recommend the use of total weight instead of length, which varies considerably due to body plasticity, or of eviscerated weight, which requires killing the animal.

Finally, although this should be subject to further investigation, the high proportion of immature individuals of both species found in Olhos de Água suggests that this site could be used as a recruitment area. In that case, Olhos de Água could be considered as a no-take zone in agreement with similar measures taken for other species which presented a similar demographic pattern (e.g. Altamirano et al. 2004).

6.1.3.2. Ecological and aquaculture implications

This thesis suggests, for the first time, phosphatidylcholine derivatives for a possible role in sea cucumber spawning. As no previous studies have been published on the identity of chemicals released by spawning sea cucumbers, this constitutes an innovative first step in the identification of the sea cucumber spawning pheromone. The complete identification of a spawning pheromone is a promising discovery as it could offer a reliable tool to induce spawning in aquaculture as an alternative to the currently applied thermal shock.

Beyond the use of pheromones in aquaculture, they could provide an environmentally safe method of pest management. The best examples of this are found in insects, in which female pheromones have been synthetized and used for decades to lure and attract males into traps and/or disrupt reproduction (Beroza and Knipling 1972; Gaston et al. 1977). In the aquatic environment, the identification of the mating pheromone of the invasive sea lamprey has contributed to successful control of populations in the Laurentian Great Lakes (USA), where they have caused the dramatic collapse of native fish communities (Li et al. 2003; Li et al. 2007; Li et al. 2002; Siefkes 2017; Smith and Tibbles 1980). In the same way, invasive sea cucumbers, such as the giant sea cucumber *Synaptula reciprocans* in the eastern Mediterranean, could thus be attracted and captured through the use of pheromones (Antoniadou C. and Vafidis 2009).

6.2. Future perspectives

Key information for management plans of natural populations of *H. arguinensis* and *H. mammata* in the Iberian Peninsula which, in some places, are facing severe threat, were obtained through this thesis. Future work should address the growth and recruitment patterns of these species as well as juvenile survivorship over longer time-scale periods to further understand the ecology of the target populations, establish effective stock assessment, and consequently contribute to their management.

Male spawning water, which was demonstrated here to induce spawning in males and females, contained phosphatidylcholine derivatives. However, not only the full chemical structure of the active compounds need to be determined, but other components of what seems to be a multicomponent pheromone need to be identified using mass spectrometry and with nuclear magnetic resonance. Furthermore, having identified the chemical structure of the putative pheromone, their biological roles need to be established through behavioural/physiological bioassays. The same procedure should be applied to the male-conditioned water, which attracted both sexes during the pre-spawning period, to determine if the compounds involved in spawning and aggregation are the same, similar or different.

The procedure for identification of bioactive compounds could be greatly accelerated if electrophysiological techniques could be deployed to allow screening of chromatography fractions and the activity of putative odorants. Based on the data presented in chapter IV, the target structures for electrophysiological experiments would be the disc of the three body appendages – tentacles, papillae and tube feet. Furthermore, a complementary approach to identify potential odorants would be the deorphanization of the putative odorant receptors isolated in this thesis. This requires the *in vitro* expression of the coding region of the receptors in an appropriate expression vector with a reporting system. An analysis of chemoreceptor expression between males and females should also be carried out, especially because males were shown to be more sensitive to spawning than females, and between individuals collected during the reproductive and non-reproductive season. In connection to this, future research should dissect the neural or neuroendocrine mechanisms associated with chemosensory responses as these are not yet known and sex differences are expected considering the specificities of oogenesis and spermatogenesis.

In summary, this thesis made significant advances into sea cucumber biology and opened new lines of research in an animal of high ecological importance but which has been object of comparatively little physiological research. It also provides an important contribution to policy making as few data were available on the reproductive biology of the species in

question, an essential component of population management strategies. Finally, the use of pheromones as an innovative population control tool is a possibility that can be tested as soon as their chemical identity is known.

6.3. References

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Holothuria arguinensis. Photograph taken by Ricardo Haponiuk.