

UNIVERSIDADE FEDERAL DE MINAS GERAIS
Instituto de Ciências Biológicas
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**MORPHOLOGY AND EVOLUTION OF WHITE SPOTS AND GONADS IN
STAUROZOA (CNIDARIA)**

Belo Horizonte
2022

Lucas Ferreira Leitão Barbosa

**MORPHOLOGY AND EVOLUTION OF WHITE SPOTS AND GONADS IN
STAUROZOA (CNIDARIA)**

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Orientadora: Profa. Dra. Lucília Souza Miranda

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ATA DE DEFESA DE DISSERTAÇÃO

LUCAS FERREIRA LEITÃO BARBOSA

Ao décimo primeiro dia do mês de agosto do ano de dois mil e vinte e dois, às nove horas, ocorreu a defesa de Mestrado da Pós-Graduação em Zoologia, de autoria do Mestrando Lucas Ferreira Leitão Barbosa intitulada: **“Morphology and evolution of white spots and gonads in Staurozoa (Cnidaria)”**. Abrindo a sessão, a Presidente da Comissão, Profa. Dra. Lucília Souza Miranda, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra para o candidato para apresentação de seu trabalho.

Esteve presente a Banca Examinadora composta pelos membros: Amanda Ferreira e Cunha, Gisele Yukimi Kawauchi, e demais convidados. Seguiu-se a arguição pelos examinadores, com a respectiva defesa do candidato.

Após a arguição, apenas os Srs. Examinadores permaneceram na sala para avaliação e deliberação acerca do resultado final, a saber: o trabalho foi **APROVADO SEM ALTERAÇÕES**.

Nada mais havendo a tratar, a Presidente da Comissão encerrou a reunião e lavrou a presente ata, que será assinada por todos os membros participantes da Comissão Examinadora.

Belo Horizonte, 11 de agosto de 2022

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Morphology and evolution of white spots and gonads in Staurozoa (Cnidaria)

LUCAS FERREIRA LEITÃO BARBOSA

Esta dissertação foi apresentada em sessão pública e submetida a avaliação em 11 de agosto de 2022, tendo sido aprovada pela Banca Examinadora composta pelos seguintes membros:

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Resumo

Staurozoa é possivelmente uma das classes de Cnidaria menos conhecidas e estudadas. É composta por aproximadamente 50 espécies que, diferentemente de grande parte dos Acraspeda, possuem fase sexuada bentônica (estauromedusa), mantendo-se aderidas a um substrato por um pedúnculo. Essa característica particular do ciclo de vida torna o grupo conhecido como medusas pedunculadas. As estauromedusas apresentam uma organização corporal simples, no entanto, determinadas regiões como os “white spots” (bolsas de nematocistos) e gônadas apresentam considerável especialização e complexidade morfológica. Apesar disso, ambas estruturas foram pouco exploradas considerando a sua diversidade morfológica, função e evolução, levando a generalizações de sua diversidade, principalmente em relação às gônadas. Assim, o presente estudo tem como objetivos: (1) descrever a organização dos “white spots” e gônadas, com base em dados da literatura e em observações dos animais; (2) avaliar o potencial uso taxonômico para características relacionadas a ambas estruturas; (3) descrever aspectos específicos sobre a morfologia dos “white spots”, com implicações para seu funcionamento; (4) reconstruir a história evolutiva de caracteres relacionados aos “white spots” e às gônadas, buscando compreender a condição ancestral desses caracteres e eventuais ganhos e/ou perdas que possam ter ocorrido ao longo da evolução do grupo. Realizamos uma ampla revisão bibliográfica sobre as duas características para todas as espécies de Staurozoa, discutindo possíveis usos taxonômicos. A morfologia neuromuscular dos “white spots” de duas estauromedusas, *Haliclystus 'sanjuanensis'* e *Manania handi*, foi estudada utilizando imunohistoquímica com anticorpos FMRFamida e α -tubulina como marcadores de neurônios e faloidina como marcador para a musculatura. Para reconstruir a história evolutiva dos “white spots” e das gônadas, análises de sinal filogenético (estatística δ) e reconstrução dos estados ancestrais, por máxima verossimilhança, foram feitas no software R, utilizando o pacote corHMM, a partir de uma filogenia molecular datada. Os resultados revelaram lacunas no conhecimento de ambas as estruturas: para as gônadas deixou evidente a sua diversidade e complexidade, com destaque para o gênero *Calvadosia*, e para os “white spots” foi possível estabelecer padrões de distribuição da estrutura na subumbrela, com potencial uso taxonômico para toda classe. Ainda para os “white spots”, a hipótese de liberação dos nematocistos contidos em seu interior para o meio externo, como forma de defesa ou ataque, ganhou força a partir da organização da musculatura e anel nervoso associado intrinsecamente à estrutura, encontrados nas imagens obtidas a partir de Microscopia Confocal de Varredura a Laser (MCVL). Por fim, nossas análises trouxeram a primeira visão evolutiva para a posição das duas estruturas, assim como a evolução das gônadas em vesículas, característica de destaque da classe. Assim, o presente estudo representa um avanço no entendimento da real diversidade morfológica de Staurozoa, abrindo perspectivas para pesquisas como relação evolutiva entre gônadas e “white spots”, mecanismos de disparo de nematocistos, além de reforçar a necessidade de estudos mais detalhados e abrangentes a respeito das gônadas, principalmente para os gêneros *Depastrum*, *Kyopoda*, *Lucernaria* e *Stylocoronella*.

Palavras-Chave: Medusas pedunculadas. Microscopia confocal. Nematocistos. Sinal filogenético. Vesículas.

Abstract

Staurozoa is possibly one of the least known and studied classes of Cnidaria. It is composed of approximately 50 species that, unlike most of the Acraspeda, have a benthic sexual phase (stauromedusa), remaining attached to a substrate by a peduncle. This particular life cycle makes the group known as stalked jellyfish. Stauromedusae have a simple body organization, however, certain regions such as the white spots (nematocysts pockets) and gonads show considerable specialization and morphological complexity. Despite this, both structures have been little explored considering their morphological diversity, function, and evolution, leading to generalizations of their diversity, especially in relation to the gonads. Thus, the present study aims to: (1) describe the organization of white spots and gonads, based on literature data and observations from animals; (2) evaluate the potential taxonomic use for features related to both structures; (3) describe specific aspects on the morphology of white spots, with implications for their function; (4) reconstruct the evolutionary history of traits related to white spots and gonads, trying to understand the ancestral condition of these traits and possible gains and/or losses that may have occurred during the evolution of the group. We conducted an extensive literature review about the two features for all species of Staurozoa, discussing possible taxonomic uses. The neuromuscular morphology of the white spots of two stauromedusae, *Haliclystus 'sanjuanensis'* and *Manania handi*, was studied using immunohistochemistry with FMRFamide and α -tubulin antibodies as markers for neurons and phalloidin as marker for musculature. In order to reconstruct the evolutionary history of white spots and gonads, phylogenetic signal analyses (δ -statistic) and reconstruction of ancestral states by maximum likelihood were performed in R software using the corHMM package based on a dated molecular phylogeny. The results revealed gaps in the knowledge of both structures: the diversity and complexity of the gonads was evident, especially in the genus *Calvadosia*, and for the white spots it was possible to establish patterns of distribution of the structure on the subumbrella, with potential taxonomic use for the entire class. Also for the white spots, the hypothesis that the nematocysts contained inside them can be released to the external environment, as a form of defense or attack, gained strength from the organization of the musculature and nerve ring intrinsically associated with the structure, found in the images obtained from Confocal Scanning Laser Microscopy (CSLM). Finally, our analyses brought the first evolutionary insight regarding the position of the two structures, as well as the evolution of the gonads into vesicles, a remarkable feature of the class. Thus, the present study represents an advance in the understanding of the real morphological diversity of Staurozoa, opening perspectives for research such as the evolutionary relationship between gonads and white spots, mechanisms of nematocyst discharge, besides reinforcing the need for more detailed and comprehensive studies regarding the gonads, especially for the genera *Depastrum*, *Kyopoda*, *Lucernaria*, and *Stylocoronella*.

Keywords: Stalked jellyfish. Confocal microscopy. Nematocysts. Phylogenetic signal. Vesicles.

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General introduction

1. Systematics and taxonomy

Staurozoa is a class (Marques & Collins, 2004) of marine cnidarians (Fig. 1), with about 50 valid species, also known as stalked jellyfishes, usually living in intertidal or subtidal waters, predominantly in temperate and polar regions (Miranda et al., 2018). They generally have a goblet-shaped body divided into two regions: an apical calyx and a basal peduncle (Fig. 1). Staurozoans have a benthic life habit, adhering by their peduncle to various types of substrates, such as algae, rocks, gravel, seagrass, shells, and even to other animals, as for example holothurians (Wietrzykowski, 1912; Kikinger & Salvini-Plawen, 1995; Salvini-Plawen, 2006; Mills & Hirano, 2007; Miranda et al., 2010, 2018).

The class Staurozoa is one of the least studied among the cnidarians (Miranda et al., 2018), historically presenting imprecise taxonomic placement. A good example for this inaccuracy is the classification of the species *Manania auricula* among the sea cucumbers (Müller, 1776).

Initially, the staurozoans were grouped with the sea anemones (Cuvier, 1817, 1830). Later, they were grouped by Sars (1846) with the medusae based on the presence of gastric filaments, and then Goette (1887) included staurozoans in the class Scyphozoa, a class with which staurozoans share similar morphological features, such as circular coronal muscles and rhopalium-like structures (Uchida, 1929). The positioning of the staurozoans as an order of Scyphozoa (i.e., Stauromedusae) remained stable for many years until disputed by Marques & Collins (2004), based on morphological and life history characters used to reconstruct the phylogenetic relationships among the medusozoan cnidarians (currently encompassing the classes Staurozoa, Cubozoa, Scyphozoa, and Hydrozoa). As a result, the Stauromedusae were recovered as a monophyletic group closely-related to the fossil group Conulatae, forming a new class, Staurozoa.

However, the hypothesis of Staurozoa composed of these two lineages was refuted a few years later based on the analysis of new fossil evidence (Van Iten et al., 2006). The fossil lineage Conulatae was recovered nested within Scyphozoa and no longer as a sister group to Stauromedusae, which in turn remained the only lineage representing the class Staurozoa (Van Iten et al., 2006). Furthermore, it was recovered as the sister group to all

other medusozoans (i.e., Cubozoa, Hydrozoa, and Scyphozoa) using both molecular (Collins et al., 2006) and morphological (Van Iten et al., 2006) data (Fig. 2A).

Staurozoa as a class composed exclusively of the order Stauromedusae is currently accepted (Miranda et al., 2016a; Kayal et al., 2018), however, their evolutionary relationship within Medusozoa remains under debate. Subsequent analyses to those mentioned above (Collins et al., 2006; Van Iten et al., 2006), using complete mitochondrial genome data (Kayal et al., 2013) and phylogenomics (Zapata et al., 2015) contradicted the previous evolutionary hypothesis for Medusozoa (Fig. 2A). Kayal et al. (2013) suggested that Staurozoa may be the sister group of Cubozoa (Fig. 2B) and Zapata et al. (2015) place them in an unresolved clade with Cubozoa and Scyphozoa (Fig. 2C). Nevertheless, more recent and complete phylogenomics analyses (Kayal et al., 2018) recovered Staurozoa as sister group to the clade formed by Cubozoa and Scyphozoa (Fig. 2D). These contrasting topologies recovered for Medusozoa have implications for the understanding of the evolution of important life cycle traits, as the origin of medusa (Kayal et al., 2018), and reinforces the need to explore each class and their characters and evolution in detail.

Staurozoan taxonomy has been based on subjective and impressive characters, many of them presenting intraspecific and ontogenetic variation (Miranda et al., 2009). Besides, until recently (Miranda et al., 2016a), the classification were based on similarities between morphological features not supported by an evolutionary analysis (Clark, 1863; Haeckel, 1879; Uchida, 1929; Carlgren, 1935). A striking morphological feature that has been used in most classifications is the claustrum, which consist of a tissue in the gastrovascular cavity delimited by a central layer of mesoglea surrounded by gastrodermis, without communication with epidermis (Fig. 3A; Miranda et al., 2016b; 2017a). Clark (1863) proposed that the stalked jellyfishes could be separated into two families: Cleistocarpidae, including the species with claustrum, and Eleutherocarpida, including the species without claustrum.

However, Collins & Daly (2005), based on a molecular analysis with limited taxon sampling, suggested that claustrum might not be a good feature for classifying subgroups of Staurozoa, since Cleistocarpidae and Eleutherocarpida resulted as non-monophyletic groups. This result was later confirmed by Miranda et al. (2016a), currently the only study that proposed a review of staurozoan classification based on phylogenetic results. Miranda et al. (2016a) included about half of the species known at the time in an

phylogenetic analyses using nuclear and mitochondrial markers, which resulted in the separation of the class into two main clades that can be diagnosed by the presence and absence of the interradial longitudinal muscle in the peduncle (Fig. 3B, C), respectively named as Myostaurida and Amyostaurida, two new suborders (Fig. 4).

Miranda et al. (2016a) also revised the taxonomy at family and genus levels, since many taxa were not recovered as monophyletic. For example, the family Kishinouyeidae (Uchida, 1929), traditionally composed of the genera *Kishinouyea*, *Sasakiella*, and *Lucernariopsis*, was recovered as monophyletic, but the genera *Kishinouyea* and *Lucernariopsis* were not. This result, in association with the absence of robust morphological characters to differentiate the proposed new lineages, has led to the synonymization of the three genera into a single one called *Calvadosia*.

Thus, the taxonomy of the class Staurozoa is now mainly based on the evolutionary history of the group (with the exception of some rare genera, see Miranda et al., 2016a), consisting of 50 species, 11 genera, 6 families, and 2 suborders (Fig. 4; Miranda et al., 2018). Although significant advances have been achieved in the classification of the group, the species richness of Staurozoa is probably underestimated, given that there are at least eight possible new species indicated by Miranda et al. (2016a) (including the recently described *Calvadosia lewisi* Miranda et al., 2017b) and considering the increasing number of new species described for the group in recent years (see Miranda et al., 2018; figure 3). Additionally, most of the new possible species of Staurozoa occur in the Southern Hemisphere, traditionally a hemisphere with few scientists dedicated to the study of stalked jellyfishes, suggesting a historical sampling and research effort bias (Miranda et al., 2018).

2. Biology

2.1. Life cycle and development

The peculiar staurozoan life cycle is perhaps one of the main characteristics of the group. As already mentioned, Staurozoa is a lineage of the subphylum Medusozoa, a group known for usually presenting two main developmental stages in the life cycle, being in most cases a benthic stage with asexual reproduction – the polyp, followed by a pelagic stage that reproduces sexually – the medusa (Collins, 2002). However, in staurozoans, the metamorphosis from polyp into medusa is gradual and occurs mainly in

the apical region (calyx), with few internal modifications in the basal region, so the stauromedusa, the sexual stage, is also benthic, living attached to the substrate by a peduncle (Kikinger & Salvini-Plawen 1995; Miranda et al., 2010), therefore, popularly called stalked jellyfishes.

The staurozoan life cycle (Fig. 5) is still enigmatic, but has been described in detail for some species. Wietrzykowski (1910, 1911, 1912) described the internal and external morphology of *Haliclystus octoradiatus*, for all its life stages. Hirano (1986) described the external anatomy (e.g., gonads, white spots, and tentacles) during the development of six species, and corroborated observations previously made by Uchida (1929) concerning the changes in the number of chambers in the peduncle during development. Kikinger & Salvini-Plawen (1995) made a significant contribution to the understanding of the life cycle of staurozoans by describing the complete development of the species *Stylocoronella riedii* and *Stylocoronella variabilis*, noting the production of lateral buds in the stauropolyp stage. More recently, Miranda et al. (2010) elucidated the life cycle of the staurozoan *Haliclystus antarcticus*, proposing that the misidentified hydrozoan *Microhydrula limopsicola* is a stage (attached larva) of the life cycle of *H. antarcticus*.

Thus, based on these previous studies, it is known that like most cnidarians, male and female stauromedusae release their gametes - previously contained in their gastrovascular cavity - through the mouth into the water column, where fertilization also occurs (Hanaoka, 1934; Otto, 1976). The fertilization is followed by the formation of a membrane around the zygote and after the processes of irregular cleavage and gastrulation, the planula larva is formed (Hanaoka, 1934; Otto, 1976). The staurozoan planula has an elongated shape composed of 16 endodermal cells surrounded by ectodermal cells and is benthic, differently from the usual medusozoan planktonic planulae (Otto, 1976). The planulae of Staurozoa creep along the substrate for a few days until they attach to the substrate (some planulae can cluster together, providing an advantage for food capture; Wietrzykowski, 1912; Miranda et al., 2010) and begin the process of metamorphosis into a stauropolyp with the consequent appearance of the eight primary tentacles (Wietrzykowski, 1912; Hirano, 1986; Kikinger & Salvini-Plawen, 1995; Miranda et al., 2010).

From this point on, the modifications in the animal body occur mainly in the calyx region (e.g., organization of arms, gastric filaments, gonads, white spots, and secondary tentacular clusters; disappearance or modification of the primary tentacles), which does

not mean the absence of modifications in the peduncle, as once suggested (Uchida, 1973). In the peduncle, the main modifications occur internally (e.g., change in the number of chambers; Uchida, 1929; Hirano, 1986). Thereby, the stauromedusa develops and when it reaches sexual maturity, a new cycle begins. It is important to highlight that there are reports of asexual reproduction in the planula and stauropolyp stages (Wietrzykowski, 1912; Kikinger & Salvini-Plawen, 1995; Jarms & Tiemann, 1996; Miranda et al., 2010).

2.2. Distribution and habitat

The stauzoans have a distribution pattern that contradicts the classic pattern of higher species richness at lower latitudes (Willig et al., 2003), since most of them are found in temperate and polar waters of the Northern Hemisphere, especially between the 40th and 70th parallels (Miranda et al., 2018). Thirty-seven out of 50 valid species are currently recorded in the Northern Hemisphere, 10 species in Southern Hemisphere, and three species in both hemispheres (Miranda et al., 2018).

The antitropicality (Crame, 1993) of the group is evident, as only three species are found in intertropical waters (Miranda et al., 2018), being two species of the genus *Calvadosia*, *C. corbini* and *C. hawaiiensis*, and one deep-sea species *Lucernaria janetae*, from the hydrothermal vent fields of East Pacific Rise (Collins & Daly 2005; Lutz et al., 2006). The explanation for this phenomenon may be related to the substrate where they are attached to.

Most of stauromedusae (8 out of 11 genera) are found attached to algae, followed by stones and seagrass as the main substrates for the species (Miranda et al., 2018). Similarly to stalked jellyfishes, algae have higher richness in temperate regions, which decreases as approaches to the tropics (Kerswell, 2006). In addition, there is a correspondence in the algal seasonal appearance with the seasonal life cycle of stalked jellyfishes, as pointed out by some authors (Uchida, 1929; Berrill, 1962; McInnes, 1989; Zagal, 2004; Miranda et al., 2018). This correspondence indicates optimal growth and nutritional conditions for these animals (Zagal, 2004). Algae also play an important role in stauromedusan camouflage, allowing them to match their surroundings (Fig. 1; Mayer, 1910; Corbin, 1978; Mills & Hirano, 2007) based mainly on a possible capacity of these animals to absorb pigments from algae (Migot, 1922). This provides further evidence that the presence of algae may be a limiting factor for the presence of stalked jellyfishes.

3. Morphology

Staurozoa is represented by relatively small organisms; with the smallest stauromedusa measuring less than 1 cm in diameter (Kramp, 1961) and the largest, *Lucernaria janetae*, reaching 10 cm in diameter (Collins & Daly, 2005). They are diploblastic animals, which means that they express two embryonic germ layers during the development, the endoderm and the ectoderm. These layers originate, respectively, the gastrodermis (innermost layer, which delimits the gastrovascular cavity) and the epidermis (outermost layer, which externally covers the animal), between these two tissue layers, there is a gelatinous extracellular matrix called mesoglea (Miranda et al., 2016b).

The stauromedusae stage (Fig. 6A, B) has polypoid and medusoid characters as a consequence of their life cycle (Collins et al., 2006). Their body can be divided into the apical calyx region, where most of the medusoid characteristics are located, such as the arms, secondary tentacles, gonads, and rhopaloids (Fig. 6A, B); and the basal peduncle, typically polypoid (Collins, 2002). The body of stauromedusae can also be divided into plans of symmetry: four perradial regions (aligned to the four corners of manubrium), four interradial regions (between the perradii), and eight adradial regions (located between a perradii and an interradii) (Fig. 6B). The establishment and recognition of these radii is fundamental for understanding and referencing the external and internal structures of the stauromedusae.

At the calyx margin there are eight adradial arms (Fig. 6A, B), which can be short or long, or even fused at the interradii, as in some species of *Calvadosia* (e.g., *Calvadosia cruciformis*) and *Lucernaria* (e.g., *Lucernaria quadricornis*) (Fig. 6C). Clusters of secondary tentacles are located at the tip of each arm, used mainly to prey capture and to assist in locomotion; the shape, quantity, and type of tentacles are often used in the taxonomy of the group (Fig. 5A, B; Uchida, 1929; Kramp, 1961; Hirano, 1997). Also along the calyx margin of some species (e.g., *Haliclystus 'sanjuanensis'*), there are adhesive structures between the arms, known as rhopaloids or anchors; such structures correspond to the eight primary tentacles that were modified during the development of the animal (Fig. 6D; Miranda et al., 2016b; Miranda & Collins, 2019). Furthermore, different authors suggested the presence of photosensitive cells associated to the rhopaloids of some species, specially of the genera *Manania* and *Stylocoronella* (Salvini-Plawen, 1966; Blumer et al., 1995; Kikinger & Salvini-Plawen, 1995; Westlake & Page, 2017; Miranda & Collins, 2019).

In the subumbrella (i.e., oral region of calyx), the gonads (Fig. 6B) - structures for the production and storage of gametes - are very evident. They usually consist of evaginations of the gastrodermis, producing vesicles, as can be observed in species of the genera *Haliclystus* and *Calvadosia* (Fig. 7A), or organized in a single layer between the gastrodermis and the epidermis, as in species of the genus *Manania* (Fig. 7B) (Uchida, 1929; Uchida & Hanaoka, 1933; Eckelbarger & Larson, 1993; Miranda et al., 2016b). There are different patterns of gonadal organization in Staurozoa and the information regarding the external and internal anatomy of the gonads is often used in the staurozoan taxonomy (Hirano, 1986; Miranda et al., 2016b, 2017a).

The reproductive structures are located within the gastric radial pockets in staurozoan species that do not have a claustrum (Fig. 7C; Miranda et al., 2017b). In species that have a claustrum, their gonads are located in the main gastric radial pocket (Fig. 7D; Miranda et al., 2017b). Importantly, as said above, the presence of claustrum evolved independently in Staurozoa (Miranda et al., 2016a), with possible implications for the organization of the gonad in the group.

Frequently associated with the gonads and scattered throughout the subumbrella, it is possible to identify rounded white structures called white spots (Figs. 6B, 7E; Miranda et al., 2016b). Structurally, the white spots are groups of nematocysts (immature nematocysts peripherally and mature nematocysts clustered in the central region) enclosed in a thin double layer of mesoglea, between a layer of epidermis (externally) and gastrodermis (internally) (Fig. 7E; Miranda et al., 2016b). The presence of white spots is widespread in different lineages of Staurozoa, occurring in all genera, and it is considered a synapomorphy of the class (Miranda et al., 2016b). However, the pattern of white spots distribution on subumbrella varies in different species, an information useful in staurozoan taxonomy (Hirano, 1997). Ontogenetic formation as well as the function of white spots is still uncertain, although there are hypotheses indicating a role for white spots in the defense and attack of prey and predators (Kling, 1879; Kassianow, 1901; Uchida & Hanaoka, 1933; Miranda et al., 2016b).

Chapter 1

Morphology and evolution of white spots of nematocysts in Staurozoa (Cnidaria)

In this chapter we described the intrinsic neuroanatomy of the white spots of nematocysts, making inferences about its functionality. We provided a broad review of the presence and distribution of these structures in all species of the class, highlighting the potential taxonomic use of white spots in the identification of species and genera. Finally, we proposed the first reconstruction of the evolutionary history of white spots distribution on the subumbrella of staurozoans.

Chapter 2

Morphological diversity and evolution of gonads in Staurozoa (Cnidaria)

In this chapter we presented an extensive review regarding the diversity of the gonads in Staurozoa. We demonstrated internal and external morphological patterns, with possible taxonomic use, and pointed out gaps in the knowledge of the reproductive structures of the class. Furthermore, the evolutionary history of staurozoan gonadal characteristics was reconstructed for the first time, evidencing their diversity.

References

- Berrill M. 1962. The biology of three New England stauromedusae, with a description of a new species. *Canadian Journal of Zoology* 40(7):1249-1262.
- Blumer MJF, Salvini-Plawen LV, Kikinger R, Büchinger T. 1995. Ocelli in a Cnidaria polyp: the ultrastructure of the pigment spots in *Stylocoronella riedli* (Scyphozoa, Stauromedusae). *Zoomorphology* 115(4):221–227.
- Carlgren O. 1935. Über eine neue Südafrikanische Lucernariidae, *Depastromorpha africana* n. gen., n. sp., nebst Bemerkungen über den Bau und die Systematik dieser Tiergruppe. *Kunglia Svenska Vetenskapsakademiens Handlingar* 15:1–24
- Clark HJ. 1863. Prodröm of the history, structure, and physiology of the order Lucernariae. *Journal of the Boston Society of Natural History* 7:531–567.
- Collins AG. 2002. Phylogeny of Medusozoa and the evolution of cnidarian life cycles. *Journal of Evolutionary Biology* 15(3):418–432.
- Collins AG, Daly M. 2005. A new deepwater species of Stauromedusae, *Lucernaria*

- janetae* (Cnidaria, Staurozoa, Lucernariidae), and a preliminary investigation of stauromedusan phylogeny based on nuclear and mitochondrial rDNA data. *Biological Bulletin* 208(3):221–230.
- Collins AG, Schuchert P, Marques AC, Jankowski T, Medina M, Schierwater B. 2006. Medusozoan phylogeny and character evolution clarified by large and small subunit rDNA data and an assessment of the utility of phylogenetic mixture models. *Systematic Biology* 55(1):97–115.
- Corbin PG. 1978. A new species of the stauromedusan genus *Lucernariopsis* (Coelenterata: Scyphomedusae). *Journal of the Marine Biological Association of the United Kingdom* 58(2):285–290.
- Crame JA. 1993. Bipolar molluscs and their evolutionary implications. *Journal of Biogeography* 20(2):145–161.
- Cuvier G. 1817. *Le Règne animal distribué d'après son organisation, pour servir de base à l'histoire naturelle des animaux et d'introduction à l'anatomie comparée*. Tome IV Paris: Chez Déterville Libraire.
- Cuvier G. 1830. *Le Règne animal distribué d'après son organisation, pour servir de base à l'histoire naturelle des animaux et d'introduction à l'anatomie comparée*. Tome III Paris: Chez Déterville Libraire.
- Eckelbarger KJ, Larson RJ. 1993. Ultrastructural study of the ovary of the sessile scyphozoan, *Haliclystus octoradiatus* (Cnidaria: Stauromedusae). *Journal of Morphology* 218(2):225–236.
- Goette A. 1887. *Abhandlungen zur Entwicklungsgeschichte der Tiere. Entwicklungsgeschichte der Aurelia aurita und Cotylorhiza tuberculata*. Hamburg und Leipzig: Verlag von Leopold Voss.
- Haeckel E. 1879. *Das system der medusen. I, 2: System der Acraspeden. Zweite Hälfte des Systems der Medusen*. Jena: Gustav Fischer.
- Hanaoka KI. 1934. Notes on the early development of a stalked medusa. *Proceedings of the Imperial Academy* 10(2):117-120.
- Hirano YM. 1986. Species of Stauromedusae from Hokkaido, with notes on their metamorphosis. *Journal of the Faculty of Science, Hokkaido University, Zoology* 24:182–201.
- Hirano YM. 1997. A review of a supposedly circumboreal species of stauromedusa, *Haliclystus auricula* (Rathke, 1806). In: den Hartog J.C., ed. *Proceedings of the 6th International Conference on Coelenterate Biology*. Leiden: National Naturhistorisch Museum, Noordwijkerhout, 247–252.
- Jarms G, Tiemann H. 1996. On a new hydrolyp without tentacles, *Microhydrula limopsicola* n. sp., epibiotic on bivalve shells from the Antarctic. *Scientia Marina* 60:109-115.

- Kassianow N. 1901. Studien über das Nervensystem der Lucernariden nebst sonstigen histologischen Beobachtungen über diese Gruppe. *Zeitschrift für Wissenschaftliche Zoologie* 69:287–377.
- Kayal E, Bentlage B, Sabrina Pankey M, Ohdera AH, Medina M, Plachetzki DC, Collins AG, Ryan JF. 2018. Phylogenomics provides a robust topology of the major cnidarian lineages and insights on the origins of key organismal traits. *BMC evolutionary biology* 18(1):1–18.
- Kayal E, Roure B, Phillippe H, Collins AG, Lavrov DV. 2013. Cnidarian phylogenetic relationships as revealed by mitogenomics. *BMC Evolutionary Biology* 13(1):5.
- Kerswell AP. 2006. Global biodiversity patterns of benthic marine algae. *Ecology* 87(10):479–2488.
- Kikinger R, Salvini-Plawen LV. 1995. Development from polyp to stauromedusa in *Stylocoronella* (Cnidaria: Scyphozoa). *Journal of the Marine Biological Association of the United Kingdom* 75:899–912.
- Kling O. 1879. Ueber *Craterolophus tethys*. Ein Beitrag zur Anatomie und Histologie der Lucernarien. *Morphologisches Jahrbuch* 5:141–166.
- Kramp PL. 1961. Synopsis of the medusae of the world. *Journal of the Marine Biological Association of the United Kingdom* 40:7–469.
- Lutz RA, Collins AG, Annis ER, Reed AJ, Bennett KF, Halanych KM, Vrijenhoek RC. 2006. Stauromedusan populations inhabiting deep-sea hydrothermal vents along the southern East Pacific Rise. *Cahiers de Biologie Marine* 47(4):409–413.
- Marques AC, Collins AG. 2004. Cladistic analysis of Medusozoa and cnidarian evolution. *Invertebrate Biology* 123(1):32–42.
- Mayer AG. 1910. *Medusae of the World. Volume III. Scyphomedusae*. Washington, D.C.: Carnegie Institution Publishing, Publication 109.
- McInnes DE. 1989. A stalked jellyfish (Stauromedusae), found at Black Rock, Port Phillip Bay. A first recording in Australia. *Victorian Naturalist* 106(3):86–92.
- Migot A. 1922. Sur le mode de fixation des Lucernaires à leur support. *Comptes Rendus des Séances de la Société de Biologie et de ses Filiales* 86:827–829.
- Mills CE, Hirano YM. 2007. Stauromedusae. In: Denny MW, Gaines SD, eds. *Encyclopedia of Tidepools and Rocky Shores*. Berkeley: University of California Press, 541–543.
- Miranda LS, Branch GM, Collins AG, Hirano YM, Marques AC, Griffiths CL. 2017b. Stalked jellyfishes (Cnidaria: Staurozoa) of South Africa, with the description of *Calvadosia lewisi* sp. nov. *Zootaxa* 4227(3):369–389.

- Miranda LS, Collins AG, Hirano YM, Mills CE, Marques AC. 2016b. Comparative internal anatomy of Staurozoa (Cnidaria), with functional and evolutionary inferences. *PeerJ* 4:e2594.
- Miranda LS, Collins AG, Marques AC. 2010. Molecules clarify a cnidarian life cycle – the “hydrozoan” *Microhydrula limopsicola* is an early life stage of the staurozoan *Haliclystus antarcticus*. *PLoS One* 5:e10182.
- Miranda LS, Collins AG. 2019. Eyes in Staurozoa (Cnidaria): a review. *PeerJ* 7:e6693.
- Miranda LS, García-Rodríguez J, Collins AG, Morandini AC, Marques AC. 2017a. Evolution of the claustrum in Cnidaria: comparative anatomy reveals that it is exclusive to some species of Staurozoa and absent in Cubozoa. *Organisms Diversity & Evolution* 17(4):753–766.
- Miranda LS, Hirano YM, Mills CE, Falconer A, Fenwick D, Marques AC, Collins AG. 2016a. Systematics of stalked jellyfishes (Cnidaria: Staurozoa). *PeerJ* 4:e1951.
- Miranda LS, Mills CE, Hirano YM, Collins AG, Marques AC. 2018. A review of the global diversity and natural history of stalked jellyfishes (Cnidaria, Staurozoa). *Marine Biodiversity* 48(4):1695–1714.
- Miranda LS, Morandini AC, Marques AC. 2009. Taxonomic review of *Haliclystus antarcticus* Pfeffer, 1889 (Stauromedusae, Staurozoa, Cnidaria), with remarks on the genus *Haliclystus* Clark, 1863. *Polar Biology* 32(10):1507–1519.
- Müller OF. 1776. *Zoologiae Danicae Prodromus, seu animalium Daniae et Norvegiae indigenarum, Characteres, Nomina et Synonyma imprimis popularium*. Havniae: Typis Hallageriis.
- Otto JJ. 1976. Early development and planula movement in *Haliclystus* (Scyphozoa, Stauromedusae). In: Mackie GO, ed. *Coelenterate Ecology and Behavior*. New York: Plenum Press, 319–329.
- Salvini-Plawen LV. 1966. Zur Kenntnis der Cnidaria des nordadriatischen Mesopsammon. *Veröffentlichungen des Instituts für Meeresforschung in Bremerhaven* 2:165–186.
- Salvini-Plawen LV. 2006. First record of a mature stauromedusa *Stylocoronella* (Cnidaria) in nature. *Cahiers de biologie marine* 47(2):219–222.
- Sars M. 1846. *Fauna littoralis Norvegiae oder Beschreibung und Abbildungen neuer oder wenig bekannter Seethiere, nebst Beobachtungen über die Organisation, Lebensweise u. Entwicklung derselben*. Christiania: Druck und Verlag von Johann Dahk.
- Uchida T. 1929. Studies on the Stauromedusae and Cubomedusae, with special reference to their metamorphosis. *Japanese Journal of Zoology* 2:103–193.

- Uchida T. 1973. The systematic position of the Stauromedusae. *Publications of the Seto Marine Biological Laboratory* 20:133–139.
- Uchida T, Hanaoka KI. 1933. On the morphology of a stalked medusa, *Thaumatoscyphus distinctus* Kishinouye. *Journal of the Faculty of Science, Hokkaido Imperial University, Series VI, Zoology* 2(3):135–153.
- Van Iten H, Leme JM, Simões MG, Marques AC, Collins AG. 2006. Reassessment of the phylogenetic position of Conulariids (?Ediacaran-Triassic) within the subphylum Medusozoa (phylum Cnidaria). *Journal of Systematic Palaeontology* 4(2):109–118.
- Westlake HE, Page LR. 2017. Muscle and nerve net organization in stalked jellyfish (Medusozoa: Staurozoa). *Journal of Morphology* 278:29–49.
- Wietrzykowski W. 1910. Recherches sur le développement des Lucernaires. *Archives de Zoologie Expérimentale et Générale, 5th Series* 5:10–27.
- Wietrzykowski W. 1911. Recherches sur le développement des Lucernaires. *Archives de Zoologie Expérimentale et Générale, 5th Series* 6:49–52.
- Wietrzykowski W. 1912. Recherches sur le développement des Lucernaires. *Archives de Zoologie Expérimentale et Générale, 5th Series* 10: 1–95.
- Willig MR, Kaufman DM, Stevens RD. 2003. Latitudinal gradients of biodiversity: pattern, process, scale, and synthesis. *Annual review of ecology, evolution, and systematics* 34:273–309.
- Zagal CJ. 2004. Population biology and habitat of the stauromedusa *Haliclystus auricula* in southern Chile. *Journal of the Marine Biological Association of the United Kingdom* 84(2):331–336.
- Zapata F, Goetz FE, Smith AS, Howison M, Siebert S, Church S, Sanders SM, Ames CL, McFadden CS, France SC, Daly M, Collins AG, Haddock SHD, Dunn C, Cartwright P. 2015. Phylogenomic analyses support traditional relationships within Cnidaria. *PLoS ONE* 10(10):e139068.

Figures

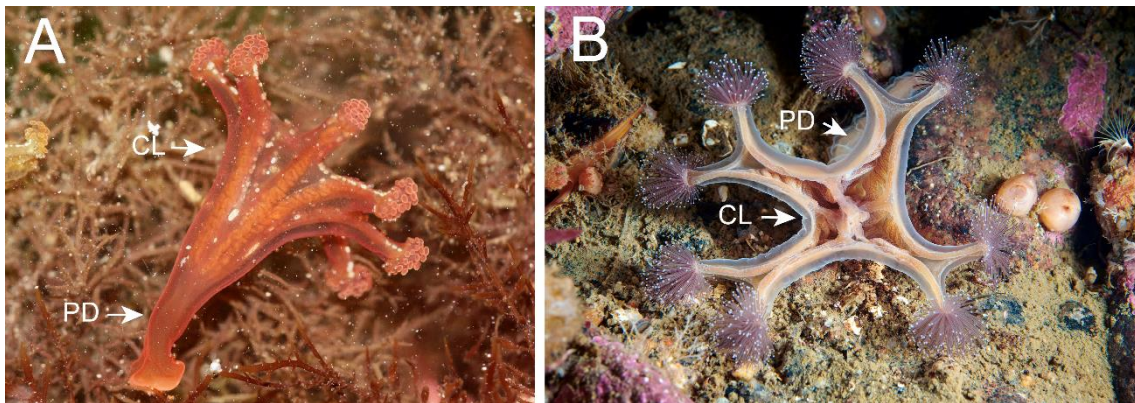


Figure 1. Main body regions of stauromedusae. **(A)** Lateral view of *Calvadosia campanulata* (photo credit: David Fenwick; scale not available). **(B)** Oral view of *Lucernaria* sp. (photo credit: Alexander Semenov; scale not available). Abbreviations: CL, calyx; PD, peduncle.

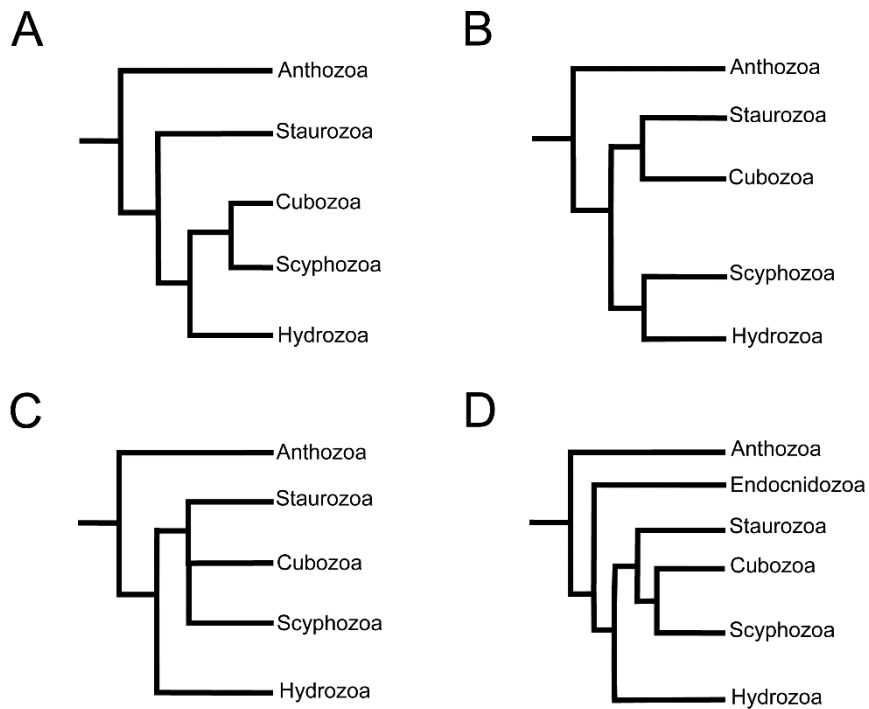


Figure 2. Phylogeny of Cnidaria and the positioning of Staurozoa in the medusozoan clade. **(A)** Staurozoa as sister group to the other lineages of Medusozoa (Collins et al., 2006; Van Iten et al., 2006). **(B)** Staurozoa as a sister group of Cubozoa (Marques & Collins, 2004; Kayal et al., 2013). **(C)** Staurozoa clustered with Cubozoa and Scyphozoa in an unresolved clade (Zapata et al., 2015). **(D)** Staurozoa as a sister group to Cubozoa+Scyphozoa forming the clade Acraspeda (Kayal et al., 2018).

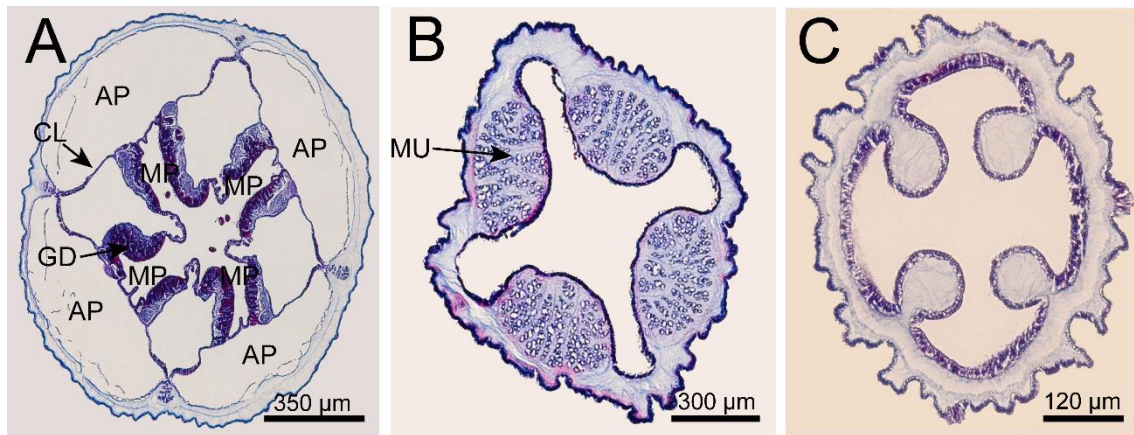


Figure 3. Microanatomy of important traits in the taxonomy of Staurozoa. **(A)** Cross section of the calyx of *Manania uchidai* showing the claustrum. **(B)** Cross section of the peduncle of *Calvadosia cruciformis* with the presence of the longitudinal interradial musculature. **(C)** Cross section of the peduncle of *Calvadosia cruciformis* without longitudinal interradial musculature. Abbreviations: AP, accessory radial pocket; CL, claustrum; GD, gonad; MP, main radial pocket; MU, musculature. Figures modified from Miranda et al. (2016b).



Figure 4. Phylogeny of Staurozoa modified from Miranda et al. (2016a). The genera *Depastrum*, *Halimocyathus*, *Stylocoronella*, and *Kyopoda*, as well as the family Kyopodidae are not represented in the phylogeny, since phylogenetic analyses considering these lineages have not yet been carried out; “?” represents these groups, with their position hypothesized from morphological evidence.

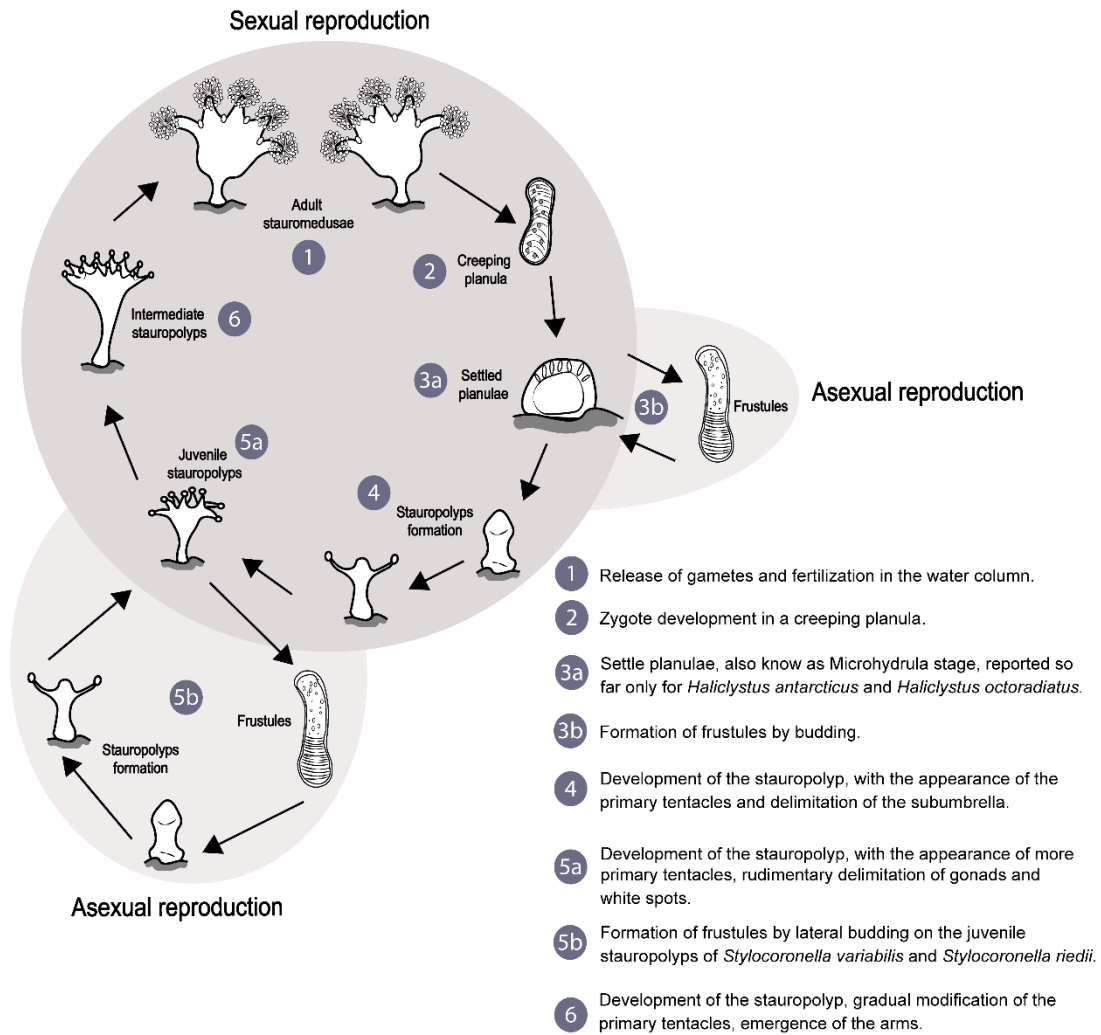


Figure 5. Hypothetic life cycle for Staurozoa, modified from Miranda et al. (2010).

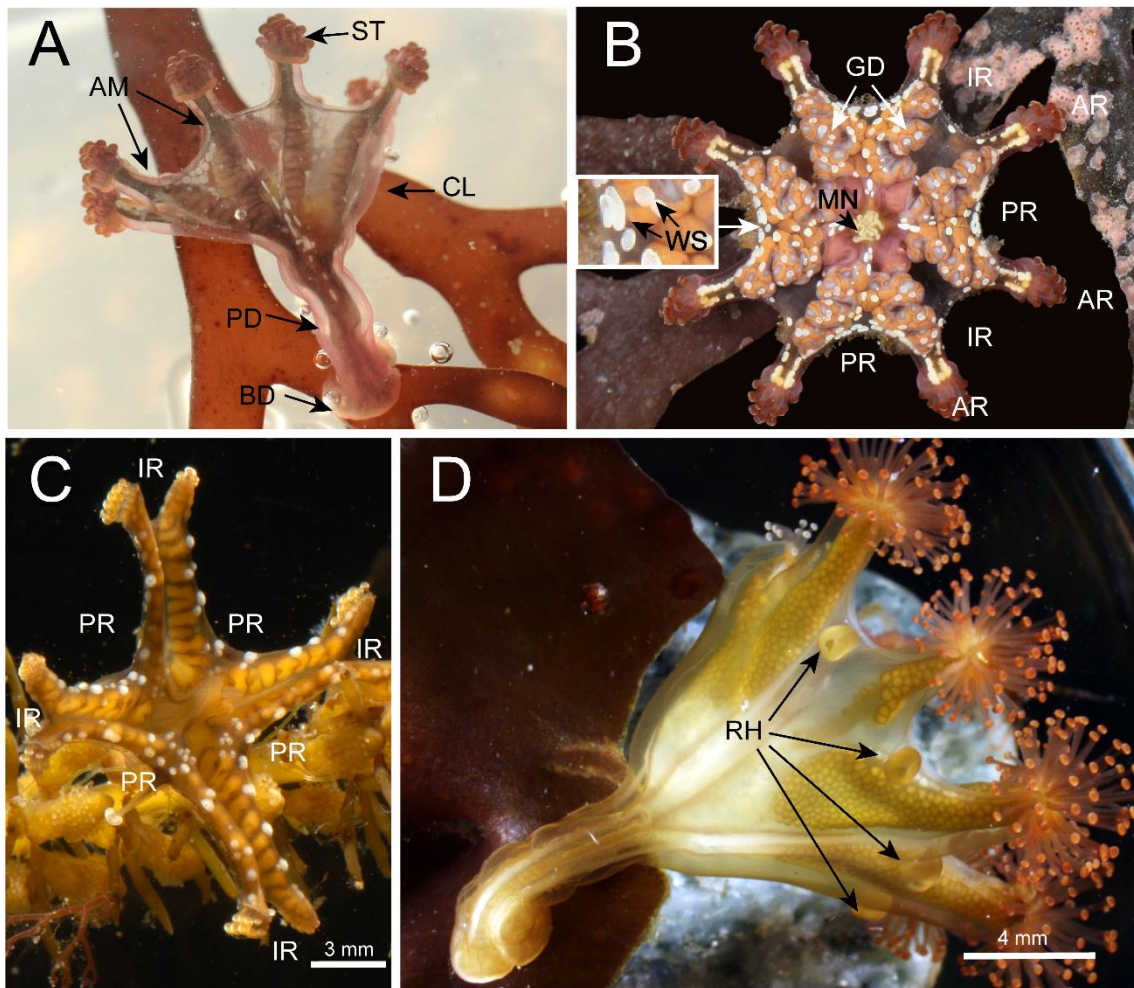


Figure 6. General external morphological aspects for Staurozoa. **(A)** Lateral view of *Calvadosia cruxmelitensis* (photo credit: David Fenwick; scale not available). **(B)** Oral view of *C. cruxmelitensis*; inset: white spots in detail (photo credit: David Fenwick; scale not available). **(C)** Oral view of *Calvadosia cruciformis*, species with paired arms in the interradia (photo credit: Yayoi Hirano). **(D)** Lateral view of *Haliclystus 'sanjuanensis'* (photo credit: Hannah Westlake). Abbreviations: AM, arms; AR, adradia; BD, basal disc; CL, calyx; GD, gonads; IR, interradia; MN, manubrium; PD, peduncle; PR, perradia; RH, rhopalioids; ST, secondary tentacles; WS, white spot.

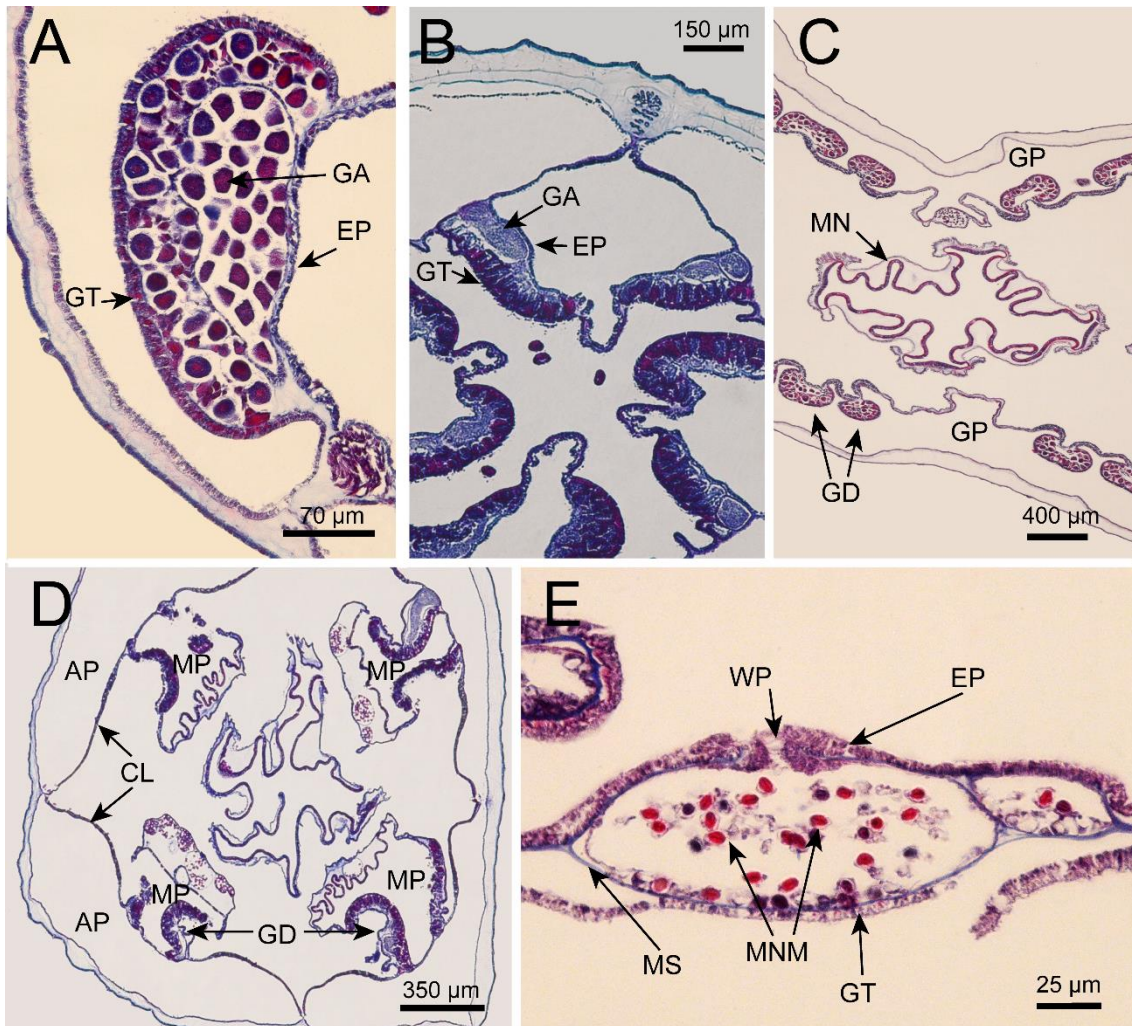


Figure 7. General internal morphological aspects for Staurozoa. (A) Cross section of female gonadal vesicle of *Haliclystus tenuis* with peripheral layer of immature oocytes, adjacent to gastrodermis, and a central layer of mature oocytes, adjacent to epidermis. (B) Cross section of the calyx of *Manania uchidai*, gonadal content between a layer of gastrodermis (adjacent to spermatocytes) and epidermis (adjacent to spermatozoa). (C) Cross section of *H. tenuis* in the manubrium region showing the four gastric radial pockets. (D) Cross section of *M. uchidai* in the manubrium region showing the claustrum dividing the gastrovascular cavity into four accessory radial pockets and four main radial pockets. (E) Longitudinal section of white spots of *Craterolophus convolvulus*, showing a thin layer of mesoglea between the gastrodermis and epidermis, interrupted by a well-defined pore, a peripheral layer of immature nematocysts and loose mature nematocysts clustered in the central region. Abbreviations: AP, accessory radial pocket; CL, claustrum; EP, epidermis; GA, gametes; GD, gonad; GP, gastric pocket; GT, gastrodermis; MN, manubrium; MNM, mature nematocysts; MS, mesoglea; MP, main radial pocket; WP, white spot pore. Figures modified from Miranda et al. (2016b).

Morphology and evolution of white spots of nematocysts in Staurozoa (Cnidaria)

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Abstract

The presence of white spots of nematocysts is a synapomorphy of Staurozoa. These structures are sacs containing central loose nematocysts and a peripheral layer of nematoblasts and are distributed over the subumbrella of stauromedusae. The internal space is delimited by a thin layer of mesoglea, adjacent to one layer of epidermis (externally) and one layer of gastrodermis (internally). The function of the white spots has been poorly discussed in the literature, but there are two main hypotheses: one suggests that this structure plays a role in defense and attack, with active release of nematocysts; the second proposes that the white spots are selective reservoirs of nematocysts that would mature and migrate out to the secondary tentacles. Further evidence, the presence of a well-delimited pore in the epidermis of white spots, favors the first hypothesis. Presence and distribution of white spots on subumbrella are relevant for the identification of species of *Halicyclstus*, although the trait has not been investigated for taxonomic use in other staurozoan genera. The present study aims to (1) analyze the neuromuscular morphology of white spots, with inferences about their function, (2) explore their taxonomic use in all genera of the class, and (3) investigate their evolution from phylogenetic signal tests and ancestral state reconstruction analyses. Based on the images obtained through confocal laser scanning microscopy, we identified muscle fibers running meridionally from the apical central spot towards the opposing pole of the white spot, as well as neurites around them, these being putative sensory cells. This organization, associated with a well-defined pore in the epidermis of white spots,

corroborates the hypothesis of the ability of these animals to actively release the nematocysts from these sacs to the external environment. The presence of white spots in the perradial axis is a conserved trait in Staurozoa, but several independent losses of interradial white spots occurred in the class, mainly in Haliclystidae. Total loss of white spots in both radii occurred only once, in the ancestral of *Haliclystus antarcticus* and *Haliclystus auricula*. Finally, we present a possible taxonomic use of white spots based on its position in the perradii and interradii. Advances obtained from this project open innovative perspectives for other studies, such as nematocyst firing mechanisms and the functional difference between nematocysts found in the white spot nematocysts and secondary tentacles.

1. Introduction

Staurozoa is a class of Cnidaria (Marques & Collins, 2004), represented by approximately 50 valid species, probably the least known and studied among Cnidaria (Miranda et al., 2018). Most species of this class occur in temperate and polar waters, in intertidal or shallow subtidal regions (Miranda et al., 2018). These animals have a life cycle with two main generations, similarly to other medusozoans, called the stauropolyp and stauromedusa (Wietrzykowski, 1912; Kikinger & Salvini-Plawen, 1995; Miranda et al., 2010, 2013). However, the metamorphosis from stauropolyp to stauromedusa is peculiar, occurring mainly (but not exclusively) in the apical region with retention of the basal polypoid peduncle in the medusoid sexual stage (Hirano, 1986; Kikinger & Salvini-Plawen, 1995; Miranda et al., 2010). Because of this characteristic life cycle, the stauromedusae are also known as stalked jellyfishes.

Although staurozoans have a somewhat simple body plan organization, with an outer layer of epidermis, an intermediate layer of mesoglea, and an inner layer of gastrodermis (lining the gastrovascular cavity) (Miranda et al., 2013, 2016a; Holst et al., 2021), there are regions of morphological complexity, with specific tissues specialized for certain functions, such as the white spots of nematocysts (Fig. 1A–D). These structures, often named in the literature as “nematocyst sacs” (Uchida, 1933; Uchida & Hanaoka, 1933, 1934; Carlgren, 1935, 1938; Gwilliam, 1956) and also erroneously as “nematocyst batteries” (Kling, 1879; Antipa, 1893; Gross, 1900; Kassianow, 1901; Carlgren, 1930; Berrill, 1962), consist of a group of nematocysts surrounded by a thin layer of mesoglea, between the epidermis and gastrodermis (Fig. 1E; Weill, 1925: figure

2; Uchida & Hanaoka, 1933; Miranda et al., 2016a). In these structures, there is a peripheral layer of immature nematocysts, adjacent to gastrodermis, and mature nematocysts clustered in the central region (Fig. 1E; Miranda et al., 2016a). The white spots are distributed on the subumbrellar surface, and are frequently associated with the gonads, calyx margin, and arms (Fig. 1A, B; Hirano, 1986, 1997; Holst et al., 2021).

Recent studies have provided advances in the morphofunctional understanding of white spots. Miranda et al. (2016a) revealed a well-delimited pore in the epidermis of white spots, with a thick central area in the epidermis associated with mesoglea interruption (Fig. 1E). This complex organization corroborates the hypothesis that the white spots can actively release their nematocysts (Fig. 1F, G) for defense or prey capture (Kling, 1879; Kassianow, 1901). In addition, the peripheral layer of nematoblasts in the white spots (Miranda et al., 2016a) corroborates that it is also a site of nematocyst production (Leuschel, 1932). On the other hand, this evidence, associated with the migrating internal layer of nematocysts (Miranda et al., 2016a), contradicts previously proposed hypotheses that relate these structures to the storage and maturation of nematocysts produced in the wall of infundibulum and maturation of nematocysts that are produced in the epidermis of the infundibulum and migrate out to the secondary tentacles (Weill, 1925, 1935). However, despite recent progress, there are specific aspects of the morphology of white spots that are still unknown, such as the mechanisms that control and open the pore.

The presence of white spots is considered a potential synapomorphy of the class (Miranda et al., 2016a), although the distribution of nematocysts on the subumbrella varies among species and genera (Hirano, 1986, 1997; Kahn et al., 2010). Specifically for the genus *Haliclystus* James-Clark, 1863 information on the presence/absence and distribution (e.g. perradial and/or interradian) of white spots on the subumbrella are used in species identification (Hirano, 1997; Kahn et al., 2010). However, the taxonomic potential of white spots in other staurozoan genera has never been analyzed. Similarly, there is no information regarding the evolutionary history of white spots in order to understand the ancestral condition of this character in different staurozoan lineages, as well as possible gains and/or losses or shifts in the white spots organization that may have occurred during the evolution of the group.

This study aims to (1) describe specific aspects of the morphology of white spots and discuss their function, using confocal laser scanning microscopy (CLSM); (2) review

the occurrence and distribution of these structures in all species of the class, based on the data available in the literature, investigating possible taxonomic use; (3) understand the evolution of white spots in Staurozoa, based on phylogenetic signal and ancestral state reconstruction analyses.

2. Material and Methods

2.1. Morphology

We investigated the neuromuscular organization of white spots from individuals of the species *Manania handi* Larson & Fautin, 1989 and *Halichystus 'sanjuanensis'* *nomen nudum*. The specimens were collected by hand at low tide in a bay near Chinese Cemetery in Victoria, British Columbia (Vancouver Island, Canada; latitude: 48°24'22.88''N, longitude: 123°19'24.48''W), between May and September of 2013–2015, under Scientific License XR 30 2013-15 issued by Department of Fisheries and Oceans Canada (see Westlake & Page, 2017). The animals were anaesthetized at 12°C, substituting gradually the local seawater with artificial seawater containing an elevated concentration of magnesium ions and reduced concentration of calcium ions (Audesirk & Audesirk, 1980).

The immunohistochemistry antibodies (anti-FMRFamide, anti- α -tubulin, anti-tyrosinated tubulin, and anti-glutamate) were used to label neurons and fluorophore-tagged phalloidin to label the F-actin of muscle fibers of the selected species. For more details on the laboratory maintenance, anaesthesia, immunolabeling, and phalloidin labeling process, see Westlake & Page (2017).

Specimens were mounted on glass slides in glycerol for imaging (see Westlake & Page, 2017). A Zeiss Axioskop compound microscope with an epifluorescence attachment and a Retiga 2000R digital camera with QCapture Pro software (version 6.0.0.412) or a Leitz Aristoplan compound microscope with an epifluorescence attachment and a Leica MC170 HD camera and Leica Application Suite V4.4 software were used to obtain images (see Westlake & Page, 2017). Confocal laser scanning microscopy (CLSM) images were made with a Nikon Eclipse TE2000-U microscope with EZ-C1 3.60 software or Olympus BX 61 WI CLSM with Olympus FluoView FV10-ASW version 1.7c software (see Westlake & Page, 2017). Image editing follows Westlake & Page (2017).

2.2. Review of white spots distribution in Staurozoa

A thorough review of data available in the literature was conducted. The review includes information on morphological (external and internal anatomy) and functional descriptions, presence/absence of white spots, and distribution patterns (perradial/interradial) of white spots on the subumbrella, for all species of Staurozoa (Table S1). Additional observations on images and preserved material for some species complemented this review (Table 1; Table S1, “this study”).

2.3. Evolution of white spots

From the staurozoan dated tree (obtained from Miranda et al., 2016b and preliminary results from Morales-Guerrero et al., *in prep.*), two analyses were performed in R version 4.1.0 (R Core Team 2021): ancestral states reconstruction and phylogenetic signal, using the package corHMM (Boyko & Beaulieu, 2021) and the code provided by Borges et al. (2019) in the GitHub branch: mrborges23/delta_statistic, respectively. For both analyses only the clade Staurozoa (28 terminals) was under investigation, so the outgroups were pruned using the package geiger (Pennell et al., 2014). We investigated the trait “distribution of white spots of nematocysts on the subumbrella”, which based on our review of the literature (Table S1), can be (1) absent, (2) present in the perradii and interradii, or (3) present only in perradii (Table 2). The evobiR package (Blackmon & Adams, 2015) was used to incorporate polymorphisms into the terminals, specifically the corresponding terminal of the *Haliclystus inabai* species.

Phylogenetic signal analysis follows the methodology recently proposed by Borges et al. (2019), which is a suitable alternative for discrete traits. The method uses the value of δ as a measure of phylogenetic signal: the higher the value δ ($\delta > 1$), the higher the level of phylogenetic signal of the discrete trait under investigation (Borges et al., 2019). We generated 1,000 random trees in order to investigate whether the value obtained in the δ statistic is significant, keeping the same terminals and proportion of character states. Subsequently, the value of δ for the simulated trees was calculated. If the value of δ obtained from the empirical analysis exceeds the 95% quantile of the simulated data, we can infer that the phylogenetic signal obtained empirically is significant (Borges et al., 2019). Akaike information criterion (using AICc correction and AICw) was

employed to fit the Mk models (Harmon, 2018). Three models with different transition rate were tested: (i) equal rates model (ER), which implies that all transitions rates have the same parameter, (ii) all rates different model (ARD), which means that all transitions rates have different parameters, and (iii) the symmetrical model (SYM), where symmetric transitions rates (forward and reverse) share the same parameters (Pennell et al., 2014). The best fit Mk model was used to reconstruct the ancestral states for each node in the dated tree using maximum likelihood method by marginal reconstruction (Joy et al., 2016).

3. Results

3.1. Morphology

3.1.1. Musculature

Halichystus 'sanjuanensis'

White spots in *H. 'sanjuanensis'* are present in the perradii only, forming a line along the perradial edges of the gonads and rarely also a few near the calyx margin (Figs. 1A, 2A; Table S1). All muscles were composed of smooth fibers; muscle bands were labeled with fluorophore-tagged phalloidin and exposed by cuts showed no evidence of striations (see more details of muscular organization in Westlake & Page, 2017). The radial musculature in the perradii is diffuse and indirectly associated with the perradial white spots. These radial muscle fibers overlaid the marginal halves of the white spots that protruded into the perradial region, but do not extend over the apices of the white spots (Fig. 2B). White spots were labeled more strongly with fluorophore-tagged phalloidin than other non-muscle tissue (Fig. 2B–D). A spot corresponding to the thick epidermis of the central portion of white spots, where the pore is located (Fig. 1E; see Miranda et al., 2016a) were labeled particularly intensely with phalloidin (Fig. 2B–D).

Manania handi

White spots in *Manania handi* were present along the calyx margin, both in perradii and interadii, and numerous white spots were present above the perradial gonads (Fig. 3A; Table S1). All muscles of *Manania handi* were also composed of smooth fibers,

and muscle bands were labeled with fluorophore-tagged phalloidin (see more details of muscular organization in Westlake & Page, 2017).

The distribution of white spots along the entire margin of the calyx and over the gonads in the perradii causes an indirect association with three muscle groups: the interradiial longitudinal, marginal, and perradial muscles. The apices of the white spots in the margin occupied a gap between the perradial muscle fibers (Fig. 3B). The final branches of the longitudinal muscle framed a triangular segment of tissue occupied by white spots near the calyx margin in each interradius (Fig. 3C). These final muscle branches always deviated around the apices of the white spots (Figs. 3B, C). The perradial muscles of *M. handi* ran in a tight band along the top of each principal radial pocket (inside which the gonads are located), overlying the white spots that are distributed above the perradial gonads (Fig. 3D). The apices of the white spots along the top of the principal radial pockets lay to either side of the muscle band (Fig. 3D). Near the edge of the calyx, the bundled perradial muscles branched out and anastomosed as they extended to the margin (Fig. 3D–F). Branches of perradial muscle either deviated around the apices (Fig. 3E) of the white spots or terminated on one side of the apex to begin on the other side, leaving a gap where the white spot was connected to the subumbrella (Fig. 3F).

Similar to *H. 'sanjuanensis'*, white spots of *M. handi* were labeled with fluorophore-tagged phalloidin. In addition, CSLM images revealed that each white spot was englobed by muscle fibers running meridionally from the apical central spot towards the opposing pole of the white spot (Fig. 3E, F); these muscle fibers were specific to the structure. The central spot (= pore) was brightly labeled with phalloidin (Fig. 3B, D–F); tangential views revealed what looked like a short, twisted tube leading from the white spot to the subumbrellar surface (Fig. 3D, inset).

3.1.2. Nervous System

Both *H. 'sanjuanensis'* and *H. handi* were immunoreactive with antibodies against FMRFamide and α -tubulin, but were not labeled by antibodies against glutamate or tyrosinated tubulin. While FMRFamide and α -tubulin antibodies each uniquely labeled some components of the nervous system, many neurites of the subumbrellar network were co-labeled with both antibodies. FMRFamide antibodies revealed neuronal cell bodies, neurites, and putative sensory cells. Sensory neurons and cell bodies were not strongly

labeled by α -tubulin antibodies. In addition to neurites, α -tubulin antibodies labeled non-neural components including nematocysts.

Halichystus 'sanjuanensis'

Neurites extended from the corners of the manubrium along the top of the radial pockets to the margin, following the radial musculature (Fig. 4A). Innervation of the perradial muscles in *H. 'sanjuanensis'* comprised a loose network (Fig. 4A). Tracts of strongly FMRFamide-immunoreactive (IR) neurons framed the white spots along the gonads on either side of the perradius (Fig. 4B). These tracts ran along the line of white spots and circumvented the central (phalloidin-labeled) spot, wrapping around it in a ring (Fig. 4B, C). Neuronal cell bodies were more numerous in this ring than in other areas and may be sensory (Fig. 4D, E). Alpha-tubulin antibodies co-labeled many neurites of the tracts, but did not co-label those FMRFamide-IR neurites that extended over the gonads to wrap around the white spots (Fig. 4D–F). Alpha-tubulin antibodies labelled the apex of the white spots as phalloidin did (Fig. 4D, F). Near the margin, the neurites innervating the perradius and white spots were continuous with neurites of the marginal muscle (Fig. 4A, B).

Manania handi

The neurites overlying the perradial muscles of *M. handi* were generally parallel to the muscle fibers (Fig. 5A). Neurites of this network also innervated the many white spots that occur above the perradial gonads (top of the principal radial pocket) (Fig. 5A). Each white spot apex was α -tubulin-IR and was encircled by FMRFamide-IR neurons (Fig. 5B). These neurites did not form a distinct tract extending from white spot to white spot as they did in *H. 'sanjuanensis'* (Fig. 5C, D). The perradial network deviated around the apex of the white spots and a ring of putative sensory neurons encircled the apex (Fig. 5C–F). The white spots along the interradian margin were similarly innervated.

3.2. Review of white spots distribution in Staurozoa

Craterolophus

Craterolophus convolvulus has white spots mainly along the perradii, above the gonads (subumbrellar epidermis of principal radial pocket) from the perradial calyx margin to the corners of the manubrium; white spots also present at the arm tips, laterally at both sides; few white spots (1–5) present in the interradial margin of the calyx (Figs. 6A, 7A; Table S1). There is no information in the literature regarding the presence of white spots in *Craterolophus macrocystis* (Table S1).

Depastromorpha

Depastromorpha africana has two rows of white spots above the perradial gonads (subumbrellar epidermis of principal radial pocket), going deeper in the calyx in the perradii (Fig. 6B, 7B; Table S1). In larger specimens some additional white spots can be present between and/or outside the rows, changing the regular organization (Table S1).

Depastrum

Depastrum cyathiforme has two irregular rows of white spots above the perradial gonads (subumbrellar epidermis of principal radial pocket), going deeper in the perradial surface of calyx (Fig. 7C; Table S1). Some specimens observed in British Museum (Table 1) have a random distribution of white spots in the perradial region above the gonads, not organized in rows.

Lipkea

Species of the genus *Lipkea* have white spots widespread on the subumbrella (Figs. 6C, 7D; Table S1). Smaller white spots generally on the inner surface of the calyx, both in the interradial and perradial. Larger white spots on the calyx margin (5–8 in *Lipkea sturdzii*, Antipa (1893)), marginal lappets, and near the gonads (in mature specimens) and manubrium (Table S1).

Calvadosia

Species of *Calvadosia* have four patterns of white spots distribution on the subumbrella (Fig. 6D-H, 8; Table S1). The first pattern is represented by white spots occurring along the calyx margin (mainly in the perradii, but also in the interradii), including the lateral tips of the arms, and above the gonads (subumbrellar epidermis of gastric radial pocket), whether the gonads are positioned on the adradial or interradii/adradial (Fig. 8A). Several white spots are present on the perradial subumbrellar sectors and close to the manubrium corner regions (Fig. 8A). Few white spots may also occur in the interradii sectors and close to interradii face of manubrium in some species (Fig. 8A; Table S1). This pattern is represented by *Calvadosia campanulata* (Fig. 6D), *Calvadosia cruxmelitensis* (Fig. 6E), *Calvadosia corbini*, *Calvadosia lewisi* (Fig. 6F), *Calvadosia tasmaniensis* (Fig. 6G), and *Calvadosia* sp. 2 NZ (see Miranda et al., 2016b). There are some differences regarding the distribution of white spots in this group of species. *Calvadosia cruxmelitensis* (Fig. 6E) has the highest concentration of white spots in oral view and it is possible to notice a characteristic “Maltese Cross” made of white spots (see species diagnosis by Corbin, 1978; Table S1). In *C. campanulata*, *C. cruxmelitensis*, and *C. tasmaniensis*, occasionally few white spots may also occur in the interradii sectors and close to interradii face of manubrium (Fig. 6D; Table S1). Specimens of *C. lewisi* seem to have an intraspecific variation in the concentration of white spots in the perradii (Miranda et al., 2017a, figure 1A; Table S1).

The second pattern is represented by *Calvadosia cruciformis* (Fig. 1B) and *Calvadosia nagatensis* (Fig. 8B; Table S1). The white spots are mainly distributed (more abundant) along the perradial margin of the calyx and arm tips, a few entering into the perradial calyx inner sectors; white spots also present in the interradii margin of the calyx and arms tips (Fig. 8B). Rarely, few white spots are present on the gonadal region of both species.

A third pattern, represented only by *Calvadosia vanhoeffeni*, has white spots mainly on perradial sectors, but a few white spots also occur in the interradii region adjacent to the gonads and along the calyx margin, especially near the base of tentacular clusters (Fig. 8C; Table 1; Table S1).

The fourth group has another unique pattern of white spots distribution on the subumbrella for the genus *Calvadosia* (Fig. 8D; Table S1). *Calvadosia* sp. 1 NZ (Miranda et al., 2016b) is the only species of this group. It is characterized by the presence of white

spots only on the perradial sectors, extending from the margin of the calyx to region near the manubrium.

There is not enough information in the literature to classify *Calvadosia hawaiiensis*, *Calvadosia tsingtaoensis*, and *Calvadosia capensis* in any of these patterns (Table S1). However, *C. capensis* has white spots distributed along calyx margin and arms (Zagal et al., 2011; Miranda et al., 2012; Table S1) and according to Carlgren (1938 p. 5, 6) the organization of the white spots would be similar to *C. campanulata*. *Calvadosia tsingtaoensis* is morphologically similar to *C. cruciformis* and *C. nagatensis* (Ling, 1937; figures 20–22) and possibly has the pattern of white spots distribution on the subumbrella similar to the other species of group 2 (Fig. 8B; Table S1).

Haliclystus

The first group includes two species, *Haliclystus antarcticus* and *Haliclystus auricula*, which do not have white spots on the subumbrellar surface (Fig. 9A; Table S1).

The second group has white spots only on the calyx margin, both in the perradii and in the interradii (Fig. 9B; Table S1). *Haliclystus borealis* (Fig. 6H) has a few white spots along the margin, may generally two on both sides of the anchors and two on the inner side of the arms (Uchida & Hanaoka, 1934; Table S1). *Haliclystus californiensis* has more white spots (5–10 in number, between each arm) with a homogeneous distribution across the entire calyx margin (Table S1). Finally, *Haliclystus sinensis* possesses white spots scattered near calyx margin (Table S1).

The third group is composed by *Haliclystus inabai* (Fig. 6I, 9C) and has white spots on the perradial inner surface (sectors) of the calyx and along the perradial margin of the calyx (Table S1). Some authors reported the rare presence of white spots also in the interradii region of calyx margin (Fig. 6I, 9C; Table S1, see Holst et al., 2019), or describe dubiously the presence of white spots in this region (Table S1, see Kishinouye, 1902; Uchida, 1929). In this study, based on our observations, we confirm the presence of a white spot in the center of the interradii calyx margin in few specimens, however, the majority of the analyzed specimens have white spots only on the perradii (Table 1; see Miranda et al., 2016b, figure 1L), thus this pattern is liable to polymorphism.

Haliclystus octoradiatus also has a unique pattern of white spots distribution, being the only representative of the fourth group. This species has few white spots along the interradial margin of the calyx (1–3), usually near the base of the arms and along perradial edges of gonads, not getting close to the manubrium. Few white spots might also occur on perradial sectors (Fig. 9D; Table S1).

Similar to *H. octoradiatus*, the representatives of the fifth group, *H. 'sanjuanensis'* (Fig. 1A, 2A) and *Haliclystus stejnegeri*, also have white spots on the perradial edges of the gonads, but they generally lack white spots in the interradia (Fig. 9E; Table S1). In *H. 'sanjuanensis'*, the white spots distribution produces a sharp perradial line from the tips of the arms to the base of manubrium. In some specimens of *H. 'sanjuanensis'*, few white spots can be seen near to the perradial calyx margin (Fig. 6J) and rarely few along the perradial and interradial (very rarely) margins (Table S1). The white spots are numerous in larger animals (Westlake, 2015, p. 42). *Haliclystus stejnegeri* has relatively sparse white spots, along the edge of gonads near calyx margin (not reaching near the manubrium), and in some specimens rarely one white spot can be seen in the interradia (Table S1; see Hirano, 1986).

The sixth group has white spots distributed in the margin of the calyx, both in the interradia and in the perradia (more numerous in the perradia), as well as in the perradial subumbrellar sectors (Fig. 9F; Table S1). Only *Haliclystus tenuis* belongs to this group.

There is no information in the literature regarding the presence and distribution of white spots in *Haliclystus kerguelensis* and *Haliclystus monstrosus* (Table S1). However, some specimens of the latter observed in British Museum (Table 1) have small and sparse white spots distributed in perradia and interradia. *Haliclystus salpinx* does not have a precise description regarding the distribution of white spots, although they are scattered on the subumbrella (Table S1). Since the information on white spots distribution is imprecise for these three species, no patterns are proposed for them.

Manania

Most species of *Manania* have a pattern of white spots distribution on the subumbrella similar to the pattern described for *C. convolvulus* (Fig. 6A, 7A). The white spots occur along the calyx margin, both in the perradia and interradia, and are more

numerous in perradii, covering most of the subumbrellar epidermis of principal radial pocket, above the perradial gonads (Fig. 10A; Table S1). *Manania handi* (Fig. 3A) presents a single continuous row of white spots bordering the calyx margin (Table S1).

All species of the genus have the pattern described above (white spots along the calyx margin and above the gonads), except *Manania uchidai* (Fig. 6K), which lacks white spots on the calyx margin. Therefore, the second pattern of white spots distribution includes only *M. uchidai* (Figs. 6K, 10B) with white spots densely distributed over the perradial gonads (subumbrellar epidermis of principal radial pocket), heading towards the manubrium, a pattern very similar to that described for the genus *Depastrum* and *Depastromorpha* (Figs. 6B, 7B, C, 10B; Table S1).

There is no information in the literature regarding white spots in *Manania hexaradiata*, but additional observations (Table 1) indicate that the white spots occur in a pattern similar to the one described for group 1 (Fig. 10A; Table S1).

Lucernaria

The information in the literature regarding the presence and distribution of white spots in *Lucernaria* is incomplete (Table S1). Although most of the species of this genus have white spots on the subumbrella (Fig. 6L; Table S1), there is no information regarding the presence/absence of these structures in *Lucernaria haeckeli* and *Lucernaria infundibulum* (Table S1). According to the literature, all species of the genus (with the exception of those species mentioned above) have white spots in both the perradii and interradii, distributed rather homogeneously on the subumbrellar surface. Therefore, based on the literature the white spots distribution is somewhat uniform in this genus.

Halimocyathus, *Kyopoda*, and *Stylocoronella*

White spots are present in all three of these genera, but descriptions of the distribution of white spots on the subumbrella are imprecise (Table S1) and, therefore, no patterns are proposed for these genera.

3.3. Evolution of white spots

No phylogenetic signal obtained in the distribution of white spots on the subumbrella of staurozoan species. The value of entropy $\delta = 10,929$ from the empirical analysis exceeds the 95% quantile of the simulated data ($\delta = 11,162$), so it can be inferred that the phylogenetic signal obtained empirically is not significant. This result indicates that closely related species do not necessarily have similar states, thus the distribution of white spots in the subumbrella is a labile trait in the evolutionary history of the class Staurozoa.

The best fit Mk model for the three-state trait was the symmetrical (SYM) (Table 3). The ancestral state reconstruction (Fig. 11) revealed that the last common ancestor of the stalked jellyfishes has the white spots in the perradii and interradii, a conserved characteristic throughout the staurozoan phylogeny. Several independent losses of white spots occurred in the interradii, more specifically, once in the genus *Calvadosia* (*Calvadosia* sp. 1 NZ), and at least four times in the family Haliclystidae: in *Manania uchidai*, *Depastromorpha africana*, *Haliclystus stejneri*, and in the common ancestor of the clade composed of *Haliclystus* ‘*sanjuanensis*’, *H. auricula*, and *H. antarcticus*. *Haliclystus inabai* has the potential to represent the fifth loss of white spots in the interradii in the family Haliclystidae, although it is polymorphic in relation to the distribution of white spots in the subumbrella, most of the specimens analyzed, have white spots only in the perradii. The clade composed by *H. antarcticus* and *H. auricula* is the only that showed a complete loss of white spots, in both perradii and interradii. This may indicate a possible initial loss of white spots in the interradii (last common ancestor of *H. ‘sanjuanensis*’, *H. antarcticus*, and *H. auricula*) followed by a loss in the perradii (last common ancestor of *H. antarcticus* and *H. auricula*).

4. Discussion

4.1. Morphology and function

4.1.1. Neuromuscular anatomy of white spots

The white spots are a synapomorphy of Staurozoa, present in all genera (Table S1; Miranda et al., 2016a). The process of white spots production during development is still obscure but reports in the literature mention that they are formed either by invagination of the subumbrella (Kassianow, 1901; Weill, 1925) or by delamination of subumbrellar epidermal cells (Leuschel, 1932; Uchida & Hanaoka, 1933). White spots appear early in development, well before the gonads differentiate (Wietrzykowski, 1912;

Hirano 1986) or simultaneously (Uchida, 1929; Hirano, 1986). The white spots appear as bubbles sandwiched between the subumbrellar epidermis and gastrodermis, surrounded by a thin layer of mesoglea except at the apex where the wall of the white spots is continuous with the subumbrellar epidermis (Fig. 1E; Miranda et al., 2016a). The nematocysts in the lumen of the white spots are similar in size and are loose and disorganized within the sac (Fig. 1E, F; Kassianow, 1901; Wietrzykowski, 1912; Weill, 1925; Miranda et al., 2016a), spilling out freely when the tissue of the white spot is pierced (Weill, 1925; Westlake, 2015; personal observations).

There are three hypothetical functions for the white spots in the literature: 1) a reservoir for nematocysts that will be used to replenish the secondary tentacle nematocysts when they are depleted (Weill, 1925, 1935); 2) a site for nematocyst production (Leuschel, 1932); or 3) structures for defense and/or attack (Keferstein, 1862; Kling, 1879; Kassianow, 1901; Uchida & Hanaoka, 1933; Berrill, 1962), specifically for protecting the gonads (Kassianow, 1901). Weill (1925) believed that all nematocysts were produced in the infundibular wall and the white spots simply served as a maturation area for the nematocysts prior to moving out to the secondary tentacles. Uchida & Hanaoka (1933) reportedly observed nematocysts migrating from the white spots into the gastrodermis and epidermis. However, the nematocysts of the white spots are distinct in size and shape from all other nematocysts (Holst et al., 2019; based on our observations they are birhopaloids, see Fig. 1G; Östman, 2000). On the other hand, Kassianow (1901), Wietrzykowski (1912), and Leuschel (1932) suggested that the white spots nematocysts were produced *in situ*, which was corroborated by recent histological analysis that confirmed the presence of peripheral layer of nematoblasts adjacent to gastrodermis in all species analyzed (Miranda et al., 2016a), which probably move to the interior of the sac when mature (Kassianow, 1901; Uchida & Hanaoka, 1933).

The epidermis around the apex of each white spot is thickened (Miranda et al., 2016a). Kassianow (1901) observed this thickening area in *C. campanulata*, *C. convolvulus*, and *H. octoradiatus*. He also found permanent pores connecting the interior of the white spot to the exterior of the subumbrella. Miranda et al. (2016a) sectioned the white spots of 10 staurozoan species from 5 genera and found an aperture (=pore) (Fig. 1E) connecting the white spots to the subumbrellar surface in *L. bathyphila* and *C. convolvulus*. In our observations of *M. handi* and *H. 'sanjuanensis'*, cells in the apices of white spots were labeled strongly with α -tubulin and phalloidin, indicating that

microtubules and microfilaments may be concentrated in these cells, which might be associated with cell movement involved in opening the pore. A particular group of cells, possibly epitheliomuscular cells, were also observed in the central opening of white spots of *L. bathyphila* (Miranda et al., 2016a, figure 32N–P, V, W).

The wall of each white spot in *M. handi* has muscle fibers running meridionally from the apex to the opposing pole of the sac (Fig. 3E, F). Although this organization was not clearly observed in *H. 'sanjuanensis'*, the walls of its white spots did label vaguely with phalloidin and it is possible that presence of the white spots muscular fibers was obscured by the overlying perradial muscle fibers (Fig. 2B–D). Kassianow (1901) also found meridional muscle fibers in the walls of the white spots in the three species that he studied. In our observations of both *M. handi* and *H. 'sanjuanensis'*, fibers of the various muscle groups adjacent to the white spots deviated around their apices (Fig. 2B, 3B–F), which was also noted by Kassianow (1901), suggesting a conserved need for the apex of the white spot to remain unobstructed.

The apices of the white spots are uniquely innervated; in both *M. handi* and *H. 'sanjuanensis'* a ring of putative sensory neurons is borne on the raised epithelium and surrounds the center of the apex (Figs. 4B–E; 5B–F). Kassianow (1901) also found a ring of neurites and a concentration of sensory neurons encircling the pore in the three species he studied.

Based on our observations, we suggest that the loose nematocysts in the white spots would be expelled through a pore by muscular activities (since the pore region of the white spots in both species was particularly intensively labeled with phalloidin) of the white spot apices and walls upon reception of a stimulus by the sensory cells surrounding the aperture, corroborating the hypothesis proposed by Kassianow (1901). Since the presence of the white spots are highly conserved in staurozoans (see “Evolution of white spots” section below) it is likely that they are engaged in important functions. Kassianow (1901) assumed that upon stimulation the nematocysts were ejected through the pore for defense or attack, and we support this hypothesis, although functional evidence in the literature is scarce. Kling (1879) observed that the irritation of the animals led to massive discharge of the nematocysts from the white spots. Berrill (1962) reported that *Artemia* were killed immediately when they came into contact with the white spots of *L. quadricornis*. Therefore, the white spots play two functions, producing and releasing the

nematocysts. Based on the complex organization of white spots, we assume that this structure can be used many times, although further investigation is necessary.

4.1.2. White spots exit pore

As was mentioned above, an exit pore in the white spots has already been reported for some species, such as *C. campanulata*, *C. convolvulus*, *H. octoradiatus*, and *L. bathyphila* (Kassianow, 1901; Miranda et al., 2016a). However, according to Leuschel (1932) the opening is not present, and the white spots are only a place where “ovoid” nematocysts are produced (Uchida & Hanaoka, 1933). Curiously, Leuschel (1932) did observe that epidermal cells were irregularly arranged in the white spots apices (Uchida & Hanaoka, 1933), i.e., the thickened region of white spots associated with the pore region (Miranda et al., 2016a). Therefore, it is likely that Leuschel (1932) did not observe opened pores, but only closed white spots (Miranda et al., 2016a, for example, only observed closed white spots for 8 out of 10 analyzed species). This data strengthens the transient nature of the pore, whose opening might be controlled by muscular action, similar to a sphincter or involving particular cell movement. Additional ultrastructural studies on the white spots are necessary to analyze the epitheliomuscular cells associated with the white spots apices in detail.

4.1.3. How do the nematocysts of white spot become functional?

Our results corroborate the hypothesis that the white spots can actively release their internal nematocysts as a form of defense or prey capture (Kling, 1879; Kassianow, 1901; Uchida & Hanaoka, 1933). However, it is not clear how the loose nematocytes would be functional as they apparently lack a cnidocil (Weill, 1925; Westlake, 2015) and are not organized in a certain direction (differently from the nematocytes from the secondary tentacle and from exumbrellar batteries, for example; Miranda et al., 2016a).

Nematocytes generally have a cnidocil, a small spine projecting from the apex of the nematocyte often surrounded by a cone of sensory stereocilia (Slautterback, 1967; Westfall, 1970; Mariscal & Bigger, 1976; Hausmann & Holstein, 1985; Bezares-Calderón et al., 2020). The cnidocil responds to physical and chemical stimulation (Watson & Hessinger, 1987; Thorington & Hessinger, 1990; Watson & Mire, 2000;

Scappaticci et al., 2010; Plachetzki et al., 2012) playing a triggering role in nematocyst discharge.

Nematocytes lacking a cnidocil are also present in another group of cnidarians, the Myxozoa (Siddall et al., 1995; Atkinson et al., 2018). Myxozoans are obligate endoparasites that have complex life cycles, with vertebrate and invertebrate hosts, with transmission via microscopic waterborne spores (Kent et al., 2001; Lom & Dyková, 2013). How the nematocysts from these animals are discharged is still under debate, but it is hypothesized that inosine (present in the mucus of fish, one of the myxozoan common hosts) facilitates nematocyst discharge by inducing conformational changes in transmembrane channels, thereby allowing the influx of water or the exocytosis of calcium (Americus et al., 2020). In *Hydra*, despite the presence of cnidocil in nematocytes, it was reported that substances released by the prey (*Artemia* or *Daphnia*) after being pierced by nematocysts, stimulate other nematocysts to discharge (Kass-Simon & Scappaticci, 2002). The nematocytes from the white spots of the stalked jellyfishes may also be sensitive to some chemical compound liberated by prey or predators. Further studies are needed to confirm a general absence of cnidocils in the nematocytes of white spots and to understand their triggering mechanism.

4.2. Review of white spots distribution in Staurozoa

Based on the literature review, we verified the presence of white spots in all staurozoan genera, which are frequently present in the perradii and interradii, and in some cases above the gonads especially in the adradii. Below we discuss particular aspects of the white spots and possible taxonomic applications.

4.2.1. Association with the gonads

A close association between white spots and gonads is recurrent in all staurozoan genera (except for the genera *Halimocyathus*, *Kyopoda*, and *Stylocoronella*, which have imprecise descriptions concerning the presence and distribution of white spots on the subumbrella) (Table S1; Zagal et al., 2011; Miranda et al., 2016a, 2017a; Holst et al., 2021), which supports the hypothesis of the role of these structures in protecting the gonadal tissue (Kling, 1879; Kassianow, 1901). However, this association varies in

different species. All genera with a claustrum (Miranda et al., 2016a, 2016b, 2017b), in which the gonads are positioned in the perradii (i.e., *Craterolophus*, *Depastromorpha*, *Depastrum*, and *Manania*) have white spots above the gonadal region (Figs. 6A, B, K; 7A–C; 10), that is, in the subumbrellar epidermis of the principal radial pocket (Miranda et al., 2017b).

In the genera without a claustrum (Miranda et al., 2016a, 2016b, 2017b), the white spots are distributed on the subumbrellar epidermis of gastric radial pocket, which also contains the gonads (Miranda et al., 2017b). Most *Calvadosia* species have white spots above the gonadal region (Figs. 6D–G; 8A), whether the gonads are positioned on the adradial or in the interradial/adradial (species with cruciform calyx, e.g., *C. lewisi*).

Collins & Daly (2005) observed nematocysts and gametes in the gonadal vesicles of *Lucernaria janetae*, which may be indicative of the presence of white spots above the gonads. Carlgren (1930) mentioned the presence of “batteries” of nematocysts on the subumbrella above the genital area in *Lucernaria australis*, although it is not possible to verify whether these structures are in fact nematocyst batteries or white spots (see Miranda et al., 2016a). Thus, some *Lucernaria* species (or specimens) may also have white spots above the gonadal region (Fig. 6L).

Species of the genus *Lipkea* have gonads close to the manubrium, deep in the calyx (Carlgren, 1933; Miranda et al., 2017a, figure 11D, G). It is possible to note the presence of white spots (*Lipkea ruspoliana*: Vogt, 1886, 1887; *Lipkea stephensoni*: Miranda et al., 2017a, figure 11) near the manubrium, and sometimes above the gonads (Miranda et al., 2017a, figure 11D).

We also verified an indirect association of white spots with gonadal region in *H. octoradiatus*, *H. 'sanjuanensis'*, and *H. stejnegeri*. These three species have white spots on the perradial margin of the gonads, but not above them (Figs. 1A; 2A; 6G; 9D, E).

4.2.2. Nematocyst batteries or white spots?

The white spots are often referred to as “nematocyst batteries” by some authors (e.g., Kling, 1879, p. 147: “Nesselbatterien”; Antipa, 1893, p. 621, 623–625, 627, 628: “Nesselbatterien”; Gross, 1900, p. 617: “Nesselbatterien”; Kassianow, 1901, p. 310, 311: “Nesselbatterien”; Carlgren, 1930, p. 7, 9, 14: “Nesselkapselbatterien”, 1933 p. 5:

“Nesselbatterien”; Berrill, 1962, p. 1252, 1255, 1260; Kikinger & Salvini-Plawen, 1995, p. 901), making older literature difficult to decipher, as ‘nematocyst battery’ now denotes simple epidermal clusters of nematocysts (Miranda et al., 2016a) that are common on the exumbrellar surface (peduncle and calyx). However, nematocyst batteries also occur on the subumbrella, as observed for the subumbrellar epidermis of the nodular gonad in *C. corbini* (Miranda et al., 2016a, figure 40H, I).

Kikinger & Salvini-Plawen (1995) described the presence of four nematocyst batteries on the subumbrella of juvenile *Stylocoronella riedli*, but there is no subsequent study to confirm if the structures are in fact nematocyst batteries or white spots, although images of the species in Salvini-Plawen (2006, figure 1) show larger white dots on the (perradial?) subumbrella, similar to the general appearance of white spots.

Species of *Lipkea* have the surface of the subumbrella covered by white spots (see Miranda et al., 2017a, figure 11), with larger spots on the lappets, calyx margin, around or above the gonads, and several small spots in the calyx sectors. Are the smaller spots in fact nematocyst batteries whereas the larger dots are white spots? Studies published so far (Vogt, 1886, 1887; Antipa, 1893; Pisani et al., 2007) did not provide further evidence, although observations in this study show that at least some smaller spots also have the typical thickening of the epidermis typical of the white spots apices (Fig. 6C).

In the description of *Kyopoda lamberti* (Larson, 1988) it is mentioned that small white spots are present not only in the calyx but also in the stalk of the animal. This statement raises doubts as to whether the author observed “true” white spots (in the sense used in this study), since these structures do not occur on stauromedusan peduncle; on the other hand, nematocyst batteries can occur in this region (Miranda et al., 2016a).

The examples mentioned above exemplify the need for more precise and detailed descriptions regarding white spots, especially for the genera *Lipkea*, *Kyopoda*, and *Stylocoronella*, highlighting the need for precise differentiation and attribution of the terms “nematocyst battery” and “white spot”.

4.2.3. Taxonomy

The use of white spots in taxonomy has not been explored in different staurozoan genera. Corbin (1978) reported that the pattern of white spots distribution in *C.*

cruxmelitensis can be used in species identification. Later, more broadly, Hirano (1997) and Kahn et al. (2010) used the presence and position (e.g. perradial *versus* perradial and interradial) of white spots to differentiate *Haliclystus* species.

Our review showed a potential taxonomic use of white spots in most staurozoan genera (Figs. 7–10). It is important to highlight that the groups proposed here are mainly based in the literature (Table S1), complemented by personal observations for some available species (Table 1). Rare variations of the patterns (e.g., the white spots in the perradial margin of the calyx in *H. 'sanjuanensis'* specimens (Figs. 6J, 9E)) may occur intraspecifically.

Hirano (1997) proposed four patterns of white spots distribution for *Haliclystus*. Here we confirm those patterns (in this work named as group 1, group 4, group 5, and group 6, which correspond to the Hirano's type 1, 2, 3, and 4, respectively; Fig. 9) and complement them by including analysis of more species (*H. antarcticus*, *H. californiensis*, *H. inabai*, *H. kerguelensis*, *H. monstrosus*, *H. salpinx*, and *H. sinensis*). Based on these analyses, we propose two new patterns for *Haliclystus*, group 2 and group 3 (Fig. 9). Most of the proposed groups are easily identified and have little or no conflicting data in the literature (Table S1). In group 3 (Fig. 9C), however, the presence of white spots in the interradia of *H. inabai* is dubious. Hirano (1986) and Zagal et al. (2011) clearly mention the presence of white spots in perradia, both on the calyx margin and on the calyx sectors. However, imprecise descriptions report the presence of white spots throughout the subumbrella, without clearly mentioning the radii (Kishinouye, 1902; Uchida, 1929; Naumov, 1961). Additional descriptions (Holst et al., 2019) and images (Miranda et al., 2016b; figure 1L) indicate the presence of white spots in the interradia (Fig. 6I). The present study confirms the presence of a white spot on the margin of the interradia in a few specimens. Nevertheless, most of the specimens analyzed have white spots only on the perradia, which indicates a polymorphism of the species in relation to the distribution of white spots. Additional studies are necessary to verify whether this variation has any reflection on the genetic diversity found in *H. inabai* (Miranda et al., 2016b) and to investigate if there is a propensity of white spot loss in the interradia for the species. The inaccuracy of information for the species *H. monstrosus* and *H. salpinx* (Table S1) may be due to taxonomic problems in the identification of these species.

The genus *Lucernaria* also has taxonomic issues (Miranda et al., 2016b) and a detailed review is necessary. Some species have little or no information in the literature

regarding the white spots (*Lucernaria haekeli*, *Lucernaria infundibulum*, *Lucernaria janetae*). In addition, only three species (*L. bathyphila*, *L. quadricornis*, and *L. sainthilairei*) were recently studied in detail using histological sections (Miranda et al., 2016a), and are the only species with more accurate information on white spots distribution (Table S1). However, they all seem to share conserved white spots distribution in both the perradii and interradii over the entire subumbrellar surface. Because of the imprecise information for *Lucernaria*, *Halimocyathus*, *Kyopoda*, and *Stylocoronella*, no patterns of white spots distribution on the subumbrella were proposed in this study for these genera, which require detailed examination.

The patterns observed in *Depastrum* and *Depastromorpha* are very similar, with white spots generally arranged in two perradial rows above the gonadal region (subumbrellar epidermis of principal radial pocket) (Fig. 6B, 7B, C). Although the white spots rows in *Depastromorpha africana* are described as regular, in larger specimens we observed few white spots outside these rows, leading to an irregular pattern (Fig. 6B). There is little information in the literature regarding the white spots in *Depastrum cyathiforme*, but Gosse (1860, figures 2, 3) presented illustrations representing the white spots irregularly distributed in two rows above the gonads, pattern also described by Clark (1863). However, according to our observations (Table 1), the white spots seem to be randomly distributed above the gonadal region, demonstrating the possibility of intraspecific variation.

Both genera mentioned above have a very similar pattern to the one observed in *M. uchidai* (Fig. 6K, 10B), which is unique within *Manania*. In other species of this genus, the white spots are present on the subumbrellar epidermis of principal radial pocket, above the perradial gonads, and also along the entire margin of the calyx, in the interradii and perradii, very similar to the pattern found in *C. convolvulus*, except that this species does not have white spots distributed along the entire calyx margin (Fig. 6A, 7A). *Manania hexaradiata* may also be an exception in the genus. Broch (1907) mentioned that groups of nematocysts are found on the exumbrella, whereas the subumbrella of this species is smooth, which could be an indication of the absence of white spots, a misinterpretation, or an oversimplified description. Indeed, this information contradicts observations (this study) of a specimen collected in Barents Sea (Table 1), which has white spots above perradial gonads and along the interradiial and perradial calyx margin, similar to most species of the genus.

Four patterns of white spots distribution were proposed for the genus *Calvadosia* (Fig. 8). Group 4 is the easiest to distinguish among them, since it comprises the only species in the genus (*Calvadosia* sp. 1 NZ) with white spots positioned only in the perradii (Fig. 8D). This species will be properly described soon in a separate manuscript. *Calvadosia vanhoeffeni* (group 3) also has a unique pattern of white spots (Fig. 8B), which are particularly abundant on perradial sectors, but also present on both perradial and interradial calyx margins and in the interradii along the gonads (Zagal et al., 2011). The remaining species of the genus are divided into two groups. Group 1 (Fig. 8A) encompasses *C. campanulata* (Fig. 6D) and *C. cruxmelitensis* (Fig. 6E), in which the white spots distribution is well described in the literature (Keferstein, 1862; Kassianow, 1901; Eales, 1938; Corbin, 1978; Mills & Hirano, 2007, figure 1E; Zagal et al., 2011; Miranda et al., 2016b, figure 1B), as well as *C. corbini*, *C. lewisi* (Fig. 6F), and *C. tasmaniensis* (Fig. 6G), for which additional observations provided in this study complement the literature and support the presence of white spots above the nodular gonads of these species. Group 2 (Fig. 8B) includes *C. cruciformis* (Fig. 1B) and *C. nagatensis*, which have white spots in the perradial and interradial calyx margins and a few on the perradial sectors, but not above the gonadal region (Uchida, 1929; Ling, 1937; this study), different from the species in group 1. In the species of group 2, some white spots appear to be present on the gonads, but are actually located on the calyx margin. This mistake may occur because the calyx margin folds inwards, covering the subumbrellar surface of the gonads, giving the impression that the white spots on the calyx margin are on top of them. Furthermore, it is possible that batteries of nematocysts occur on the gonads, as observed in *C. corbini*, whereas white spots are apparently restricted only to the calyx margins and perradial sectors for group 2 species (Table 1).

4.3. Evolution of white spots

There are several methods to measure the tendency for closely related species to resemble each other more than less related taxa, as a result of shared evolutionary history (i.e., phylogenetic signal), such as Pagel's λ and Blomberg's K (Pagel, 1999; Blomberg et al., 2003). Both metrics mentioned above were developed for continuous traits, using Brownian motion as a model of trait evolution (Kamilar & Cooper, 2013), which is difficult to translate to discrete traits (Fritz & Purvis, 2010; Borges et al., 2019). An alternative for discrete traits is the D statistic proposed by Fritz & Purvis (2010), which

is only applicable for binary traits. The δ statistic (Borges et al., 2019), a newly proposed method for evaluating the degree of phylogenetic signal between a phylogeny and discrete traits, allows multistate data analysis, thus being the best alternative to measure the phylogenetic signal of the white spots distribution on the subumbrella of staurozoan species.

The result obtained in the phylogenetic signal analysis of the investigated trait (Table 2) was not significant, since the value of δ obtained from the empirical analysis (10,929) did not exceed the 95% quantile of the simulated data (11,162). This result can be justified by the high convergence in the loss of white spots in the interradii, as well as also to the polymorphism found in *H. inabai*, increasing the uncertainty in the reconstruction of the ancestral states of the phylogeny, a necessary step in the adopted methodology (Borges et al., 2019). The independent loss of white spots in the interradii mainly within the family Haliclystidae stands out, occurring only once outside this clade, in the genus *Calvadosia* (Fig. 11). Only two species of Staurozoa (*H. antarcticus* and *H. auricula*) completely lost the white spots on the subumbrella. Our results also indicated the loss of the white spots in the perradii in the ancestor of both species, a sign that the total loss of the white spots in the subumbrella may be related to an initial gradual loss in the interradii (Fig. 11).

It is curious that, in general, there are few similar morphological characteristics among the genera that convergently lost the white spots in the interradii. Features such as calyx shape, presence of claustrum, and position of the gonads are quite different among the genera *Haliclystus*, *Depastromorpha*, *Manania*, and *Calvadosia* (Miranda et al., 2016b); this might indicate that there is no particular body pattern that leads to the loss of the white spots in the interradii. However, it is notable that species that lost white spots in the interradii maintain white spots associated with the gonads. In *M. uchidai* and *D. africana*, the gonads are positioned in the perradii inside the principal radial pockets, and these are covered by white spots in the same radii. In *H. 'sanjuanensis'* and *H. stejnegeri*, which have the gonads positioned in the perradii/adradii inside the gastric radial pockets, the white spots are distributed along the perradial gonadal margin. A possible evolutionary association of white spots with gonads requires further analyses and would test the hypothesis that the white spots acts specifically in gonadal protection (Kling, 1879; Kassianow, 1901). If the potential gonadal protection provided by the white spots is significant, an additional question is, taking into account a long history of conservation

of this trait, what are the pressures (or lack thereof) that might have caused the complete loss of white spots in the ancestor of *H. antarcticus* and *H. auricula*?

Another possible function of white spots is in prey capture. Although the stalked jellyfishes mainly use the secondary tentacles to capture prey and subsequently bend their arms to bring prey attached to secondary tentacular clusters to the manubrium (Hyman 1940; Larson 1980), there is another form of prey capture described for these animals. Larson (1980) described an occasional “flip over” behavior in *C. corbini*: after the prey was captured by the secondary tentacles, the stalk basal disc was released from the substrate and the oral side (subumbrella) was pushed against the substrate (e.g., seagrass), trapping the prey against it until the prey was swallowed. The same behavior was observed for *H. 'sanjuanensis'* (Westlake, 2015), when large prey or several small prey were offered. Therefore, the white spots could release nematocysts directly on the trapped prey. Since this behavior seems to be occasional, apparently related to larger or several smaller prey, the huge amount of nematocysts released from white spots directly onto the prey may be a valuable resource for subduing food. Therefore, it could also be interesting to evaluate the feeding behavior of *H. antarcticus* and *H. auricula*, since the absence of white spots might also be related to this behavior.

Although it is a challenge to speculate about the possible causes that could explain independent losses of white spot in interradial or a complete loss in those two species, we have demonstrated that shared ancestry affects the distribution of white spots on the subumbrella and evidenced a highly conserved trait where most species of Staurozoa and their ancestors have had white spots in periradial and interradial.

5. Conclusions

In this study, we explored the neuromuscular morphology of the white spots of nematocysts. In addition, we presented a thorough literature review regarding the white spots distribution on the subumbrella of all staurozoan species.

We found an intrinsic meridional musculature englobing the white spots, and musculature associated with the white spots pore, which is possibly related to the opening and closing of the structure, as well as putative sensory neurons surrounding them. Fibers from other muscular groups near the white spots always deviate around the apex of the

structure, where the opening is located, indicating that the pore needs to be unobstructed. These observations corroborate the hypothesis that the white spots can actively release their internal nematocysts. Researches focusing on the activation of these nematocysts might provide further clues on the white spots functioning.

The literature review provides important taxonomic information for different genera in Staurozoa. We identify well-established patterns of white spots for the genera *Craterolophus*, *Calvadosia*, *Depastromorpha*, *Depastrum*, *Haliclystus*, and *Manania*, which can be relevant in the identification of different taxonomic levels (species and genera). Some small intraspecific variations may occur in the distribution of white spots (e.g., the rare presence of white spots in the perradial margin of the calyx of *H. 'sanjuanensis'* as shown in Fig. 6J) as well as the apparent polymorphism in *H. inabai*. This reinforces the need for further morphological and taxonomic studies on these genera, as well as on the other genera for which no patterns were proposed (i.e., *Halimocyathus*, *Kyopoda*, *Lucernaria*, and *Stylocoronella*). We noticed a frequent association of white spots with the gonads, either directly, where white spots occurred on top of them (e.g., in some *Calvadosia*), or indirectly, where white spots occurred on the perradial margin closest to the gonads (e.g., in some *Haliclystus*). The possible role of white spots in gonadal protection requires additional research.

Based on the literature review, we also investigated for the first time the evolutionary history of white spots distribution. The presence of white spots on both axes (i.e., perradial and interradial) is conserved in Staurozoa, with some independent losses of white spots on the interradia, especially in the family Haliclystidae but also in one species of *Calvadosia*. A complete loss of the white spots in *H. antarcticus* and *H. auricula* was preceded by loss of the white spots in the interradia of the exclusive common ancestor shared by these two species and *H. 'sanjuanensis'*. The possible triggers for the (independent) interradial and (unique) complete losses of white spots are still not clear, but macroevolutionary studies encompassing morphology and ecology could provide interesting evidence.

References

- Americus B, Lotan T, Bartholomew JL, Atkinson S D. 2020. A comparison of the structure and function of nematocysts in free-living and parasitic cnidarians (Myxozoa). *International Journal for Parasitology* 50:763–769.

- Antipa G. 1893. Eine neue Stauromeduse (*Capria n. sturdzii* n.). *Mittheilungen aus der Zoologischen Station zu Neapel* 10:618–632.
- Atkinson SD, Bartholomew JL, Lotan T. 2018. Myxozoans: ancient metazoan parasites find a home in phylum Cnidaria. *Zoology* 129:66–68.
- Audesirk G, Audesirk T. 1980. Complex mechanoreceptors in *Tritonia diomedea*. *Journal of comparative physiology* 141(1):111–122.
- Berrill M. 1962. The biology of three New England stauromedusae, with a description of a new species. *Canadian Journal of Zoology* 40(7):1249–1262.
- Bezares-Calderón LA, Berger J, Jékely G. 2020. Diversity of cilia-based mechanosensory systems and their functions in marine animal behavior. *Philosophical Transactions of the Royal Society B* 375(1792):20190376.
- Blackmon H, Adams RA. 2015. EvobiR: tools for comparative analyses and teaching evolutionary biology. *Zenodo*.
- Blomberg SP, Garland Jr T, Ives AR. 2003. Testing for phylogenetic signal in comparative data: behavioral traits are more labile. *Evolution* 57(4):717–745.
- Borges R, Machado JP, Gomes C, Rocha AP, Antunes A. 2019. Measuring phylogenetic signal between categorical traits and phylogenies. *Bioinformatics* 35(11):1862–1869.
- Boyko JD, Beaulieu JM. 2021. Generalized hidden Markov models for phylogenetic comparative datasets. *Methods in Ecology and Evolution* 12(3):468–478.
- Broch H. 1907. Hydroiden und medusen. *Report of the Second Norwegian Arctic Expedition in the “Fram” 1898–1902* 2:1–12.
- Carlgren O. 1930. Die Lucernariden. *Further Zoological Results of the Swedish Antarctic Expedition 1901–1903* 2:1–18.
- Carlgren O. 1933. Zur Kenntnis der Lucernariiden *Lipkea*, *Capria* und *Brochiella*. *Kungliga Fysiografiska Sällskapets Handlingar* 44:1–19.
- Carlgren O. 1935. Über eine neue Südafrikanische Lucernariidae, *Depastromorpha africana* n. gen., n. sp., nebst Bemerkungen über den Bau und die Systematik dieser Tiergruppe. *Kunglia Svenska Vetenskapsakademiens Handlingar* 15:1–24.
- Carlgren O. 1938. Eine neue Südafrikanische Lucernariidae, *Lucernariopsis capensis*. *Kungliga Fysiografiska sällskapets i Lund Förhandlingar* 8:1–6.
- Clark HJ. 1863. Prodomus of the history, structure, and physiology of the order Lucernariae. *Journal of the Boston Society of Natural History* 7:531–567.
- Collins AG, Daly M. 2005. A new deepwater species of Stauromedusae, *Lucernaria janetae* (Cnidaria, Staurozoa, Lucernariidae), and a preliminary investigation of stauromedusan phylogeny based on nuclear and mitochondrial rDNA data.

- Biological Bulletin* 208(3):221–230.
- Corbin PG. 1978. A new species of the stauromedusan genus *Lucernariopsis* (Coelenterata: Scyphomedusae). *Journal of the Marine Biological Association of the United Kingdom* 58(2):285–290.
- Eales NB. 1938. *Lucernaria discoidea*, a new species from the Channel Islands. *Journal of the Marine Biological Association of the United Kingdom* 23(1):167–170.
- Fritz SA, Purvis A. 2010. Selectivity in mammalian extinction risk and threat types: a new measure of phylogenetic signal strength in binary traits. *Conservation Biology* 24(4):1042–1051.
- Gosse PH. 1860. LII. On the *Lucernaria cyathiformis* of Sars. *Annals and Magazine of Natural History* 5(30):480–481.
- Gross I. 1900. Zur Anatomie der Lucernariden. *Jenaischen Zeitschrift für Naturwissenschaft* 33:611–624.
- Gwilliam GF. 1956. Studies on West Coast Stauromedusae. Ph.D. Dissertation. Berkeley: University of California.
- Harmon L. 2018. *Phylogenetic comparative methods: learning from trees*.
- Hausmann K, Holstein T. 1985. Bilateral symmetry in the cnidocil-nematocyst complex of the freshwater medusa *Craspedacusta sowerbii* Lankester (Hydrozoa, Limnomedusae). *Journal of ultrastructure research* 90(1):89–104.
- Hirano YM. 1986. Species of Stauromedusae from Hokkaido, with notes on their metamorphosis. *Journal of the Faculty of Science, Hokkaido University, Zoology* 24: 182–201.
- Hirano YM. 1997. A review of a supposedly circumboreal species of stauromedusa, *Halicystus auricula* (Rathke, 1806). In: den Hartog J.C., ed. *Proceedings of the 6th International Conference on Coelenterate Biology*. Leiden: National Natuurhistorisch Museum, Noordwijkerhout, 247–252.
- Holst S, Heins A, Laakmann S. 2019. Morphological and molecular diagnostic species characters of Staurozoa (Cnidaria) collected on the coast of Helgoland (German Bight, North Sea). *Marine Biodiversity* 49(4):1775–1797.
- Holst S, Miranda LS, Meyer P, Michalik P, Sötje I. 2021. Morphological analyses of the adult and juvenile stages of the stalked jellyfish *Craterolophus convolvulus* (Johnston, 1835) (Cnidaria: Staurozoa: Stauromedusae: Craterolophidae) using micro-CT. *Zoologischer Anzeiger* 292:240–260.
- Hyman LH. 1940. Observations and experiments on the physiology of medusae. *Biological Bulletin* 79(2): 282–296.
- Joy JB, Liang RH, McCloskey RM, Nguyen T, Poon AF. 2016. Ancestral reconstruction. *PLoS Computational Biology* 12(7):e1004763.

- Kahn AS, Matsumoto GI, Hirano YM, Collins AG. 2010. *Haliclystus californiensis*, a “new” species of stauromedusa (Cnidaria: Staurozoa) from the northeast Pacific, with a key to the species of *Haliclystus*. *Zootaxa* 2518:49–59.
- Kamilar JM, Cooper N. 2013. Phylogenetic signal in primate behaviour, ecology and life history. *Philosophical Transactions of the Royal Society B: Biological Sciences* 368(1618):20120341.
- Kassianow N. 1901. Studien über das Nervensystem der Lucernariden nebst sonstigen histologischen Beobachtungen über diese Gruppe. *Zeitschrift für Wissenschaftliche Zoologie* 69:287–377.
- Kass-Simon GSAA, Scappaticci JrAA. 2002. The behavioral and developmental physiology of nematocysts. *Canadian Journal of Zoology* 80(10):1772–1794.
- Keferstein W. 1862. Untersuchungen über niedere Seethiere. *Zeitschrift für wissenschaftliche Zoologie* 12:1–147.
- Kent ML, Andree KB, Bartholomew JL, El-Matbouli M, Desser SS, Devlin RH, Feist SW, Hedrick RP, Hoffmann RW, Khattra J, Hallett SL, Lester RJ, Longshaw M, Palenzeula O, Siddall ME, Xiao C. 2001. Recent advances in our knowledge of the Myxozoa. *Journal of Eukaryotic Microbiology* 48(4):395–413.
- Kikinger R, Salvini-Plawen LV. 1995. Development from polyp to stauromedusa in *Stylocoronella* (Cnidaria: Scyphozoa). *Journal of the Marine Biological Association of the United Kingdom* 75:899–912.
- Kishinouye K. 1902. Some new Scyphomedusae of Japan. *Journal of the College of Science, Imperial University of Tokyo* 17:1–17.
- Kling O. 1879. Ueber *Craterolophus tethys*. Ein Beitrag zur Anatomie und Histologie der Lucernarien. *Morphologisches Jahrbuch* 5:141–166.
- Larson RJ. 1980. A new stauromedusa, *Kishinouyeya corbini* (Scyphozoa, Stauromedusae) from the tropical western Atlantic. *Bulletin of Marine Science* 30(1):102–107.
- Larson RJ. 1988. *Kyopoda lamberti* gen.nov., sp.nov., an atypical stauromedusa (Scyphozoa, Cnidaria) from the eastern Pacific, representing a new family. *Canadian Journal of Zoology* 66(10):2301–2303.
- Larson RJ, Fautin DG. 1989. Stauromedusae of the genus *Manania* (= *Thaumatoscypus*) (Cnidaria, Scyphozoa) in the northeast Pacific, including descriptions of new species *Manania gwilliamsi* and *Manania handi*. *Canadian Journal of Zoology* 67:1543–1549.
- Leuschel H. 1932. Beiträge zur Histologie und Physiologie der Lucernariden. *Zoologische Jahrbücher* 52:338–388.

- Ling SW. 1937. Studies on Chinese Stauromedusae. I. Stauromedusae from Tsingtao. *Amoy Marine Biological Bulletin* 3: 1–35.
- Lom J, Dyková I. 2013. Myxozoan genera: definition and notes on taxonomy, life-cycle terminology and pathogenic species. *Folia Parasitologica* 53(1):1–36.
- Mariscal RN, Bigger CH. 1976. A comparison of putative sensory receptors associated with nematocysts in an anthozoan and a scyphozoan. *Coelenterate Ecology and Behavior*. Boston: Springer, 559–568.
- Marques AC, Collins AG. 2004. Cladistic analysis of Medusozoa and cnidarian evolution. *Invertebrate Biology* 123(1):32–42.
- Mills CE, Hirano YM. 2007. Stauromedusae. In: Denny MW, Gaines SD, eds. *Encyclopedia of Tidepools and Rocky Shores*. Berkeley: University of California Press, 541–543.
- Miranda LS, Branch GM, Collins AG, Hirano YM, Marques AC, Griffiths CL. 2017a. Stalked jellyfishes (Cnidaria: Staurozoa) of South Africa, with the description of *Calvadosia lewisi* sp. nov. *Zootaxa* 4227(3):369–389.
- Miranda LS, Collins AG, Marques AC. 2010. Molecules clarify a cnidarian life cycle – the “hydrozoan” *Microhydrula limopsicola* is an early life stage of the staurozoan *Haliclystus antarcticus*. *PLoS One* 5:e10182.
- Miranda LS, Collins AG, Marques AC. 2013. Internal anatomy of *Haliclystus antarcticus* (Cnidaria, Staurozoa) with a discussion on histological features used in staurozoan taxonomy. *Journal of Morphology* 274(12):1365–1383.
- Miranda LS, Collins AG, Hirano YM, Mills CE, Marques AC. 2016a. Comparative internal anatomy of Staurozoa (Cnidaria), with functional and evolutionary inferences. *PeerJ* 4:e2594.
- Miranda LS, García-Rodríguez J, Collins AG, Morandini AC, Marques AC. 2017b. Evolution of the claustrum in Cnidaria: comparative anatomy reveals that it is exclusive to some species of Staurozoa and absent in Cubozoa. *Organisms Diversity & Evolution* 17(4):753–766.
- Miranda LS, Haddad MA, Mills CE, Marques AC. 2012. *Lucernariopsis capensis* Carlgren, 1938 (Cnidaria, Staurozoa) in Brazil: first record outside its type locality in South Africa. *Zootaxa* 3158(1):60–64.
- Miranda LS, Hirano YM, Mills CE, Falconer A, Fenwick D, Marques AC, Collins AG. 2016b. Systematics of stalked jellyfishes (Cnidaria: Staurozoa). *PeerJ* 4:e1951.
- Miranda LS, Mills CE, Hirano YM, Collins AG, Marques AC. 2018. A review of the global diversity and natural history of stalked jellyfishes (Cnidaria, Staurozoa). *Marine Biodiversity* 48(4):1695–1714.

- Naumov DV. 1961. Stsifoidnye meduzy morei SSSR (Scyphozoan medusae in the sea of USSR). *Opredeliteli po Faune SSSR* 75:1–98.
- Östman C. 2000. A guideline to nematocyst nomenclature and classification, and some notes on the systematic value of nematocysts. *Scientia Marina* 64(S1):31–46.
- Pagel M. 1999. Inferring the historical patterns of biological evolution. *Nature* 401:877–884.
- Pennell MW, Eastman JM, Slater GJ, Brown JW, Uyeda JC, FitzJohn RJ, Alfaro ME, Harmon LJ. 2014. geiger v2.0: an expanded suite of methods for fitting macroevolutionary models to phylogenetic trees. *Bioinformatics* 30:2216–2218.
- Pisani V, Otero-Ferrer F, Lotto S, Maurel P, Goy J. 2007. *Lipkea ruspoliana* Vogt, 1887 (Stauromedusae, Scyphozoa, Cnidaria) dans les aquariums du Musée Océanographique de Monaco. *Bulletin de la Société Zoologique de France* 132:183–190.
- Plachetzki DC, Fong CR, Oakley TH. 2012. Cnidocyte discharge is regulated by light and opsin-mediated phototransduction. *BMC Biology* 10(1):1–10.
- R Core Team. 2020. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>.
- Salvini-Plawen LV. 2006. First record of a mature stauromedusa *Stylocoronella* (Cnidaria) in nature. *Cahiers de Biologie Marine* 47(2):219–222.
- Scappaticci Jr AA, Kahn F, Kass-Simon G. 2010. Nematocyst discharge in *Hydra vulgaris*: differential responses of desmonemes and stenoteles to mechanical and chemical stimulation. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 157(2):184–191.
- Siddall ME, Martin DS, Bridge D, Desser SS, Cone DK. 1995. The demise of a phylum of protists: phylogeny of Myxozoa and other parasitic Cnidaria. *The Journal of Parasitology* 81(6):961–967.
- Slautterback DB. 1967. The cnidoblast-musculoepithelial cell complex in the tentacles of *Hydra*. *Zeitschrift für Zellforschung und Mikroskopische Anatomie* 79(2):296–318.
- Thorington GU, Hessinger DA. 1990. Control of cnida discharge: III. Spirocysts are regulated by three classes of chemoreceptors. *The Biological Bulletin* 178(1):74–83.
- Uchida T. 1929. Studies on the Stauromedusae and Cubomedusae, with special reference to their metamorphosis. *Japanese Journal of Zoology* 2:103–193.
- Uchida T. 1933. Eine neue Becherqualle aus Hokkaido. *Proceedings of the Imperial Academy* 9(8):450–452.

- Uchida T, Hanaoka KI. 1933. On the morphology of a stalked medusa, *Thaumatoscyphus distinctus* Kishinouye. *Journal of the Faculty of Science, Hokkaido Imperial University, Series VI, Zoology* 2(3):135–153.
- Uchida T, Hanaoka KI. 1934. Anatomy of two stalked medusae with remarks on the distribution of the Stauromedusae in Japan. *Journal of the Faculty of Science, Hokkaido Imperial University, Series VI, Zoology* 2(4):211–239.
- Vogt C. 1886. Sur une médusaire sessile, *Lipkea ruspoliana*. *Archives des Sciences* 16: 356–362.
- Vogt C. 1887. *Sur un nouveau genre de médusaire sessile Lipkea ruspoliana* c.v. Imprimerie Centrale Genevoise, Genève, 53 p.
- Watson GM, Hessinger DA. 1987. Receptor-mediated endocytosis of a chemoreceptor involved in triggering the discharge of cnidae in a sea anemone tentacle. *Tissue and Cell* 19(6):747–755.
- Watson GM, Mire P. 2000. Stereocilia based mechanoreceptors of sea anemones. *Cell and Molecular Biology of the Ear*. Boston: Springer, 19–39.
- Weill R. 1925. Foyers de formation et voies de migration des nématocystes de *Halyclistus* [SIC] *octoradiatus* Clark. Existence, sur leur parcours, de réservoirs sélectifs. *Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences* 180:327–330.
- Weill R. 1935. Les “réservoirs sélectifs” et la maturation des nématocystes de *Lucernaria campanulata* Lam. *Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences* 120:774–777.
- Westfall JA. 1970. The nematocyte complex in a hydromedusan, *Gonionemus vertens*. *Zeitschrift für Zellforschung und Mikroskopische Anatomie* 110(4):457–470.
- Westlake H. 2015. A Comparative Immunohistochemical Study of the Neuromuscular Organization of *Halichlystus 'sanjuanensis'* and *Manania handi* (Cnidaria: Staurozoa). Thesis, University of Victoria.
- Westlake HE, Page LR. 2017. Muscle and nerve net organization in stalked jellyfish (Medusozoa: Staurozoa). *Journal of Morphology* 278:29–49.
- Wietrzykowski W. 1912. Recherches sur le développement des Lucernaires. *Archives de Zoologie Expérimentale et Générale, 5th Series* 10:1–95.
- Zagal CJ, Hirano YM, Mills CE, Edgar GJ, Barrett NS. 2011. New records of Staurozoa from Australian coastal waters, with a description of a new species of *Lucernariopsis* Uchida, 1929 (Cnidaria, Staurozoa, Stauromedusae) and a key to Australian Stauromedusae. *Marine Biology Research* 7(7):651–666.

Figures

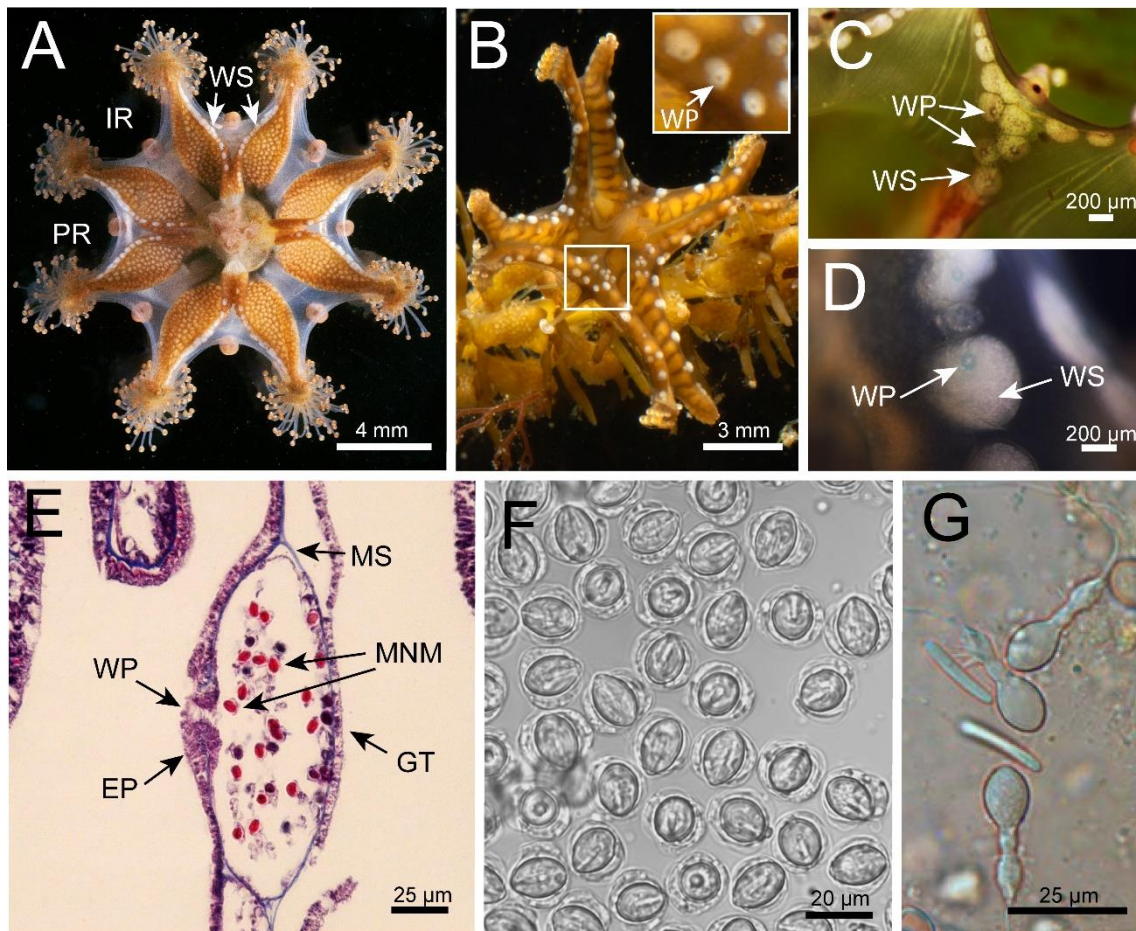


Figure 1. General aspects of white spots of nematocysts. **(A)** White spots distribution on the subumbrella of *Haliclystus 'sanjuanensis'* (photo credit: Ron Larson). **(B)** White spots distribution on subumbrella of *Calvadosia cruciformis* (modified from Miranda et al., 2018). **Inset:** pore of the white spots. **(C, D)** Ring of pigmentation near the pore of each white spot in *Manania handi* and *H. 'sanjuanensis'*, respectively. **(E)** Longitudinal section of white spots of *Craterolophus convolvulus*, showing a thin layer of mesoglea between the gastrodermis and epidermis, interrupted by a well-defined pore, a peripheral layer of immature nematocysts and loose mature nematocysts clustered in the central region (modified from Miranda et al., 2016a). **(F)** Brightfield images of nematocysts that were released from a cut white spot. **(G)** Discharged nematocysts of white spots, birhopaloid (discharged shaft with one distal and one proximal dilatation, see Östman, 2000). Abbreviations: EP, epidermis; GT, gastrodermis; IR, interradial; MNM, mature nematocyst; MS, mesoglea; PR, perradial; WP, white spot pore; WS, white spot.

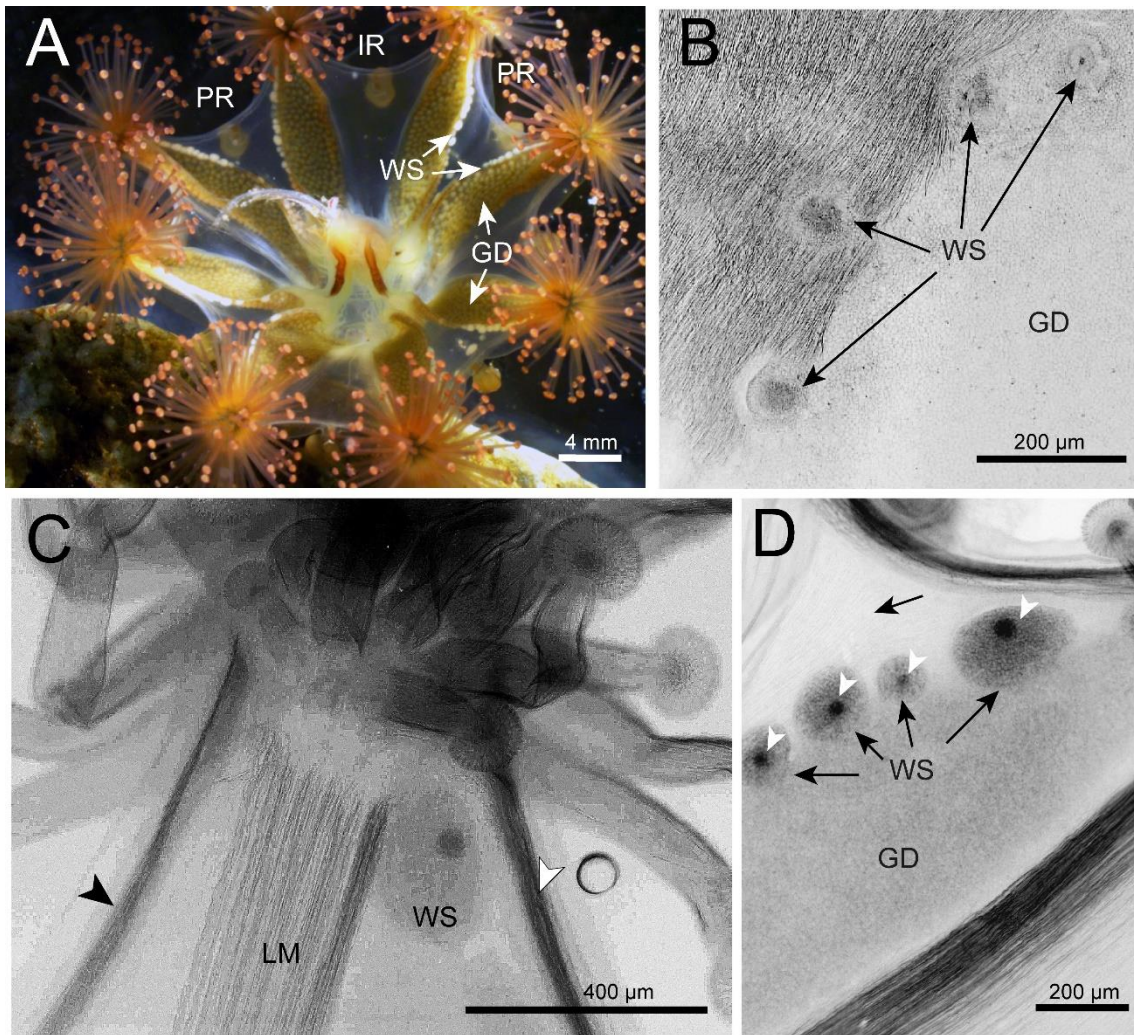


Figure 2. Fluorophore-tagged phalloidin labelling of the perradial musculature of *Haliclystus 'sanjuanensis'*. **(A)** General subumbrellar view of *H. 'sanjuanensis'*. **(B)** Fibers of the perradial muscle extended over one half of each perradial white spot lining the edge of a gonad. Fibers do not extend over the apices of the white spots or the gonad tissue. **(C)** An interradial (black arrowhead) and perradial (white arrowhead) segment of marginal muscle runs up the sides of an arm to the tentacles. A white spot near the longitudinal muscle band, which terminates abruptly at the base of the tentacles. **(D)** Density of perradial muscle fibers (black arrow). The white spots labelled strongly with fluorophore-tagged phalloidin; a strongly labelled central spot corresponded to the apex of each white spot (arrowheads). Abbreviations: IR, interradial; GD, gonads; LM, longitudinal muscle; PR, perradial; WS, white spots.

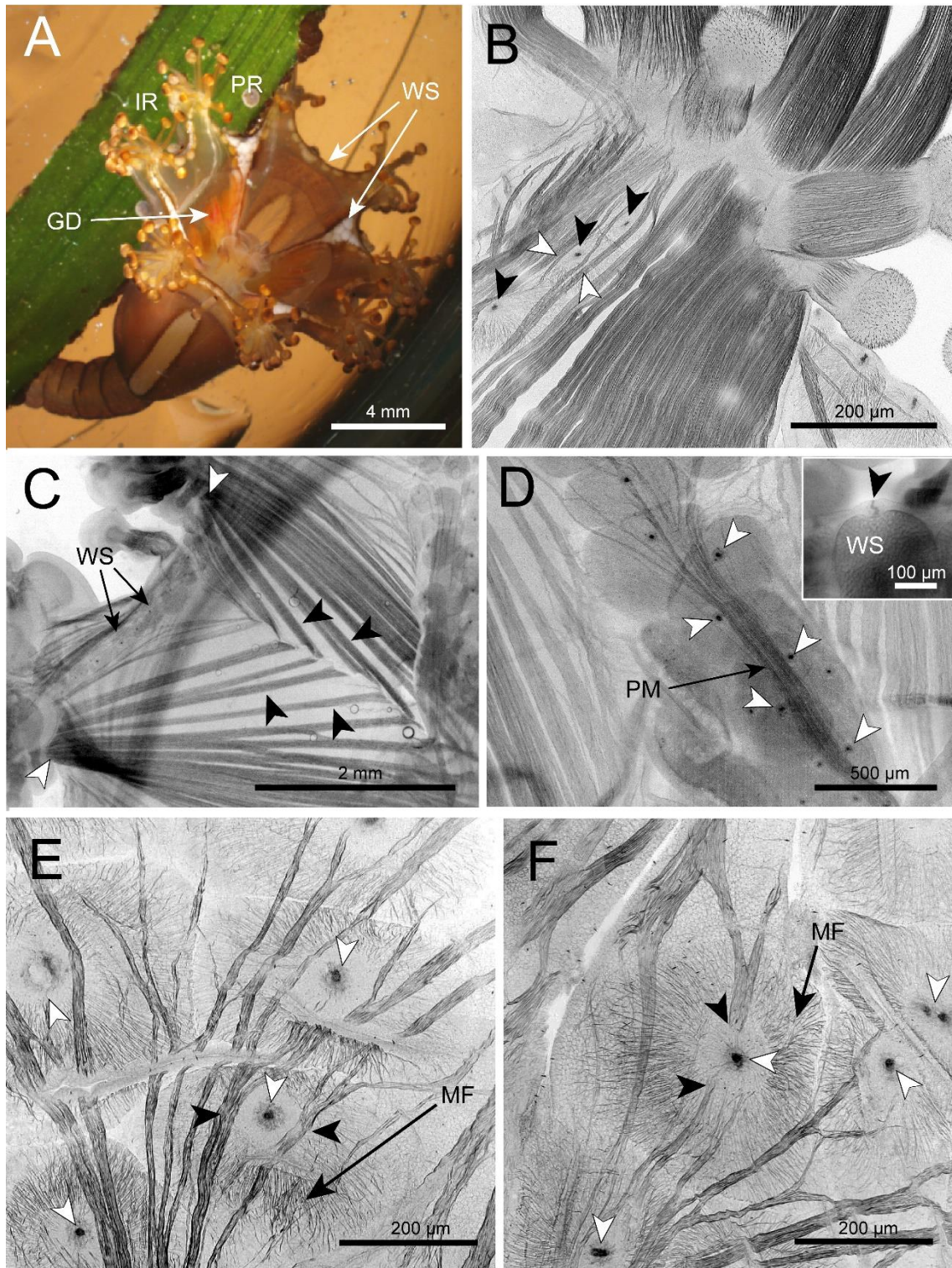


Figure 3. Fluorophore-tagged phalloidin labelling of the interradial longitudinal, marginal, and perradial muscles of *Manania handi*. **(A)** General subumbrellar view of *M. handi*. **(B)** Subumbrellar view of the end of an arm. The apices of the white spots (black arrowheads) occupied a gap between the perradial muscle fibers (white arrowheads). **(C)** Oblique bands of longitudinal muscle extended from a central interradial axis (black arrowheads). The final branches of the muscle framed white spots situated between the longitudinal muscle and margin. The longitudinal muscle bands terminated at the end of the adjacent adradial arms (white arrowheads). **(D)** The tightly bundled perradial muscle ran along the top of the perradial pocket; where the pocket joined the calyx wall, the muscle began to branch, fan out and anastomose. Along the top of the perradial pocket, the apices of the white spots (white arrowheads) labelled

with phalloidin lay to either side of the muscle band. **Inset:** tangential view of a white spot apex with what appears to be a short tubular structure (black arrowhead) connecting the white spot to the subumbrellar surface. **(E, F)** Perradial muscle branches that anastomosed around or were interrupted (black arrowheads) leaving gaps for the apices of the white spots (white arrowheads) near the margin. Meridional muscle fibers (black arrows) wrapped around each white spot from the apices. Abbreviations: GD, gonads; IR, interradia; MF, meridional muscle fibers; PR, perradial muscle; PM, perradial muscle; WS, white spot.

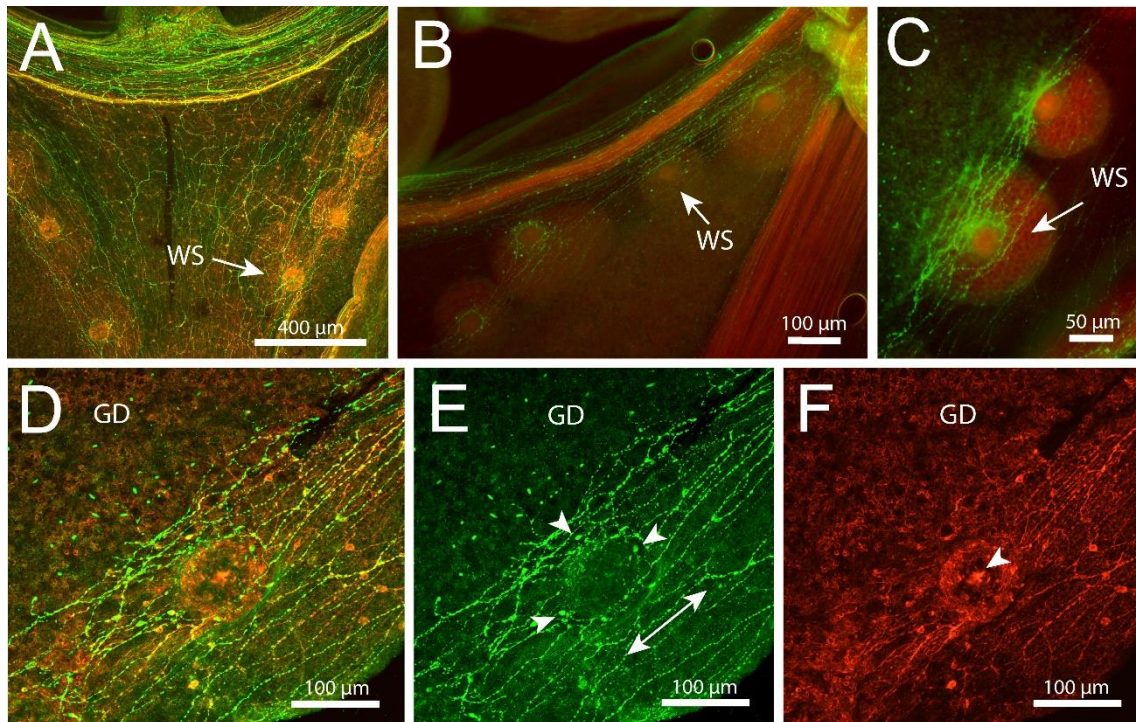


Figure 4. Innervation associated with the white spots in *H. 'sanjuanensis'*. **(A)** The loose FMRFamide-IR network (green) of the perradius. **(B)** Tracts of strongly FMRFamide-IR neurites (green) ran along the lines of white spots and circumvented their apices; these neurites were continuous with those of the margin and tentacles. **(C)** Neurites circling white spots apices in detail. **(D)** Ring of FMRFamide-IR neurites (green) and cell bodies encircles the α -tubulin-IR apex of the white spot (red). **(E)** FMRFamide-IR neurons encircled the apex of the white spot. A ring of cell bodies surrounded the central spot (arrowheads), and neurites extended over the gonad tissue adjacent to the white spot. Neurites overlying the perradial musculature were largely parallel to the muscle fibers (arrow indicates direction of musculature). Note punctate labelling of neurites. **(F)** Alpha-tubulin labelled neurites in the perradius around the white spot, and strongly labelled the apex of the white spot (arrowhead). Alpha-tubulin-IR neurites did not extend over the gonad tissue as FMRFamide-IR neurites did. Abbreviations: GD, gonad; WS, white spot.

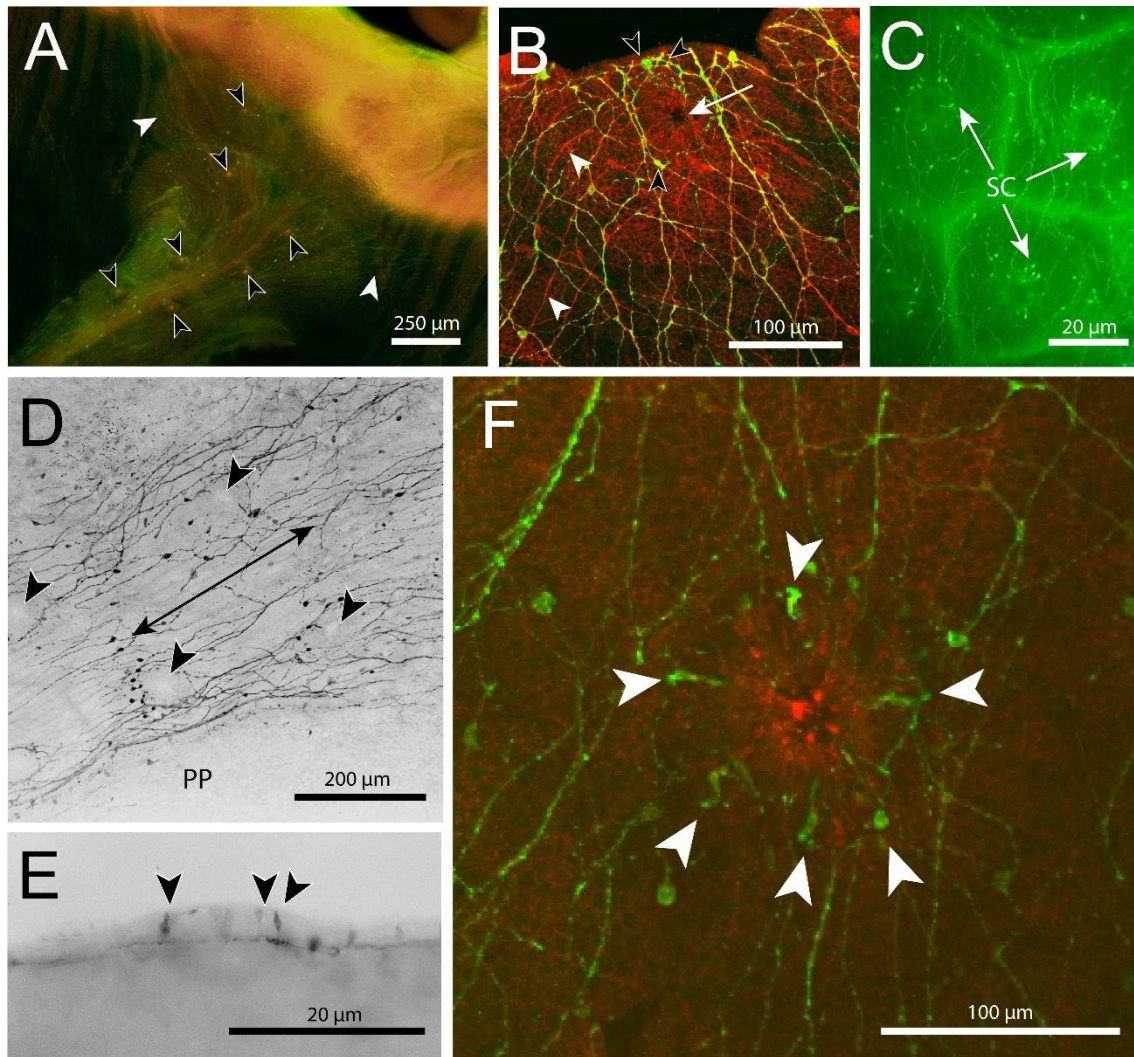


Figure 5. Innervation associated with the white spots in *M. handi*. **(A)** FMRFamide-IR (green) neurites on the top of the perradial pocket were generally parallel to phalloidin-labelled muscles (red). The apices of the white spots (black arrowheads) lie to either side of the perradial muscle band. Where the perradial pocket joins the calyx wall (white arrowheads), the neurites of the perradius fan out as they extend towards the margin. **(B)** FMRFamide-IR neurites (green) were co-labelled by α -tubulin (red) antibodies, save for those neurites and cell bodies (black arrowheads) directly encircling the apex of the white spot (white arrow). Many neurites were independently α -tubulin-IR (white arrowheads). **(C)** FMRFamide-IR (green) neurites encircle the apex of the white spot, putative sensory cells. **(D)** FMRFamide-IR neurons on the top of the perradial pocket; neurites were no more concentrated over the muscle band (directionality indicated by the arrow) than they were around the white spots on either side of it. Neurites do not overlie apices of the white spots. Neurites do not extend down to the sides of the perradial pocket, in which the gonads are embedded. **(E)** Tangential view of FMRFamide labelling showing elongate putative sensory cell bodies (arrowheads) occupying the thickened epidermis of a white spot apex. **(F)** Detail of FMRFamide immunoreactivity of the apex of a white spot (red); putative sensory neurons (arrowheads) encircle the apex. Abbreviations: PP, perradial pocket; SC, sensory cell.

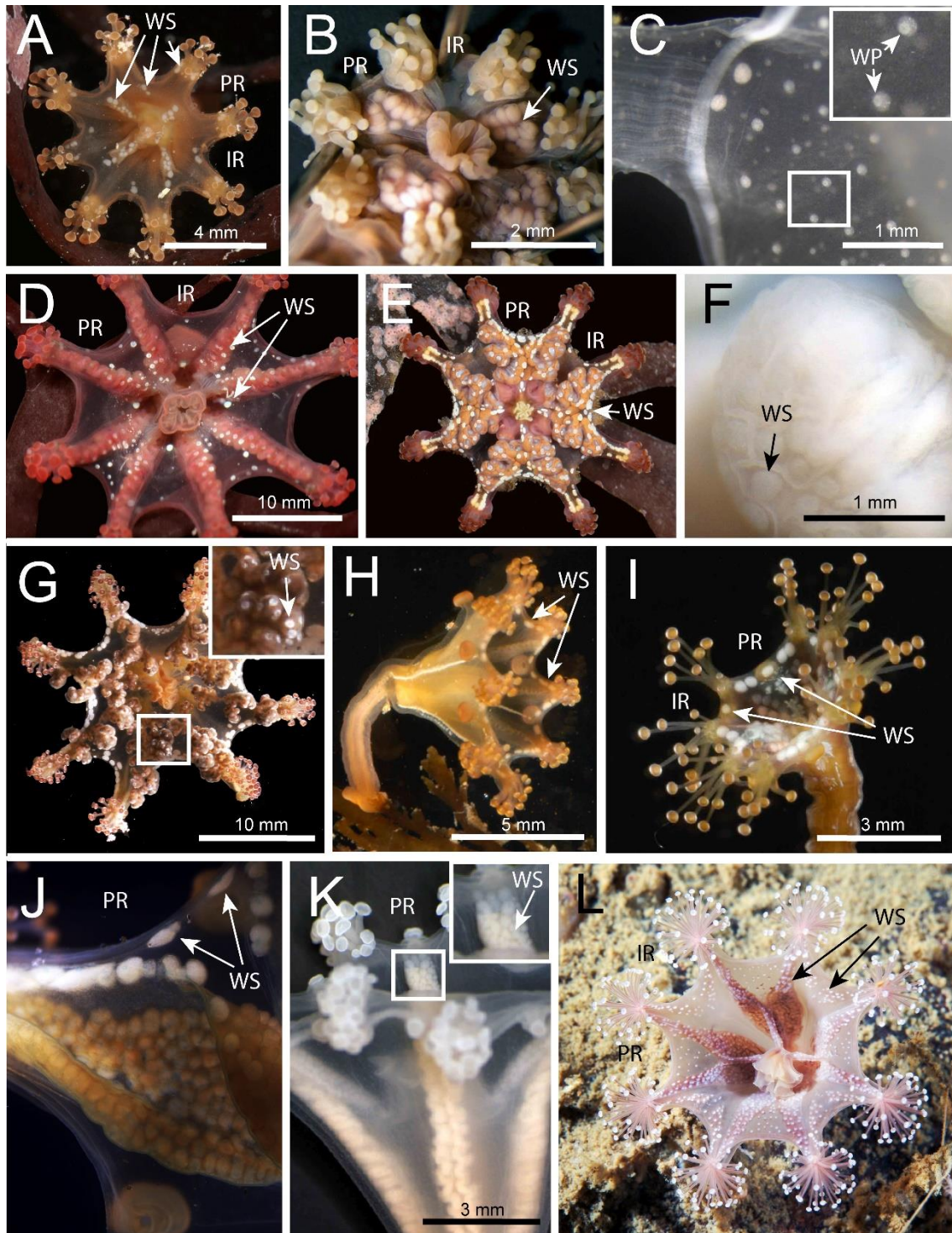


Figure 6. White spots on the subumbrella of staurozoans. (A) White spots distributed on the gonads in the perradii, near the apex of the arms and on the margin of the calyx of *Craterolophus convolvulus* (photo credit: David Fenwick). (B) White spots above the perradial gonads, forming (generally) two regular rows in *Depastromorpha africana* (photo credit: Yayoi Hirano). (C) Close-up of the subumbrella of *Lipkea* sp. with widely distributed white spots. Inset: opening pore of the white spot (photo credit: Yayoi Hirano). (D) White spots distributed on the *Calvadosia campanulata* subumbrella; note the presence of a white spot near the manubrium in the interradii (photo credit: David Fenwick). (E) Pattern of white spots in the subumbrella of *Calvadosia cruxmelitensis* forming a Maltese Cross (photo credit: David Fenwick; scale not available). (F) Close-up of the white spots on the gonadal nodes of *Calvadosia lewisi* (photo credit: Yayoi Hirano). (G) Pattern of white spots in the subumbrella of *Calvadosia tasmaniensis* (photo credit: Yayoi Hirano).

Inset: Close-up of the white spots on the gonadal nodes of *C. tasmaniensis*. **(H)** White spots present only on the calyx margin of *Haliclystus borealis* (photo credit: Yayoi Hirano). **(I)** White spots in *Haliclystus inabai* present inside the calyx only in the perradii, on the calyx margin, both in the perradii and interradii, with only one white spot occurring in the center of the interradial calyx margin (photo credit: Yayoi Hirano). **(J)** Rare white spots present on the perradial margin of the calyx of *Haliclystus 'sanjuanensis'* (scale not available). **(K)** White spots present only above the perradial gonads of *Manania uchidai* (subumbrellar epidermis of principal radial pocket) (photo credit: Yayoi Hirano). **(L)** White spots widely distributed in the subumbrella of *Lucernaria* sp., possibly on top of the gonads (photo credit: Alexander Semenov; scale not available). Abbreviations: IR, interradii; PR, perradii; WP, white spot pore; WS, white spot.

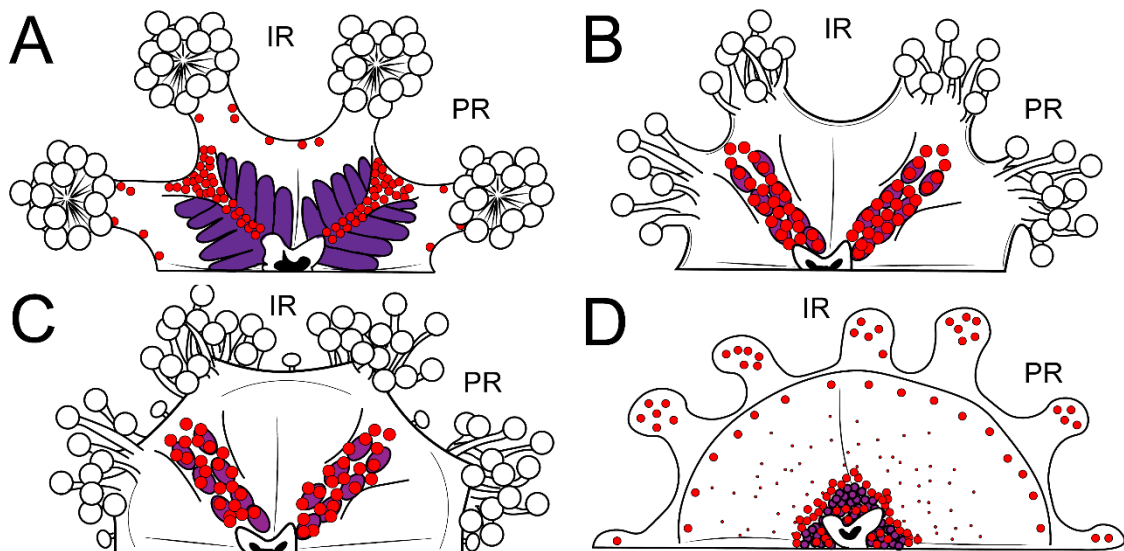


Figure 7. Scheme of the distribution pattern of white spots on the subumbrella of the genera *Craterolophus*, *Depastromorpha*, *Depastrum*, and *Lipkea*. **(A)** *Craterolophus convolvulus*, white spots above the gonads in the perradii, on the arms and in the calyx margin in the interradii. **(B)** *Depastromorpha africana*, white spots above the gonads in the perradii, organized in two regular rows in some specimens. **(C)** *Depastrum cyathiforme* (based on Gosse (1860), figure 3), white spots above the gonads in the perradii, organized in two irregular rows or randomly distributed. **(D)** *Lipkea* sp., white spots widespread on the subumbrella. Smaller white spots generally on the inner surface of the calyx, both in the interradii and perradii. Larger white spots on the calyx margin, marginal lappets, near the gonads and manubrium. Abbreviations: IR, interradii PR; perradii; gonads in purple; white spots in red.

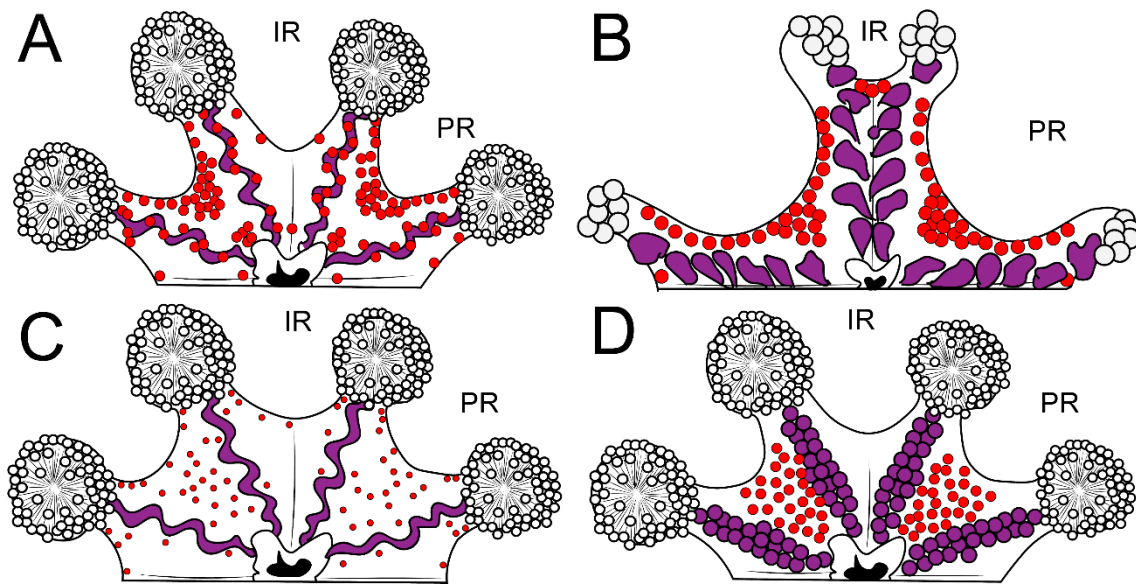


Figure 8. Scheme of the distribution pattern of white spots on the subumbrella of the genus *Calvadosia*. (A) First pattern represented by *Calvadosia campanulata*, *Calvadosia cruxmelitensis*, *Calvadosia corbini*, *Calvadosia lewisi*, *Calvadosia tasmaniensis*, and *Calvadosia* sp. 2 NZ, white spots occurring along the calyx margin (mainly in the perradii, but also in the interradii) and above the gonads. Several white spots are present on the perradial subumbrellar sectors and close to the manubrium corner regions. Few white spots may also occur in the interradii sectors and close to interradii face of manubrium in some species. (B) Second pattern represented by *Calvadosia cruciformis* and *Calvadosia nagatensis*, white spots along the perradial margin of the calyx and arms tips, a few entering into the perradial calyx inner sectors, white spots also present in the interradii margin of the calyx and arms tips. (C) Third pattern represented by *Calvadosia vanhoeffeni*, white spots mainly on perradial sectors, but few white spots also occur in the interradii region adjacent to the gonads. (D) Fourth pattern represented by *Calvadosia* sp. 1 NZ, white spots only on the perradial sectors, extending from the margin of the calyx to region near the manubrium. Abbreviations: IR, interradii; PR, perradii; gonads in purple; white spots in red.

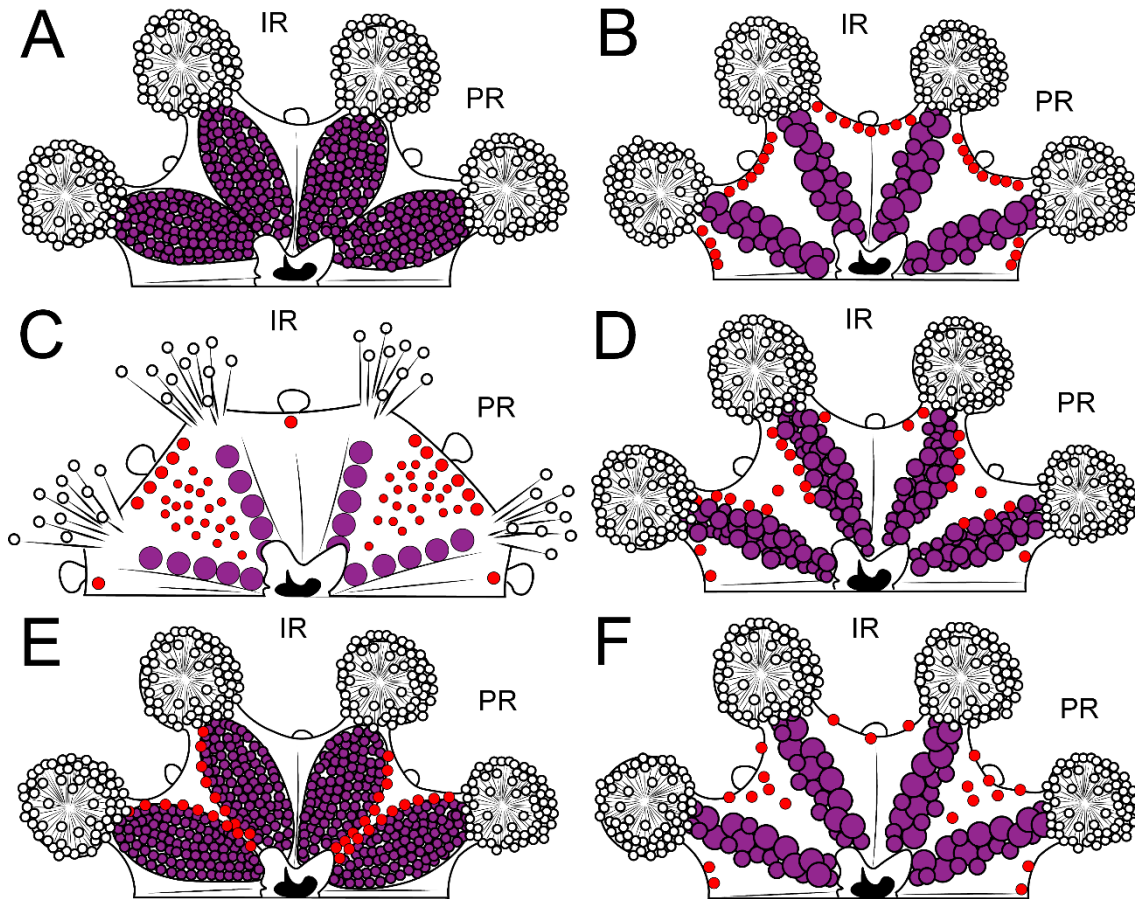


Figure 9. Scheme of the distribution pattern of white spots on the subumbrella of the genus *Haliclystus*. (A) First pattern represented by *Haliclystus auricula* and *Haliclystus antarcticus*, white spots absent on the subumbrellar surface. (B) Second pattern represented by *Haliclystus borealis*, *Haliclystus californiensis*, and *Haliclystus sinensis*, white spots only on the calyx margin, both in the perradii and in the interradii. (C) Third pattern represented by *Haliclystus inabai*, white spots on the perradial sectors of the calyx and along the perradial margin of the calyx; a single white spot in the center of the interradial calyx margin is present in some specimens. (D) Fourth pattern represented by *Haliclystus octoradiatus*, few white spots along the interradial margin of the calyx (1–3), usually near the base of the arms and along perradial edges of gonads, not getting close to the manubrium. Few white spots might also occur on perradial sectors. (E) Fifth pattern represented by *Haliclystus 'sanjuanensis'* and *Haliclystus stejnegeri*, white spots present only on the perradial margins of the gonads. In *H. stejnegeri* the white spots do not run along the entire perradial margin of the gonads, as in *H. 'sanjuanensis'*. (F) Sixth pattern represented by *Haliclystus tenuis*, white spots distributed in the margin of the calyx, in the interradii and perradii, but more numerous in the perradii; white spots also present in the perradial subumbrellar sectors. Abbreviations: IR, interradii; PR, perradii; gonads in purple; white spots in red.

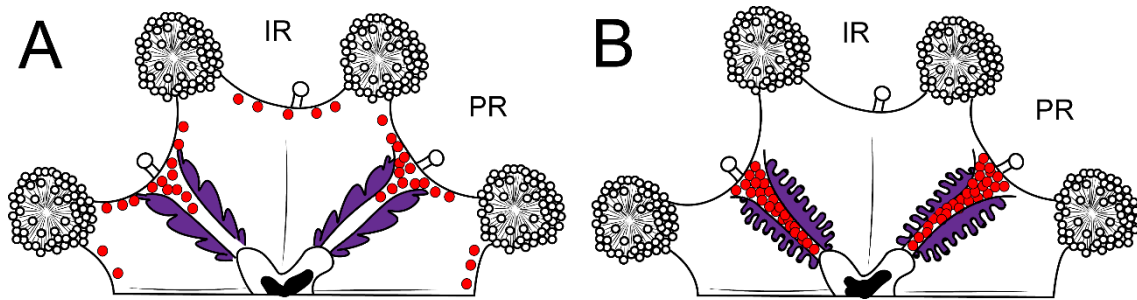


Figure 10. Scheme of the distribution pattern of white spots on the subumbrella of the genus *Manania*. **(A)** First pattern represented by *Manania atlantica*, *Manania auricula*, *Manania distincta*, *Manania gwilliamsi*, and *Manania handi*, white spots occur along the calyx margin, both in the perradii and interradii, and are more numerous in perradii, covering most of the subumbrellar epidermis of principal radial pocket, above the perradial gonads. **(B)** Second pattern represented by *Manania uchidai*, white spots only densely distributed over the subumbrellar epidermis of principal radial pocket, above the perradial gonads. Abbreviations: IR, interradii; PR perradii; gonads in purple; white spots in red.

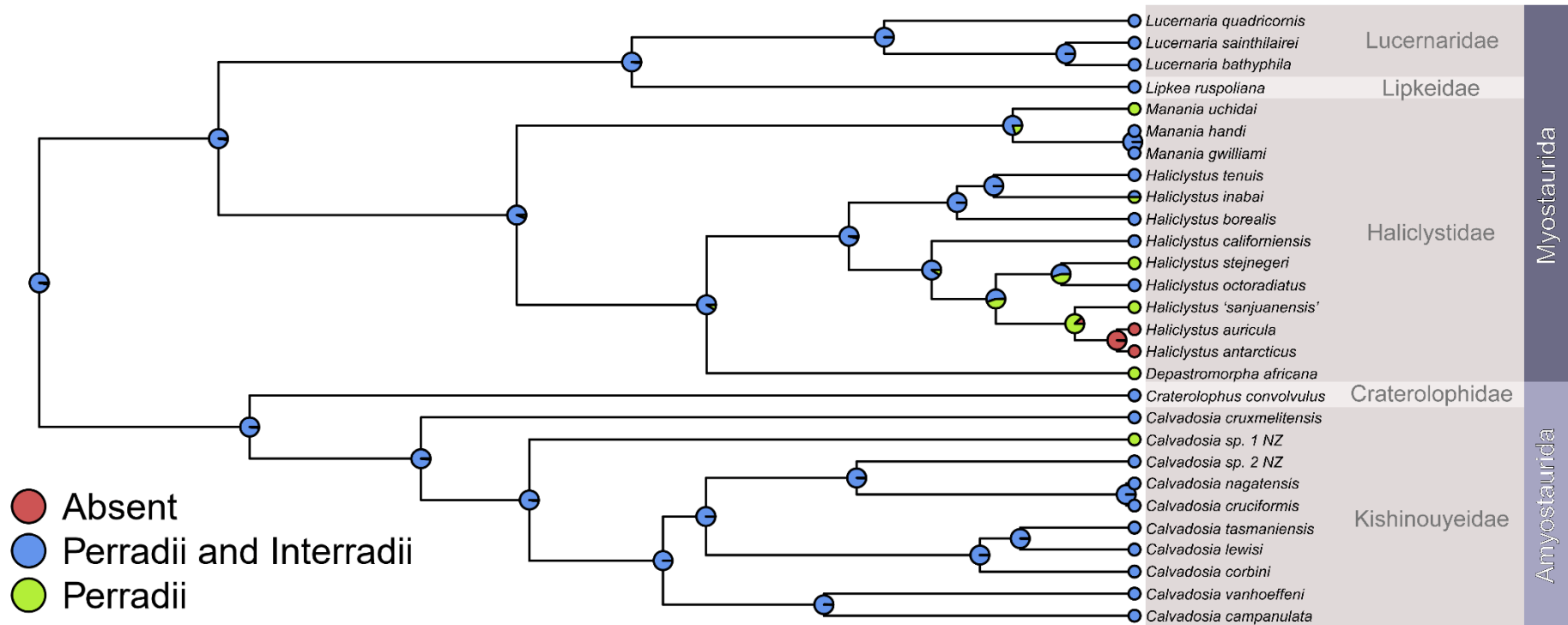


Figure 11. Ancestral state reconstruction for the distribution of white spots on subumbrella. The charts represent the probability of the ancestral state, terminals with more than one state represent polymorphism. Red indicates that the white spots are completely absent; blue indicates that the white spots are present in both the perradii and interradii; green indicates that the white spots are present only in the perradii.

Tables

Table 1. Species of staurozoans analyzed in this study.

Species	Voucher catalog number	Locality
<i>Calvadosia campanulata</i>	USNM 1233741	Verte Island, La Ciotat, Roscoff, France
<i>Calvadosia corbini</i>	MZUSP 1563; MZUSP 1564; MZUSP 1565	Praia dos Padres, Aracruz, Espírito Santo, Brazil
	USNM 54479; USNM 54480	La Parguera, Puerto Rico
<i>Calvadosia cruciformis</i>	Personal collection	Oshoro Bay, Hokkaido, Japan
	Personal collection	Obira, Hokkaido, Japan
<i>Calvadosia cruxmelitensis</i>	USNM 1233742	Wembury Bay, Plymouth, England, United Kingdom
	LABEC 16	Penzance, United Kingdom
<i>Calvadosia lewisi</i>	MZUSP 3415; MZUSP 3416	Simons' Town, Cape Town, Western Cape, South Africa
	Personal collection	Cape Town(?), South Africa (locality details unknown)
	CMNH ZG7819	A-Frame, False Bay, Cape Town, Western Cape, South Africa
<i>Calvadosia nagatensis</i>	LABEC 8; LABEC 9	Amatsura, Kamogawa, Chiba, Japan
	Personal collection	Uchiura Bay, Kamogawa, Chiba, Japan
<i>Calvadosia tasmaniensis</i>	USNM 1233739	Fortescue Bay, Tasmania, Australia
	USNM 1233740	Gerloff Bay, South Australia, Australia
	Personal collection	Blackfellows Cave, South Australia, Australia
<i>Calvadosia vanhoeffeni</i>	LABEC 6	PROANTAR IV, st 4872, Antarctica
	British Museum Catalog No. 1949.2.1.12; No. 1949.2.1.13	Cape Adare, Antarctica
<i>Calvadosia</i> sp. 1 NZ	USNM 1233747	South Island, Kaikoura Bay, New Zealand
	NIWA 62040	Island Bay, New Zealand
<i>Calvadosia</i> sp. 2 NZ	NIWA 86808; NIWA 86809	Tuputerenga Reserve, station Z15699, Wellington, New Zealand
	NIWA 86810; NIWA 86811	Tuputerenga Reserve, station Z15700, Wellington, New Zealand
	NIWA 79477	station Z15652, New Zealand
<i>Craterolophus convolvulus</i>	USNM 54321	Woods Hole, Nobska Point, Massachusetts, USA
	LABEC 7	Helgoland Island, Germany
<i>Depastromorpha africana</i>	MZUSP 3418	Sea Point, Cape Town, Western Cape, South Africa
	MAZUSP 3419	Kalk Bay, Cape Town, Western Cape, South Africa
	USNM 1233748	Gerloff Bay, South Australia, Australia
	Personal collection	Castle Rocks, False Bay, Cape Town, Western Cape, South Africa
<i>Depastrum cyathiforme</i>	British Museum Catalog No. 1934.7.28.1-10	Carnlough, Ireland
<i>Haliclystus antarcticus</i>	MZUSP 1551; MZUSP 1554; MZUSP 1555; MZUSP 1556; MZUSP 1557	Pieter Lenie Beach, Copacabana Refuge (USA), Admiralty Bay, King George Island, Antarctica
	MZUSP 1552	Shag Point, Arctowski Polish Station, Admiralty Bay, King George Island, Antarctica
	MZUSP 1559; MZUSP; 1561	Los Molinos, Valdivia, Chile
<i>Haliclystus borealis</i>	Personal collection	next to Denshin Beach, Muroran, Hokkaido, Japan
	Personal collection	Cape Erimo, Hokkaido, Japan
<i>Haliclystus inabai</i>	USNM 1233743	Otsuchi Bay, Iwate, Japan

	USNM 1233744	Oshoro Bay, Hokkaido, Japan
	USNM 1233745	Boso Peninsula, Uchiura Bay, Chiba, Japan
	Personal collection; CMNH	Uchiura Bay, Kamogawa, Chiba, Japan (20060625Kominato); Cape Futtsu, Chiba, Japan (20180419Futtsu); Shimoda (Izu Peninsula, Shizuoka), Misaki (Kanagawa), Tateyama (Chiba), Otsuchi Bay (Iwate), and Oshoro Bay & Obira (Hokkaido)
<i>Haliclystus tenuis</i>	CMNH	Cape Futtsu, Chiba, Japan
<i>Haliclystus monstrosus</i>	British Museum Catalog No. 1962.10.10.46	Southern Kuril Strait, Kurile Islands, Russia
<i>Haliclystus 'sanjuanensis'</i>	LABEC 11; LABEC 12; LABEC 14; LABEC 15	Castle Point, Washington, USA
	LABEC 13	MacGinities shoreline, Washington, USA
	Personal collection	Chinese Cemetery, Victoria, BC, Canada (Vancouver Island 48°24'22.88"N, 123°19'24.48"W)
<i>Haliclystus stejnegeri</i>	LABEC 1	E. Kamchatka, Avancha Bay, Russia
<i>Lipkea ruspolitana</i>	LABEC 10	Aquariums du Musée océanographique de Monaco, Monaco
<i>Lipkea</i> sp. 1	LABEC 5	North Carolina Aquarium at Fort Fisher, North Carolina, USA
<i>Lipkea</i> sp. 2	USNM 1315325; USNM 1315326	Coastal Branch of the Natural History Museum and Institute Chiba, Katsuura, Chiba, Japan
<i>Lucernaria haeckeli</i>	Zoological Museum's collection, Natural History Museum of Denmark	Bay opposite to Red Island, Rode Fjord, Greenland
<i>Manania handi</i>	Personal collection	Chinese Cemetery, Victoria, BC, Canada (Vancouver Island 48°24'22.88"N, 123°19'24.48"W)
<i>Manania hexaradiata</i>	British Museum Catalog No. 1972.11.28.3	Barents Sea (72°23'30"N, 52°41'E)
<i>Manania uchidai</i>	Personal collection	Rausu, Hokkaido, Japan

Table 2. Distribution of white spots on subumbrella for each staurozoan species (see Table S1 for review and references).

Species	Distribution of white spots on subumbrella
<i>Craterolophus convolvulus</i>	perradial and interradial
<i>Calvadosia campanulata</i>	perradial and interradial
<i>Calvadosia corbini</i>	perradial and interradial
<i>Calvadosia cruciformis</i>	perradial and interradial
<i>Calvadosia cruxmelitensis</i>	perradial and interradial
<i>Calvadosia nagatensis</i>	perradial and interradial
<i>Calvadosia tasmaniensis</i>	perradial and interradial
<i>Calvadosia vanhoeffeni</i>	perradial and interradial
<i>Calvadosia lewisi</i>	perradial and interradial
<i>Calvadosia</i> sp. 1 NZ	perradial
<i>Calvadosia</i> sp. 2 NZ	perradial and interradial
<i>Depastromorpha africana</i>	perradial
<i>Haliclystus antarcticus</i>	absent
<i>Haliclystus auricula</i>	absent
<i>Haliclystus borealis</i>	perradial and interradial
<i>Haliclystus californiensis</i>	perradial and interradial
<i>Haliclystus inabai</i>	perradial and interradial or perradial
<i>Haliclystus octoradiatus</i>	perradial and interradial
<i>Haliclystus 'sanjuanensis'</i>	perradial
<i>Haliclystus stejneri</i>	perradial
<i>Haliclystus tenuis</i>	perradial and interradial
<i>Manania gwilliami</i>	perradial and interradial
<i>Manania handi</i>	perradial and interradial
<i>Manania uchidai</i>	perradial
<i>Lipkea ruspoliana</i>	perradial and interradial
<i>Lucernaria bathyphila</i>	perradial and interradial
<i>Lucernaria quadricornis</i>	perradial and interradial
<i>Lucernaria sainthilairei</i>	perradial and interradial

Table 3: MK models tested for ancestral reconstruction of distribution of white spots on subumbrella.

Model	AICc	AICw
ER	49.597	0.182
ARD	48.160	0.373
SYM	47.818	0.443

Supplemental material

Table S1 - Tables containing the complete literature review on white spots for all species of Staurozoa.

<https://drive.google.com/drive/folders/1UytlSOGXDKkIHeMJcQPhyqVbIPbKVpf8?usp=sharing>

Morphological diversity and evolution of gonads in Staurozoa (Cnidaria)

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Abstract

Staurozoa is a class of benthic cnidarians with 50 described species, generally found in temperate and polar waters. They are known as stalked jellyfish, since the metamorphosis from polyp to medusa occurs mainly in the apical region, with retention of the basal peduncle in the medusoid sexual stage. Despite having a relatively simple body organization, the stauromedusa presents regions of tissue specialization, including the gonads. Features such as the presence of gametoducts, follicular cells, compartmentalization of mature and immature gametes, and the wide variation in the macroscopic arrangement of the gonads confer morphological complexity to the class. However, the understanding of the morphological diversity of the gonads in Staurozoa is still superficial and mainly focused on the genus *Haliclystus*. The present study aims to (1) investigate the morphological diversity of the gonads for all species of the class and (2) understand the evolutionary history of the presence of vesicles and the positioning of the gonads in Staurozoa. An extensive literature review was conducted, highlighting the macroscopic (external) and microscopic (internal) organization of the gonads for all species of Staurozoa, as well as their arrangement in the subumbrella. Also, phylogenetic signal analyses (δ -statistic) and reconstruction of ancestral states, by maximum likelihood, were performed in R software, using the corHMM package, from a dated molecular phylogeny of Staurozoa. The literature review provided a more comprehensive perspective of the gonads for the class, making evident its diversity and complexity, with emphasis on the genus *Calvadosia* as the most heterogeneous group concerning the gonadal organization. The results also pointed to the potential taxonomic use of new gonadal features and indicated gaps in the knowledge about the gonads of some genera, such as *Depastrum*, *Kyopoda*, *Lucernaria*, and *Stylocoronella*, and in the understanding of the internal complexity of the vesicles in *Lucernaria*. The reconstruction of the evolutionary history of the presence of vesicles and positioning of the gonads revealed a

phylogenetic signal, further indicating that the ancestral condition is the presence of vesicles located on the adradial axis. This study highlights the morphological diversity of the gonads in Staurozoa, providing subsidies for further researches on gonadal characteristics in taxonomy, evolutionary correlations with other structures, such as the claustrum, and the evolution of the gonads in Cnidaria.

1. Introduction

Staurozoa (Marques & Collins, 2004) is a class of Cnidaria with approximately 50 valid species, found mainly in temperate and polar waters, with higher richness documented in the Northern Hemisphere (Miranda et al., 2018). These animals (Fig. 1A, B) have a particular life cycle: unlike other Medusozoa (Collins et al., 2006), the metamorphosis from polyp to medusa occurs gradually, mainly in the apical region (Hirano, 1986; Kikinger & Salvini-Plawen, 1995; Miranda et al., 2010). As a consequence, the benthic medusoid sexual stage (stauromedusa) retains polypoid characteristics (Collins et al., 2006; Miranda et al., 2013, 2016a), attaching the peduncle (Fig. 1B) to many different substrates, mainly macroscopic algae and rocks (Miranda et al., 2018).

Despite having this prominent life cycle feature, stalked jellyfishes are possibly the least known and studied group among the cnidarians (Miranda et al., 2018). As a result, other outstanding features of the class are subject to generalizations, such as their gonads. The gonads of the stalked jellyfishes (Fig. 1C) have been described in several articles (e.g., Uchida, 1929; Eckelbarger & Larson, 1993; Miranda et al., 2016a), which showed that these structures usually consist of evaginations of the gastrodermis, frequently resulting in the formation of vesicles (Fig. 1D), as can be observed in species of *Haliclystus* and *Calvadosia*. However, there is variation, and some species present organization of the gonads in a single layer between the gastrodermis and the epidermis, as species of *Manania* (Fig. 1E; Uchida & Hanaoka, 1933; Miranda et al., 2016a). These reproductive structures are located within the gastric radial pocket in staurozoan species that do not have a claustrum (Fig. 1F). Claustrum is a structure formed by a central mesoglea layer surrounded by double gastrodermis layers, which divides the gastrovascular cavity into eight gastric pockets (Miranda et al., 2017a). In species with a claustrum, their gonads are located in the main radial gastric pocket (Fig. 1G; Miranda et al., 2017a). Importantly, the presence of claustrum evolved independently in Staurozoa

(Miranda et al., 2016a), with possible implications for the organization of the gonad in the group. Other features that indicate the complexity of the gonad in Staurozoa include the frequent gonadal compartmentalization into regions of mature and immature female/male gametes (Miranda et al., 2016a); the presence of gametoduct, a channel that connects the region of mature gametes to the gastrovascular cavity (Miranda et al., 2016a); and the presence of follicular cells that surround the oocytes so as to position the mature gametes in the central region of the ovaries, a feature possibly unique to Staurozoa (Eckelbarger, 1994, but see question in Tiemann & Jarms, 2010).

However, gonadal information has never been evaluated in an evolutionary context within Staurozoa. The low data sampling, with higher representativeness for the genus *Haliclystus* (Uchida & Hanaoka, 1933, 1934; Eckelbarger & Larson, 1993; Eckelbarger, 1994), leads to generalizations that may impair the understanding about the real diversity and evolution of the gonad in Staurozoa and, consequently, in Cnidaria.

In taxonomy, it is evident that the inadequate data sampling regarding the other genera is detrimental, considering that again, only researchers on *Haliclystus* traditionally use gonadal characteristics in the identification and delimitation of species based on the number of vesicles and on the rows of vesicles that compose its gonads (Clark, 1863; Haeckel, 1879; Pfeffer, 1889; Mayer, 1910; Amor, 1962; Hirano, 1986; Miranda et al., 2009; Kahn et al., 2010). Thus, the present study, based on a broad literature review, aims (1) to evidence the real gonadal diversity of Staurozoa, pointing out gaps in knowledge and highlighting new sources of characters that can be used in the taxonomy of the class; (2) to understand the evolutionary history of gonadal traits through phylogenetic signal analysis and reconstruction of ancestral states.

2. Material and Methods

2.1. Review of the morphological diversity of gonads in Staurozoa

An extensive literature review has been conducted regarding the morphology of the gonads for all species of the class Staurozoa. The review includes features of the macroscopic (external) organization, such as form and position of the gonads (perradii, adradii, interradii/adradii), and microscopic (internal) organization, such as the presence of vesicles, gametoducts, and follicular cells (Table S1). Additional observations on

images and preserved material for some species complemented this review (Table 1; Table S1, “this study”).

2.2. Evolution of gonads

In order to evaluate the evolutionary history of gonadal traits for the class Staurozoa, two analyses were performed in R version 4.1.0 (R Core Team 2021): ancestral states reconstruction and phylogenetic signal, using the package corHMM version 2.8 (Boyko & Beaulieu, 2021) and the code provided by Borges et al. (2019) in the GitHub branch: `mrborges23/delta_statistic`, respectively. We selected two gonadal traits evidenced in the literature review (Table S1; Table 2). The most-recent staurozoan dated tree (obtained from Miranda et al., 2016b and preliminary results from Morales-Guerrero et al., *in prep.*) was used in the analyses. Only the clade Staurozoa (28 terminals) was under investigation, so the outgroups were pruned using the package `geiger` (Pennell et al., 2014). Terminals within the clade Staurozoa that have no data for the analyzed trait were also pruned.

The methodology recently proposed by Borges et al. (2019) was used to investigate the presence of phylogenetic signal between the traits and the tree. The method reconstructs the ancestral states of the trait, and from the uncertainty level of the reconstruction, it calculates the value of δ , which indicates the phylogenetic signal; the higher the value δ ($\delta > 1$), the higher the level of phylogenetic signal of the discrete trait under investigation (Borges et al., 2019). We generated 1,000 random trees in order to investigate whether the value obtained in the δ statistic is significant, keeping the same terminals and proportion of character states. Subsequently, the value of δ for the simulated trees was calculated. If the value of δ obtained from the empirical analysis exceeds the 95% quantile of the simulated data, we can infer that the phylogenetic signal obtained empirically is significant (Borges et al., 2019).

Three models with different transition rate were tested for each trait: (i) equal rates model (ER), which implies that all transitions rates have the same parameter, (ii) all rates different model (ARD), which means that all transitions rates have different parameters, and (iii) the symmetrical model (SYM), where symmetric transitions rates (forward and reverse) share the same parameters (Pennell et al., 2014). The best fit Mk model, based in the Akaike information criterion (AICc correction and AICw), was used to reconstruct

the ancestral states for each node of each trait in the dated tree using maximum likelihood method by marginal reconstruction (Joy et al., 2016).

3. Results

3.1. Review of gonadal morphology in Staurozoa (Table S1)

3.1.1. General description of the gonads in Staurozoa

Staurozoa presents a morphological diversity of their reproductive structures. Their arrangement in the subumbrella establishes three different patterns of gonadal distribution. They can be (1) positioned in the adradii (along the arms) (e.g., in species of *Haliclystus*; Fig. 1A); (2) they can be positioned in the interradii/adradii for species that have the arms paired in the interradii (e.g., *Calvadosia cruciformis*; Fig. 2A); and (3) positioned in the perradii (e.g., *Craterolophus convolvulus*; Fig. 2B).

In addition, there are two major groups of gonadal morphotypes: (1) gonads in a sinuous row (Fig. 2C), whether positioned in the adradii, interradii/adradii, or perradii; and (2) gonads in rows or clusters of vesicles (gametes contained between a layer of epidermis and a layer of evaginated gastrodermis; Fig. 1C, D), positioned in the adradii or perradii. The first group encompasses genera such as *Calvadosia* (except *Calvadosia* sp. 1 NZ), *Craterolophus*, and *Manania*, and the second group includes *Depastromorpha*, *Haliclystus*, and *Lipkea* (Table 3).

The internal organization of the gonadal tissue also varies in stauromedusae. *Haliclystus* and some species of *Calvadosia* are traditionally characterized as possessing vesicles (Table S1), since they present a well-defined evagination of the gastrodermis and evident compartmentalization of gametes, which is different from the internal organization observed in other genera. Therefore, in the evolutionary analyses performed in this study, the vesicles of *Haliclystus* and *Calvadosia* will be considered as "type 1 vesicles" (Fig. 1D; Table 2). Differently, the gonadal internal organization of some species of *Lucernaria* will be considered as "type 2 vesicles" (Fig. 2D; Table 2), based on their imprecise vesicular organization, contrasting to the "traditional" vesicles (like those found in *Haliclystus*). The "vesicles" of the genus *Lucernaria* will be discussed in more detail in the next section. A third internal organization is the complete absence of vesicles, as found in *Craterolopus* and *Manania* (Fig. 1E).

3.1.2. Morphological description of the gonads for each staurozoan genus

Calvadosia

The genus *Calvadosia* shows a high variety of form and organization of gonads (Figs. 3, 4; Tables 2, 4; Table S1). In general, species of the genus has the gonads positioned either in the adradial (Figs. 3A, B; 4A, B; Tables 2, 4; e.g., *Calvadosia campanulata* and *Calvadosia* sp.1 NZ) or in the interradial/adradial, depending on whether the species has paired arms in the interradial (Figs. 2A; 3C; 4C-E; e.g., *C. cruciformis*, *Calvadosia tasmaniensis*, and *Calvadosia lewisi*) (Tables 2, 4; Table S1). The gonads extend from the subumbrellar region near the manubrium to the tips of the arms in most species, except *Calvadosia vanhoeffeni*, in which the gonads do not reach the apex of the arms. The genus has no claustrum, so the gonads are contained in radial gastric pockets (Table S1).

Only *Calvadosia* sp. 1 NZ does not have gonads arranged in a sinuous row. This species has gonads organized a series of circular vesicles that are organized in rows (Figs. 3B; 4B), very similar to the gonadal arrangement in *Halicyclotus* (Fig. 1A, C; Table S1). The other species of the genus, however, have gonads in the form of a single (more or less) sinuous row, usually forming an outgrowth on the subumbrellar epidermis, which can give the appearance of a “ladder” in exumbrellar view in some species, as in *C. cruciformis* (Figs. 2A; 4C). Moreover, in *Calvadosia capensis*, *Calvadosia corbini*, *C. lewisi*, *C. tasmaniensis*, and possibly *Calvadosia hawaiiensis*, along the sinuous row, nodules protrude into the subumbrella of the animal (Figs. 3C; 4D, E; Table S1). The nodules consist of evaginations of the subumbrellar epidermis lining the gonads and can assume different shapes, an information useful in taxonomy (Miranda et al., 2017b). *Calvadosia corbini* and *C. tasmaniensis* that have erect, irregular (raisin-like)-shaped, and in some cases branched nodules (Fig. 4D), whereas *C. lewisi* has curved and smooth nodules (Figs. 3C; 4E; Table S1).

Calvadosia corbini, a species with nodular gonads, has the internal gonadal tissue organized in vesicles, similarly to *C. cruciformis*, *Calvadosia nagatensis*, and *C. vanhoeffeni* (Figs. 3D-F; Tables 2, 4; Table S1). However, for the other species of the genus, there is no information or precise information regarding this feature (Tables 2, 4),

although it is worth noting that the morphological similarity of *Calvadosia tsingtaoensis* with *C. cruciformis* and *C. nagatensis* (Ling, 1937) may be an indication of the presence of vesicles in *C. tsingtaoensis* (Table S1). There is a regionalization of gametes in each vesicle, where the mature gametes are adjacent to the epidermis, surrounded by the immature gametes, which are positioned adjacent to the gastrodermis of the vesicle (Fig. 3D, E; Miranda et al., 2016a; Table S1). Gametoducts are present and are formed from the fusion of putative follicular cells (of gastrodermal origin) and cells of the vesicular gastrodermis (Fig. 3F; Miranda et al., 2016a; Table S1).

Haliclystus

The genus *Haliclystus* has a very homogenous pattern of gonads. This pattern consists of 8 leaf-shaped (lanceolate) gonads positioned on each adradial, extending from the subumbrellar region near the manubrium to the tips of the arms (Fig. 1A, C). These gonads are composed of rows of rounded vesicles (Fig. 1D), which may vary in number among species and individuals, generally having the largest number of rows in their middle portion (Figs. 1B; 5A, B; Table 5; Table S1). The gonads are distributed in the gastric radial pockets, since species of this genus lacks the claustrum (Fig. 1F). Each vesicle has a regionalization of gametes, where the mature gametes are in the central region of the vesicle, being surrounded by the immature gametes, which are positioned adjacent to the gastrodermis of the vesicle (Fig. 1D; Table S1). Gametoducts are present and are formed from the fusion of putative follicular cells (of gastrodermal origin) and cells of the vesicle gastrodermis (Miranda et al., 2016a; Table S1).

Differences found among the species of the genus are mainly related to the number of rows of vesicles and vesicles in each gonad (Fig. 5A, B; Table 5; Table S1). *Haliclystus sinensis* presents only one row of vesicles, whereas *Haliclystus inabai* and *Haliclystus borealis* can also present only one to 3 or 6 rows, respectively (Table 5; Table S1). *Haliclystus 'sanjuanensis'* has the largest and most variable number of vesicle rows in the genus (10 - 22), as well as the largest number of vesicles per gonad, ranging from 200 to 300 (Table 5; Table S1).

Lipkea

The gonads of the genus *Lipkea* are restricted to the inner regions of the calyx near the manubrium. The vesicles that compose them are grouped in a circular shape and positioned in the adradial (Figs. 5C, 6A; Table S1). There is no robust information regarding regionalization of gametes in the vesicles or presence of gametoducts, but Carlgren (1933) mentions and illustrates (Carlgren, 1933: figure 12) the presence of channels in each vesicle that open in the gastrovascular cavity (Table S1).

Craterolophus

Craterolophus has eight gonads laterally folded in longitudinal rows (sinuous rows) in the perradii, extending from the lower inner calyx near manubrium to the calyx margin (Figs. 2B, C; 7A). The number of sinuosities can vary between 10 and 16 (Mayer, 1910; Table S1). The gonads are in the main radial gastric pocket (due to the presence of the claustrum), and their content (gametes) is restricted to a layer of gastrodermis and epidermis, not forming vesicles (Fig. 6B). Immature gametes lie closer to the gastrodermis, while mature gametes are adjacent to the epidermis, indicating a regionalization of gametes along the gonads (Fig. 6B; Table S1). Gametoducts are present and are formed by putative follicular cells (of gastrodermal origin) which merges with gastrodermis of gastrovascular cavity (Miranda et al., 2016a; Table S1).

Gonadal layers of same main radial pocket are formed by lateral tissue of two different interradial septa (two adjacent septa), which gives a herringbone shape (or a feathery shape; Table S1) to the gonads (in exumbrellar view) when these two sinuous rows of gonads meet at the perradii (Figs. 2B, C; 7A). In *C. convolvulus*, the gonads along the subumbrella narrows at both ends (near the manubrium and near the calyx margin), evidencing its herringbone shape. There are no specific descriptions for the other species of the genus, *Craterolophus macrocystis*, but some authors indicate that they are similar to *C. convolvulus* (Table S1).

Depastromorpha

Depastromorpha africana, the single species of the genus, has the gonad organized in two parallel rows of vesicles. Given the presence of the claustrum, two well

defined homogeneous rows of vesicles are visible on each perradii (Figs. 6C, 7B; Table S1). The rows extend longitudinally along the entire body of the animal, approaching the margin of the calyx. The gonads are located in the main gastric radial pocket due the presence of a claustrum (Miranda et al., 2016a). There is no information regarding the regionalization of gametes in the vesicles or the presence of gametoducts.

Manania

Manania has eight gonads laterally folded in longitudinal rows (sinuous rows) in the perradii, extending from the lower calyx to the calyx margin (Figs. 6D, E, F; 7C, D; Table 6). The gonads are located in the main gastric radial pocket (due to the presence of the claustrum), and their content (gametes) is restricted to a layer of gastrodermis and epidermis, not forming vesicles (Fig. 1E). Immature gametes lie closer to the gastrodermis, while mature gametes are adjacent to the epidermis, indicating a regionalization of gametes along the gonads (Fig. 1E; Table S1), and gametoducts are present.

Gonadal layers of same main radial pocket formed by lateral tissue of two different interradial septa (two adjacent septa), which gives a herringbone shape to the gonads (in exumbrellar view) when these two sinuous rows of gonads meet at the perradii (Figs. 6D, E, F; 7C, D; Table 6). The herringbone pattern of gonads can be more or less homogeneous; in *Manania atlantica*, *Manania handi*, *Manania gwilliami* (Fig. 6D), and *Manania distincta* (Fig. 6E) the gonads narrow at both ends (near the manubrium and near the calyx margin), thus being less homogeneous (Fig. 7C) in appearance than the gonads of *Manania uchidai* (Fig. 6F), which remain with an homogeneous shape throughout the calyx (Fig. 6F; Table 6). This homogeneous pattern means that *M. uchidai* does not have a remarkable herringbone shape of the gonads. *Manania hexaradiata* has gonads described as “horseshoe”-shaped (Couch, 1844; Clark, 1863; Table S1), differently from the herringbone pattern described for the other species of the class. As the descriptions are not precise and no illustrations were provided (Table 6; Table S1), it is difficult to attest whether in fact this could be a different pattern from the other species of the genus. *Manania auricula* do not present precise descriptions regarding its gonads, although it apparently has gonads more similar to those of *M. uchidai*, with a more

homogeneous herringbone pattern (Table 6; Table S1; Miranda & Collins, 2019: figure 2).

The pattern of the gonads in *Manania* (Fig. 7C, D, E) strongly resembles that of *Craterolophus* (Fig. 7A; Table S1).

Depastrum

The information in the literature regarding the gonads of *Depastrum cyathiforme*, the single species of the genus, is imprecise and fragmented. The gonads are organized in two sinuous rows, positioned in the perradii, from the region near the manubrium but not extending to the margin of calyx. Due to the presence of a claustrum (Mayer, 1910), the gonads are located in the main gastric radial pocket. The general gonadal pattern in exumbrellar view (Fig. 6G) of *D. cyathiforme* is similar to species of *Craterolophus* (Figs. 2B, C; 7A) and *Manania* (Figs. 6D, E, F; 7C, D), although there is not enough information in the literature to provide a robust description (Table S1).

Halimocyathus

There is little information regarding the gonads of *Halimocyathus platypus*, the single species of the genus, but it is possible to infer that the horseshoe-shaped gonads are positioned in the perradii, due to the presence of the claustrum (see Clark, 1863; Table S1). The gonads extend to the calyx margin in a sinuous row (12-17 sinuosities), where each sinuosity gradually increases the closer they are to the calyx margin (Table S1).

Kyopoda

Descriptions of the gonads of *Kyopoda lamberti*, the single species of the genus, are scarce and imprecise in the literature. The species has the gonads located in the peduncle, instead of the calyx, which does not occur in any other stauromedusa. They are positioned in the adradialia possibly organized in two parallel rows of vesicles (probably) (Table S1; Larson, 1988: figure 1C).

Lucernaria

Lucernaria presents fragmented and confusing information regarding the gonads. It is possible to divide the genus into two groups, based on the species that have paired arms at the interradii (*Lucernaria quadricornis*, *Lucernaria sainthilairei*, and *Lucernaria walteri*) and those that do not (*Lucernaria australis*, *Lucernaria bathyphila*, *Lucernaria haeckeli*, and *Lucernaria janetae*). For the first group, the gonads occur in the interradii (near the manubrium) and gradually diverge into the adradia, where they reach the tips of the arms. In the second group, the gonads are positioned in the adradia (Table 2; Table S1).

The gonads have a vesicle-like organization, however they are not very well defined (Fig. 2D; Miranda et al., 2016a; Table S1), so more detailed studies are necessary to clarify the vesicular nature of internal gonadal organization in the genus. In general, each “vesicle” has a regionalization of gametes, where the mature gametes are in the central region of the “vesicle”, being surrounded by the immature gametes, which are positioned adjacent to the gastrodermis of the “vesicle” (but see Miranda et al., 2016a: figure 27; Table S1). Gametoducts are present and are formed from the fusion of putative follicular cells (of gastrodermal origin) and cells of the vesicle gastrodermis (Miranda et al., 2016a; Table S1).

Stylocoronella

There is little information regarding the gonads of the genus *Stylocoronella* (Fig. 6H; Table S1). *Stylocoronella riedli* has four pairs of V-shaped gonads (Fig. 6G; Table S1), positioned on the adradia, not reaching calyx margin. In exumbrellar view, the gonads resemble raisins, possibly formed by a cluster of rounded vesicles (Table S1). There is no mention of the gonads of *Stylocoronella variabilis* in the literature.

3.2. Extent of knowledge of the gonads of Staurozoa

The literature review revealed the real extent and major gaps of knowledge of the gonadal structures of Staurozoa (Fig. 8) and confirmed that generalizations made for the entire class are based mainly on the characteristics found in the genus *Haliclystus* (e.g., Eckelbarger & Larson, 1993; Eckelbarger, 1994).

Regarding the organization of the gonads into vesicles (Fig. 8A), more than half of the species of the class have no information or imprecise information in the literature. Only four species present unequivocal reports of the absence of vesicles in their gonads (*Craterolophus convolvulus*, *Manania atlantica*, *Manania handi* and *Manania uchidai*), while 17 species clearly possess them (12 species of *Haliclystus*, four of *Calvadosia* and *Depastromorpha africana*).

The scenario is somewhat different for the positioning of the gonads on the subumbrella (Fig. 8B). Only five species do not have precise information about their positioning, including *Calvadosia hawaiiensis*, *Kyopoda lamberti*, *Halimocyathus platypus*, *Stylocoronella riedli*, and *Stylocoronella variabilis*. The adradial position is the most recurrent, followed by perradial and interradial/adradial.

The presence of follicular cells (Fig. 8C; Table 7) and gametoducts (Fig. 8D; Table 7) are the most unknown gonadal features among the species of Staurozoa. Only seven out of the 50 species in the class show possible follicle cells and 13 undoubtedly present gametoducts. *Depastromorpha africana* does not show gametoducts, according to Carlgren (1935), although this statement requires further and detailed investigation, considering that eight out nine species analyzed by Miranda et al. (2016a), from different genera, have gametoducts.

The gaps in knowledge of gonadal structures for several species mentioned above can be justified by the limited number of studies published so far about gonadal morphological descriptions for each genus (Fig. 8E). The discrepancy is remarkable between the number of studies produced for the genera *Haliclystus* and *Calvadosia* in relation to lesser-known and diverse genera, such as *Kyopoda*, *Halimocyathus*, and *Stylocoronella*. These gaps reinforces that the knowledge of gonadal pattern of organization in Staurozoa are based on generalizations of few genera, which can undermine homology relations and inferences on evolutionary history of gonads in Cnidaria.

3.3. Evolution of gonads

We found a phylogenetic signal in all tests performed for all gonadal traits (Table 2). The value of entropies from the empirical analysis of every test exceeds the 95%

quantile of the simulated data so it can be inferred that the phylogenetic signal obtained empirically is significant (Table 8). This result indicates that all traits distribution under investigation is non-random and highly conserved throughout the phylogeny and may be considered as a result of shared ancestry, where more closely related species tend to have similar states.

The best fit Mk model for the presence of vesicles was the equal rates (ER) (Table 8). Reconstruction of the evolutionary history of this trait (Fig. 9) revealed that the last ancestor of stalked jellyfishes would have a gonad consisting of type 1 vesicles. They were lost independently three times during the staurozoan evolution, including the total loss in the genera *Craterolophus* and *Manania*, and a modification in *Lucernaria*, with type 2 vesicles.

The positioning of the gonads in the subumbrella also showed equal rates (ER) as best fit Mk model (Table 8). The ancestral states reconstruction revealed that the last common ancestor of stalked jellyfishes had their gonads along the arms in the adradii (Fig. 10). The trait was independently modified twice to the interradii/adradii positioning, once in the genus *Calvadosia* and once in *Lucernaria*, then returned to the adradii positioning in *Lucernaria bathyphila*. Greatest uncertainty occurs in the last common ancestor of the family Haliclystidae, with very close probabilities of the positioning of the gonads in the adradii or the perradii (Fig. 10). The presence of the claustrum is related to the perradial positioning of the gonads in *Manania* and *Depastromorpha*, so it is necessary to discuss the evolution of this structure for a better understanding of the perradial positioning of the gonads in this family. Perradial gonads also appeared independently in the genus *Craterolophus* (Fig. 10), also related to the presence of claustrum.

4. Discussion

4.1. Diversity of gonads in Acraspeda

The gonads of Staurozoa are described in several papers (Haeckel, 1882; Mayer, 1910; Uchida, 1929; Berrill, 1963; Eckelbarger & Larson, 1993; Eckelbarger, 1994; Miranda et al., 2013, 2016a) and some of them highlight their morphological complexity in comparison to the other cnidarians (Eckelbarger & Larson, 1993; Eckelbarger, 1994),

especially when compared to species of Acraspeda (clade including Staurozoa, Cubozoa, and Scyphozoa) (Kayal et al., 2018).

The class Scyphozoa differs from the other classes regarding the morphology of the gonads mainly because their gametes are immersed in the mesoglea of the gonads, which is lined by gastrodermis (Widersten, 1965; Eckelbarger, 1994; Morandini & Silveira, 2001), differently from Cubozoa that present their gametes somewhat linearly compartmentalized between the mesoglea and gastrodermis in a thin independent layer (Tiseo et al., 2019). These organizations are mostly different from gonadal morphology observed in Staurozoa, which presents a more evident compartmentalization, having their gametes contained in a space delimited by a thin membrane, keeping the immature gametes closer to the gastrodermis, and the mature gametes in the central region (Eckelbarger, 1994; Miranda et al., 2016a).

Specializations in the release of mature gametes from the gonads are also different in Acraspeda. The release of gametes apparently occurs in a relatively similar way in Cubozoa and Scyphozoa. In cubomedusae the gametes are released through a rupture of the gastrodermis, as observed and described to *Carybdea xaymacana*, *Tamoya haplonema*, and *Chiropsalmus quadrumanus* (Conant, 1898; Tiseo et al., 2019). Scyphomedusae also release the gametes through a rupture of the gastrodermis at a more fragile point, as hypothesized for *Lychnorhiza lucerna* (Schiariti et al., 2012), or may have a pore in the gastrodermis lining their gonads that allows the passage of mature gametes into the gastrovascular cavity, as occurs in *Periphylla periphylla* (Tiemann & Jarms, 2010). On the other hand, the stauromedusae have well-delimited gametoducts, which are ciliated and responsible for carrying the mature gametes into the gastrovascular cavity of the animal (Miranda et al., 2016a). These gametoducts can be highly branched (see the discussion in Haeckel, 1882). The structure has been described in several older papers (Clark, 1878; Haeckel, 1882; Wietrzykowski, 1912) and was observed in a recent histological study by Miranda et al. (2016a) in eight out of nine species sectioned, further attesting the complexity of the gonads in Staurozoa.

Many of the attributions concerning the gonadal complexity of Staurozoa is due to the presence of follicle cells, which are associated with gametes (Eckelbarger & Larson, 1993; Eckelbarger, 1994; Miranda et al., 2016a), being considered as a potential synapomorphy of the class (Marques & Collins, 2004; Collins & Daly, 2005; Collins et

al., 2006; Van Iten et al., 2006). Although their nature and function are still not clear in the literature, its exclusivity to class Staurozoa has been questioned, as apparently it can also occur in Scyphozoa (Tiemann & Jarms, 2010). However, more detailed studies are needed to describe and trace homologies regarding these cells.

Therefore, it is possible to conclude that there is indeed an apparent higher level of gonadal complexity in Staurozoa due to the presence of gametoducts and compartmentalization of gametes. However, it is fundamental to emphasize that macro and microanatomical comparative studies including the three classes of Acraspeda, addressing their diversity, are essential to establish homologies and correct the inappropriate generalizations regarding gonadal complexity.

4.2. General morphology of the gonads of Staurozoa

The data survey conducted in the present study (Fig. 8; Table S1) provides a justification for the generalization often made regarding the gonads of Staurozoa. The genus with the largest number of studies on gonads is *Haliclystus*; not surprisingly, the gonadal characteristics of the group, such as the presence of vesicles and follicular cells, are extrapolated to general characteristics of the class (Eckelbarger & Larson, 1993; Eckelbarger, 1994). Yet, within the genus *Haliclystus* little or nothing is known about the gonads of different species, including the rare *Haliclystus kerguelensis* and *H. monstrosus*, for which there is only information about the positioning of the structure in the subumbrella (Vanhöffen, 1908; Mayer, 1910; Ling, 1937; Naumov, 1961; Kahn et al., 2010; Table S1). Thus, the knowledge of the gonads of *Haliclystus*, despite being the most studied genus (Fig. 8), still presents some points to be explored, especially the microanatomy of its gonads, since only five out of the 13 species of the genus present the internal organization of the gonads precisely described (Uchida, 1929; Uchida & Hanaoka, 1934; Miranda et al., 2013, 2016a; Table S1).

Perhaps the genus that better exemplifies the generalization bias mentioned above is *Calvadosia* (Figs. 3, 4). The gonads are presented either in a sinuous form along the adradial or in rows of vesicles (as in the case of *Calvadosia* sp. 1 NZ; Fig. 3B). Some species can also present nodular gonads, a unique feature among the Staurozoa (Figs. 3C; 4D, E), a characteristic that may have high taxonomic value for the genus (Miranda et al., 2017b). The generalizations become even more rough when the gonads of *Kyopoda*

lamberti are analyzed. Larson (1988) described the gonads of the species as very different from those found in other stauromedusae, which are located in the peduncle rather than the calyx. The gonads are supposedly paired in the adradial; however, in figure 3C (Larson, 1988), there is a structure dividing the peduncle cavity that looks similar to the claustrum, in spite of Larson (1988) classifying the species as “Eleutherocarpida”, a former group of species without claustrum (Miranda et al., 2016a, 2016b). Besides, the species seems to have gonadal vesicles (?) positioned in two distinct cavities (could these be the four accessory radial pockets and four main radial pockets?). Therefore, this species needs to be anatomically reassessed for a better understanding of its internal organization, including gonadal anatomy.

The genera *Manania* and *Craterolophus* have very similar gonads. First, both gonads are positioned in the perradial, due to the presence of the claustrum (Figs. 1F; 2B, C; 6D, E, F; 7A, C, D). Second, both are organized in a sinuous row, which forms, in exumbrellar view, a herringbone-like pattern (Figs. 2B, C; 6D, E, F; 7A, C, D; Table S1). Finally, the gametes are contained between a layer of gastrodermis and epidermis, without the formation of vesicles (Figs. 1D; 6B; Miranda et al., 2016a; Holst et al., 2019; 2021). Some divergence, however, is found in the literature regarding this general pattern for *Manania*. *Manania auricula* and *Manania uchidai* do not show a remarkable herringbone gonadal pattern (Table S1; Figs. 6F, 7C; Miranda & Collins, 2019: figure 2), possibly indicating that the sinuosities (lateral gonadal projections) can be more or less conspicuous, as observed when they are compared to *Manania gwilliami* (Fig. 6D) and *Manania distincta* (Fig. 6E), for example. The description of gonads in *Manania hexaradiata* is not precise, as its taxonomic status (Miranda et al., 2016b). Descriptions of the gonads of the species are very old (Broch, 1907; Mayer, 1910) and lack illustrations, which makes morphological understanding difficult. They are described, however, as a horseshoe shape, not extending to the coronal musculature (Broch, 1907; Mayer, 1910), just as *Depastrum cyathiforme* (Fig. 6G) and *Halimocyathus platypus* were described by the same author (Mayer, 1910), possibly reflecting the gonadal similarity in these two species. Nevertheless, further studies addressing these species are necessary for a more precise comparison.

Based on our review, the only genus that has a claustrum and vesicle organization is *Depastromorpha*. Its gonads are organized in two rows of vesicles, very clearly seen from the exumbrella (Figs. 6C, 7B), along the stauromedusa body. Carlgren (1935), in

his description of the species, illustrated (Carlgren, 1935: figures 8, 10 and 11) the vesicle-shaped male gonads, as well as the presence of a claustrum dividing the gastrovascular cavity (Carlgren, 1935: figure 11). Carlgren (1935) also mentioned that he did not observe gametoducts in the analyzed specimen, information also reported for *L. sainthilairei* (Miranda et al., 2016a). This raises the question of whether the presence of gametoducts vary in different species and whether the structure are present throughout the life of the animal or their appearance is related to the level of gonadal maturity. The vesicles of the specimen of *L. sainthilairei* analyzed had low numbers of internal mature gametes, possibly indicating an immature male (Miranda et al., 2016a: figure 36), unlike the male gonads of *D. africana*, illustrated by Carlgren (1935). Although there is no mention of the level of gonadal maturity, the illustrations (Carlgren, 1935: figures 8, 10 and 11) indicate that the animal is sexually mature. In order to make any more precise inference, however, it would be necessary to section different individuals at different stages of gonadal maturity to verify this information.

4.3. After all, what do we know about the gonads of *Lucernaria*?

The genus *Lucernaria* is without a doubt one of the most enigmatic within the class Staurozoa. It was the first to be proposed (Müller, 1776), with the description of *L. quadricornis*, and after that until today, it underwent several taxonomic changes (Kramp, 1961; Kikinger & Salvini-Plawen, 1995; Collins & Daly, 2005), evidencing gaps in knowledge that need to be filled in the genus.

Reproductive structures are a prime example of one of the “knowledge gaps” in the genus. The gonads of *Lucernaria* have been discussed since the late nineteenth century, especially by Haeckel (1882), Antipa (1892), and Mayer (1910). With the study of Miranda et al. (2016a), new information has been brought into the discussion. Miranda et al. (2016a) performed a series of sections in specimens of *L. bathyphila* (Fig. 11A), *L. sainthilairei* (Fig. 2D), and *L. quadricornis* (Fig. 11B), highlighting that their gonads present a form of organization in vesicles somewhat irregular, compared to the well-delimited vesicles found in other genera, as in *Haliclystus*. It is possible to notice in the images obtained from *L. bathyphila* that the male gametes are localized in “chambers” contained in the mesoglea, between a layer of evaginated gastrodermis and a layer of epidermis (Fig. 11A; Miranda et al., 2016a: figures 31D-Q). Each of these chambers

apparently has a ciliated duct that would be responsible for transporting the gametes to a main chamber, where through the main gametoduct they would reach the gastrovascular cavity of the animal to be released to the external environment (Fig. 11A; Miranda et al., 2016a: figures 31O-Q).

Haeckel (1882) described in his study the female gonads of *L. bathyphila* noticing the presence of secondary chambers that contain the gametes, each with a duct that would be responsible for carrying the gametes to a main chamber (Fig. 11C), somewhat similar to the images of the male (Miranda et al., 2016a). This “complex” form of gonadal organization made Haeckel propose a new genus for the species, *Lucernosa*. Years later, following Haeckel’s (1882) proposal, Antipa (1892) classified three more species in the genus *Lucernosa*, under the justification of the complexity of their reproductive structures, as *Lucernosa walteri*, *Lucernosa kukenthalii* (later synonymized with *L. walteri* by Thiel, 1928), *Lucernosa haeckeli*. Just as in the illustrations by Haeckel (1882), it is possible to observe in the illustrations by Antipa (1892), now representing the male gonads, a “vesicle” composed of a series of secondary chambers that carry the gametes through ducts to a main chamber (Fig. 11D). Almost two decades later, Mayer (1910) synonymized the genus *Lucernosa* with *Lucernaria*, arguing that one should avoid characteristics that require dissection of the animal to classify it, despite recognizing the difference in vesicular “complexity” of *Lucernosa* and *Lucernaria*. Twenty years later, Carlgren (1930), in his description of a female *Lucernaria australis*, reported the presence of a series of “sacs” (secondary chambers?) with a common excretory duct (main chamber?) inside the gonadal vesicle (Carlgren, 1930: figure 17), showing the gonadal complexity in yet another species of *Lucernaria*.

Sections performed on a female *L. quadricornis* by Miranda et al. (2016a: figure 27; Fig. 11B) neither show the organization illustrated by Haeckel (1882) for a female of *L. bathyphila* (Fig. 11C) nor the Carlgren’s (1930) description of a female *L. australis*. In figure 27I by Miranda et al. (2016a), it is possible to observe a series of ducts in the vesicle, but the gametes are somewhat internally disorganized, not showing a maturation pattern as observed in other genera, where there is a concentration of immature gametes at the periphery of the gonad (near the gastrodermis) and mature gametes located more in the center. This different organization raises the question if the general pattern of gametes maturation is in fact expressed in all species of the class.

However, additional observations in this study revealed a slight similarity of some vesicles of *L. quadricornis* with the illustration by Haeckel (1882), with mature gametes compartmentalized in secondary chambers, as well as the presence of the ducts in each of them (Fig. 11E), a little different from what was presented by Miranda et al. (2016a) (Fig. 11B). Doubts about this “more complex” pattern of the internal organization of vesicles increase when we analyze sections of a male *C. vanhoeffeni* by Miranda et al. (2016a: figure 48; Fig. 3F) and additional observations of a male *C. nagatensis* (Fig. 11F). The sectioned individuals showed the same complex pattern of secondary chambers and ducts, very similar to the illustration by Antipa (1892; Fig. 11D), possibly indicating that this vesicle organization, at least in males, may not be restricted to *Lucernaria*. Illustrations of male (Fig. 11G) and female (Fig. 11H) vesicles of *C. campanulata* by Claus (1883) strengthens this hypothesis, since he represented the same “complex” organization for the male, while the female apparently has a simple compartmentalization of gametes, as in the other genera. In addition, illustration of a male vesicle of *Haliclystus tenuis* by Uchida (1929) (as *Haliclystus auricula*) is very similar to the representation by Claus (1883; Fig. 11G, H), indicating that this male form of organization may extend beyond *Lucernaria* and *Calvadosia*.

However, it is not possible to attest if such organization of the male vesicle is indeed a different pattern from that found in the other species of *Haliclystus* (e.g., *H. antarcticus*; Miranda et al., 2013: figure 10C, E) and some species of *Calvadosia* (e.g., *C. corbini*; Miranda et al., 2016a: figure 40). It is necessary to perform sections of different specimens (males and females), at different stages of development, to confirm that this is a meaningful difference and not a result of levels of gonadal maturation.

We compiled for the species that have vesicular gonads, information in the literature regarding their “complexity” (Table 9). They were separated into two distinct groups: (1) multilocular, including species with the so-called “complex” vesicles, with series of ducts and secondary chambers, as described by Haeckel (1882) and Antipa (1892); (2) unilocular, including species with “simpler” organization, where the immature gametes are concentrated in the periphery near the gastrodermis and the mature gametes in the center, in a chamber (Table 9). Nevertheless, as already mentioned, it is critical that further and more detailed studies address the hypothesis of different vesicular organizations.

4.4. Use of gonads in the taxonomy of Staurozoa

Gonadal characteristics have long been used in the taxonomy of the class. Historically, as mentioned above, their internal organization was used for the separation of the two former genera *Lucernaria* and *Lucernosa*, due to their internal vesicular “complexity” (Haeckel, 1882; Antipa, 1892; Mayer, 1910). However, nowadays the use of gonads in taxonomy is almost exclusively restricted to *Haliclystus*. The gonads of the genus are well conserved among species, showing variations mainly in the number of vesicles and the number of rows of vesicles that compose them (Kramp, 1961; Hirano, 1997; Miranda et al., 2009; Zagal et al., 2011), traits used in their taxonomy (Table 5; Table S1).

However, from the literature review, it is possible to evidence a variation and overlap in the number of vesicles and vesicle rows among the species, which hampers their taxonomic use. For example, *H. borealis*, *H. inabai*, and *H. sinensis* may possess the gonads organized in only one row (Table 5; Uchida & Hanaoka, 1934; Ling, 1937, 1939; Kahn et al., 2010). The wide spectrum in the number of vesicles (Table 5; Table S1) in the species *Haliclystus antarcticus* (60 -150 vesicles), *H. borealis* (30 - 100), *H. 'sanjuanensis'* (200 - 300 vesicles) and especially *Haliclystus stejnegeri* (30 - 240 vesicles) also demonstrates the high plasticity that species can express regarding their gonads, varying mainly according to the maturity stage (Uchida, 1929). Another characteristic used in taxonomy that can be variable due to the same reason is the irregular shape of their vesicle rows. Species such as *Haliclystus monstrosus*, *H. borealis*, *H. stejnegeri*, and *H. 'sanjuanensis'* are described as presenting irregularly arranged rows by some authors (Uchida, 1929; Uchida & Hanaoka, 1934; Naumov, 1961; Miranda et al., 2009).

Recently some gonadal characteristics of *Calvadosia* have been used for taxonomic purpose (Zagal et al., 2011; Miranda et al., 2017b). Some species of *Calvadosia* have nodular gonads (Table 4), characteristic that shows variations in its structure among species of the genus, which can be explored taxonomically. For instance, the recently described *C. lewisi* (Fig. 3C; Miranda et al., 2017b) presents smooth rounded nodules, while other closely-related species (e.g., *C. corbini* and *C. tasmaniensis*) present nodules with a raisin-like irregular appearance (Miranda et al., 2017b).

The microanatomy of the gonads is rarely used in taxonomy, although it has been used in *Lucernaria* as mentioned above (Haeckel, 1882; Antipa, 1892) until the beginning of the 20th century, when Mayer (1910) suggested to avoid characteristics that require dissection of the animal. However, nowadays, histological techniques are more accessible and precise, which facilitates their use, and can be a useful tool. Few species of the class have a detailed description of their microanatomy, but only with the information already provided in the literature (e.g., Haeckel, 1882; Carlgren, 1933; Uchida & Hanaoka, 1934; Eckelbarger & Larson 1993; Eckelbarger, 1994; Miranda et al., 2013, 2016a) it is possible to predict a potential taxonomic use, as for example, the presence of vesicles and their internal organization (see section 4.1.3). Thus, gonadal characters in staurozoan taxonomy can be used in a more extensive way.

4.5. Evolution of the gonads in Staurozoa

The vesicle-organized gonads proved to be a conserved trait within the phylogeny of the Staurozoa, being lost independently in the genera *Manania* and *Craterolophus* (Fig. 9). Thus, the first question that arise is what may have led to the loss of these structures in these lineages.

Comparing morphologically the two genera, the main feature that they have in common is the presence of the claustrum (Hertwig & Hertwig, 1879; Gwilliam, 1956; Berrill, 1962, 1963; Miranda et al., 2016a, 2017a; Holst et al., 2021), so their gonads are located in the main gastric radial pocket, in the perradii. It would be intuitive to say that there might be an evolutionary correlation between both structures. However, *D. africana* (belonging to the family Haliclystidae, as *Manania*), another species that has a claustrum (Carlgren, 1935; Miranda et al., 2016a), has the gonads organized in vesicles (Carlgren, 1935; Miranda et al., 2017a), differently from *Craterolophus* and *Manania*. Nevertheless, the correlation between absence of vesicles and presence of claustrum should not be totally discarded, since some genera with claustrum (*Depastrum* and *Halimocyathus*) needs further investigation regarding the presence of vesicles and all the species without claustrum that were considered in our analysis have vesicle-organized gonads, either type 1 or type 2.

Just as for the independent loss of the vesicular organization, the possible appearance of vesicles in the last common ancestor of Staurozoa and its maintenance in

most extant species do not yet have an evolutionary justification. There is no information in the literature that indicates an evolutionary advantage that vesicles provided for species that possess them. However, Mills & Hirano (2007) point out that stauromedusae spawn daily over a month or more, indicating that not all gametes are released at once to the external environment. Indeed, not all vesicles observed in an individual are necessarily full or empty of mature gametes (Miranda et al., 2016a: figure 8B; this study). Thus, the vesicles may confer a selectivity on the gametes that are ready to be released by the animal. New studies describing the reproductive behavior for the class, comparing species with different forms of gonad organization, are essential to answer these questions.

The positioning of the gonads in the subumbrella of the stauromedusae (Fig. 10) is directly related to the presence of the claustrum and the positioning of the arms. The last common ancestor of the Staurozoa possibly had the gonads positioned at adradial, a trait well conserved throughout the phylogeny. The perradial position of the gonads may have appeared once in *Craterolophus* and once in the ancestor of the family Haliclystidae, as already hypothesized by Miranda et al. (2016b). However, the probability of the ancestor of Haliclystidae having the gonads positioned in the adradial is still slightly higher, suggesting that the perradial gonads and the claustrum appeared independently at least three times in Staurozoa (*Craterolophus*, *Manania*, and *Depastromorpha*). Analysis including more representatives of *Manania* may increase the probability that the perradial gonads appeared in the ancestor of Haliclystidae.

5. Conclusions

In this study we review and deepen the knowledge about the staurozoan gonads. For the first time in the literature, we provide an evolutionary perspective on the presence of vesicles and the positioning of the gonads in the subumbrella.

The literature review revealed that there is no information or precise information regarding the gonads of most species in the class. Information regarding the presence of vesicles for example is restricted almost entirely to *Haliclystus*. This can be explained by the disproportionate number of studies conducted on this genus in relation to the others. Follicular cells and gametoducts are other features that, despite being remarkable in Staurozoa, have not been reported for most species of the class.

Also based on the review, and with the additional observation of specimens (Table 1), it was possible to establish morphological patterns for the gonads of most genera of Staurozoa (Figs. 4, 5, 7). They may be positioned in the (1) adradii, as found in *Haliclystus* and some species of *Calvadosia*; besides being the probable position of the gonad in staurozoan ancestor (Fig. 10); (2) interradii/adradii in species with paired arms in the interradii (e.g., *C. cruciformis*, *L. quadricornis*); (3) in the perradii, due to the presence of the claustrum. Gonads organized in vesicles is ancestral in the evolutionary history of Staurozoa (Fig. 9), occurring mainly in species lacking claustrum, with *Depaстроморpha africana* being the single exception so far.

Nevertheless, the vesicle-organized gonad categorization is not clear for *Lucernaria*; the information available in the literature for the genus regarding gonads is fragmented and confusing, as it is for other genera such as *Depastrum*, *Halimocyathus*, *Kyopoda*, and *Stylocoronella*. However, recent sections of the gonads of some species of *Lucernaria* (Miranda et al., 2016a), have revealed an irregular shape of vesicles, differing from the traditional vesicles found in *Haliclystus*. These observations raise questions regarding the micro-anatomical diversity of vesicles in the class, since this study revealed that different species, not only from the genus *Lucernaria*, exhibit a "more complex" internal organization than previously described for species of *Haliclystus* and *Calvadosia* (Table 8; Fig. 11). Further sectioning of animals at different life stages and with a greater representation of genera are fundamental to address the real morphological diversity of staurozoan gonads.

The taxonomic use of gonads is restricted to *Haliclystus*, mainly considering the number of vesicles and rows of vesicles in the gonads. However, our review indicated possible variation and overlapping information for these characteristics (e.g., *H. borealis*, *H. inabai*, and *H. sinensis*; Table 5), which may be a result of the maturity level of the specimen analyzed. In *Calvadosia*, however, there are features such as the texture of the nodular gonads that are clearly different among species (e.g., *C. lewisi* and *C. tasmaniensis*) that should be explored in the taxonomy of the group.

This study presents the current state of knowledge of gonadal diversity in Staurozoa, indicating possible steps that are necessary to improve the morphological understanding for the class. It instigates promising researches about the reproduction behavior, the gonadal development, and the evolution of cnidarians, such as the transience

of gametoducts, the correlation between claustrum and vesicles and the development of these structures during the life cycle, and the establishment of homologies of gonads in Acraspeda.

References

- Amor A. 1962. Sobre Stauromedusae del litoral patagonico *Haliclystus auricula* (Rathke). *Notas del Museo de la Universidad Nacional de La Plata, Zoología* 20:89–96.
- Antipa G. 1892. Die lucernariden der bremer expedition nach ostspitzbergen im jahre 1889. *Zoologische Jahrbücher Abtheilung Für Systematik, Geographie und Biologie der Thiere* 6:377–397.
- Antipa G. 1893. Eine neue Stauromeduse (*Capria* n. *sturdzii* n.). *Mittheilungen aus der Zoologischen Station zu Neapel* 10:618–632.
- Berrill M. 1962. The biology of three New England stauromedusae, with a description of a new species. *Canadian Journal of Zoology* 40(7):1249–1262.
- Berrill M. 1963. Comparative functional morphology of the stauromedusae. *Canadian Journal of Zoology* 41(5):741–752.
- Bigelow HB. 1920. *Mollusks, Echinoderms, Coelenterates, etc. Part H Medusae and Ctenophora*. T. Mulvey.
- Borges R, Machado JP, Gomes C, Rocha AP, Antunes A. 2019. Measuring phylogenetic signal between categorical traits and phylogenies. *Bioinformatics* 35(11):1862–1869.
- Boyko JD, Beaulieu JM. 2021. Generalized hidden Markov models for phylogenetic comparative datasets. *Methods in Ecology and Evolution* 12(3):468–478.
- Broch H. 1907. Hydroiden und medusen. *Report of the Second Norwegian Arctic Expedition in the “Fram” 1898–1902* 2:1–12.
- Browne ET. 1910. *Reports of the Natural History Results of the Voyage of the SS Discovery in the Antarctic Regions in 1901, under Captain R F Scott RN. Coelentera. V. Medusae*. London: London British Museum (Natural History).
- Carlgren O. 1930. Die Lucernariden. *Further Zoological Results of the Swedish Antarctic Expedition 1901–1903* 2:1–18.
- Carlgren O. 1933. Zur Kenntnis der Lucernariiden *Lipkea*, *Capria* und *Brochiella*. *Kungliga Fysiografiska Sällskapets Handlingar* 44:1–19.

- Carlgren O. 1935. Über eine neue Südafrikanische Lucernariidae, *Depastromorpha africana* n. Gen., n. Sp., nebst Bemerkungen über den Bau und die Systematik dieser Tiergruppe. *Kunglia Svenska Vetenskapsakademiens Handlingar* 15:1–24.
- Clark HJ. 1863. Prodrusus of the history, structure, and physiology of the order Lucernariae. *Journal of the Boston Society of Natural History* 7:531–567.
- Clark HJ. 1878. Lucernariae and their allies. A memoir on the anatomy and physiology of *Halicystus auricula* and other Lucernarians, with a discussion of their relations to other Acalephae; to beroids, and polypi. *Smithsonian Contributions to Knowledge* 242:1–130.
- Claus C. 1883. *Untersuchungen über die Organisation und Entwicklung der Medusen*. Freytag.
- Collins AG, Daly M. 2005. A new deepwater species of Stauromedusae, *Lucernaria janetae* (Cnidaria, Staurozoa, Lucernariidae), and a preliminary investigation of stauromedusan phylogeny based on nuclear and mitochondrial rDNA data. *Biological Bulletin* 208(3):221–230.
- Collins AG, Schuchert P, Marques AC, Jankowski T, Medina M, Schierwater B. 2006. Medusozoan phylogeny and character evolution clarified by large and small subunit rDNA data and an assessment of the utility of phylogenetic mixture models. *Systematic Biology* 55(1): 97–115.
- Conant FS. 1898. The Cubomedusae. *Memoirs of the Biological Laboratory of the Johns Hopkins University*. Vol. 4. Baltimore: The Johns Hopkins Press, 1–61.
- Corbin PG. 1978. A new species of the stauromedusan genus *Lucernariopsis* (Coelenterata: Scyphomedusae). *Journal of the Marine Biological Association of the United Kingdom* 58(2):285–290.
- Couch RQ. 1844. *A Cornish fauna: being a compendium of the natural history of the country. Part III. The Zoophytes and calcareous corallines*. Truro: Royal Institution of Cornwall, L.E. Gillet, 83-84.
- Eales NB. 1938. *Lucernaria discoidea*, a new species from the Channel Islands. *Journal of the Marine Biological Association of the United Kingdom* 23(1):167–170.
- Eckelbarger KJ. 1994. Oocyte nutrition in the lower Metazoa: the Scyphozoa. In: Wilson WH, Stricker SA, Shinn GL, eds. *Reproduction and Development of Marine Invertebrates*. Baltimore: John Hopkins University Press, 15–28.
- Eckelbarger KJ, Larson RJ. 1993. Ultrastructural study of the ovary of the sessile scyphozoan, *Halicystus octoradiatus* (Cnidaria: Stauromedusae). *Journal of Morphology* 218(2):225–236.
- Elmhirst R. 1922. Notes on *Lucernaria quadricornis*, Müller, and related species. *The Annals and Magazine of Natural History* 10(56):221–224.

- Falconer A. 2013. A stalked jellyfish *Stenoscypus inabai* (Kishinouye, 1893) (Stauromedusae), found at The Jawbone, Port Phillip Bay, Victoria. *Victoria Naturalist* 130:202–207.
- Gellerman MP. 1926. Medusae of the San Juan archipelago. Masters thesis, University of Washington, Seattle, Washington.
- Grohmann PA, Magalhaes MP, Hirano YM. 1999. First record of the order Stauromedusae (Cnidaria, Scyphozoa) from the tropical southwestern Atlantic, with a review of the distribution of Stauromedusae in the southern hemisphere. *Species Diversity* 4(2):381–388.
- Gwilliam GF. 1956. Studies on West Coast Stauromedusae. Ph.D. dissertation. Berkeley: University of California.
- Haeckel E. 1879. Das system der medusen. I, 2: System der Acraspeden. Zweite Hälfte des Systems der Medusen. Jena: Gustav Fischer.
- Haeckel E. 1882. Report on the deep-sea medusae dredged by H.M.S. Challenger during the years 1873–1876. Report on the scientific results of the voyage H.M.S Challenger during the years 1873–1876. *Zoology* 4:1–154.
- Hertwig O, Hertwig R. 1879. *Die Actinien: anatomisch und histologisch, mit besonderer Berücksichtigung des Nervenmuskelsystems* (Vol. 1). Gustav Fischer.
- Hirano YM. 1986. Species of Stauromedusae from Hokkaido, with notes on their metamorphosis. *Journal of the Faculty of Science, Hokkaido University, Zoology* 24: 182–201.
- Hirano YM. 1997. A review of a supposedly circumboreal species of stauromedusa, *Haliclystus auricula* (Rathke, 1806). In: den Hartog J.C., ed. *Proceedings of the 6th International Conference on Coelenterate Biology*. Leiden: National Naturhistorisch Museum, Noordwijkerhout, 247–252.
- Holst S, Heins A, Laakmann S. 2019. Morphological and molecular diagnostic species characters of Staurozoa (Cnidaria) collected on the coast of Helgoland (German Bight, North Sea). *Marine Biodiversity* 49(4):1775–1797.
- Holst S, Miranda LS, Meyer P, Michalik P, Soetje I. 2021. Morphological analyses of the adult and juvenile stages of the stalked jellyfish *Craterolophus convolvulus* (Johnston, 1835) (Cnidaria: Staurozoa: Stauromedusae: Craterolophidae) using micro-CT. *Zoologischer Anzeiger* 292:240–260.
- Joy JB, Liang RH, McCloskey RM, Nguyen T, Poon AF. 2016. Ancestral reconstruction. *PLoS Computational Biology* 12(7):e1004763.
- Kahn AS, Matsumoto GI, Hirano YM, Collins AG. 2010. *Haliclystus californiensis*, a “new” species of stauromedusa (Cnidaria: Staurozoa) from the northeast Pacific, with a key to the species of *Haliclystus*. *Zootaxa* 2518:49–59.

- Kayal E, Bentlage B, Sabrina Pankey M, Ohdera AH, Medina M, Plachetzki DC, Collins AG, Ryan JF. 2018. Phylogenomics provides a robust topology of the major cnidarian lineages and insights on the origins of key organismal traits. *BMC Evolutionary Biology* 18(1):1–18.
- Keferstein W. 1862. Untersuchungen über niedere Seethiere. *Zeitschrift für wissenschaftliche Zoologie* 12:1–147.
- Kikinger R, Salvini-Plawen LV. 1995. Development from polyp to stauromedusa in *Stylocoronella* (Cnidaria: Scyphozoa). *Journal of the Marine Biological Association of the United Kingdom* 75:899–912.
- Kishinouye K. 1902. Some new Scyphomedusae of Japan. *Journal of the College of Science, Imperial University of Tokyo* 17:1–17.
- Kishinouye K. 1910. Some medusae of Japanese water. *Journal of the College of Science, Imperial University of Tokyo* 27:1–35.
- Kramp PL. 1961. Synopsis of the medusae of the world. *Journal of the Marine Biological Association of the United Kingdom* 40:7–469.
- Larson RJ. 1980. A new stauromedusa, *Kishinouyea corbini* (Scyphozoa, Stauromedusae) from the tropical western Atlantic. *Bulletin of Marine Science* 30(1):102–107.
- Larson RJ. 1988. *Kyopoda lamberti* gen.nov., sp.nov., an atypical stauromedusa (Scyphozoa, Cnidaria) from the eastern Pacific, representing a new family. *Canadian Journal of Zoology* 66(10):2301–2303.
- Larson RJ, Fautin DG. 1989. Stauromedusae of the genus *Manania* (= *Thaumatoscypus*) (Cnidaria, Scyphozoa) in the northeast Pacific, including descriptions of new species *Manania gwilliami* and *Manania handi*. *Canadian Journal of Zoology* 67:1543–1549.
- Lechuga GR, Alamo MAF. 2005 Primer registro de *Kishinouyea corbini* Larson, 1980 (Cnidaria: Scyphozoa, Stauromedusae) para México. *Revista de la Sociedad Mexicana de Historia Natural* 7:107–110.
- Ling SW. 1937. Studies on Chinese Stauromedusae. I. Stauromedusae from Tsingtao. *Amoy Marine Biological Bulletin* 3:1–35.
- Ling SW. 1939. Studies on Chinese stauromedusae. II. Further studies on some stauromedusae from China. *Lingnan Science Journal* 18(3,4):281-291, 495-503.
- Marques AC, Collins AG. 2004. Cladistic analysis of Medusozoa and cnidarian evolution. *Invertebrate Biology* 123(1):32–42.
- Mayer AG. 1910. Scyphomedusae. *Medusae of the World*, vol. III. Washington, D.C.: Carnegie Institution Publishing. Publication 109.

- Mills CE, Hirano YM. 2007. Stauromedusae. In: Denny MW, Gaines SD, eds. *Encyclopedia of Tidepools and Rocky Shores*. Berkeley: University of California Press, 541–543.
- Miranda LS, Branch GM, Collins AG, Hirano YM, Marques AC, Griffiths CL. 2017b. Stalked jellyfishes (Cnidaria: Staurozoa) of South Africa, with the description of *Calvadosia lewisi* sp. nov. *Zootaxa* 4227(3):369–389.
- Miranda LS, Collins AG. 2019. Eyes in Staurozoa (Cnidaria): a review. *PeerJ* 7:e6693.
- Miranda LS, Collins AG, Hirano YM, Mills CE, Marques AC. 2016a. Comparative internal anatomy of Staurozoa (Cnidaria), with functional and evolutionary inferences. *PeerJ* 4:e2594.
- Miranda LS, Collins AG, Marques AC. 2010. Molecules clarify a cnidarian life cycle – the “hydrozoan” *Microhydrula limopsicola* is an early life stage of the staurozoan *Haliclystus antarcticus*. *PLoS One* 5:e10182.
- Miranda LS, Collins AG, Marques AC. 2013. Internal anatomy of *Haliclystus antarcticus* (Cnidaria, Staurozoa) with a discussion on histological features used in staurozoan taxonomy. *Journal of Morphology* 274(12): 1365–1383.
- Miranda LS, García-Rodríguez J, Collins AG, Morandini AC, Marques AC. 2017a. Evolution of the claustrum in Cnidaria: comparative anatomy reveals that it is exclusive to some species of Staurozoa and absent in Cubozoa. *Organisms Diversity & Evolution* 17(4):753–766.
- Miranda LS, Haddad MA, Mills CE, Marques AC. 2012. *Lucernariopsis capensis* Carlgren, 1938 (Cnidaria, Staurozoa) in Brazil: first record outside its type locality in South Africa. *Zootaxa* 3158(1):60–64.
- Miranda LS, Hirano YM, Mills CE, Falconer A, Fenwick D, Marques AC, Collins AG. 2016b. Systematics of stalked jellyfishes (Cnidaria: Staurozoa). *PeerJ* 4:e1951.
- Miranda LS, Mills CE, Hirano YM, Collins AG, Marques AC. 2018. A review of the global diversity and natural history of stalked jellyfishes (Cnidaria, Staurozoa). *Marine Biodiversity* 48(4):1695–1714.
- Miranda LS, Morandini AC, Marques AC. 2009. Taxonomic review of *Haliclystus antarcticus* Pfeffer, 1889 (Stauromedusae, Staurozoa, Cnidaria), with remarks on the genus *Haliclystus* Clark, 1863. *Polar Biology* 32(10): 1507–1519.
- Morandini AC, Da Silveira FL. 2001. Sexual reproduction of *Nausithoe aurea* (Scyphozoa, Coronatae). Gametogenesis, egg release, embryonic development, and gastrulation. *Scientia Marina* 65(2):139–149.
- Müller OF. 1776. *Zoologiae Danicae Prodromus, seu animalium Daniae et Norvegiae indigenarum, Characteres, Nomina et Synonyma imprimis popularium*. Havniae: Typis Hallageriis, 227 p.

- Naumov DV. 1961. Stsifoidnye meduzy morei SSSR (Scyphozoan medusae in the sea of USSR). *Opredeliteli po Faune SSSR* 75:1–98.
- Oka A. 1897 Sur une nouvelle espèce Japonaise du genre *Lucernaria*. *Annotationes Zoologicae Japonenses* 1:141–145.
- Okubo T. 1917. Preliminary note on a new genus of Stauromedusae from Hokkaido. *Zoological Magazine, Tokyo* 29:317–322.
- Pennell MW, Eastman JM, Slater GJ, Brown JW, Uyeda JC, FitzJohn RJ, Alfaro ME, Harmon LJ. 2014. geiger v2.0: an expanded suite of methods for fitting macroevolutionary models to phylogenetic trees. *Bioinformatics* 30:2216–2218.
- Pfeffer G. 1889. Zur Fauna von Süd-Georgien. *Jahrbuch der Hamburgischen Wissenschaftlichen Anstalten* 6:37–55.
- Pisani V, Otero-Ferrer F, Lotto S, Maurel P, Goy J. 2007. *Lipkea ruspoliana* Vogt, 1887 (Stauromedusae, Scyphozoa, Cnidaria) dans les aquariums du Musée Océanographique de Monaco. *Bulletin de la Société Zoologique de France* 132:183–190.
- Quezada AE. 1969. *Haliclystus auricula* (Rathke 1806) (Coelenterata, Scyphozoa, Stauromedusae) en el golfo de Arauco (Chile). *Boletín de la Sociedad Biológica de Concepción* 42: 75–80.
- Sars M. 1829. *Bidrag til Söedyrenes Naturhistorie*. Bergen: Med Sex Illuminerede Steentryktavler, 60 p.
- Schiariti A., Christiansen E, Morandini AC, da Silveira FL, Giberto DA, Mianzan HW. 2012. Reproductive biology of *Lychnorhiza lucerna* (Cnidaria: Scyphozoa: Rhizostomeae): individual traits related to sexual reproduction. *Marine Biology Research* 8(3):255–264.
- Stiasny G. 1919. Die Scyphomedusen-Sammlung des Naturhistorischen Reichsmuseums in Leiden: II. Stauromedusae, Coronatae, Semaestomeae. *Zoologische Mededelingen* 5(9):66–99.
- Thiel ME. 1928. Die Scyphomedusen des Zoologischen Staatsinstituts und Zoologischen Museums in Hamburg: I, Cubomedusae, Stauromedusae und Coronatae. *Mitteilungen aus dem Zoologischen Museum Hamburg* 43:1–34.
- Tiemann H, Jarms G. 2010. Organ-like gonads, complex oocyte formation, and long-term spawning in *Periphylla periphylla* (Cnidaria, Scyphozoa, Coronatae). *Marine Biology* 157(3):527–535.
- Tiseo GR, García-Rodríguez J, Zara FJ, Ames CL, Marques AC, Morandini AC. 2019. Spermatogenesis and gonadal cycle in male *Tamoya haplonema* and *Chiropsalmus quadrumanus* (Cnidaria, Cubozoa). *Zoologischer Anzeiger* 279:59–67.

- Uchida T. 1929. Studies on the Stauromedusae and Cubomedusae, with special reference to their metamorphosis. *Japanese Journal of Zoology* 2: 103–193.
- Uchida T. 1933. Eine neue Becherqualle aus Hokkaido. *Proceedings of the Imperial Academy of Japan* 9:450–452
- Uchida T, Hanaoka KI. 1933. On the morphology of a stalked medusa, *Thaumatoscyphus distinctus* Kishinouye. *Journal of the Faculty of Science, Hokkaido Imperial University, Series VI, Zoology* 2(3): 135–153.
- Uchida T, Hanaoka KI. 1934. Anatomy of two stalked medusae with remarks on the distribution of the Stauromedusae in Japan. *Journal of the Faculty of Science, Hokkaido Imperial University, Series VI, Zoology* 2(4): 211–239.
- Van Iten H, Leme JM, Simões MG, Marques AC, Collins AG. 2006. Reassessment of the phylogenetic position of Conulariids (?Ediacaran-Triassic) within the subphylum Medusozoa (phylum Cnidaria). *Journal of Systematic Palaeontology* 4(2):109–118.
- Vanhöffen E. 1908. Die Lucernariden und Scyphomedusen der Deutschen Südpolar-Expedition 1901–1903. *Deutsche Südpolar-Expedition* 10:25–49.
- Westlake H. 2015. A Comparative Immunohistochemical Study of the Neuromuscular Organization of *Haliclystus 'sanjuanensis'* and *Manania handi* (Cnidaria: Staurozoa). Thesis, University of Victoria.
- Widersten B. 1965. Genital organs and Fertilization in some Scyphozoa. *Zool. Bidr. Uppsala* 37:45–58.
- Wietrzykowski W. 1912. Recherches sur le développement des Lucernaires. *Archives de Zoologie Expérimentale et Générale, 5th Series* 10:1–95.
- Zagal CJ, Hirano YM, Mills CE, Edgar GJ, Barrett NS. 2011. New records of Staurozoa from Australian coastal waters, with a description of a new species of *Lucernariopsis* Uchida, 1929 (Cnidaria, Staurozoa, Stauromedusae) and a key to Australian Stauromedusae. *Marine Biology Research* 7(7):651–666.

Figures

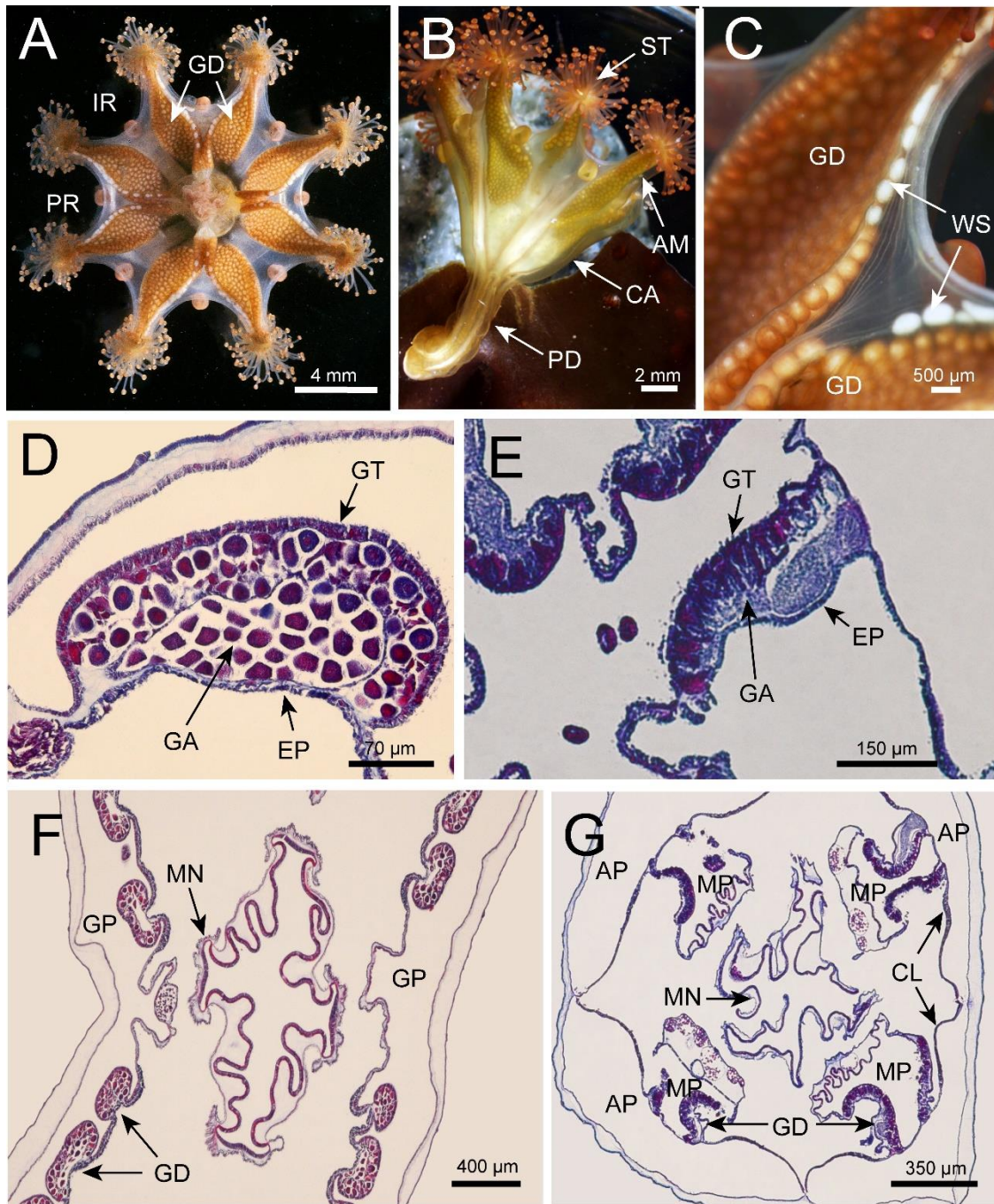


Figure 1. General aspects of gonads. (A) Gonads on the subumbrella of *Haliclystus 'sanjuanensis'* (photo credit: Ron Larson). (B) *H. 'sanjuanensis'* in exumbrellar view (photo credit: Hannah Westlake). (C) Close-up of the gonads of *H. 'sanjuanensis'*, showing its composition of vesicles arranged in rows (photo credit: Hannah Westlake). (D) Cross section of the female gonads of *Haliclystus tenuis*; notice the evagination of the gastrodermis forming a vesicle (modified from Miranda et al., 2016a). (E) Cross section of the male gonads of *Manania uchidai*, notice that the gametes are between a layer of epidermis and gastrodermis without evagination, not forming vesicles (modified from Miranda et al., 2016a). (F) Cross section of the calyx of *H. tenuis*, gonads contained in gastric radial pockets (modified from Miranda et al., 2016a). (G) Cross section of the calyx of *Craterolophus convolvulus*, with the presence of the claustrum dividing the gastrovascular cavity into four accessory radial pockets and four principal radial pockets, which contain the gonads (modified from Miranda et al., 2016a). Abbreviations: AM, arm; AP, accessory radial pocket; CA, calyx; CL, claustrum; EP, epidermis; GA, gametes; GD, gonads; GP, gastric pocket; GT, gastrodermis; IR,

interradii; MN, manubrium; MP, main radial pocket; PD, peduncle; PR, perradii; ST, secondary tentacles, WS, white spot.

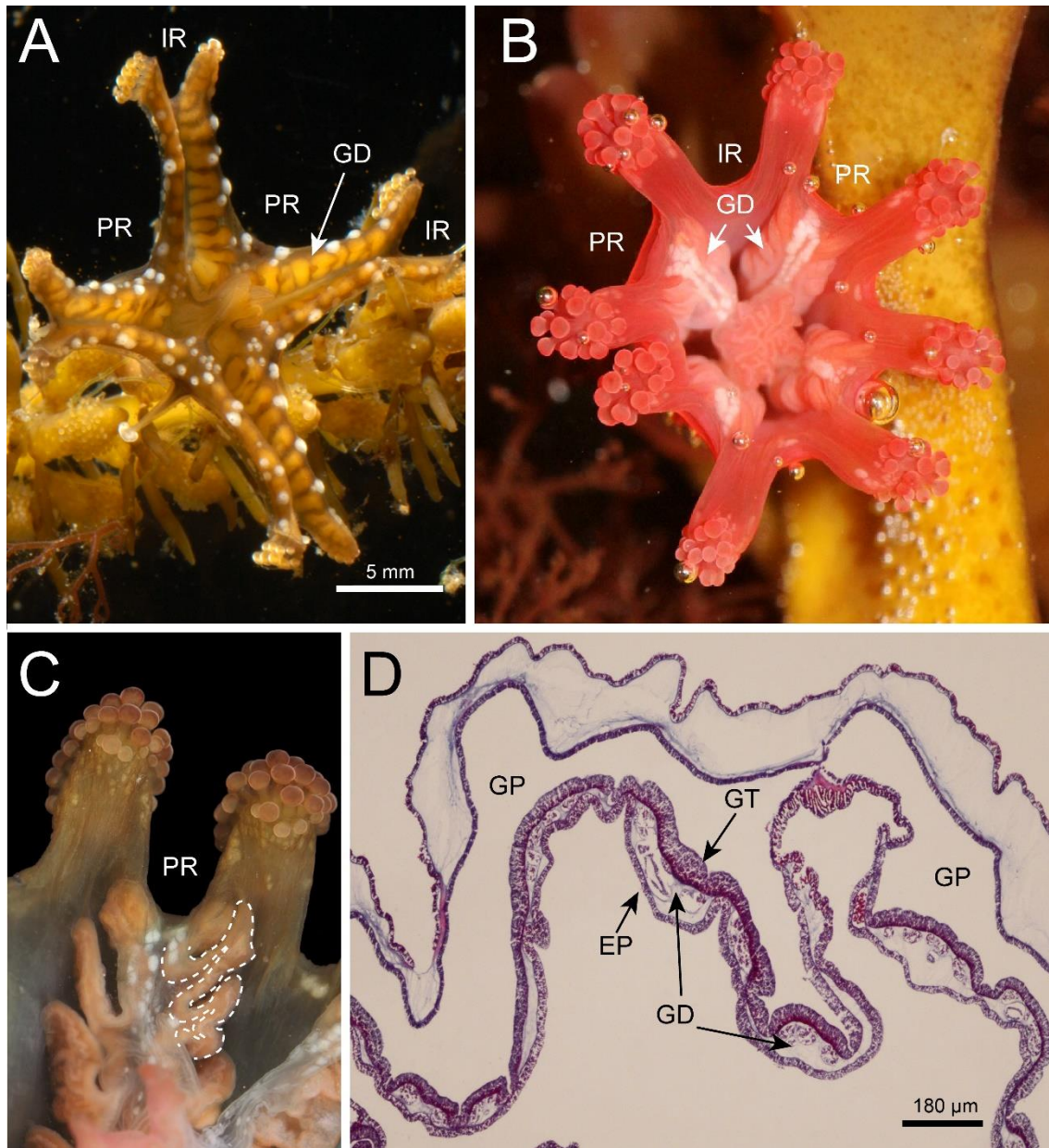


Figure 2. General aspects of gonads. **(A)** Gonads positioned in the interradi/adradii of *Calvadosia cruciformis* (photo credit: modified from Miranda et al., 2018). **(B)** Gonads positioned in the perradii of *Craterolophus convolvulus* (photo credit: David Fenwick; scale not available). **(C)** Close-up of the perradii of *C. convolvulus* showing the gonads in a sinuous row (dashed line) (photo credit: David Fenwick; scale not available). **(D)** Cross section of the gonads of *Lucernaria sainthilairei*; this type of vesicular organization, for the purposes of this paper, will be referred to as "type 2 vesicles", since the vesicles are less obvious and not well-delimited (modified from Miranda et al., 2016a). Abbreviations: EP, epidermis; GD, gonads; GP, gastric pocket; GT, gastrodermis; IR, interradii; PR, perradii.

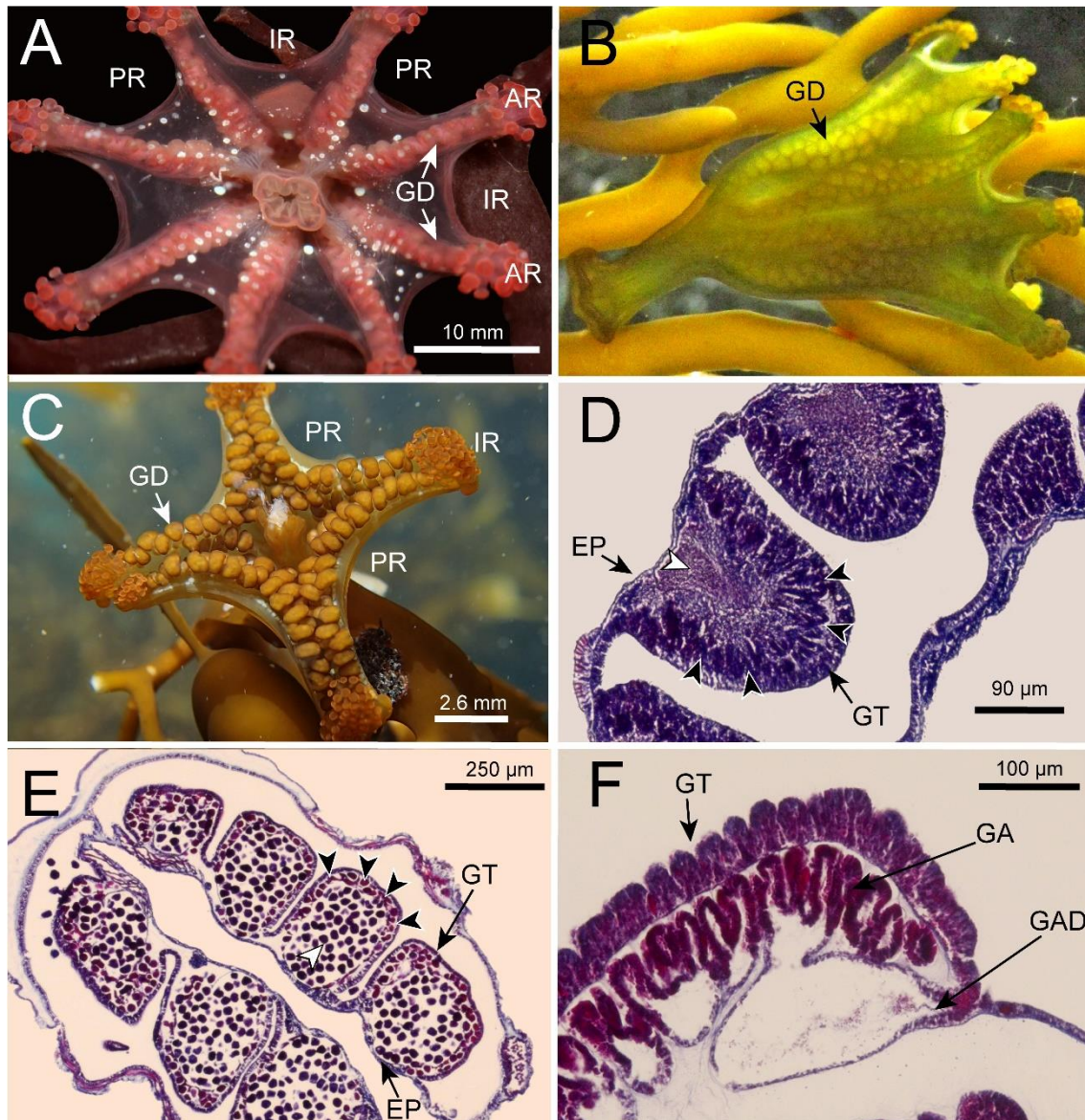
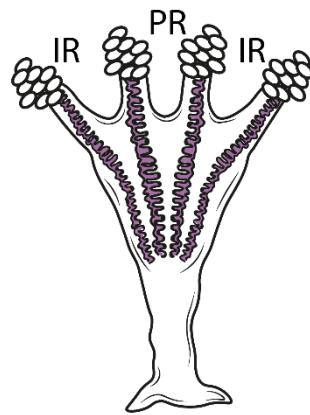
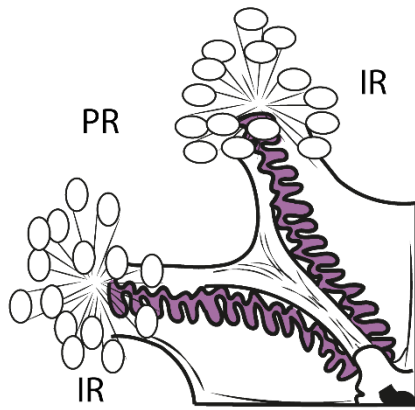
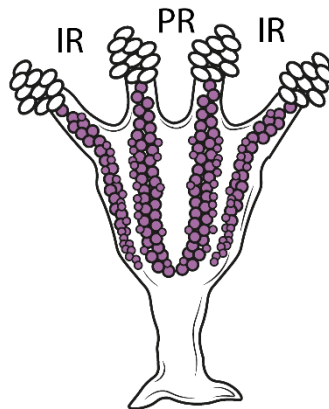
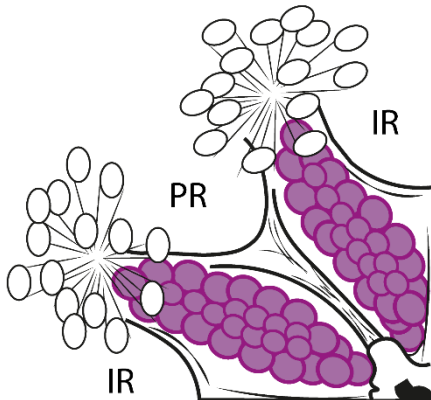


Figure 3. Gonads of the genus *Calvadosia*. (A) Gonads positioned on the adradial arms of *Calvadosia campanulata* (photo credit: David Fenwick). (B) Gonads of *Calvadosia* sp. 1 NZ organized in rows of vesicles in the adradial arms (scale not available). (C) Nodular gonads of *Calvadosia lewisi* (modified from Miranda et al., 2017b). (D) Cross section of the male nodular gonads of *Calvadosia corbini*, showing the presence of vesicles with compartmentalization of gametes, spermatozoa (white arrowhead) in the center and spermatocytes (black arrowheads) near the gastrodermis (modified from Miranda et al., 2016a). (E) Cross section of the female gonads of *Calvadosia cruciformis* showing the presence of vesicles with compartmentalization of gametes, mature oocytes (white arrowhead) in the center and immature oocytes (black arrowheads) near the gastrodermis (modified from Miranda et al., 2016a). (F) Cross section of the female gonads of *Calvadosia vanhoeffeni*, showing the presence of vesicles and gametoduct (modified from Miranda et al., 2016a). Abbreviations: AR, adradial arms; GA, gametes; GAD, gametoduct; GD, gonads; GT, gastrodermis; IR, interradial arms; PR, perradii.

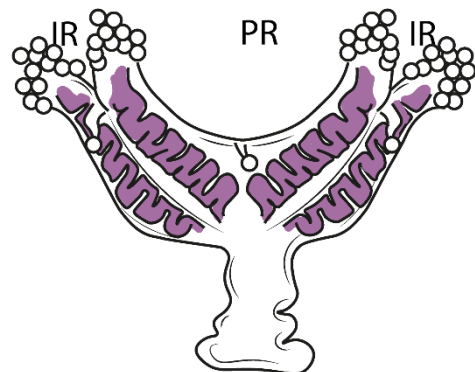
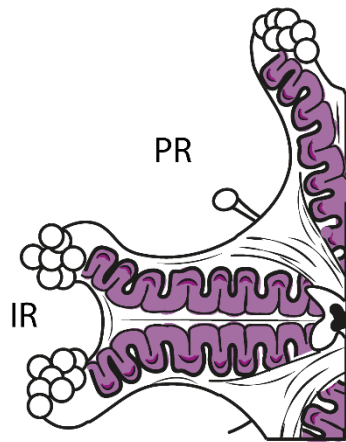
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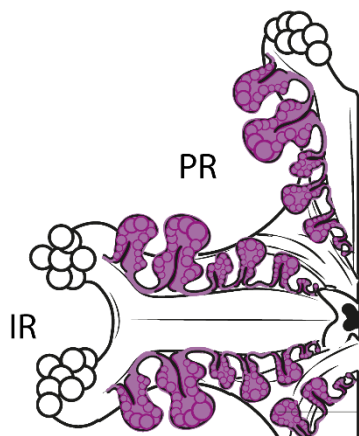
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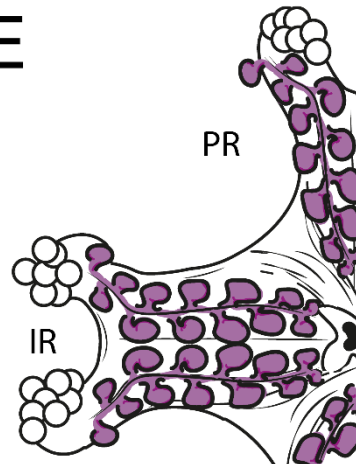


Figure 4. Scheme of morphological patterns of gonads of the genus *Calvadosia*; **(A-C)** Images on the left represents the gonads in subumbrellar view and on the right the exumbrellar view. **(A)** Representation of the gonads of *Calvadosia campanulata*, *Calvadosia cruxmelitensis*, and *Calvadosia vanhoeffeni*. The gonads are positioned in the adradial along the arms in a sinuous row. **(B)** Representation of the gonads of *Calvadosia* sp.1 NZ, the gonads are positioned in the adradial along the arms, formed by a series of rows of vesicles. **(C)** Representation of the gonads of *Calvadosia cruciformis* and *Calvadosia nagatensis*, the gonads are positioned in the interradial (near the manubrium) and in the adradial (toward the tips of the arms). They are formed by a sinuous row that in exumbrellar view resemble the steps of a ladder. **(D)** Representation of the gonads (subumbrellar view) of *Calvadosia capensis*, *Calvadosia corbini*, *Calvadosia lewisi*, and *Calvadosia tasmaniensis*. The gonads are positioned in the interradial (near the manubrium) and in the adradial (toward the tips of the arms). They are formed by a sinuous row, which protrude into the epidermis forming irregular nodules. **(E)** Representation of the gonads (subumbrellar view) of *Calvadosia lewisi*, the gonads are positioned in the interradial (near the manubrium) and in the adradial (toward the tips of the arms). They are formed by a sinuous row, which protrude into the epidermis forming smooth nodules. Abbreviations: IR, interradial; PR, perradial, gonads in purple.

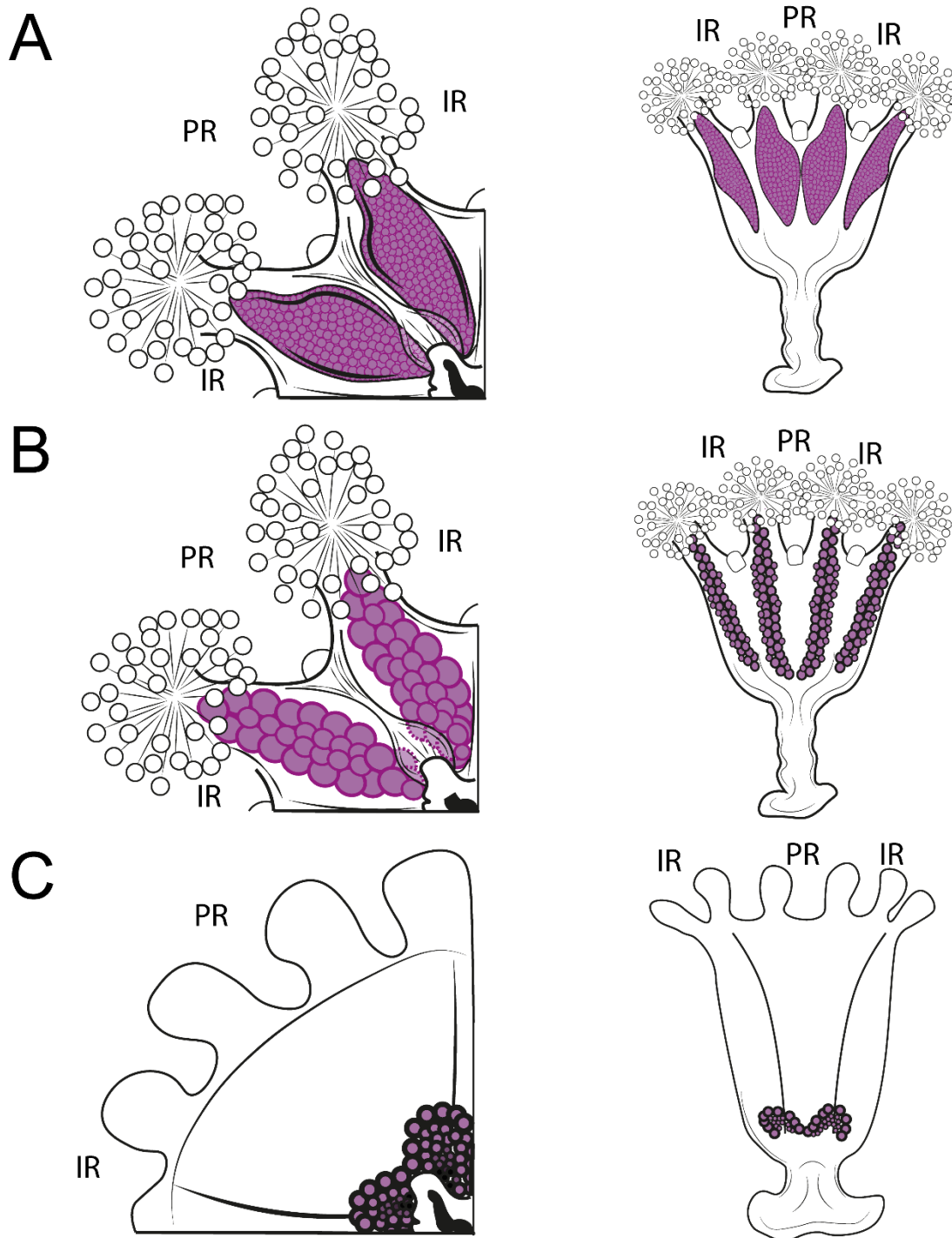


Figure 5. Scheme of morphological patterns of gonads of the genera *Haliclystus* and *Lipkea*. Images on the left represents the gonads in sumbrellar view and on the right in exumbrellar view. (A, B) Generalized representation of the gonads of all species of *Haliclystus*, gonads positioned in the adradial along the arms, formed by a series of rows of vesicles, with more (A) or less (B) vesicles in each species. It is possible to notice the leaf shape of the gonads in exumbrellar view. (C) Generalized representation of the gonads of all species of *Lipkea*, gonads formed by a cluster of vesicles in the adradial, in the inner region of the calyx near the manubrium. Abbreviations: IR, interradial; PR, periradial, gonads in purple.

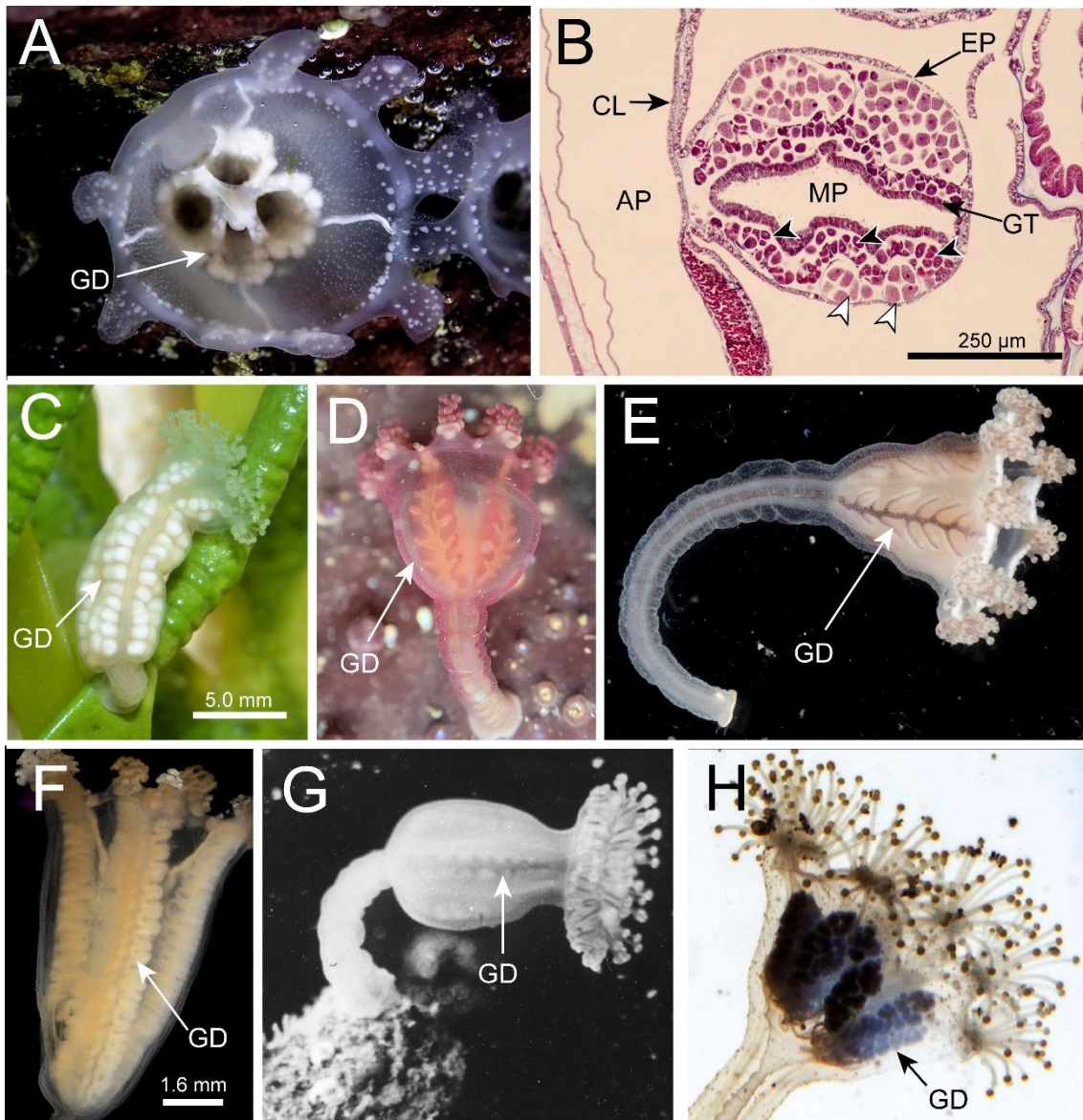


Figure 6. Gonads of the genera *Lipkea*, *Craterolophus*, *Depastromorpha*, *Manania*, *Depastrum*, and *Stylocoronella*. (A) Vesicle clusters inside the calyx of *Lipkea stephensoni* (?) (photo credit: Lisa Beasley; scale not available). (B) Cross section of the female gonads of *Craterolophus convolvulus*, notice that the gametes are between a layer of epidermis and gastrodermis without evagination, not forming vesicles, mature oocytes (white arrowhead) in the center and immature oocytes (black arrowheads) near the gastrodermis (modified from Miranda et al., 2016a). (C) *Depastromorpha africana* in exumbrellar view, showing the two rows of vesicles along its calyx (modified from Miranda et al., 2017b). (D) Gonads of *Manania gwilliami* (?) in exumbrellar view, notice the herringbone shape (photo credit: Alex Heyman; scale not available). (E) Gonads of *Manania distincta* in exumbrellar view (photo credit: Gustav Paulay, Florida Museum of Natural History; scale not available), notice the herringbone shape a little more slender than *M. gwilliami* (D), and with sinuosities more conspicuous than those of the gonads of *Manania uchidai* (F), in exumbrellar view; notice a homogeneous herringbone shape along the calyx (modified from Miranda et al., 2016a). (G) Gonads of *Depastrum cyathiforme* in exumbrellar view (photo credit: P. G. Corbin; scale not available). (H) V-shaped gonads of *Stylocoronella* sp. in exumbrellar view (photo credit: Ruth Sharratt,

edition: David Fenwick; scale not available). Abbreviations: AP, accessory radial pocket; CL, claustrum; EP, epidermis; GD, gonads; GT, gastrodermis; MP, main radial pocket.

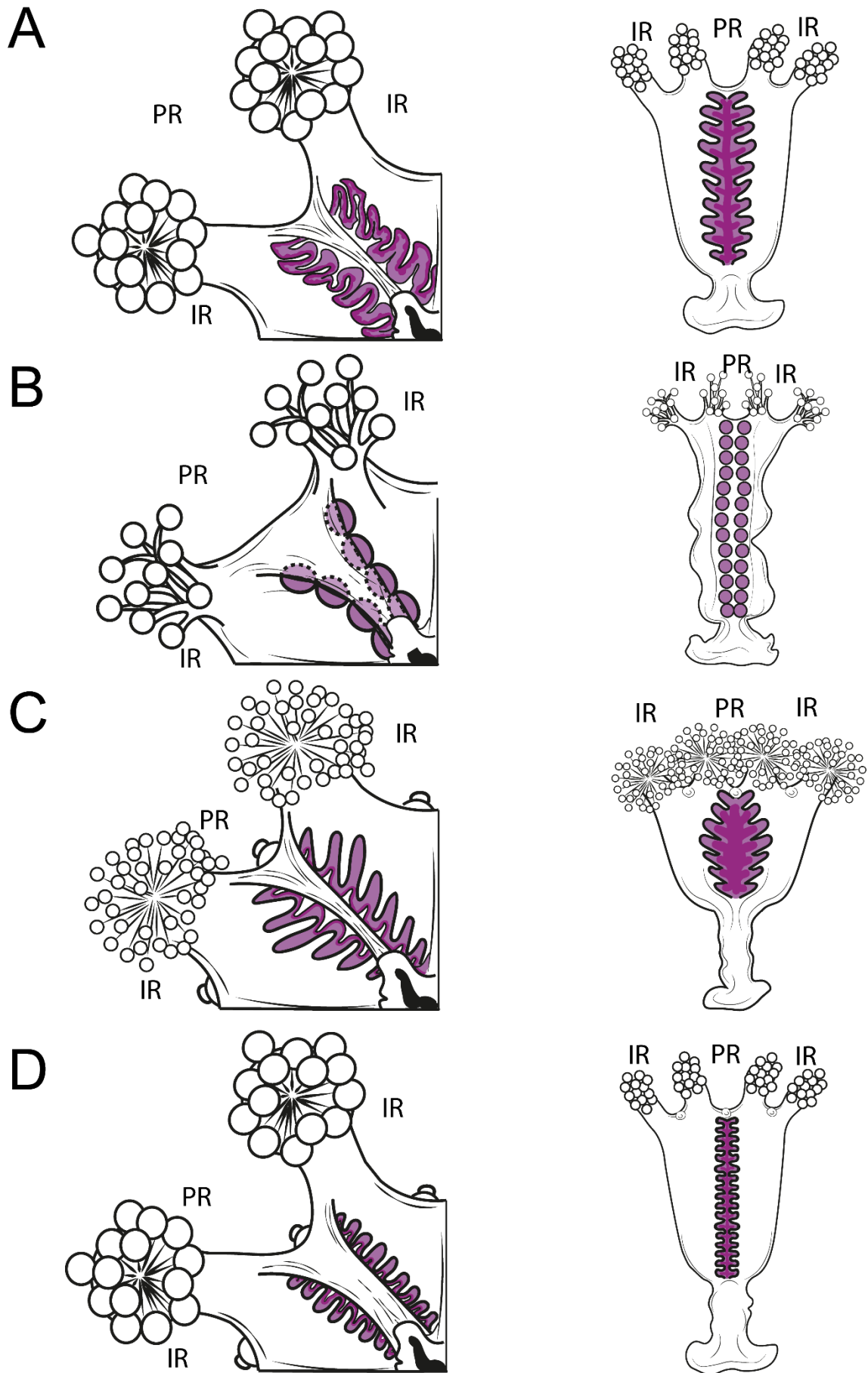


Figure 7. Scheme of morphological patterns of gonads of the genera *Craterolophus*, *Depastromorpha*, and

Manania. Images on the left represents the gonads in subumbrellar view and on the right in exumbrellar view. **(A)** Representation of the gonads of *Craterolophus convolvulus*, the gonads are positioned in the perradii in a sinuous row; notice the herringbone shape of the gonads in exumbrellar view. **(B)** Representation of the gonads of *Depastromorpha africana*, the gonads are positioned in the perradii in a single row of round vesicles. **(C, D)** Representation of the gonads of the species of *Manania*; the gonads are positioned in the perradii in a sinuous row; notice the herringbone shape of the gonads in exumbrellar view. **(C)** The gonads narrows at both ends (near the manubrium and near the calyx margin) in *Manania handi*, *Manania distincta*, and *Manania gwilliami* in contrast to **(D)** *Manania uchidai* and *Manania auricula* (?), in which the gonads remain homogeneous along the calyx. Abbreviations: IR, interradii; PR, perradii, gonads in purple.

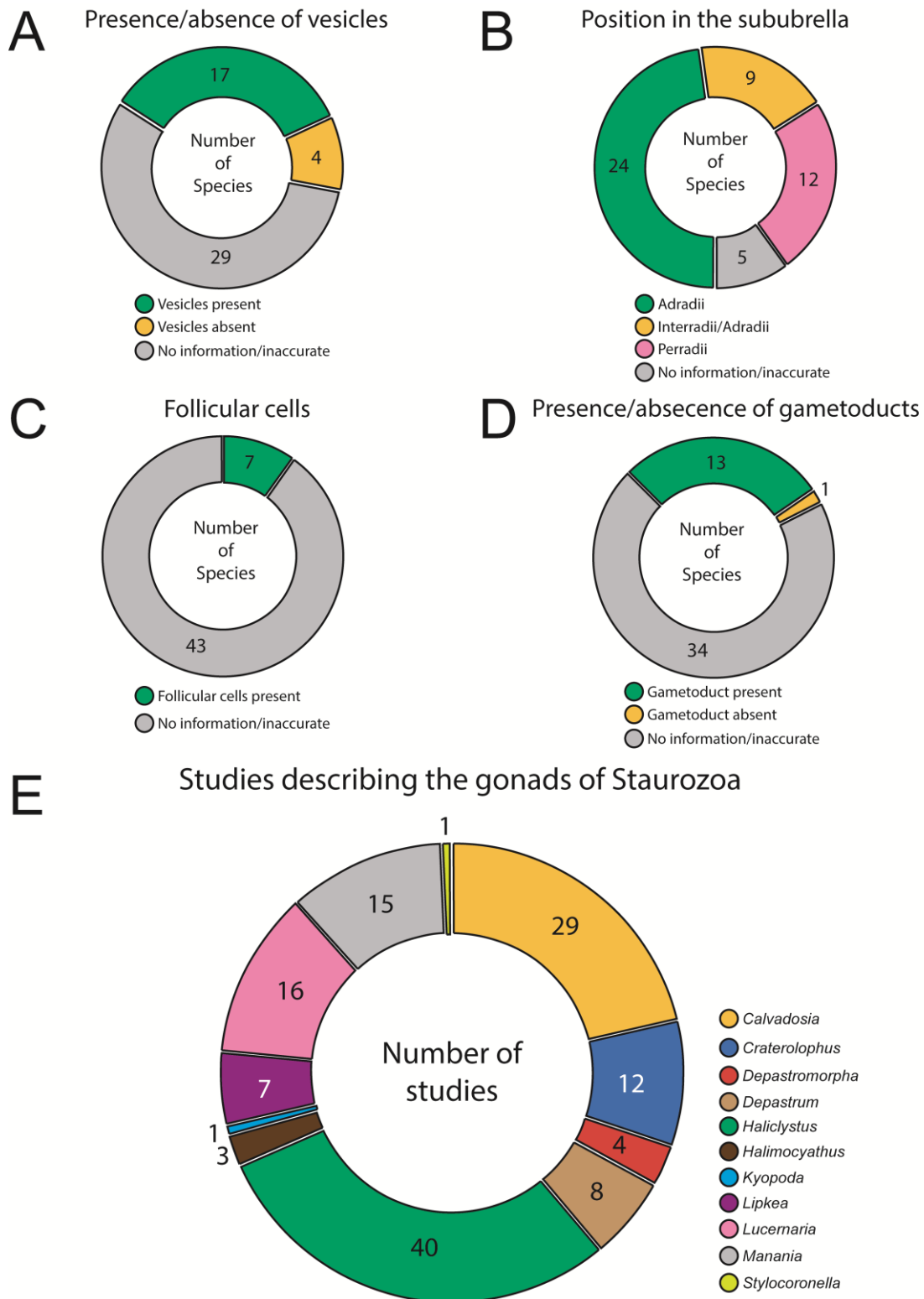


Figure 8. Extent of knowledge of the gonads of Staurozoa. **(A)** Presence of vesicles in all species of Staurozoa. **(B)** Position of the gonads in the subumbrella of all species of Staurozoa. **(C)** Presence of follicular cells in all species of Staurozoa. **(D)** Presence of gametoducts in all species of Staurozoa. **(E)** Number of studies conducted between 1806 and 2021 that mentioned descriptions of gonadal structures relative to each genus of Staurozoa.

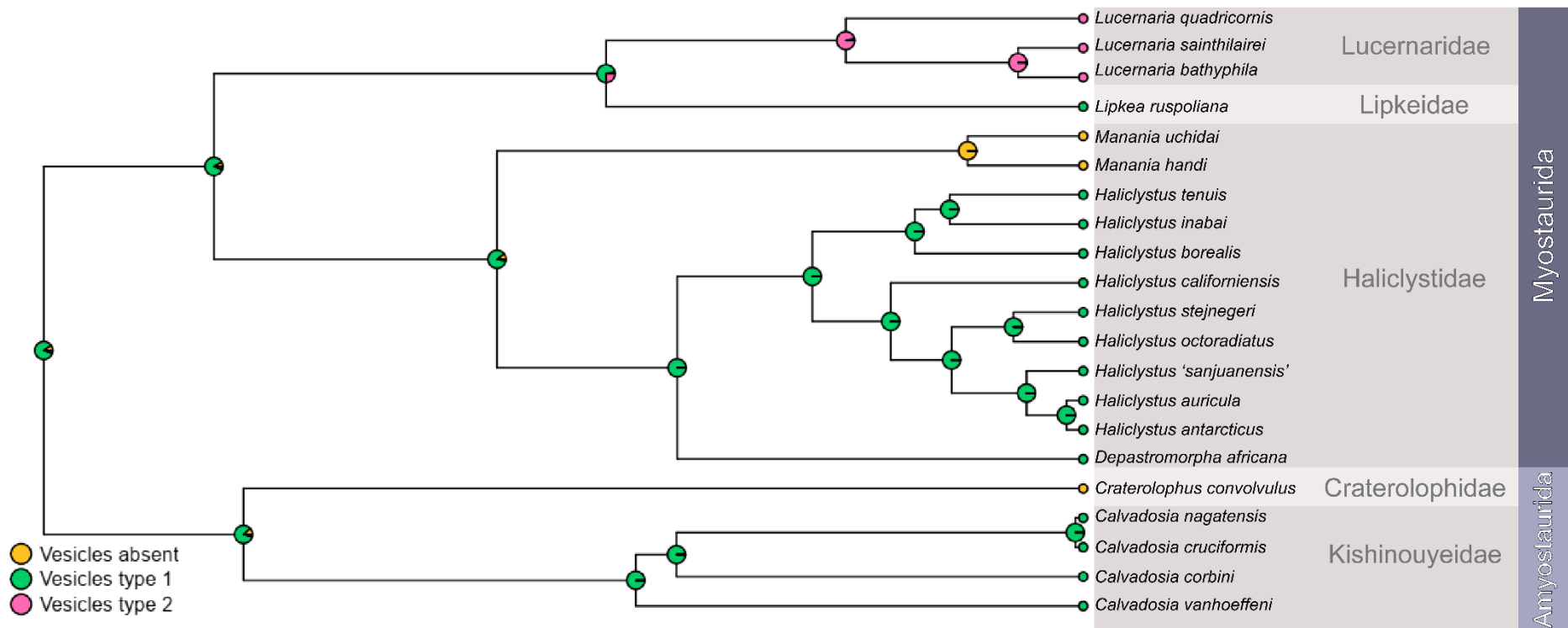


Figure 9. Ancestral state reconstruction for the presence of vesicles. The charts represent the probability of the ancestral state. Yellow indicates that the vesicles are completely absent; green indicates the presence of vesicles type 1; pink indicates the presence of vesicles type 2 (see text for more information).

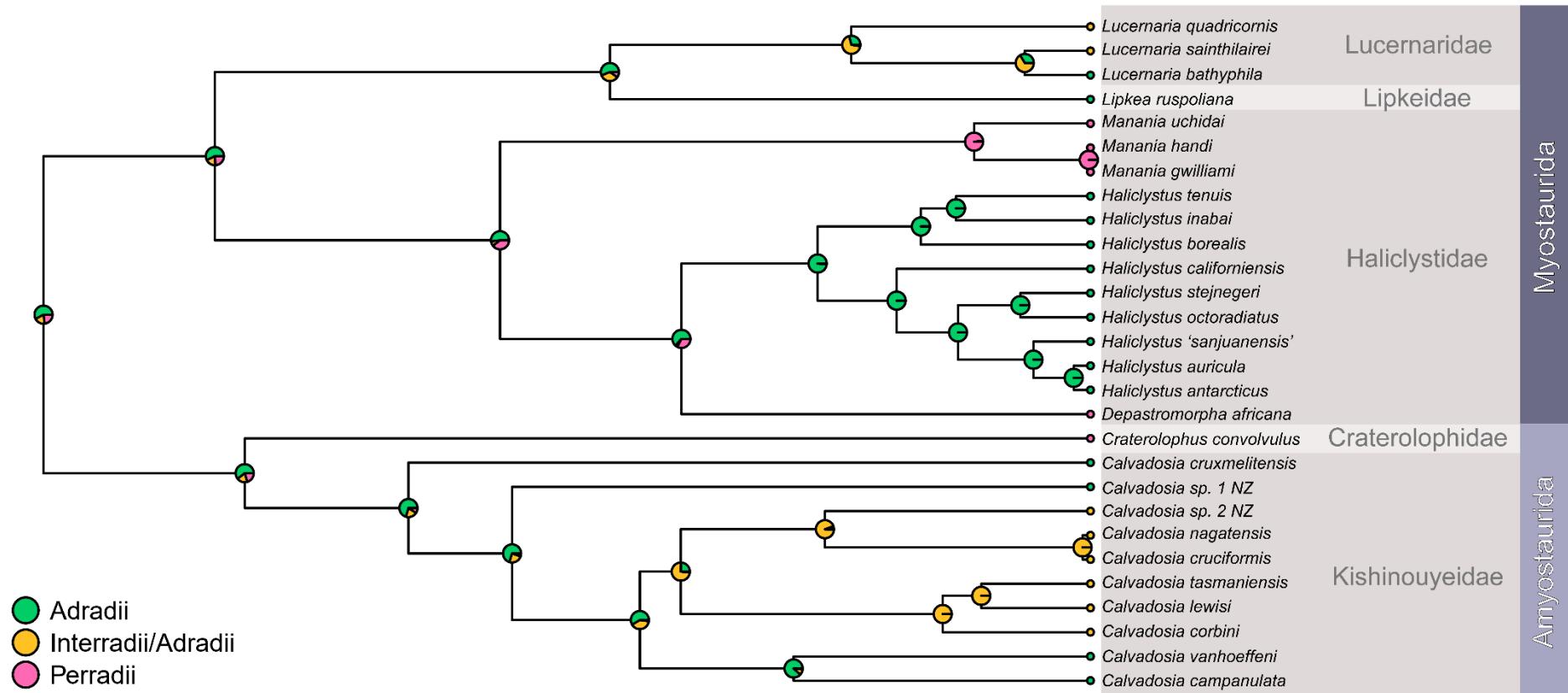


Figure 10. Ancestral state reconstruction for the distribution of the gonads on subumbrella. The charts represent the probability of the ancestral state. Green indicates that the gonads are positioned on the adradii along the stauromedusan arms; yellow indicates that the gonads are positioned in the interradii/adradii; pink indicates that the gonads are positioned in the perradii.

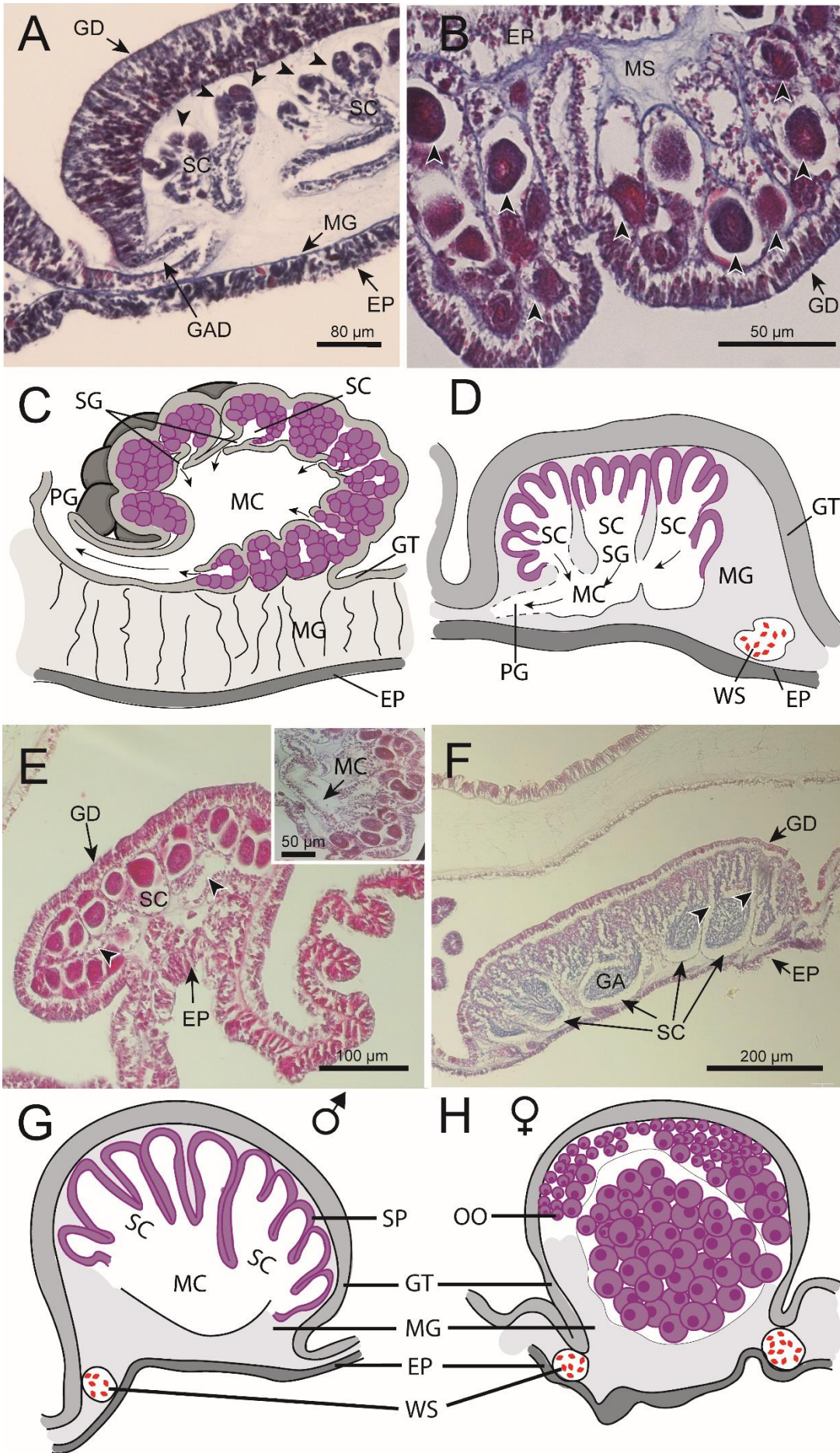


Figure 11. Internal organization of *Lucernaria* and *Calvadosia* vesicles. The arrows represent the trajectory of the mature gamete to the exit through the primary gametoduct. **(A)** Section of a male vesicle of *Lucernaria bathyphila*, notice the spermatocytes (arrowheads) localized in “chambers” contained in the mesoglea, between a layer of evaginated gastrodermis and a layer of epidermis, and the gametoduct (modified from Miranda et al., 2016a). **(B)** Section of a female vesicle of *Lucernaria quadricornis*, notice that the gametes (arrowheads) are somewhat disorganized inside the vesicle (modified from Miranda et al., 2016a). **(C)** Modified from Haeckel (1882); representation of the internal organization of the female vesicle of *L. bathyphila*, with the presence of secondary chambers and ducts. **(D)** Modified from Antipa (1892); representation of the internal organization of the male vesicle of *Lucernaria walteri*, with the presence of secondary chambers and ducts. **(E)** Section of a female vesicle of *L. quadricornis*, with the presence of secondary chambers, ducts (arrowheads) and the main chamber (inset). **(F)** Section of a male vesicle of *Calvadosia nagatensis*, with the presence of secondary chambers and ducts (arrowheads). Modified from Claus (1883); male **(G)** and female **(H)** vesicles of *Calvadosia campanulata*, the male with a more complex organization, as in **B**, while the female displays an organization similar to that found in *Halicylistus* (see Fig. 1C). Abbreviations: EP, epidermis; GA, gametes, GAD, gametoduct; GT, gastrodermis; MG, mesoglea; MC, main chamber; OO, oocytes; PG, primary gametoduct; SC, secondary chamber; SG, secondary gametoduct; SP, Spermatocytes; WS, white spot; gametes in purple; nematocysts of white spots in red.

Tables

Table 1. Species of Staurozoa analyzed in this study.

Species	Voucher catalog number	Slides catalog number	Locality
<i>Calvadosia campanulata</i>	USNM 1233741	-	Verte Island, La Ciotat, Roscoff, France
<i>Calvadosia corbini</i>	MZUSP 1563; MZUSP 1564; MZUSP 1565	LEM 14	Praia dos Padres, Aracruz, Espírito Santo, Brazil
<i>Calvadosia cruciformis</i>	USNM 1106656	LEM 15	Muroran, Hokkaido, Japan
<i>Calvadosia cruxmelitensis</i>	USNM 1233742	-	Wembury Bay, Plymouth, England, United Kingdom
	LABEC 16	-	Penzance, England, United Kingdom
<i>Calvadosia lewisi</i>	MZUSP 3415; MZUSP 3416	-	Simons's Town, Cape Town, Western Cape, South Africa
<i>Calvadosia nagatensis</i>	LABEC 8; LABEC 9	-	Amatsura, Kamogawa, Chiba, Japan
<i>Calvadosia tasmaniensis</i>	USNM 1233739	-	Fortescue Bay, Tasmania, Australia
	USNM 1233740	-	Gerloff Bay, South Australia, Australia
<i>Calvadosia vanhoeffeni</i>	LABEC 6	-	PROANTAR IV, st 4872, Antarctica
	USNM 79939	LEM 16	Janus Island, Palmer Archipelago, Antarctica
<i>Calvadosia</i> sp. 1 NZ	USNM 1233747	-	South Island, Kaikoura Bay, New Zealand
	NIWA 62040	-	Island Bay, New Zealand
<i>Calvadosia</i> sp. 2 NZ	NIWA 86808; NIWA 86809	-	Taputeranga Reserve, station Z15699, Wellington New Zealand
	NIWA 86810; NIWA 86811	-	Taputeranga Reserve, station Z15700, Wellington New Zealand
	NIWA 79477	-	station Z15652, New Zealand
<i>Craterolophus convolvulus</i>	USNM 54321	LEM 16	Woods Hole, Nobska Point, Massachusetts, USA
	LABEC 7	-	Helgoland Island, Germany
<i>Depaстроморpha africana</i>	MZUSP 3418	-	Sea Point, Cape Town, Western Cape, South Africa
	MAZUSP 3419	-	Kalk Bay, Cape Town, Western Cape, South Africa
	USNM 1233748	-	Gerloff Bay, South Australia, Australia
<i>Halicystus antarcticus</i>	MZUSP 1551; MZUSP 1554; MZUSP 1555; MZUSP 1556; MZUSP 1557	LEM 01-08	Pieter Lenie Beach, Copacabana Refuge (USA), Admiralty Bay, King George Island, Antarctica
	MZUSP 1552		Shag Point, Arctowski Polish Station, Admiralty Bay, King George Island, Antarctica
	MZUSP 1559; MZUSP; 1561		Los Molinos, Valdivia, Chile
<i>Halicystus inabai</i>	USNM 1233743	-	Otsuchi Bay, Iwate, Japan
	USNM 1233744	-	Oshoro Bay, Hokkaido, Japan
	USNM 1233745	-	Boso Peninsula, Uchiura Bay, Chiba, Japan
<i>Halicystus 'sanjuanensis'</i>	LABEC 11; LABEC 12; LABEC 14; LABEC 15	-	Castle Point, Washington, USA
	LABEC 13	-	MacGinities shoreline, Washington, USA
<i>Halicystus stejnegeri</i>	LABEC 1	-	E. Kamchatka, Avancha Bay, Russia
<i>Halicystus tenuis</i>	USNM 1106652	LEM 09	Muroran, Hokkaido, Japan
<i>Lipkea ruspoliana</i>	LABEC 10	-	Aquariums du Musée océanographique de Monaco, Monaco
<i>Lipkea</i> sp. 1	LABEC 5	-	North Carolina Aquarium at Fort Fisher, North Carolina, USA
<i>Manania uchidai</i>	USNM 1106645	LEM 10	Muroran, Hokkaido, Japan
<i>Lucernaria quadricornis</i>	USNM 1106240	LEM 11	Chupa Inlet, Kandalaksha Bay, Russia
<i>Lucernaria bathyphila</i>	USNM 1106643	LEM 12	Nicol'skaya Inlet, Kandalaksha Bay, Russia
<i>Lucernaria saintilairei</i>	USNM 1102446	LEM 13	Cross Islands, close to the Biological Station of Moscow State University, Russia

Table 2. Gonad-related traits obtained from the literature review (Table S1) that were used in the evolutionary analyses. "?" represents absence of information or uncertainty; species with this symbol were not used in the analyses.

Species	Position of the gonads on the subumbrella	Presence of vesicles
<i>Craterolophus convolvulus</i>	Perradial	Absent
<i>Calvadosia campanulata</i>	Adradial	?
<i>Calvadosia corbini</i>	Interradial/adradial	Present - Vesicle type 1
<i>Calvadosia cruciformis</i>	Interradial/adradial	Present - Vesicle type 1
<i>Calvadosia cruxmelitensis</i>	Adradial	?
<i>Calvadosia nagatensis</i>	Interradial/adradial	Present - Vesicle type 1
<i>Calvadosia tasmaniensis</i>	Interradial/adradial	?
<i>Calvadosia vanhoeffeni</i>	Adradial	Present - Vesicle type 1
<i>Calvadosia lewisi</i>	Interradial/adradial	?
<i>Calvadosia</i> sp. 1 NZ	Adradial	Present - Vesicle type 1
<i>Calvadosia</i> sp. 2 NZ	Interradial/adradial	?
<i>Depastromorpha africana</i>	Perradial	Present - Vesicle type 1
<i>Haliclystus antarcticus</i>	Adradial	Present - Vesicle type 1
<i>Haliclystus auricula</i>	Adradial	Present - Vesicle type 1
<i>Haliclystus borealis</i>	Adradial	Present - Vesicle type 1
<i>Haliclystus californiensis</i>	Adradial	Present - Vesicle type 1
<i>Haliclystus inabai</i>	Adradial	Present - Vesicle type 1
<i>Haliclystus octoradiatus</i>	Adradial	Present - Vesicle type 1
<i>Haliclystus 'sanjuanensis'</i>	Adradial	Present - Vesicle type 1
<i>Haliclystus stejnegeri</i>	Adradial	Present - Vesicle type 1
<i>Haliclystus tenuis</i>	Adradial	Present - Vesicle type 1
<i>Manania gwilliami</i>	Perradial	?
<i>Manania handi</i>	Perradial	Absent
<i>Manania uchidai</i>	Perradial	Absent
<i>Lipkea ruspoliana</i>	Adradial	Present - Vesicle type 1
<i>Lucernaria bathyphila</i>	Adradial	Present - Vesicle type 2
<i>Lucernaria quadricornis</i>	Interradial/adradial	Present - Vesicle type 2
<i>Lucernaria sainthilairei</i>	Interradial/adradial	Present - Vesicle type 2

Table 3. Morphotypes of the gonads of the genera of Staurozoa. "?" represents absence of information or uncertainty.

Genera	Morphotypes	References
<i>Calvadosia</i>	Sinuuous row and rows of vesicles	Keferstein, 1862; Clark, 1863; Haeckel, 1879; Kishinouye, 1902; Mayer, 1910; Okubo, 1917; Stiansy, 1919; Uchida, 1929; Ling, 1937; Eales, 1938; Kramp, 1961; Larson, 1980; Hirano, 1986; Lechuga & Alamo, 2005; Zagal et al., 2011; Miranda et al., 2012, 2016a, 2017b; This study.
<i>Craterolophus</i>	Sinuuous row	Mayer, 1910; Kramp, 1961; Miranda et al., 2016a; Holst et al., 2021; This study.
<i>Depastromorpha</i>	Rows of vesicles	Carlgren, 1935; Zagal et al., 2011; Miranda et al., 2017b, This study
<i>Depastrum</i>	?	?
<i>Haliclystus</i>	Rows of vesicles	Sars, 1829; Haeckel, 1879; Kishinouye, 1902, 1910; Mayer, 1910; Bigelow, 1920; Gellerman, 1926; Uchida, 1929, 1933; Uchida & Hanaoka, 1934; Ling, 1937, 1939; Gwilliam, 1956; Amor, 1962; Berrill, 1962; Clark, 1863; Quezada, 1969; Hirano, 1986; Eckelbarger & Larson, 1993; Eckelbarger, 1994; Hirano, 1997; Mills & Larson, 2007; Miranda et al., 2009; Kahn et al., 2010; Falconer, 2013; Westlake, 2015; Miranda et al., 2016a; Holst et al., 2019; This study.
<i>Halimocyathus</i>	Cluster of vesicles?	Mayer, 1910; Haeckel, 1879.
<i>Kyopoda</i>	?	?
<i>Lipkea</i>	Cluster of vesicles	Antipa, 1893; Carlgren, 1933; Pisani et al., 2007; Miranda et al., 2017b.
<i>Lucernaria</i>	Sinuuous row and rows of vesicles?	Haeckel, 1879; Haeckel, 1882; Antipa, 1892; Mayer, 1910; Berrill, 1963; Collins & Daly, 2005; Miranda et al., 2016a, This study.
<i>Manania</i>	Sinuuous row	Mayer, 1910; Uchida, 1929; Uchida & Hanaoka, 1933; Gwilliam, 1956; Naumov, 1961; Hirano, 1986; Larson & Fautin, 1989; Westlake, 2015; Miranda et al., 2016a; This study.
<i>Stylocoronella</i>	Cluster of vesicles?	This study.

Table 4. General characteristics of the gonads of *Calvadosia*. "?" represents absence of information or uncertainty.

Species	Distribution	Morphotypes	Vesicles	References
<i>C. campanulata</i>	AR	Sinuuous row	?	Keferstein, 1862; Clark, 1863; Haeckel, 1879; Stiansy, 1919; Eales, 1938; Zagal et al., 2011; This study.
<i>C. capensis</i>	AR	Sinuuous row (nodular)	?	Miranda et al., 2012, 2017b.
<i>C. corbini</i>	IR/AR	Sinuuous row (nodular)	Yes	Larson, 1980; Grohmann et al., 1999; Lechuga & Alamo, 2005; Miranda et al., 2016a, 2017b; This study.
<i>C. cruciformis</i>	IR/AR	Sinuuous row	Yes	Okubo, 1917; Uchida, 1929; Ling, 1937; Kramp, 1961; Hirano, 1986; Miranda et al., 2016a; This study.
<i>C. cruxmelitensis</i>	AR	Sinuuous row	?	Corbin, 1978; Mills & Hirano, 2007; Zagal et al., 2011.
<i>C. hawaiiensis</i>	?	?	?	-
<i>C. lewisi</i>	IR/AR	Sinuuous row (nodular)	?	Miranda et al., 2017b; This study.
<i>C. nagatensis</i>	IR/AR	Sinuuous row	Yes	Oka, 1897; Kishinouye, 1902; Mayer, 1910; Uchida, 1929; Ling, 1939; This study
<i>C. tasmaniensis</i>	IR/AR	Sinuuous row (nodular)	?	Zagal et al., 2011; Miranda et al., 2017b; This study
<i>C. tsingtaoensis</i>	IR/AR?	?	?	Ling, 1937.
<i>C. vanhoeffeni</i>	AR	Sinuuous row	Yes	Browne, 1910; Carlgren, 1930; Zagal et al., 2011; Miranda et al., 2016a; This study.
<i>Calvadosia</i> sp. 1 NZ	AR	Rows of vesicles	Yes	This study.
<i>Calvadosia</i> sp. 2 NZ	IR/AR	Sinuuous row	Yes	This study.

Table 5. General characteristics of the gonads of *Halicyclystus*. "?" represents absence of information or uncertainty.

Species	Distribution	Morphotype	Number of rows of vesicles per gonad	Number of vesicles	References
<i>H. antarcticus</i>	AR	Rows of vesicles	6 - 12	60 - 150	Pfeffer, 1889; Mayer, 1910; Thiel, 1928; Amor, 1962; Quezada, 1969; Miranda et al., 2009; This study
<i>H. auricula</i>	AR	Rows of vesicles	2 - 8	100 -150	Clark, 1863; Haeckel, 1879; Mayer, 1910; Miranda et al., 2009; This study
<i>H. borealis</i>	AR	Rows of vesicles	1 - 6	30 - 100	Uchida & Hanaoka, 1934; Naumov, 1961; Hirano, 1986, Miranda et al., 2009; This study
<i>H. californiensis</i>	AR	Rows of vesicles	3 - 4 (abreast in the widest portion of the gonad)	40 - 50	Gwilliam, 1956; Miranda et al., 2009; Kahn et al., 2010; This study
<i>H. inabai</i>	AR	Rows of vesicles	1 - 3	40 - 70	Kishinouye, 1902; Mayer, 1910; Uchida, 1929; Ling, 1939; This study
<i>H. kerguelensis</i>	AR	?	?	?	Miranda et al., 2009; This study
<i>H. monstrosus</i>	AR	Rows of vesicles	4 - 6	?	Naumov, 1961; Miranda et al., 2009; This study
<i>H. octoradiatus</i>	AR	Rows of vesicles	2 - 4	10 - 70	Clark, 1863; Haeckel, 1879; Mayer, 1910; Miranda et al., 2009; This study
<i>H. salpinx</i>	AR	Rows of vesicles	4?	20 - 50?	Sars, 1829; Clark, 1863; Haeckel, 1879; Gwilliam, 1956; Berrill, 1962; Mills & Larson, 2007; Miranda et al., 2009; This study
<i>H. 'sanjuanensis'</i>	AR	Rows of vesicles	10 - 22	200 - 300	Gellerman, 1926; Gwilliam, 1956; Miranda et al., 2009; This study
<i>H. sinensis</i>	AR	Rows of vesicles	1	17 - 20	Ling, 1937; Miranda et al., 2009; Kahn et al., 2010; This study
<i>H. stejnegeri</i>	AR	Rows of vesicles	3 - 10	90 - 240	Kishinouye, 1902; Mayer, 1910; Bigelow, 1920; Uchida & Hanaoka, 1934; Naumov, 1961; Hirano, 1986; Miranda et al., 2009; This study
<i>H. tenuis</i>	AR	Rows of vesicles	2 - 6	30 - 50	Hirano, 1986; Miranda et al., 2009; This study

Table 6. General characteristics of the gonads of *Manania*. "?" represents absence of information or uncertainty.

Species	Distribution	Morphotype	Form	References
<i>M. atlantica</i>	PR	Sinuuous row	Herringbone	Berrill, 1962; 1963.
<i>M. auricula</i>	PR?	?	Herringbone (homogeneous)?	Miranda & Collins, 2019; figure 2C, D
<i>M. distincta</i>	PR	Sinuuous row	Herringbone	Kishinouye, 1910.
<i>M. gwilliami</i>	PR	Sinuuous row	Herringbone	Gwilliam, 1956; Larson & Fautin, 1989.
<i>M. handi</i>	PR	Sinuuous row	Herringbone	Gwilliam, 1956; Larson & Fautin, 1989; Westlake, 2015
<i>M. hexaradiata</i>	PR?	?	Horseshoe?	Broch, 1907; Mayer, 1910.
<i>M. uchidai</i>	PR	Sinuuous row	Herringbone (homogeneous)	Uchida, 1929; Uchida & Hanaoka, 1933; Naumov, 1961; Hirano, 1986; Miranda et al., 2016a; This study

Table 7. Species of Staurozoa that are reported to have follicular cells and gametoduct.

Species	Follicle cells	Gametoduct	Reference
<i>Calvadosia corbini</i>	?	Yes	Miranda et al., 2016a
<i>Calvadosia cruciformis</i>	Yes	Yes	Miranda et al., 2016a
<i>Calvadosia vanhoeffeni</i>	?	Yes	Miranda et al., 2016a
<i>Craterolophus convolvulus</i>	Yes	Yes	Hertwig & Hertwig, 1879; Miranda et al., 2016a
<i>Depastromorpha africana</i>	?	No	Carlgren, 1935
<i>Haliclystus antarcticus</i>	Yes	?	Miranda et al., 2013
<i>Haliclystus 'sanjuanensis'</i>	Yes	?	Eckelbarger & Larson, 1993
<i>Haliclystus salpinx</i>	Yes	?	Eckelbarger, 1994
<i>Haliclystus tenuis</i>	Yes	Yes	Miranda et al., 2016a
<i>Lipkea stephensoni</i>	?	Yes	Carlgren, 1933
<i>Lipkea sturdzii</i>	?	Yes	Antipa, 1893
<i>Lucernaria australis</i>	?	Yes	Carlgren, 1930
<i>Lucernaria bathyphila</i>	?	Yes	Haeckel, 1882; Miranda et al., 2016a
<i>Lucernaria haeckeli</i>	?	Yes	Antipa, 1892
<i>Lucernaria quadricornis</i>	Yes	Yes	Elmhirst, 1922; Berrill, 1963; Miranda et al., 2016a
<i>Lucernaria walteri</i>	?	Yes	Antipa, 1892
<i>Manania uchidai</i>	?	Yes	Miranda et al., 2016a

Table 8. MK models tested for ancestral reconstruction and values of entropy (δ) for both traits analyzed.

Trait	Model	AICc	AICw	δ empirical	δ simulated
Presence of vesicles	ER	23.398	0.914	2.089	1.469
	ARD	28.145	0.085		
	SYM	37.587	0.000		
Position of the gonads on the subumbrella	ER	42.420	0.841	1.135	0.925
	ARD	53.057	0.004		
	SYM	45.808	0.154		

Table 9. Internal organization of the vesicles of Staurozoa. "?" represents absence of information or uncertainty.

Species	Male	Female	References
<i>Calvadosia campanulata</i>	Multilocular	Unilocular	Claus, 1883 - Plate 10, figures 65, 66.
<i>Calvadosia corbini</i>	Unilocular	?	Miranda et al., 2016a - figure 40; This study.
<i>Calvadosia cruciformis</i>	?	Unilocular	Miranda et al., 2016a - figure 44; This study.
<i>Calvadosia nagatensis</i>	Multilocular	?	This study.
<i>Calvadosia vanhoeffeni</i>	Multilocular	?	Miranda et al., 2016a - figure 48; This study.
<i>Calvadosia</i> sp. 1 NZ	?	Unilocular	This study.
<i>Calvadosia</i> sp. 2 NZ	Multilocular	?	This study.
<i>Depastromorpha africana</i>	Unilocular	?	Carlgren, 1935 - figures 8, 10, 11
<i>Haliclystus antarcticus</i>	Unilocular	Unilocular	Miranda et al., 2013 - figure 10; This study.
<i>Haliclystus borealis</i>	Unilocular?	Unilocular	Uchida & Hanaoka, 1934 - figures 4, 5, 10, 11.
<i>Haliclystus 'sanjuanensis'</i>	?	Unilocular	This study.
<i>Haliclystus stejnegeri</i>	Unilocular?	Unilocular	Uchida & Hanaoka, 1934 - figure 19.
<i>Haliclystus tenuis</i>	Multilocular	Unilocular	Uchida, 1929 - figures 12, 14, 22, 23; Miranda et al., 2016a; This study.
<i>Lipkea stephensoni</i>	?	Unilocular?	Carlgren, 1933 - figure 12.
<i>Lucernaria australis</i>	?	Multilocular	Carlgren, 1930 - figure 17.
<i>Lucernaria bathyphila</i>	Multilocular	Multilocular ?	Haeckel, 1882 - Plate 17, figure 18; Miranda et al., 2016a - figure 31; This study.
<i>Lucernaria haeckeli</i>	Multilocular	?	Antipa, 1892 - Plate 18, figure 11-14.
<i>Lucernaria infundibulum</i>	Multilocular ?	Multilocular ?	Haeckel, 1879.
<i>Lucernaria quadricornis</i>	?	Multilocular ?	Miranda et al., 2016a - figure 27; This study.
<i>Lucernaria sainthilairi</i>	Multilocular ?	?	Miranda et al., 2016a - figure 36; This study.
<i>Lucernaria walteri</i>	Multilocular	?	Antipa, 1892 - Plate 17, figures 7, 9.

Supplemental material

Table S1 - Tables containing the complete literature review on gonads for all species of Staurozoa.

https://drive.google.com/drive/folders/1aJTxyvO3KAMKnOCysytMQO8waj_u6OAB?usp=sharing

General Conclusion

This study aimed to explore overlooked questions regarding the morphology of stalked jellyfishes, specifically the white spots and gonads. Both structures have been described in the literature for the past nearly 200 years (Kling, 1879; Haeckel, 1879; Kassianow 1901; Mayer 1910; Hirano, 1986, 1997; Miranda et al., 2016), however never in depth, broadly, and in a comparative way for all species of the class.

Based on our literature review it was possible to obtain important information regarding white spots and gonads, such as the distribution patterns of white spots in the subumbrella, which have taxonomic value for the class, and the morphological "complexity" observed inside the gonadal vesicles of some species (e.g., *Lucernaria walteri*), indicating that there may be an intrinsic variation of the internal organization of these vesicles among species of the class, which means that the gonadal diversity in Staurozoa may be underestimated (Eckelbarger & Larson, 1993; Eckelbarger, 1994). Furthermore, from the literature it was also possible to highlight genera that have received little or no attention in morphological studies, as *Depastrum*, *Kyopoda*, *Lucernaria*, and *Stylocoronella*. The genus *Lucernaria* stands out, since it is a relatively diverse genus in number of species (eight species described) but presents fragmented and confusing information about its gonads and white spots, masking important information to understand the diversity and evolution of the class, such as the aforementioned internal morphological complexity of the vesicles.

Concerning the white spots, besides discussing the taxonomic use for the structure, our results corroborate the capacity to release the nematocysts contained in the structure as a form of defense and attack of stauromedusae, as previously hypothesized (Kling, 1879; Kassianow, 1901). The presence of a circular nerve ring and muscle fibers intrinsic to the structure indicate a sensitivity to external stimuli followed by the opening of the white spot pore (Miranda et al., 2016) by the action of the muscular fibers and, consequently, the nematocysts release to the external environment.

For the first time, the evolutionary history of white spots and gonads of Staurozoa were analyzed. The presence of white spots on the perradial and interradial axis were shown to be conserved through staurozoan evolutionary history, with a gradual loss of the structure on the interradial followed by the perradial in the family Haliclystidae, until

the complete absence of the structure in *Haliclystus antarcticus* and *Haliclystus auricula*. It is not possible to raise plausible explanations for this event, however, the frequent association of the structure with the gonads and even feeding behavior may be directions for future studies.

For the gonads, not only their positioning in the subumbrella was reconstructed, but also the presence of vesicles. The analyses showed that the last common ancestor of Staurozoa had the gonads positioned at adradial and possess type 1 vesicles (similar to those found in the genus *Haliclystus*). The positioning of the structure changes throughout the evolutionary history of the class possibly as a consequence of two traits, (1) the pairing of the animal's arms at the interradial (e.g., *Lucernaria quadricornis* and *Calvadosia cruciformis*), leading to interradial/adradial gonads, and (2) the presence of the claustrum, leading to perradial gonads. Gonads positioned at perradial do not have vesicles, except *Depastromorpha africana*, raising questions about factors related to vesicle expression.

Hence, the present study contributes to a more comprehensive understanding of white spots and gonads in Staurozoa, opening perspectives for future researches about gonadal development and diversity, mechanisms of nematocyst release and functioning, and new sources of characters for the taxonomy of the group. It also indicates gaps in the knowledge of the class that must be assessed through more broad approaches, including comparative studies with other cnidarian classes that address the complexity and morphological diversity existing in the group of stalked jellyfishes.

References

- Eckelbarger KJ, Larson RJ. 1993. Ultrastructural study of the ovary of the sessile scyphozoan, *Haliclystus octoradiatus* (Cnidaria: Stauromedusae). *Journal of Morphology* 218(2):225–236.
- Eckelbarger KJ. 1994. Oocyte nutrition in the lower Metazoa: the Scyphozoa. In: Wilson WH, Stricker SA, Shinn GL, eds. *Reproduction and Development of Marine Invertebrates*. Baltimore: John Hopkins University Press, 15–28.
- Haeckel E. 1879. *Das system der medusen. I, 2: System der Acraspeden. Zweite Hälfte des Systems der Medusen*. Jena: Gustav Fischer.
- Hirano YM. 1986. Species of Stauromedusae from Hokkaido, with notes on their metamorphosis. *Journal of the Faculty of Science, Hokkaido University, Zoology* 24:182–201.

- Hirano YM. 1997. A review of a supposedly circumboreal species of stauromedusa, *Haliclystus auricula* (Rathke, 1806). In: den Hartog J.C., ed. *Proceedings of the 6th International Conference on Coelenterate Biology*. Leiden: National Naturhistorisch Museum, Noordwijkerhout, 247–252.
- Kassianow N. 1901. Studien über das Nervensystem der Lucernariden nebst sonstigen histologischen Beobachtungen über diese Gruppe. *Zeitschrift für Wissenschaftliche Zoologie* 69:287–377.
- Kling O. 1879. Ueber *Craterolophus tethys*. Ein Beitrag zur Anatomie und Histologie der Lucernarien. *Morphologisches Jahrbuch* 5:141–166.
- Mayer AG. 1910. Scyphomedusae. *Medusae of the World*, vol. III. Washington, D.C.: Carnegie Institution Publishing. Publication 109.
- Miranda LS, Collins AG, Hirano YM, Mills CE, Marques AC. 2016a. Comparative internal anatomy of Staurozoa (Cnidaria), with functional and evolutionary inferences. *PeerJ* 4:e2594.