DEPARTMENT OF BIOLOGICAL AND
ENVIRONMENTAL SCIENCES

REPRODUCTION AND ADAPTED HISTOLOGY FOR YELLOW HEDGEHOG SPONGES

A study of Polymastia boletiformis in the Koster Sea

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Abstract

The Koster Sea is Sweden's most sponge rich area, hosting around 160 sponge species. Sponges are filter feeding invertebrates that belong to one of the oldest animal phyla on earth, *Porifera*. Concerning their body structure, they are regarded to be the simplest of multicellular animals, built up by rather independent cells with a low degree of specialization.

This study has been looking into reproduction among the *Polymastia*. A genus with two known species in Swedish waters – *Polymastia mamillaris* and *Polymastia boletiformis*. So far, there has been no detailed reproductive study for either of those commonly found species in this region. Also, no knowledge of specific asexual reproductive structures is documented for the species.

Major aims for this study were to identify collected sponges to species and to troubleshoot a repeatable methodology for histological processing of reproductive material in *Polymastia*. Reproductive studies in sponges are rare, and so methodology for good histology is still cryptic.

Polymastia specimens collected in the Koster Sea during 2023 were examined histologically to see if there were any differences in reproductive structures (asexual as sexual) regarding size, location in sponge bodies and seasonality. Together with *Polymastia* specimens from Gothenburg Museum of Natural History all sponges were examined for signs of asexual reproduction.

Sponges collected in this study belonged to the same species, the yellow hedgehog sponge *P. boletiformis.* Three specimens were identified as reproductive females with early oocytes with significantly different sizes. They were collected in April and the presence of small oocytes indicated that the species might reproduce during summer in the Koster Sea. No signs of asexual reproduction were found among the collected specimens nor the museum specimens.

This study contributes to the field of basic science within sponge reproduction with a fully adapted histology protocol for *P. boletiformis*. Furthermore, it contributes with the first observations of reproductive *Polymastia* individuals in Swedish waters.

Sammanfattning

Kosterhavet utmärker sig nationellt genom sin osedvanligt rika mångfald av svampdjur, med omkring 160 påträffade arter i området. Svampdjur är filtrerande evertebrater som hör till ett av de äldsta fylumen inom djurriket som vi känner till – *Porifera*. De anses vara de enklaste av multicellulära djur då deras konstitution består av relativt självständiga celler med endast ett fåtal specialiserade celltyper.

Denna studie behandlar reproduktion inom släktet *Polymastia*. Ett släkte med två vanligt förekommande arter i Kosterhavet – *Polymastia mamillaris* och *Polymastia boletiformis*. Hitintills har inga regionala studier om reproduktion utförts för någon av dessa arter. Därtill saknas det kännedom om arternas förmåga att föröka sig asexuellt.

Huvudsakliga syften inom studien var att artbestämma de insamlade svampdjuren och att experimentera fram en anpassad metodologi för histologisk undersökning av reproduktiva strukturer för *Polymastia*. Reproduktiva studier av svampdjur är sällsynta och därmed är histologisk metodologi för denna djurgrupp ännu bristfällig och i behov av vidare utveckling.

Polymastia svampdjur som samlades in från Kosterhavet under 2023 undersöktes histologiskt för att se om det förelåg en skillnad mellan reproduktiva strukturer (asexuella såsom sexuella) gällande storlek, förekomst i svampdjurens kroppar och reproduktiv säsong. Tillsammans med *Polymastia* individer från Göteborgs Naturhistoriska Museum granskades svampdjuren efter tecken på asexuell reproduktion.

Samtliga insamlade svampdjur tillhörde arten *P. boletiformis*. Tre individer identifierades som reproduktiva honor med signifikant olikstora oocyter i ett tidigt utvecklingsstadie. De reproduktiva svampdjuren samlades in i april och förekomsten av små oocyter indikerar att arten möjligen reproducerar sig under sommarhalvåret i Sverige. Inga tecken på asexuell reproduktion påträffades hos museimaterialet eller hos de för studien insamlade svampdjuren.

Studien bidrar till vidare grundforskning inom svampdjursreproduktion genom ett frambearbetat histologiskt protokoll för *P. boletiformis* och genom att sammanfatta tidigare känd information om artens reproduktion. Därutöver bidrar den med en första dokumentation av reproduktiva *Polymastia* individer i svenska vatten.

1. Introduction

Sponges belongs to the ancient phylum *Porifera* and includes mainly marine, sessile, filter feeding invertebrates that are divided into four classes – *Calcarea*, *Demospongiae*, *Hexactinellida* and *Homoscleromorpha* (Maldonado & Riesgo, 2008). So far, more than 9000 sponge species are described globally (LaDouceur, 2021) of which up to 85 % of the species belongs to *Demospongiae* (Maldonado & Riesgo, 2008).

Morphologically, extant sponge species are diverse in both size and body shape (LaDouceur, 2021). Regarding their inner body composition, sponges are characterised by the absence of true tissues and organs (Moen & Svensen, 2009). However, they have a sophisticated network of channels and voids building up their aquiferous system (LaDouceur, 2021). Water flows in through small pores called ostia, further through a system of tubes and out through wider openings named oscula (Moen & Svensen, 2009). Due to this filtering capacity, sponges are regarded as key ecosystem engineers – facilitating food particles and shelter to other invertebrates and to symbiotic microorganisms (Folkers & Rombouts, 2020).

Sponges generally consists of three main regions. The outermost part, the ectosome, contains the ostia, a variety of microorganisms and specialized cells called pinacocytes. They create a special mucus and work as a protective barrier to the surrounding environment. The choanoderm is the inner cell lining of the sponge body that comprises the aquiferous system and choanocytes. Choanocytes are flagellated cells that, among other functions, produce the currents through the aquiferous system. These cells are often located in specific choanocyte chambers. The middle part of a sponge is called the mesohyl. It is a constantly changing structure, where a mixture of special cell types such as amoebocytes (i.e., pluripotent stem cells) moves around in an extracellular matrix of collagen or spongin fibres, skeletal structures called spicules (made up by silica or calcium carbonate) and symbiotic prokaryotes (LaDouceur, 2021).

Sponges can reproduce both sexually and asexually. Hermaphroditism is common among brooding sponges while spawning sponges tend to be gonochoric (LaDouceur, 2021). Brooding species often break down their aquiferous system for the development of embryos in their mesohyl or in temporary follicles. Larvae are released through oscula. For spawning species unfertilized eggs or zygotes are released. Gametogenesis occurs usually through the differentiation of an amoebocyte into an oocyte and of a choanocyte into a spermatocyte. Asexual reproduction takes place through fragmentation, budding or the creation of special reproductive structures called gemmules (LaDouceur, 2021).

Increasing exposure from human related activities such as bottom trawling, acidification and global warming are threatening sponge populations and their habitats worldwide. To turn this trend of decline and ensure a favourable conservation status of sponge species, inventory studies of their distribution and reproduction can contribute with valuable knowledge for sponge management, restoration and conservation (Vetenskapsrådet, n.d). Conservation of sponges will also contribute to maintain future possible sources of natural products from sponges used within biotechnology (Maldonado & Riesgo, 2008). Due to sponges' potential use within medicine, there is steadily growing demands for more and relevant research. Secondary metabolites produced by sponges are for example applicable as possible antibiotics, treatment for Alzheimer's decease and as cytotoxins (LaDouceur, 2021). Yearly, around 200 new sponge metabolites are discovered (Uppsala Universitet, 2024).

1.1 Polymastiidae in the Koster Sea

The Koster Sea is known since the late 19th century for its sponge grounds with a remarkable high biodiversity of species (Alander, 1942). Around 160 species are documented from the area (Uppsala Universitet, 2024) where of 60 species are only present here in Sweden. The rich sponge fauna is sustained by the steady inflow of a cold and salty deep-water current from the

Atlantic, that is channelled along the 247 m deep and sediment rich Koster trench (an old fault line) which runs through the area (Länsstyrelsen Västra Götaland, n.d.).

In the Koster Sea there are two common representatives of *Polymastia*: the yellow hedgehog sponge, *Polymastia boletiformis* (syn. *robusta*) and the papillate sponge, *Polymastia mamillaris* (SLU Artdatabanken, n.d.). *P. boletiformis* is a yellow to orange, globular to bulbose cushion shaped sponge with its body surface covered by protrusions, called papillae, of the same colour as the body. It is known to thrive on smooth bedrock or hard rocks without much of a sediment layer. *P. mamillaris* is a yellow to grey, flat cushion shaped sponge with white or slightly yellow papillae. It is usually observed in a habitat borderline, between bedrock and sediment rich sea floor with its body well-covered in sediment or in rock hollows with sediment (Moen & Svensen, 2009; Ackers et al., 2007). Single individuals of other species within the *Polymastiidae* have been noted from Skagerrak, such as *Polymastia penicillus, Sphaerotylus capitatus* and *Spinularia spinularia*, though yet not reported in the Koster area (Plotkin et al., 2018). One *Polymastia conigera* has been collected in Säcken in the northern part of the Koster Sea (see Appendix 3) though the species identification of the specimen is still uncertain.

1.2 Reproduction

Polymastiidae is a family within *Demospongiae* with little knowledge about their reproductive traits (Boury-Esnault, 2002). The genus *Polymastia* was described by J. S. Bowerbank in 1864 and is the only genus within *Polymastiidae* where some studies have been conducted about their reproduction (Boury-Esnault, 2002). They are known to be spawning and gonochoric (Maldonado & Riesgo, 2008). Oocytes are released through oscula located on the outer tip of some papillae. Oocytes remain in the mucus of the ectosome where fertilization occurs and the development into benthic larvae proceeds. Asexual reproduction is only described within *Polymastia arctica*, through budding from their papillae (Boury-Esnault, 2002).

1. 2. 1 Advantages of budding in sponge reproduction

Earlier studies of influences on asexual reproduction in sponges have made a connection between abiotic disturbances, sexual seasonality, body constitution and the intensity of budding (Battershill & Bergquist, 1985; Cardone et al., 2010; Plotkin & Ereskovsky, 1997).

Environments that expose sponges to abiotic stress increases some species tendencies to go into asexual reproduction mode. In *Tethya citrina*, repeating budding events are enhanced by exposure to prolonged environmental stressors from living in the intertidal zone. Such specimens studied *ex situ* in stable aquarium conditions ceases to produce buds (Cardone et al., 2010). A study on *Polymastia* sponges in the southern hemisphere shows that there is a clear correlation between a disturbance, such as a storm, and its influence on success of sexual or asexual reproduction strategies in sponges. In areas that are affected by storms, sediment is rearranged and made to cover hard surfaces. Thereby ruling out the preconditions of the attachment of benthic larvae that needs to “glue” themselves to surfaces free from too much soft and unstable sediment. Contrary to this, asexually produced buds were observed to attach to sandy surfaces. (Battershill & Bergquist, 1985). Accordingly, budding observed in *P. arctica* sponges is triggered by exposure to sediment destabilisation stress (Plotkin & Ereskovsky, 1997).

So far, there has been no detailed study for either of the commonly found (or not yet documented) *Polymastia* species in the Koster Sea. Also, no knowledge of the specific asexual reproductive structures is documented from the Swedish species (Boury-Esnault, 2002). Compared with other invertebrate phyla, the knowledge level about reproduction in *Porifera* is still rather inadequate. Thereby, further development of histology methods adapted for the study of reproductive structures among different sponge species is needed (Maldonado & Riesgo, 2008).

2. Aim

This study was designed to fulfil three major aims.

1) To examine specimens of *Polymastia* collected in the Koster Sea for identification to species through morphological studies.

2) To develop and describe a methodology for histological investigation of reproductive structures in *Polymastia* sponges.

3) To identify and measure reproductive structures and infer potential seasonality of reproduction and location of gametes in *Polymastia.*

a) Is there a difference in reproductive structures (gametes and asexual structures) regarding size, location in sponge bodies and seasonality in the sampled *Polymastia*?

H_o: there is no significant difference between the means of gamete sizes between the reproductive individuals within the same *Polymastia* species.

3. Material and Methods

3.1 Sponge samples

This study was based on museum specimens as well as newly collected *Polymastia* sponges from the Swedish Westcoast.

3. 1. 1 Museum sponges
The historical material belongs to the collections of Gothenburg Natural History Museum (GNM) and contained 28 specimens (where of one *P. mamillaris*, one *P. conigera* and the rest *P. boletiformis).* They were collected along the coast of Bohuslän between 1964 and 2010 and preserved in 80% EtOH.

3. 1. 2 Sampling for new sponges
During two occasions with scientific trawling in the Koster Sea (see Figure 1) a total of thirteen *Polymastia* sponges were collected. Nine specimens were collected during a field day in spring (24th of April 2023) to Berggylteskär 58.8282 N, 11.0856 E at 160 m depth. In autumn (17th of October 2023) during a field day to Björns Rev 58.8780 N, 11.0826 E (outside the trawling prohibited area), yet four more specimens were brought up from a depth between 100-120 m. See Table 1 for the material data of the collected sponges.

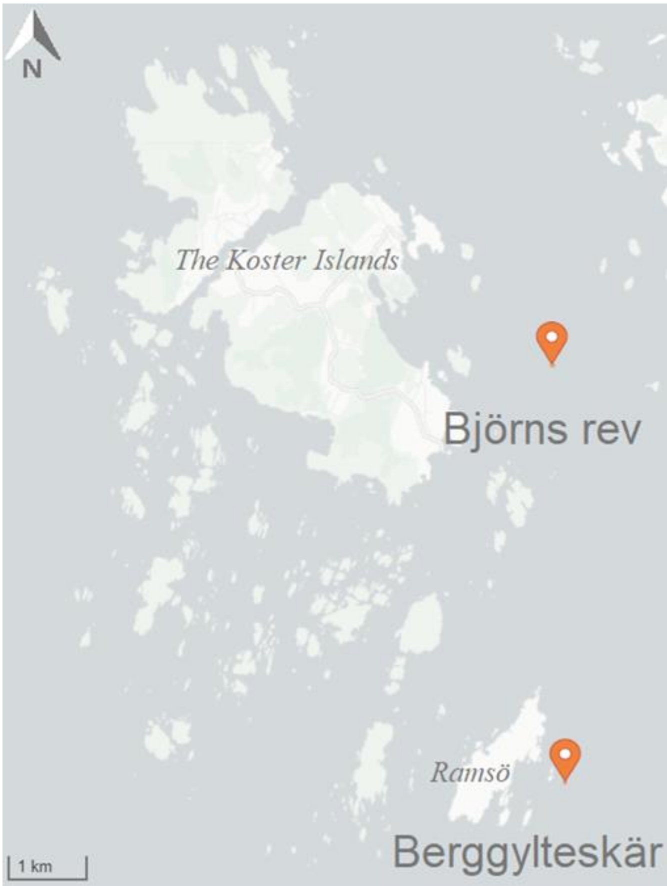

Figure 1*. Map with locations where Polymastia sponges were collected during 2023 in the Koster Sea. Map from Karthavet, Havs-och Vattenmyndigheten, HaV, 2024.*

3.2 Asexual reproduction, metrics and fixation

Every specimen of the museum material was examined during a visit in November 2023. First, they were morphologically examined with a Leica stereo microscope for signs of asexually reproductive structures. Such as buddings or other deviant structures on their papillae and ectosome. Photos for ID and measurements of their length, width and height were made before small samples of tissue for DNA- and histological analysis were taken (though not processed in this study).

Sponges collected in April during a University of Gothenburg course were kept in a circulatory water system for two weeks. Thereafter, four sponges were fixed in 2,5% GA (Glutaraldehyde) and five sponges in EMA (EtOH 95%, Methanol 100%, Acetic acid, 3:1:1) to examine how the different fixatives alter shrinkage, quality and viewability of the biological material when used for histology. After two weeks in fixative the sponges were transferred into 70% EtOH.

Measurements (diameter, height and width) of the sponges were taken after fixation. See Table 6 in Appendix 2. Papillae were examined with a ZEISS Stemi 305 stereo microscope for signs of budding or other possible structures that could be involved in asexual reproduction. All sponges were sketched to further observe their external appearance.

Figure 2. *Polymastia sponges collected in the Koster Sea at Björns rev during October 2023.*

Sponges collected in autumn were kept in an aquarium for four days (see Figure 2). Before fixation, the sponges were examined for buds with a stereo microscope, measured (diameter, height and width) and cleaned from silt sediment before being photographed. Fixation was made with EMA except from half a sponge that was fixated in formalin, for a histological comparison. After three days of fixation, they were transferred into 70% EtOH. To see how much they had shrunken during fixation they were measured once more. DNA samples were taken from all but one sponge prior to preservation (not used in this study).

Table 1. *Polymastia specimens that were collected in the Koster Sea during 2023. Table showing their individual specimen numbers (used in a sponge collection database – P for Polymastia and the following number represent the order of non-genera specific sponges being collected and added to the database), sponge colour and data about the collecting. LMB= Lara Maleen Beckmann and EB= Emelia Börjesson. Short dash indicates lack of measurements.*

Specimen number	***Colour of body (of papillae)***	***Location***	***Depth (m)***	***Date (D/M/Y)***	***Collector***
P0043	Orange (orange)	Berggylteskär	160	4/04/23	LMB
P0045	Yellow (yellow)	Berggylteskär	160	4/04/23	LMB
P0047 A	-	Berggylteskär	160	4/04/23	LMB
P0047 B	-	Berggylteskär	160	4/04/23	LMB
P0047 C	-	Berggylteskär	160	4/04/23	LMB
P0047 D	-	Berggylteskär	160	4/04/23	LMB
P0058	Orange (orange)	Berggylteskär	160	4/04/23	LMB
P0059	Yellow (yellow)	Berggylteskär	160	4/04/23	LMB
P0060	Orange (orange)	Berggylteskär	160	4/04/23	LMB
P0131	Yellow (yellow)	Björns rev	100–120	17/10/23	EB
P0132	Yellow (yellow)	Björns rev	100–120	17/10/23	EB
P0133	Orange (orange)	Björns rev	100–120	17/10/23	EB
P0134	Orange (orange)	Björns rev	100–120	17/10/23	EB

3.3 Species identification of sponges collected in the Koster Sea

3. 3. 1 Spicule preparations

For each sponge two 3 mm^3 tissue pieces were cut. One from the ectosome and one from the choanosome. The tissue pieces were put into labelled Eppendorf tubes with sodium hypochloride bleach for 1 hour until all tissue were dissolved. The bleach was pipetted off and spicule supernatant was cleaned from bleach by resuspending it with distilled water for 10 minutes before distilled water was removed and added again. This was repeated five times to make sure all remnants of the bleach were washed off, before the Eppendorf tubes were refilled with 70% EtOH. Slides were preheated by adding them to a slide dryer before 0,5 µl of each spicule solution were pipetted onto the slides and left to dry. Finally, a droplet of DPX mountant and a cover slip were placed on top of the dried spicules.

3. 3. 2 Measuring spicules

Photos of different types of spicules from each slide were taken with a Lumenera Infinity 1 camera through an Olympus CX31 microscope using 10x and 40x magnification. A software program, Infinity Analyze 7, was used to control the camera and adjust settings of the photos. For every tissue type (ectosomal and choanosomal) twenty spicules of each spicule type (ectosomal tylostyles, intermediate tylostyles and principle tylostyles) were measured with FIJI ImageJ (4-bit Java 8). Measurements of spicule length, proximal tyle diameter and maximal shaft diameter were taken for each spicule (See figure 3). A total of 120 spicules were measured per specimen.

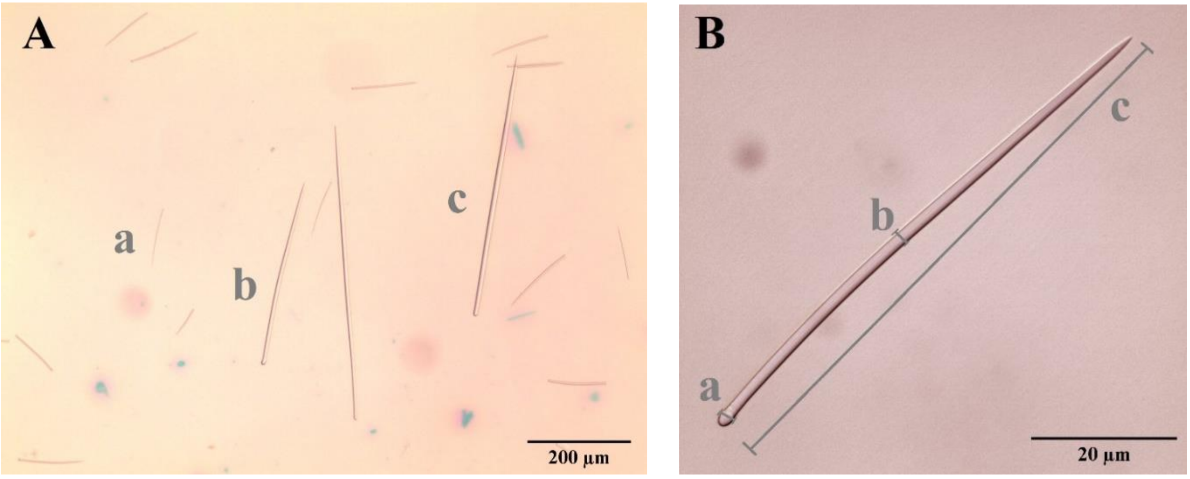

Figure 3 A. *Three different spicule types were identified. Scattered spicules in 10x magnification.* ***a***. *Ectosomal tylostyle* ***b***. *Intermediary tylostyle* ***c***. *Principal tylostyle* ***B***. *The measurements that were taken for each spicule. Ectosomal tylostyle in 40x magnification.* ***a***. *Proximal tyle diameter* ***b***. *Shaft diameter* ***c***. *Spicule length.*

3. 3. 3 Analysing spicules

The spicule data was organized in Excel before it was transferred into SPSS (IBM SPSS Statistics 28) for a descriptive analysis of size ranges. Thereafter, ranges for all specimens were compared with ranges in literature (Plotkin et al., 2018) for spicule length, proximal diameter and shaft diameter for each spicule type to conclude about which *Polymastia* species the collected sponges might belong to.

3.4 Histological investigation

To develop a histological protocol adapted for *Polymastia* sponges, an initial comparative pilot study was carried out.

One specimen fixed in glutaraldehyde (2,5%) and one fixed in EMA were chosen and two 5 mm^3 pieces were cut from each sponge and put into labelled histocassettes. A comparison was made between embedding the tissues in agarose or not – for an eventual improvement of the positioning of the tissues in the blocks (see Figure 4.A.). Other aspects that were compared were concentration of the desilicifying agent (2% or 5% hydrofluoric acid), thickness of the sectioning (20 µm, 10 µm, 8 µm, 7 µm, 6 µm and 5 µm) (see Figure 4.B.) and choice of staining – Gomori's Trichrome Stain or Hematoxylin and Eosin Staining (H&E). See details about the histological procedure further down (3.5). For every tissue block 5 slides were produced. The sections on the slides were examined with an Olympus CX31 microscope in 10x and 40x magnification and different structures – as ectosome, choanocyte chambers, tracts, ostia and parasites (incorrectly interpreted as oocytes, see Figure 5.A.) – were photographed for comparison of methods.

Between the different batches that were processed later, more experimentation was done. One issue was to get air out of the void rich sponge tissues since air bubbles creates holes in the wax sections. To see if it was possible to decrease the amount of air, the beaker with all histocassettes from one batch was placed on a shaker during the 100% EtOH overnight step and during the first step of wax embedding. Another batch were made using 15 mm^3 to see if the same concentration of hydrofluoric acid that were used for smaller pieces also would work for desilicifying all spicules in larger tissue pieces.

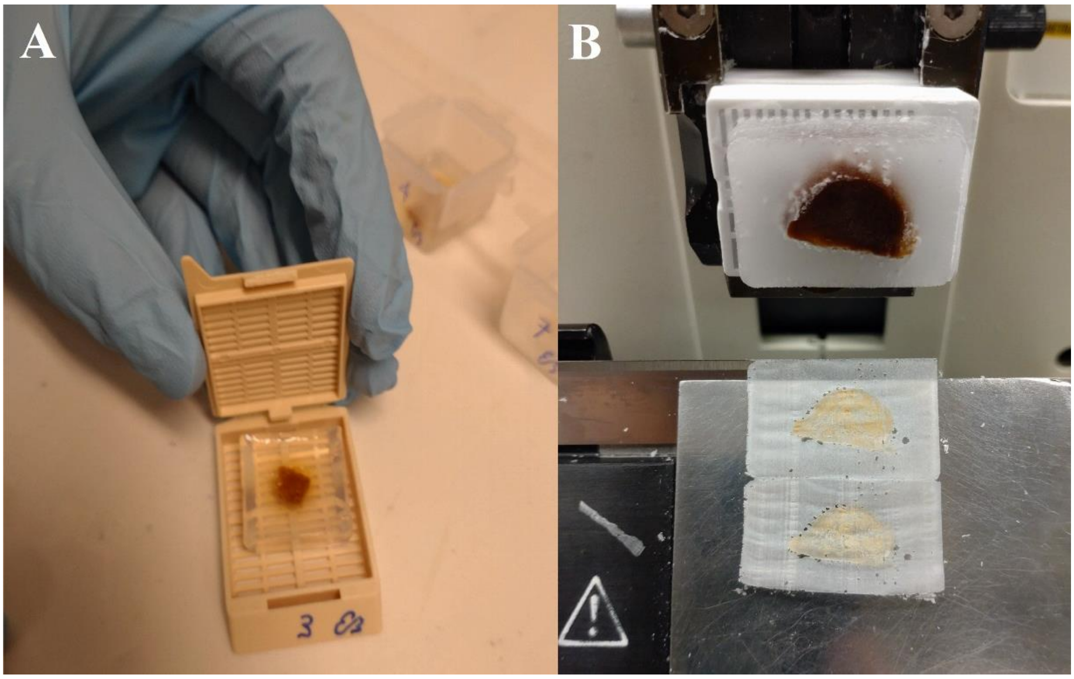

***Figure 4**. **A.** Wax block containing a larger tissue piece fixed in 2,5% glutaraldehyde. The block was sliced on the microtome with 7 µm thick sections that lined up in a ribbon. **B.** Smaller tissue piece embedded in agarose to minimise movement that might damage or reorientate the tissue in the histocassette.*

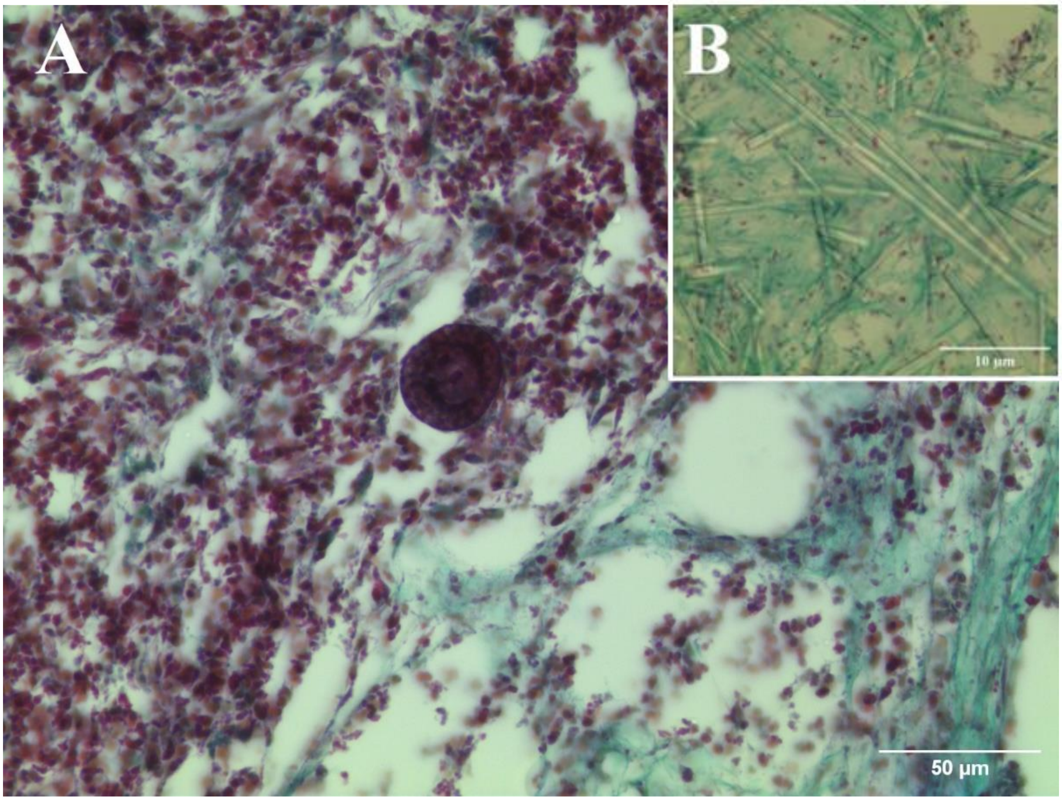

***Figure 5. A**. During the pilot study parasitic cells were initially interpreted as oocytes and used for comparing different staining methods to distinguish gametes among other cell types. Tissue fixed in EMA and stained with Gomori Trichrome Stain. **B.** Tissue desilicified with an inadequate concentration of hydrofluoric acid – leaving undissolved glass spicules that might damage tissue and microtome knife blade.*

3.5 Adapted histology protocol for ***Polymastia***

The histological methodology followed a basic protocol with some adjustments. See full Histology Protocol in Appendix 1.

Tissue pieces (between 7 mm^3 and 15 mm^3 in size) were cut from each sponge and put into labelled histocassettes and put into a beaker with 70% EtOH. For each sponge four to six tissue pieces were cut, depending on sponge body size. Pieces were cut in one or two transects from dorsal to ventral side and from periphery to interior part of the sponge, for a detailed determination of location of potential gametes.

3. 5. 1 Desilicification

A crucial step was to remove all spicules in the sponge tissue through desilicification. Otherwise, the spicules might split the histological sections or damage the knife used for sectioning. Before the samples were desilicificated the ethanol needed to be washed off by letting the histocassettes soak in distilled water for 15 minutes. The histocasettes were then transferred into a beaker with 2% hydrofluoric acid. After one hour, they were moved into another beaker with 2% hydrofluoric acid and soaked for two more hours. To wash off the acid, they were soaked in distilled water for 15 minutes.

3. 5. 2 Dehydration

After the desilicification a series of dehydration steps followed. The tissues were gradually exposed to higher concentrations of ethanol, from 30% to 100%. This to remove all the water in the tissues for increasing the permeability of paraffin wax used later in the process. The dehydration series comprised of 30 minutes soaks in 30%, 40%, 50%, 60% and 70% EtOH. Followed by three shorter soaks á 10 minutes each in 80% and then in 90% EtOH and two 10 minutes soaks in 95% EtOH. In the 100% EtOH soak they were left overnight (overnight equals a minimum of 8 hours – preferably 12 hours or more) in approximately double the volume of EtOH used in earlier steps. Finally, another soak in 100% EtOH for 30 minutes ended the dehydration series.

3. 5. 3 Clearing

Since ethanol is not miscible with paraffin wax, the ethanol needed to be cleared off with a solution that mixes well with the wax before the infiltration took place. This was made using Safeclear (a Xylene substitute) as a clearing agent and the tissues were let to soak in the solution for two hours with a change of solution after one hour.

3. 5. 4 Wax embedding

The embedding of the tissues in paraffin wax were made in a wax oven in 58°C. It included a three-stage procedure as the histocassettes were moved between three beakers with increasingly cleaner wax (last stage containing no traces of clearing agent). The first stage lasted overnight, the second stage for 8 hours and the third stage overnight. When the tissues were completely infiltrated with wax, they were ready to be embedded into blocks. This was done by pouring a small amount of molten wax into the mould, enough to cover the bottom surface. With the help of warm tweezers, the tissue was placed into correct position before the histocassette (with lid removed) was placed on top of the mould and it was filled up with wax to the brim and let to solidify. For the avoidance of getting air bubbles into the block – especially close to the tissue piece – the molten wax was poured in the corner of the mould with the wax beaker held close.

3. 5. 5 Microtome sectioning

After solidification the blocks were removed from the moulds and prepared for sectioning by lying on ice for 15 to 30 minutes. The cold treatment ensured that the hardness of the wax and the tissue were approximately the same and enabled the microtome to slice thin and smooth sections of the blocks. Sectioning was made using a Leica RM2255 Microtome (occasionally a Leica RM2155 was also used) with a C.L. Sturkey Inc. D554P High profile microtome knife blade.

Each block was placed into the block holder of the microtome and trimmed with 30 µm sections until the tissue was reached. The blocks were sliced with 7 µm sections and after every third or fourth section a trimming of 17 µm was made. Wax ribbons containing three to four subsequent sections were moved with tweezers to a 40°C water bath, to float out all the wrinkles of the wax. From the water bath the sections were scoped up with a glass slide in a vertical angel and put onto a slide dryer for 30 minutes. Out of each block 15 slides were produced.

Important factors that were taken into considerations during the procedure were to make sure that the knife was firmly fastened and cleaned in between sectioning. This to avoid errors like getting small cracks in the sliced tissue due to vibrations or the splitting of wax in the sections due to debris on the knife. The board beneath the knife holder had to be cleaned from wax when needed for the avoidance of the sections to stuck and get damaged. All steps that involved transfer between different solutions, were done by moving the histocassettes with forceps to reduce the time that tissues were exposed to air. Histocassettes were shaken now and then to let go of air trapped in the tissues. The volume of different solutions used in each step were adapted after the number of histocassettes that were processed. Generally, an amount of 100 ml or 200 ml were enough to cover the histocassettes.

3. 5. 6 Staining and mounting

The dried-up slides were put into racks and stained with Gomori’s Trichrome Stain (see appendix 1) which stains structures with mitochondria red (Ganten et al., 2006). One or two droplets of DPX mountant were placed on the stained tissue sections. Coverslip was added in an angle to try and make sure that no air bubbles were created. Then the slides were let to dry.

3.6 Identifying reproductive individuals and analysis of gametes

Slides with sections from all sponges were analysed with an Olympus CX31 microscope in 10x and 40x magnification. One section on every slide was chosen and searched through after gametes. New tissue pieces from the reproductive individuals were then processed and five slides from each block were produced, stained and mounted. One section per slide was chosen and gametes (oocytes with a distinct non-amoebic shape and visible nucleus, see Figure 8) were photographed with the Lumenera Infinity 1 camera. The distribution of gametes in the sponge were noted – if they were present in the base, the top or throughout the whole of the sponge body.

For every individual the feret diameter of 100 randomly chosen oocytes were measured with the software FIJI ImageJ by marking the oocytes circumference on a graphics tablet.

3. 6. 1 Statistical Analysis

Measurements of gamete sizes (oocyte feret diameter) were compiled in Excel before transferred into SPSS (IBM SPSS Statistics 28). The measurements for each specimen were first analysed with descriptive statistics to get an overview of the data. Followed by a Kolmogorov-Smirnov Normality test and a Levene’s test of homogeneity of variances. The assumption that the mean oocyte sizes among the reproductive *Polymastia* sponges were equal was challenged by running the data through a One-Way Welch’s ANOVA test. To pin out between which individuals a potential difference might lie a Post Hoc Games-Howell test was conducted.

4. Results

4.1 Spicule analysis to determine species

Measurements for all collected specimens were concordant with literature measurements for *Polymastia boletiformis* (Lamarck, 1815:332), the yellow hedgehog sponge.

In the spicule analysis a total of 1536 spicules were measured. They were classified into three spicule type categories – ectosomal tylostyles (n= 517), intermediary tylostyles (n= 493) and principal tylostyles (n= 526). See table 2 for the specimens' compiled spicule measurements.

Length of spicule types from all specimens had following ranges: ectosomal tylostyles 59 – 274 µm with an average of 147 ± 27 µm. Intermediary tylostyles 202 – 559 µm (410 ± 66 µm). Principal tylostyles 406 – 718 µm (542 ± 53 µm).

Proximal tyle diameter for all specimens ranged: ectosomal tylostyles 1,0 – 8,5 µm (3,8 ± 0,9 µm). Intermediary tylostyles 1,2 – 13,5 µm (5,7 ± 1,7 µm). Principal tylostyles 3,0 – 12,9 µm (6,5 ± 1,7 µm).

Spicule shaft diameters for all specimens were within a range of: ectosomal tylostyles 1,0 – 7,8 µm (3,7 ± 1,0 µm). Intermediary tylostyles 1,9 – 13,4 µm (7,2 ± 2,0 µm). Principal tylostyles 3,6 – 15,4 µm (9,2 ± 2,0 µm).

***Table 2**. Descriptive measurements of spicule length, proximal tyle diameter and shaft diameter for the different spicule types. n=4608.*

Spicule type	*Minimum (µm)*	*Average (µm)*	*Maximum (µm)*
		Spicule length	
Ectosomal	59	147	274
Intermediary	202	410	559
Principal	406	542	718
		Proximal tyle diameter	
Ectosomal	1,0	3,8	8,5
Intermediary	1,2	5,7	13,5
Principal	3,0	6,5	12,9
		Shaft diameter	
Ectosomal	1,0	3,7	7,8
Intermediary	1,9	7,2	13,4
Principal	3,6	9,2	15,4

The spicules were derived from two different tissue types – the ectosome (n=770) and the choanosome (n= 766). Figure 6 displays the length distribution of the different spicule types from the ectosome and the choanosome from all specimens.

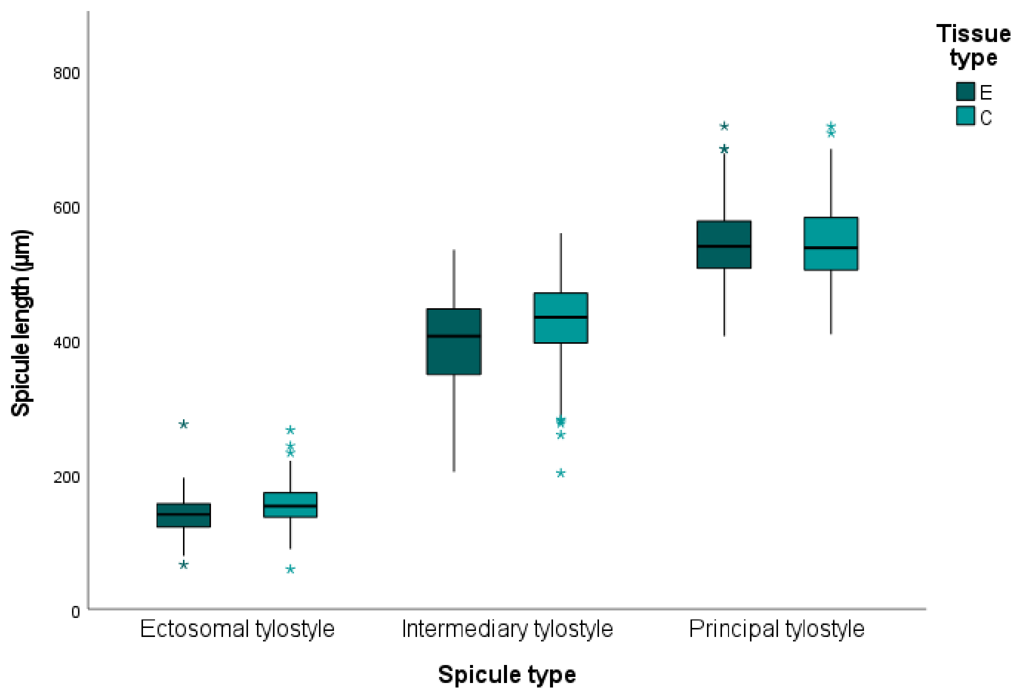

Figure 6. *Overview of the length for the different spicule types of the ectosome (E) and choanosome (C) from all the collected sponges. n(ectosome)=770, n(choanosome)=766, n(total)=1536.*

4.2 Adapted Histology for *Polymastia boletiformis*

See the full histology protocol generated in this study for *P. boletiformis* sponges in Appendix 1.

4. 2. 1 Histological investigation

The different experimentations that took place had following results.

Agarose restricted tissue pieces from moving around in the histocassettes during processing but the viewability of cells on the slides needed thinner sections than it was possible to slice, without getting wrinkly wax. Therefore, agarose was chosen to not be used.

The usage of large tissue pieces in small sized histocassettes helped with keeping the tissues in place during processing – but since they were compressed in the histocassettes, they continued to stay in the compressed shape when they were poured out into their moulds. This due to paraffin wax in all cells that rapidly hardened when exposed to room temperature. They were then put into larger histocassettes and went through wax embedding once more to see if the tissues could reshape back into original sizes. Alas, they did not, so the study continued with processing smaller tissue pieces.

For the 5 mm^3 tissue pieces a concentration of 2% hydrofluoric acid was sufficient to desilicify all spicules. For 5 mm^3 pieces in agarose and for larger tissue pieces (15 mm^3) a concentration of 5% hydrofluoric acid was needed. Pieces used for most of the histological processing had a size between 7 mm^3 and 15 mm^3 and by shaking the histocassettes when soaking in 2% hydrofluoric acid the spicules dissolved, and so the lower concentration was used for the protocol.

The technique of shaking the beaker to release trapped air (during last step of dehydration and first step of paraffin wax embedding) was chosen to not be included in the protocol. This since the method was similarly efficient with the shaking of histocassettes with tweezers during tissue processing.

Thickness of sections was determined to be 7 µm. Thinner sectioning made fragile wax sections that easily wrinkled and became destroyed.

Staining with Hematoxylin and Eosin (H&E), specimens fixed in EMA or glutaraldehyde gave similar results regarding level of viewability between different structures as choanocytes and areas with spicule tracts. Reproductive specimens were never stained with H&E so how gametes distinguish with this staining method remains unclear.

Visible result of staining with Gomori's Trichrome varied depending on chemical fixative used for the specimens. For sponges fixated in EMA, Gomori's gave clear contrast between structures as choanocytes and areas with spicule tracts. For specimens in glutaraldehyde the staining result for those structures resembled that of H&E. Oocytes and oocyte nucleus were easy to discover and distinguish from other cells due to clear contrast for both tissues fixated in EMA as well as in glutaraldehyde. Accordingly, Gomori's Trichrome was chosen as staining method for the protocol.

4.3 Reproduction

Out of the thirteen collected *P. boletiformis* sponges three specimens (P0047A, P0047D and P0059), all collected the 4th of April 2023, were identified as reproductive females by the presence of oocytes. See table 3. Sexes for the other specimens remains unknown.

Table 3. *In this study three reproductive females were identified by the presence of oocytes. Table shows oocyte descriptive data, location of oocytes (abbreviations regarding observations of vertical distribution in sponge body **T:** top; **M:** middle; **B:** base), number of papillae examined for asexual reproduction and observations of budding among the specimens. Short dash indicates lack of measurements. A number of 100 oocytes were measured per specimen.*

Specimen number	***Scientific name***	***Sex***	***Max*** *(µm)*	***Min*** *(µm)*	***$\bar{x}$*** *(µm)*	***SD*** *(µm)*	***Location of oocytes***	***Papillae*** *(№)*	***Budding***
			Oocyte feret diameter						
P0043	*P. boletiformis*	-	-	-	-	-	-	110	No
P0045	*P. boletiformis*	-	-	-	-	-	-	60	No
P0047 A	*P. boletiformis*	♀	43,2	10,1	20,1	5,6	T, M, B	52	No
P0047 B	*P. boletiformis*	-	-	-	-	-	-	42	No
P0047 C	*P. boletiformis*	-	-	-	-	-	-	66	No
P0047 D	*P. boletiformis*	♀	22,0	10,8	14,6	2,4	T, M, B	37	No
P0058	*P. boletiformis*	-	-	-	-	-	-	34	No
P0059	*P. boletiformis*	♀	25,1	14,3	18,9	2,4	T, M, B	50	No
P0060	*P. boletiformis*	-	-	-	-	-	-	80	No
P0131	*P. boletiformis*	-	-	-	-	-	-	58	No
P0132	*P. boletiformis*	-	-	-	-	-	-	107	No
P0133	*P. boletiformis*	-	-	-	-	-	-	45	No
P0134	*P. boletiformis*	-	-	-	-	-	-	83	No

Oocyte feret diameters (see Figure 7) ranged from 10,1 – 43,2 µm with a mean of 20,1 ± 5,6 µm for specimen P0047A (n=100). For P0047D oocyte feret diameter were within a range of 10,8 – 22,0 µm with a mean of 14,6 ± 2,4 µm (n=100). Specimen P0059 had an oocyte feret diameter size range of 14,3 – 25,1 µm with a mean of 18,9 ± 2,4 µm (n=100).

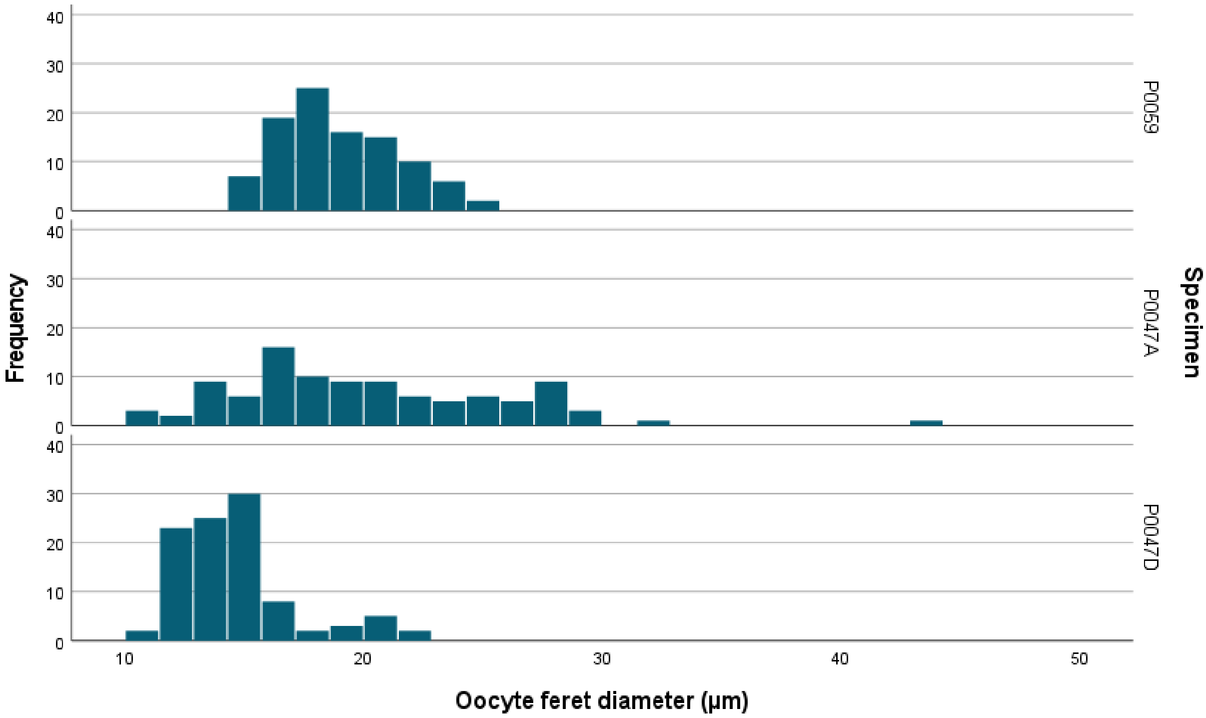

Figure 7. *The size distribution of oocyte feret diameters for the females that were collected in April 2023. A number of 100 oocytes were measured for each specimen.*

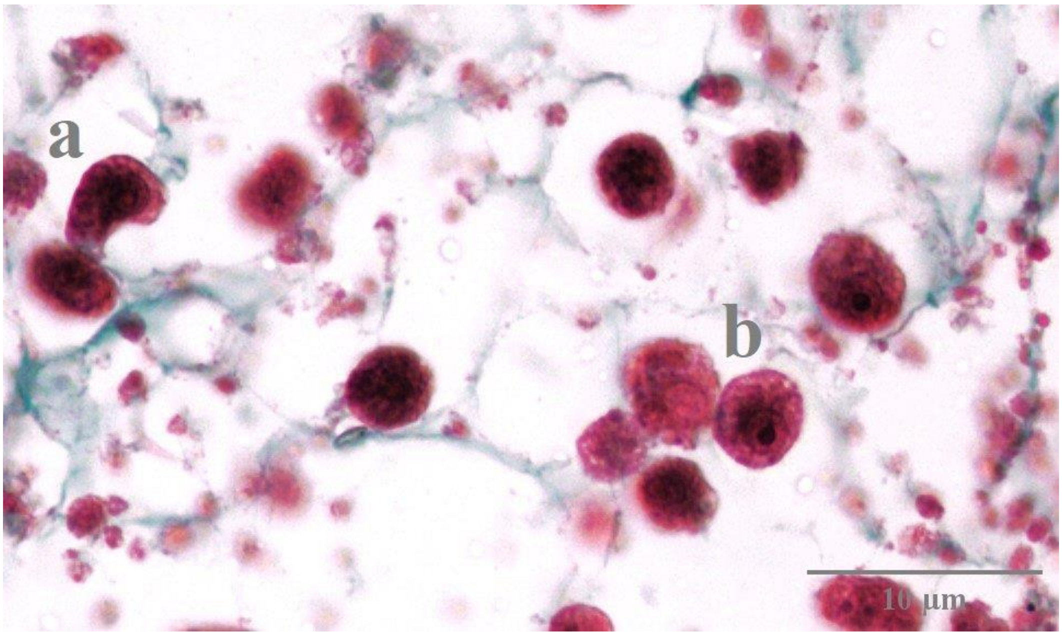

Figure 8. *Polymastia oocytes.* ***a.*** *Amoebocytic oocytes* ***b.*** *Oocytes with developed nucleus and clear cell membrane were measured.*

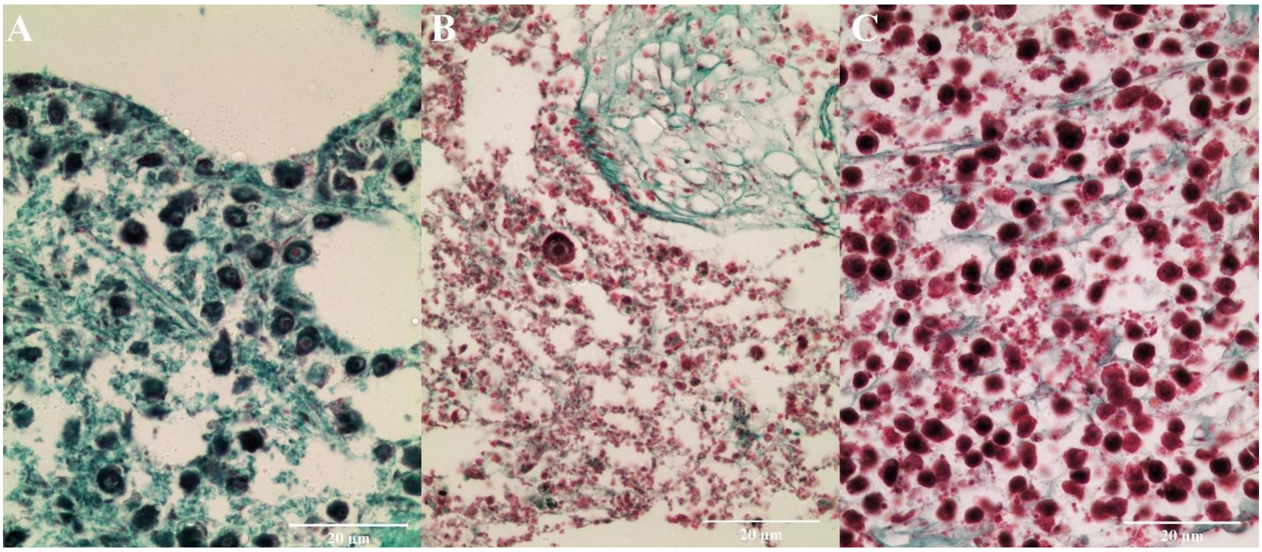

Figure 9. A. *Oocytes from P0059 were scattered all over choanosome. Those with an amoeboocytic shape were not measured.* ***B.*** *Specimen P0047A had scarce oocytes scattered one by one. The choanosome still had a lot of choanocytes organised into choanochambers.* ***C.*** *Specimen P0047D with an abundance of oocytes scattered across all tissue except from spicule tracts and ectosome. Choanocyte chambers dissolved into loose choanocytes or differentiated into amoebocytes and oocytes. Scale bares 20 µm.*

Oocytes were present in top, middle and base parts of choanosome – and in choanosomal parts of papillae – for all specimens. The impression of oocyte quantity noted by eyesight when overlooking the sections ranged the specimens from highest number of oocytes to lowest followingly: P0047D, P0059 and P0047A. In P0047D oocytes and amoebocytes (see Figure 9.C. and Figure 10.B.) were everywhere in the choanosome with a low number of choanocytes ordered in choanocyte chambers. Chambers were generally present close to ectosome. P0059 had oocytes in all parts of the choanosome and a low number of choanocytes ordered in choanochambers (see Figure 9.A. and Figure 10.A.). In specimen P0047A, the majority of choanocytes were arranged in choanocyte chambers with single oocytes diffused one by one all over the choanosome (See Figure 9.B).

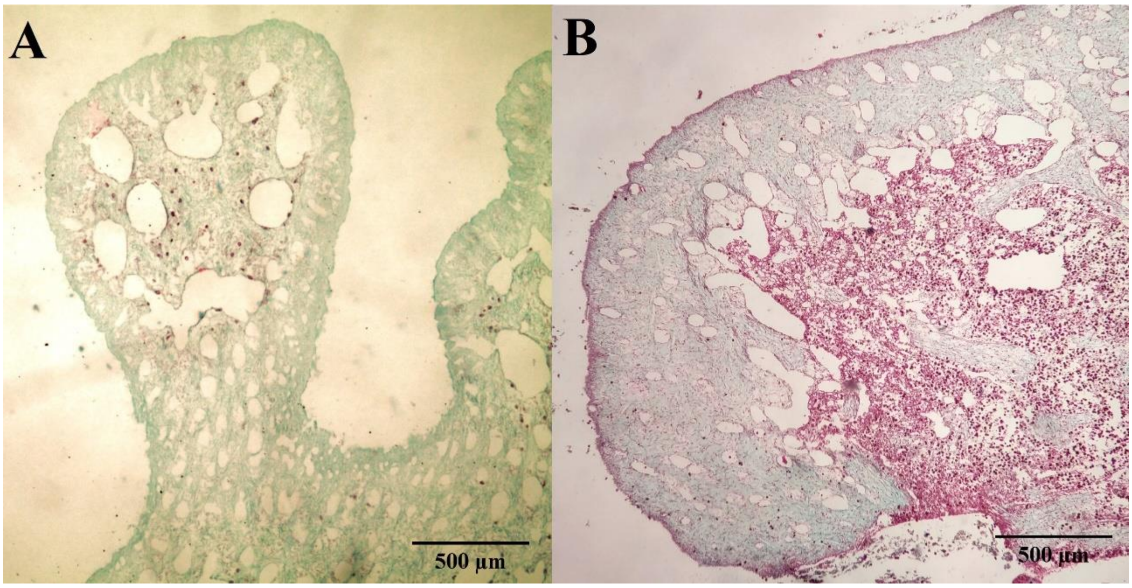

Figure 10. A. *Oocytes in the choanosomal, interior part of a papillae from specimen P0059.* ***B.*** *Cross section displaying choanosome close to ectosome with oocytes occupying majority of choanosomal tissue space. Choancoyte chambers present close to the ectosome.*

4. 3. 1 Statistic analysis

The statistical analysis examined the assumption of working hypothesis H_0: there is no significant difference between the means of gamete sizes between the individuals.

The p-values generated from the Kolmogorov-Smirnov test that was used to check for normality of data were for specimen P0059 D(100)= 0,050; for specimen P0047A D(100)= 0.,007; for specimen P0047D D(100)= 0,001. All values of p < 0,05 indicates that the data were not normally distributed. Followed by a Levene's test to check for homogeneity of variances the p-value were 0,001 which indicates when p < 0,05 that there is a significant difference of variance.

According to the assumptions of nonparametric data and heterogeneous variances the analysis continued with running a Welch's ANOVA test to test the null hypothesis (See table 4). A conclusion to reject H_0 was made since p-value < 0,05. There was a statistically significant difference between the oocyte sizes among the collected *P. boletiformis* specimens.

Table 4. *Results of Welch's ANOVA test. df2= 185, 258, d.f=2, p < 0,05. The null hypothesis regarding equal means of oocyte sizes were rejected. A statistically significant difference was indicated among reproductive specimens.*

Welch's ANOVA			
Oocyte feret diameter	***df***	***df2***	***Sig.***
Welch	2	185, 258	< 0,001

Table 5. *The multiple comparing results of the Games-Howell Post Hoc test.*

Games-Howell Post Hoc Test Results						
ID	**ID**	**MD**	**SE**	**Sig.**	**95% Confidence Interval**	
					Lower Bound	*Upper Bound*
P0059	P0047A	-1,223610	0,608517	0,113	-2,66567	0,21845
	P0047D	4,309720*	0,342492	<0,001	3,50093	5,11851
P0047A	P0059	1,223610	0,608517	0,113	-0,21845	2,66567
	P0047D	5,533330*	0,607848	<0,001	4,09280	6,97386
P0047D	P0059	-4,309720*	0,342492	<0,001	-5,11851	-3,50093
	P0047A	-5,533330*	0,607848	<0,001	-6,97386	-4,09280

* The mean difference (MD) is significant at the 0,05 level.

To detect where differences lied, a Games-Howell test was used (See Table 5). Between P0059 and P0047D there was a statistically significant difference, p<0,05, as well for between P0047A and P0047D. Between individual P0059 and P0047A there was no statistically significant difference, p>0,05.

No obvious papillae buddings or other structures that could be related with asexual reproduction were identified for neither museum specimens (see Table 7 in Appendix 2) nor for the specimens collected in 2023 (see Table 3).

Deviant structures were observed on a few specimens. Some had inhalant papillae with thin and long branches. An observation that raised interest was that of a thin branch that fell off from the main papillae, during careful examination of a sponge fixated in April (specimen P0047 C). Thin and pointy tips (see Figure 11) were observed on papillae for some of the specimens, one collected in April and two in October. Three museum specimens collected in late August and middle of September were also exhibiting pointy papillae. A papilla with a budlike tip was also noted on specimen P0060. Some of the museum specimens had aged in their preservatives and deformed characters did not allow valid examination for asexual structures.

5. Discussion

5.1 Species identification

5. 1. 1 Spicule analysis
The combination of living features of the collected sponges and measurements of their spicule skeleton were used for the identification to species. Features regarded were body shape and size, having papillae with similar colour as the body and the presence of fusiform papillae for some of the specimens. These features together with results of the length of maximum principal tylostyles < 800 µm with a total mean of 542 µm – indicates that they belong to the species *P. boletiformis*, the yellow hedgehog sponge. The species is known to have principal tylostyles with an average of 540 µm (Plotkin et al., 2018) whereas *P. mamillaris*, the papillate sponge, is recorded to have an average of 1052 µm (Morrow & Boury-Esnault, 2000).

The measurements of principal tylostyles should be seen as a biased compilation of larger principal tylostyles. This since *P. boletiformis* is regarded to only have two spicule types – ectosomal and principal tylostyles – not three as this study erroneously divided the spicules into. An improvement of the spicule analysis would accordingly to this be to remeasure spicules or rearrange the ranges of types into two size categories and then to comprise the descriptive measurements before comparing with literature. An even number of measured spicules for each specimen would also improve the accuracy of the description of spicules. In this study there were four specimens with a lack of data due to preparation slides with a low number of spicules of certain types. Repeated spicule preparation from the specimens could have corrected this.

For all specimens, the length distributions of spicules were similar for the two tissue types, with only a small difference in ranges. This gives strength to the conclusion about determining the specimens to *P. boletiformis* since a difference is not noted in literature as a character for the species. In case of a larger difference, this way of comparing spicules could have contributed to the identification of potential *P. mamillaris* or other *Polymastia* species unknown for the area.

5. 1. 2 Collecting specimens
Aspects of why only one species was collected might lie in the fact that the distribution of species could be strictly related to preferred microhabitats, conditioned by the marine geologic landscape of the two sampling locations. Due to *P. mamillaris* occurring in hollows and at edges, they might be common but more difficult to collect. Depending on techniques of scientific trawling, the size and distribution of sponge populations from year of collection and the probability of trawling over a sponge population might explain the absence of *P. mamillaris* in this study. Both Björns rev and Berggylteskär are known for earlier collected *P. mamillaris* (Morrow & Boury-Esnault, 2000) and considered to be suitable habitats for both species.

Another possible explanation to the absence of *P. mamillaris* could be effects of the regular bottom trawling for northern shrimp, *Pandalus borealis,* along the Koster fault line during the 20th and early 21st century. For sponges, bottom trawling is regarded as a major threat. The increased sedimentation and deposition of sediment can lead to population declines due to suffocation when their aquiferous system fills up with sediment particles (OSPAR Commission, 2010). This could have led to a higher mortality within the *P. mamillaris* population in the area due to its preference for areas were sediment settles, compared with the more current-exposed areas where *P. boletiformis* thrive.

Improvements to further reproductive studies of the Swedish *Polymastia* would be to collect more species. Especially *P. mamillaris* would be interesting to collect since no reproductive study of this species has been conducted before.

5.2 Histology of reproductive material

The resulting histological protocol for reproductive structures of *P. boletiformis* produces slides of such a quality that it can be used for identifying and measuring oocytes. Additional investigation needs to be made to see if the procedure is adequate for studies of spermatocytes, as none were observed in this study.

To further refine and develop the methodology it would be adequate to find out how to produce thinner sections for an even better viewability of the gametes and their cell organelles. An alternative to paraffin wax embedding would be to use araldite (Boury-Esnault & Bézac, 2007) or epoxy resin (Plotkin et al., 2018). Advantages of plastic embedding are the possibilities to slice thinner sections (WebPath, n.d.) and the lower shrinkage level of tissues (Nielsen et al., 1995) since other solutions are used for dehydration and clearing (WebPath, n.d.).

Regarding fixative used in the study, using an alcoholic fixative such as EMA or glutaraldehyde is preferred to formalin out of health perspectives for the conservator and out of the more rapid processing (Rahman et al., 2022) The fixative impact on shrinkage of tissues is preferred to be as negligible as possible to get accurate measurements of the reproductive structures. If a shrinkage percentage is known, it might be applicated to get measurements closer to reality. In this study the number of specimens that were measured before fixation were too few for any conclusions to be drawn. Specimens fixed in glutaraldehyde were never measured before fixation so comparisons with EMA are not possible to do. Measurements for specimens fixed in October in EMA and half a specimen fixed in formalin had a similar shrinkage level (1-3 mm), but further studies needs be conducted on more specimens to evaluate the factor of shrinkage through statistical analysis. Both EMA and glutaraldehyde were adequate as fixatives for studying and identifying oocytes in histological tissue sections.

Gomori’s Trichrome differentiated sponge tissue structures more clearly compared with Hematoxylin and Eosin, hence it was chosen as staining technique in this study. Though, to compare the difference of gamete staining a further study on gametes (and not parasitic cells) could be conducted.

5.3 Sexual reproduction

This study contributes with the first ever made documentation of reproductive individuals of *P. boletiformis* sponges in Swedish waters, by the revelation of oocytes in three of the specimens collected in April in the Koster Sea. Oogenesis occurred in the choanosomes in the females. No oocytes were detected in the ectosome. Oocyte average diameters from the females were small, compared with the known average of 100 µm for mature *P. boletiformis* oocytes from specimens collected in September 1965 in Roscoff (Borojevic, 1967). Indicating that the oocytes in this study may be early oocytes. Observations of amoebocytic oocytes in all specimens were supporting this conclusion.

5. 3. 1 Oocyte size

One of the reproductive specimens, individual P0047D, had significantly smaller oocytes compared with the others (P0059 and P0047A). An explanation could be that the specimens were slightly asynchronously triggered to go into their sexual reproduction mode, and that P0047D had a later onset of its gametogenesis. If oocytes grow with a certain rate after ending the amoebocytic stage, oocytes may be smaller due to a shorter time span of differentiation and vitellogenesis compared with the other specimens that might had begun to differentiate earlier.

A size difference might otherwise be due to the abundancy of oocytes. The specimen showed massive oogenesis occurring in the choanosome, with few choanocyte chambers to be seen except from along the edges of the ectosome. It had the highest abundancy of observed oocytes and the lowest of choanocytes. Oocytes are known to engulf nurse cells, which are often comprised of choanocytes (LaDouceur, 2021). A synchronous differentiation within a specimen with a high number of oocytes, might lead to the engulfment of almost all available choanocytes surrounding the oocytes. Energy resources split over a vast number of oocytes might decrease he oocyte growth rate, compared with an individual with a lower ratio of oocytes per available nursing cells. To make more valid speculations, future studies would gain on incorporate a study of fecundity for reproductive specimens.

The high variation of oocyte sizes and observed large outliers of P0047A could depend on errors of measuring or by an asynchronous oogenesis pattern within the individual. Oogenesis might begin at different times for the amoebocytes and therefore display oocytes with a variety of sizes. The low abundance of oocytes dispersed in the choanosome in this specimen could depend on this asynchronous onset of oogenesis.

Additionally, it could be a connection between asynchronous gametogenesis within a specimen and its microbial biodiversity. Amoebocytic oocytes are known to move around and engulf microbials so that, if fertilization happens, the larvae will have a rich arsenal of symbiotic microorganisms from the start (Maldonado & Riesgo, 2008). For an individual, the variation of microbial diversity and mass could possibly affect how long time it takes for the amoebocytic oocytes to gather enough microorganisms. If a specimen has a lower abundancy of microorganisms – for some amoebocytes it might take a longer period to incorporate enough microorganisms than for others – causing a variation of oocyte sizes within the specimen.

5. 3. 2 Seasonality

A study of *Polymastia sp.* sponges in the Mediterranean has shown that gametogenesis occurs once a year during a specific period and that oocytes can be present in female specimens several months before spawning. The appearance of spermatocytes in males seemed to be synchronised with each other within a population, and with the final maturation stage of oocytes (Bergquist, 1978). Since the females were in an early stage of gametogenesis its reasonable that no males and spermatocytes were identified in this study.

Whether the other six specimens collected in April at Berggylteskär were all males, or some males and females with a later onset of sexual reproduction mode or only females with an asynchronously onset of oogenesis remains unknown. Sexes of specimens collected in October were not possible to determine without any observations of gametes – presumably since their sexually reproductive period were over. This indicated by the presence of early oocytes in April and earlier studies about a distinct reproduction period within the genus. Though more studies are needed to define if this also correlates with the Swedish *Polymastia* species sexual reproduction.

The rise of temperature is known to be a major triggering factor for gametogenesis in sponges and does affect the rate of the development and release of oocytes for some species (Maldonado

& Riesgo, 2008). Even though surface water temperature rise in spring in the Koster Sea – at the depths from where the specimens were collected, the temperature is relatively stable, around 6°C, all year round (SMHI, 2014). So, the onset of gametogenesis would probably not be triggered by temperature alterations.

A possibility is that the capability of reproduction in *P. boletiformis* is phylogenetically constrained – following a circannual rhythm that is endogenously programmed in extant specimens. A rhythm of reproduction that evolved from environmental conditions that affected their ancestors (Eckelbarger & Watling, 1995). If so, such a rhythm could have evolved in *P. boletiformis* sponges that were adapted to marine shallower areas with a yearly temperature flux. That still determines and synchronizes the onset of sexual reproduction of the species regardless of their locations depth and exposure to temperature rise or not.

An abiotic factor that is known to activate oogenesis especially in more stable environments is the increase of food availability (Maldonado & Riesgo, 2008). Phytoplankton primary production and biomass peak in the shift between Mars and April in temperate oceans of the northern hemisphere due to the returning sunlight (LibreTexts, n.d.). The Atlantic current that is channelled through the Koster Sea will probably contain a higher amount of phytodetritus during spring. Since oogenesis requires a certain energy investment, this could be a reliable explanation of the onset of gametogenesis seen in the three specimens. If spawning, fertilization, larval and zygote development and post-spawning restoration of the adult sponge happens in late spring or later during the summer – there will probably still be advantageous amounts of detritus for growth to filter feed for both juvenile and adult sponges. So, due to a peak in food availability – an early gametogenesis might be advantageous in the area.

Since the rate of oocyte development is unclear for the species it is not possible to determine when spawning occurs. It is likely to be in or around the summer months, possible between May and September – but to fully determine reproductive seasonality for *P. boletiformis* and other *Polymastia* species in Swedish waters further investigation is needed through specimen collection over a full year. Furthermore, specimens sampled monthly over a year could also be studied to see whether they have a synchronous or asynchronous spawning, to determine their mature gamete sizes and to compare the ratio between number of oscula, size and fecundity.

5.4 Asexual reproduction

5.4. 1 Observations

Examination of museum sponges and the collected sponges from the Koster Sea were made looking for asexual structures similar to the buds known from *P. arctica*. Deviant structures were only observed briefly and no valid conclusions about their function can be made from these observations.

Some papillae of the observed *P. boletiformis* had long and thin branches. A single observation of a branch that fell off from one of the specimens could indicate that they have brittle papillae as a strategy of enhancing fragmentation due to physical disturbance. Oocytes were observed in some of the papillae, and it is known that some sponge species maximise their dispersal capacity by combining the propagules of asexual and sexual reproduction (Maldonado & Riesgo, 2008).

To investigate possible gains of brittle papillae branches further, *P. boletiformis* are often observed growing on upward facing rocks (Ackers et al., 2007) – a type of environment where also a lot of other invertebrate species thrive, like larger crustaceans that could cause physical stress on a sponge. Or brittle papillae could be a result of mutualism between sponge and crustacean – a hypothesis based on observations of *P. boletiformis* growing on crustaceans (Moen & Svensen, 2009). Living opportunistically on a mobile invertebrate could be advantageous in regards of mitigating dispersal of sponges to new areas. Though, the observations of *P.*

boletiformis on crustaceans could be of specimens that attached as benthic larvae. A third hypothesis explaining this possible brittleness could be that it's a good trait of a papillae to be slightly brittle to ease the process of pinching of buds. Other explanations with high probability are that the papilla was damaged during collection or before that, through EtOH preservation or that the technique to handle the sponge when searching for asexual structures was not so delicate as perceived. More observations of this phenomenon are needed for any conclusions to be drawn.

Prolonged tips observed on some of the *P. boletiformis* could be a sign of early budding or post-budding on papillae. If the species can create buds similar to *P. arctica* it could be signs of either of the phases. Those buds are known to be created by the excretion of amoebocytes outside the papilla, where the differentiation into a bud occurs. For budding similar to the observation of a larger single bud on a *P. boletiformis* collected in 1908, pointy tips could be related with post-budding. The description of the creation of the bud was by an expansion within the papilla, caused by amoebocyte organisation and differentiation and by the ligation of the proximal part of the papilla until the tip got its spherical budlike shape (Arnesen, 1918). After complete ligation of a budding event of this type, a papilla might get a pointy tip.

Figure 11. *Inhalant papillae with pointy tips on one of the specimens collected in October 2023. Photo by Maël Grosse.*

The deviant bud found on specimen P0060 were slightly similar to the illustrations in Arnesens publication from 1918 but since it was horizontally attached to the sponge body it is more likely that the budlike top is another papilla, and that the whole structure is an anastomosed papilla.

5.4.2 Likelihood of budding among Polymastia in the Koster Sea and further questions
Whether the *Polymastia* species along the Swedish Westcoast and species found in the Koster Sea are likely to differentiate buds or not must be viewed out of several perspectives. Since *Polymastia* sponges in the area regardless of species are living beneath the 10-20 metres halocline, they live in rather stable environmental conditions (SMHI, 2014), where probably the main strategy for propagation is sexual reproduction due to the lack of major abiotic stressors.

Possible abiotic stressors that could affect the reproductive mode of the sponges in the Koster Sea might be events such as marine heatwaves and sediment stress from the regular physical disturbance of the seafloor from local shrimp trawling. Or from seafloor turbidity currents and submarine slides created by the seldom events of earthquakes along the Koster fault line. Several large seismic activities were occurring in the early 20th century in the Oslo Rift Zone affecting the whole of the Swedish Westcoast (Bungum et al., 2009). The sponge grounds in the Koster Sea would probably have been exposed to submarine slides and turbidity currents due to the area's proximity to these earthquakes and their aftershocks. Effecting all marine landscapes – and probably suspending sediments from the Koster fault line. According to the timing of these earthquakes, one in 1907 could hypothetically have induced the formation of the bud on the *P. boletiformis* collected in 1908 described by Arnesen.

The capacity to produce buds are probably different depending on *Polymastia* species and their niches (regarding ecology, preferred habitats and morphology of adult and juvenile). The species might also possess different abilities to adapt instead of getting into survival mode when exposed to different stressors. A stressor for one species, might not be such a big stressor for the other. In an *ex situ* modelling with different kinds of exposure to loose sediment, a *P. mamillaris* with its long papillae would probably not become just as stressed as a *P. boletiformis* with usually shorter papillae, if covered with a thick layer of sediment. Such an experimental set up can examine if budding can be induced by sediment stress in the Swedish *Polymastia* species and compare factors as sponge size, papillae length and number of inhalant and exhalant papillae (with oscula) and the sex of the sponges.

Though, future studies are needed to reveal any, if existing, connections between such possible stressors in the area and the onset of asexual reproduction. By collecting more sponges over the whole of the year a potential budding seasonality could be defined. The absence of buds in this and in future studies could signal the absence of exposure to stressors to specimens or a lack of reproductive plasticity within a species – that they do not reproduce asexually at all, even though exposed to abiotic stressors.

A risk of producing buds living in areas with strong currents might be that the buds can be brought far away to areas that is not suitable as habitat. Since *Polymastia* sponges are known to produce benthic larvae, this might indicate that they have specific habitat requirements. Benthic larvae could be a strategy to combat the risk of random pelagic dispersal and enabling higher survival numbers of the larvae. Already positioned in a suitable environment, the larvae can search for a suitable location to settle (this behaviour have also been noted for buds). Benthic larvae might create a high individual number in a population and a relative proximity of individuals in an area. The latter could be to an important factor for higher rates of sexual fertilization. All these factors might answer why budding in *P. boletiformis* are so rare, if ever present.

Another question is if they have a specific seasonality for asexual reproduction – this might be important to know since shown in *P. arctica* and in *T. citrina* the amoebocytes used for the creations of buds are needed for gametogenesis. When gametogenesis begins it turns budding into a phase of stagnation (Plotkin & Ereskovsky, 1997; Cardone et al., 2010). After spawning, in the end of October, it was discovered in *T. citrina* that high levels of amoebocytes present in the choanosome gave rise to a continuation of budding in winter until early spring. Sponges with high levels of budding intensity were also noted to not increase in size (Cardone et al., 2010). So, the mode of reproduction of a *Polymastia* sponge might differ depending on season, size and the level and type of abiotic stress.

If the species only reproduce with benthic larvae, they must be regarded to have low dispersal rates. Questions arise about how and when the different *Polymastia* species dispersed into the area (or maybe evolved in the case of *P. mamillaris*?) after the end of last ice age. This could be studied by analysing spicules of marine sediment deposits in areas lifted above sea level due to land rise. If some species could be determined, it could set an approximative time when they were established in the area and a rapid dispersal into the area might be a sign of asexual reproduction. A more realistic thing to study regarding dispersal rates of the *Polymastia* species would be to survey the new establishments of artificial hard rock habitats in offshore wind farms, already created or soon to be created along the Swedish Westcoast (Länsstyrelsen, 2024). If *Polymastia* sponges establish rather quickly without any known close populations, it might be a sign that they sometimes disperse by asexually produced propagules.

6. Conclusions

This study contributes to the still rather scarcely studied knowledge field of sponge biology with an adapted histology protocol for *Polymastia boletiformis*, the yellow hedgehog sponge, to be used in future studies of their reproduction and other morphological observations. Early oocytes were observed in three *P. boletiformis* specimens collected in April suggesting that the species might reproduce during the summer months in the Koster Sea and along the Swedish Westcoast. The slight difference in oocyte sizes between the sexually reproductive individuals possibly indicates an asynchronous onset of gametogenesis in the beginning of the spawning season. No reliable signs of asexual reproduction, such as budding, were observed among the *Polymastia* specimens examined in this study. More inventories of the Swedish *Polymastia* species and their reproduction through further sampling over a full year are essential to better fill up the voids of our knowledge and understanding of the Scandinavian sponge fauna.

In times with rapid ecological and environmental changes in our biosphere – fundamental knowledge like reproduction and ecological data can contribute to actions within conservation and habitat management of marine ecosystems to increase ecological resilience. *P. boletiformis* is a representative of an important ecosystem, the deep-sea sponge grounds of the Koster Sea, and information of its reproduction can be used as a baseline for future studies.

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Appendices

Appendix 1

Polymastia boletiformis Histology Protocol

1. DESILICIFY

Distilled water	15 min ☐	
Hydroflouric acid 2%	1 hour ☐ 2 hours ☐	
Distilled water	15 min ☐	
2. DEHYDRATE	30%	30 min ☐
Ethanol	40%	30 min ☐
	50%	30 min ☐
	60%	30 min ☐
	70%	30 min ☐
	80%	10 min ☐ 10 min ☐ 10 min ☐
	90%	10 min ☐ 10 min ☐ 10 min ☐
	95%	10 min ☐ 10 min ☐
	100%	Overnight ☐ 30 min ☐
3. CLEARING		1 hour ☐ 1 hour ☐
Xylene substitute/Safeclear		
4. EMBEDDING		Overnight ☐ 8 hours ☐ Overnight ☐
Paraffin wax		

5. POUR INTO MOLDS

6. MICROTOME SLICING

7. STAINING

Safeclear	5 min	☐
Safeclear	5 min	☐
100% EtOH	5 min	☐
70% EtOH	5 min	☐
Distilled water	5 min	☐
Gomori's Trichrome Stain	5 min	☐
95% EtOH	Rinse	☐
100 % EtOH	3 min	☐
100 % EtOH	3 min	☐
Safeclear	5 min	☐
Safeclear	5 min	☐

8. MOUNTING

DPX Mountant

Appendix 2

Table 6. *Measurements of specimen body sizes before and after fixation with 2,5 % GA (glutaraldehyde), EMA or formalin. Short dash indicates lack of measurements. Abbreviations;* ***d****: diameter;* ***w****: width;* ***h****: height.*

Specimen number	***Fixation***	***d*** (mm)	***w*** (mm)	***h*** (mm)	***d*** (mm)	***w*** (mm)	***h*** (mm)
		Size before fixation			*Size after fixation*		
P0043	2,5% GA	-	-	-	36	25	21
P0045	2,5% GA	-	-	-	24	18	12
P0047 A	2,5% GA	-	-	-	26	23	11
P0047 B	2,5% GA	-	-	-	20	15	8
P0047 C	2,5% GA	-	-	-	24	21	17
P0047 D	2,5% GA	-	-	-	22	19	14
P0058	EMA	-	-	-	18	9	11
P0059	EMA	-	-	-	18	12	11
P0060	EMA	-	-	-	34	29	20
P0131	EMA	27	25	15	27	24	13
P0132	EMA	37	27	18	35	25	18
P0133	EMA	28	19	12	28	17	12
P0134 (½)	EMA	27 (-)	21	13	26 (12)	19	12
P0134 (½)	Formalin	27 (-)	21	13	26 (14)	18	12

Appendix 3

Table 7. *Material data of museum specimens from Gothenburg Natural History Museum examined for signs of asexual reproduction. Abbreviations:* ***d:*** *diameter;* ***h:*** *height;* ***w:*** *width.*

Specimen number	***GNM-PORIF Catalog number***	***Scientific name***	***Date (D/M/Y)***	***d* (mm)**	***h* (mm)**	***w* (mm)**	***Buds***
P0142	758	*P. mamillaris*	23/07/1964	54	9	41	No
P0143	753	*P. boletiformis*	22/08/2009	40	15	26	No
P0144	748 (A)	*P. boletiformis*	27/08/2007	16	7,5	12,5	No
P0145	748 (B)	*P. boletiformis*	27/08/2007	47	10,5	47	No
P0146	748 (C)	*P. boletiformis*	27/08/2007	51	21	29	No
P0147	756 (A)	*P. boletiformis*	25/08/2009	25	12	20	No
P0148	756 (B)	*P. boletiformis*	25/08/2009	40	8	22	No
P0149	756 (C)	*P. boletiformis*	25/08/2009	36,5	13	24,5	No
P0150	752 (A)	*P. boletiformis*	22/08/2009	27	10,5	15	No
P0151	752 (B)	*P. boletiformis*	22/08/2009	38	18	33	No
P0152	660	*P. boletiformis*	23/08/2009	48	33	32	No
P0153	908	*P. boletiformis*	07/08/2015	32	15	25,5	No
P0154	749	*P. boletiformis*	27/08/2007	13	5	14	No
P0155	750 (A)	*P. boletiformis*	27/08/2007	13	4,5	8	No
P0156	750 (B)	*P. boletiformis*	27/08/2007	10,5	9	14	No
P0157	751 (A)	*P. boletiformis*	09/06/2008	23	7	15	No
P0158	751 (B)	*P. boletiformis*	09/06/2008	35	-	-	No
P0159	746	*P. boletiformis*	01/06/2007	32,5	8	18	No
P0160	747	*P. boletiformis*	21/08/2007	19	2,5	11	No
P0161	461	*P. boletiformis*	16/09/2010	24	10	19	No
P0162	457	*P. boletiformis*	15/09/2010	17	5	10	No
P0163	456	*P. boletiformis*	15/09/2010	30	9	19	No
P0164	458	*P. boletiformis*	16/09/2010	29	7	25	No
P0165	459	*P. boletiformis*	16/09/2010	28	8	12	No
P0166	759	*P. boletiformis*	10/08/1972	9	3	10	No
P0167	755	*P. boletiformis*	24/08/2009	22	10,8	18	No
P0168	279	*P. boletiformis*	09/06/2008	23	8	-	No
P0169	758	*P. conigera?*	01/07/1972	28	12	21	No