# The Molecular Machinery of Gametogenesis in *Geodia* Demosponges (Porifera): Evolutionary Origins of a Conserved Toolkit across Animals

Vasiliki Koutsouveli,*[1,2] Paco Cárdenas,[2] Nadiezhda Santodomingo,[1] Anabel Marina,[3] Esperanza Morato,[3] Hans Tore Rapp,[4] and Ana Riesgo*[1]

[1]Department of Life Sciences, The Natural History Museum of London, London, United Kingdom

[2]Pharmacognosy, Department of Medicinal Chemistry, Uppsala University, BMC, Uppsala, Sweden

[3]Servicio de Proteómica, Centro de Biología Molecular Severo Ochoa (CBMSO), Universidad Autónoma de Madrid (UAM) and Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain

[4]Department of Biological Sciences, University of Bergen, Bergen, Norway

***Corresponding authors:** E-mails: v.koutsouveli@nhm.ac.uk; a.riesgo@nhm.ac.uk.

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## Abstract

**All animals are capable of undergoing gametogenesis. The ability of forming haploid cells from diploid cells through meiosis and recombination appeared early in eukaryotes, whereas further gamete differentiation is mostly a metazoan signature. Morphologically, the gametogenic process presents many similarities across animal taxa, but little is known about its conservation at the molecular level. Porifera are the earliest divergent animals and therefore are an ideal phylum to understand evolution of the gametogenic toolkits. Although sponge gametogenesis is well known at the histological level, the molecular toolkits for gamete production are largely unknown. Our goal was to identify the genes and their expression levels which regulate oogenesis and spermatogenesis in five gonochoristic and oviparous species of the genus *Geodia*, using both RNAseq and proteomic analyses. In the early stages of both female and male gametogenesis, genes involved in germ cell fate and cell-renewal were upregulated. Then, molecular signals involved in retinoic acid pathway could trigger the meiotic processes. During later stages of oogenesis, female sponges expressed genes involved in cell growth, vitellogenesis, and extracellular matrix reassembly, which are conserved elements of oocyte maturation in Metazoa. Likewise, in spermatogenesis, genes regulating the whole meiotic cycle, chromatin compaction, and flagellum axoneme formation, that are common across Metazoa were overexpressed in the sponges. Finally, molecular signals possibly related to sperm capacitation were identified during late stages of spermatogenesis for the first time in Porifera. In conclusion, the activated molecular toolkit during gametogenesis in sponges was remarkably similar to that deployed during gametogenesis in vertebrates.**

***Key words:*** **oogenesis, spermatogenesis, evolution, Porifera, transcriptomics, proteomics.**

## Introduction

Sex is a fundamental trait for eukaryote evolution. It allows the cellular and molecular scenario for recombination, underpinning major fitness advantages in eukaryotes (Barton and Charlesworth 1998). Evidences for sexual reproduction (i.e., meiosis, recombination, and syngamy) have been detected in choanoflagellates (Levin and King 2013) and other unicellular eukaryotes (Ramesh et al. 2005; Malik et al. 2007; Logsdon 2008), with the meiotic molecular machinery already in place (Ramesh et al. 2005; Carr et al. 2010). In sexually reproducing animals, gametogenesis is the process by which gametes—ova in females and spermatozoa in males—are produced by cell differentiation of the germ line (McCarrey 2018). Cytologically, the process of gametogenesis is very conserved across all metazoans, including the same steps in both sexes: one round of DNA replication followed by two rounds of meiosis (McCarrey 2018). One of the most important stages of gametogenesis is the first meiotic prophase where pairing of homologous chromosomes and genetic recombination happens before the completion of meiosis I (Haschek et al. 2010). In oogenesis of many animal groups, including mollusks, ascidians, and humans (Russo et al. 1998), a meiotic cell cycle arrest is a very crucial step in which growth and maturation of the oocyte occurs (Telfer and McLaughlin 2007). All the processes after this arrest progress very quickly and cannot be distinguished histologically (McCarrey 2018). In contrast, in spermatogenesis, all phases of gametogenesis are morphologically very distinct and highly conserved in all Metazoa, from sponges to humans (Bellve et al. 1977; Bonilla and Xu 2008; White-Cooper and Bausek 2010). Major processes in spermatogenesis are the decrease in cell







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size (cytoplasmic loss), chromatin compaction, and flagellum and acrosome formation for sperm capacitation (Auger 2018).

Gametogenesis occurs in gonadal tissues (ovaries/testes) in most animal groups, including early-branching metazoans such as Ctenophora and Cnidaria. Gonads are usually composed of a germinative cell layer with a simple follicle and surrounded by accessory cells (Eckelbarger 1993; Vargas-Ángel et al. 2006; Shikina and Chang 2016). However, Porifera, the earliest splitting metazoan lineage (Dohrmann and Wörheide 2013; Feuda et al. 2017; Zumberge et al. 2018; Laumer et al. 2019), do not have gonads and the female and male gametes, which derive from archaeocytes and choanocytes (Simpson 1984), are widespread in the internal tissue (mesohyl) sometimes enveloped by a follicle layer, with male gametes often grouped in spermatic cysts (e.g., Riesgo, Taylor, et al. 2007; Maldonado and Riesgo 2008; Koutsouveli et al. 2018). In sponges, are the molecular pathways involved in the promotion of gametogenesis different, due to the absence of gonads?

The cytological progression of gametogenesis in Porifera has been described in detail for many sponge groups (see review by Ereskovsky [2010]), highlighting great similarities to other metazoans. Sponge oogenesis consists of two major cellular stages: ameboid-like previtellogenic (PV) oocytes and mature round vitellogenic oocytes. Most of oocyte differentiation and vitellogenesis in sponges occurs during the first meiotic arrest (Maldonado and Riesgo 2008). All the different stages of spermatogenesis (spermatogonia, primary and secondary spermatocytes, spermatids, and spermatozoa) have been observed in sponges (e.g., Riesgo et al. 2008; Lanna and Klautau 2010; Riesgo 2010). Therefore, from an evolutionary point of view, the morphological conservation of oogenesis and spermatogenesis strongly suggests an ancient molecular toolkit. Common molecular markers of gametogenesis (germ cell development, oogenesis, and spermatogenesis) are present in early diverging metazoans such as Placozoa and Cnidaria (Extavour 2009; Eitel et al. 2011; Shikina and Chang 2016), proving that the molecular regulation of gametogenesis appeared very early in animal evolution. Porifera share many genes with the rest of the Metazoa (Srivastava et al. 2010; Riesgo et al. 2014), but regarding the molecular toolkits for gametogenesis, very little is known. For germline determination, the entire complement of germ line markers or the Germline Multipotency Program (GMP) was found in several lineages of sponges (Riesgo et al. 2014; Fierro-Constaín et al. 2017). GMP genes have been expressed not only in somatic multipotent cells but also in gametes of two sponge lineages, indicating that some of those genes may still play a role in the sponge gametogenesis (Leininger et al. 2014; Fierro-Constaín et al. 2017), whereas some others may participate in the development and the maintenance of primordial stem cells (Solana 2013). Interestingly, some genes involved in the oocyte maturation processes, and more precisely in the regulation of the oocyte meiosis arrest, such as *mos*, are considered to be absent in sponges (Extavour 2009). Using a single cell RNAseq approach in *Amphimedon queenslandica*, Sebé-Pedrós et al. (2018) identified cells with transcriptomic profiles concordant with spermatozoa, but they did not confirm if those cells were truly sperm. All in all, many uncertainties and gaps remain in our knowledge on the molecular toolkit involved in gametogenesis in sponges.

In the present study, our goal was to identify the molecular toolkit in Porifera during female and male gametogenesis using RNAseq and proteomics. The species of the genus *Geodia* are appropriate models for studying the molecular aspects associated with gametogenesis, for several reasons. They have a common reproductive strategy due to phylogenetic constrains (Liaci and Sciscioli 1969; Mercurio et al. 2007; Spetland et al. 2007; personal observations) that includes consistent gonochorism (maintaining their sex for life) and oviparity (Liaci and Sciscioli 1969, 1970; Watanabe 1978; Mercurio et al. 2007; Spetland et al. 2007). Besides their consistent reproductive strategy, we selected *Geodia* spp. for this study because they are dominant species, in terms of size and biomass, of deep-sea boreo-Arctic sponge grounds in the North Atlantic (Klitgaard and Tendal 2004; Cárdenas et al. 2013). These *Geodia*-dominated grounds provide essential ecosystem services (Maldonado et al. 2016) and due to their vulnerability to anthropogenic activities, they are now considered Vulnerable Marine Ecosystems (UNGA 2006; FAO 2009). Knowledge about the reproduction of these key North Atlantic species will therefore benefit the conservation of these Vulnerable Marine Ecosystems. In particular, we selected these five species of *Geodia* (order Tetractinellida, family Geodiidae) because they have developed slightly shifted reproductive cycles at the same habitat (Koutsouveli V, unpublished data). So, at time of collection, the different species were at different moments of their life cycles, and by targeting them all we could cover as many developmental stages of gametogenesis as possible. These five species are *Geodia hentscheli* (Cárdenas et al. 2010), *Geodia phlegraei* (Sollas 1880), *Geodia barretti* (Bowerbank 1858), *Geodia macandrewii* (Bowerbank 1858), and *Geodia atlantica* (Stephens 1915).

## Results and Discussion

In our study, we observed a highly conserved molecular toolkit participating in the physiological process of gametogenesis in all studied *Geodia* species. Even though some cytological features of gametogenesis have become more complex with evolutionary time, the molecular regulation appears to be very similar from early-branching metazoans to humans.

### Cytology of Gametogenesis in *Geodia* spp

For the subsequent analyses, oocytes were identified as PV, vitellogenic I (Vi_I), II (Vi_II), and III (Vi_III), based on their size and amount of yolk they contained during histological observations. Spermatic cysts were also labeled as SP_I or SP_II according to level of maturation, with SP_I to be present in early spermatogenesis (containing spermatogonia and primary spermatocytes) and SP_II to be in late spermatogenesis (containing mostly secondary spermatocytes, spermatids, and spermatozoa and also few spermatogonia and primary spermatocytes). Accordingly, the individuals of each species were grouped based on the maturation stage of their oocytes and spermatic cysts as assessed at the time of our histological analysis.

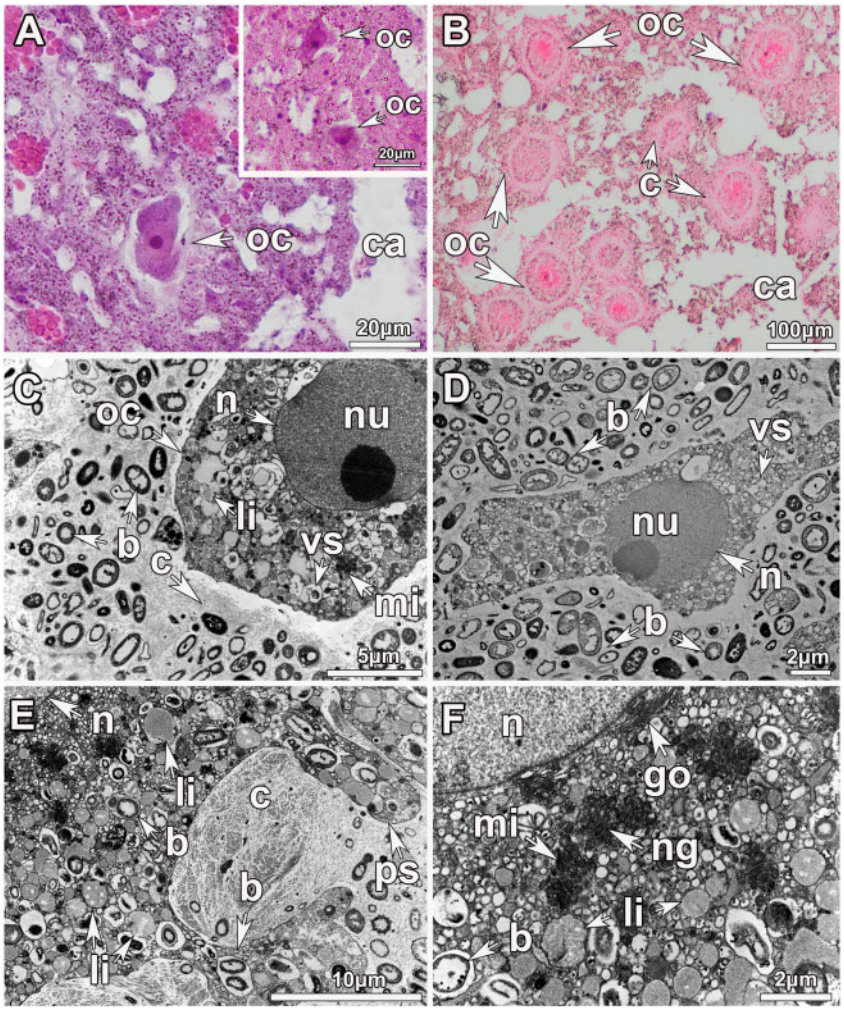


**Fig. 1.** Morphological features of oocytes in sponge tissues of *Geodia* species. (*A*) Light microscopy image showing a PV oocyte (oc) of *Geodia phlegraei* ($\leq$20 $\mu$m in diameter) (insert in the upper right) and a vitellogenic stage I (Vi_I) oocyte in the tissue of *Geodia barretti* ($\sim$30$\mu$m in diameter). (*B*) Light microscopy image of several vitellogenic oocytes of maturation stage III (Vi_III) in *Geodia atlantica* close to the canals (ca), ready to be released. A thick layer of collagen (c) surrounds the mature oocytes. (*C*) Transmission Electron Micrograph (TEM) of a PV oocyte of *Geodia hentscheli*. The nucleolated (nu) nucleus (n) occupies most of the oocyte. Many vesicles (vs), few lipid droplets (li) as well as mitochondrial clouds (mi) can be seen in the ooplasm. A thin layer of collagen is deposited around the oocyte. Many bacterial (b) symbionts were observed in the mesohyl, none inside the oocyte. (*D*) TEM image of an ameboid shape PV oocyte of *G. hentscheli* with a large nucleolated nucleus and the ooplasm full of vesicles but no yolk or lipid droplet. (*E*) TEM image of a vitellogenic oocyte (Vi_II) of *Geodia macandrewii* full of lipid yolk (li) and bacterial symbionts in vesicles. The oocyte has pseudopodia (ps) to acquire further bacteria and nutrients for the maternal tissue. A thick layer of collagen surrounds the oocyte. (*F*) Close-up of (*E*) of *G. macadrewii*. Dense accumulations of mitochondria are observed in the ooplasm, close to the nucleus, and among those the nuage (ng) granules. The Golgi apparatus (go) can be seen in the periphery of the nucleus.

### *Oogenesis*

The oocytes within each individual were consistently found in the same developmental stage. PV oocytes of all *Geodia* spp. were smaller ($18 \pm 3$ $\mu$m) (fig. 1*A*) than the most mature (Vi_III) oocytes (100 $\mu$m in largest diameter) (fig. 1*B*). PV oocytes (fig. 1*C* and *D*) had few small lipid droplets (fig. 1*C* and *D*), whereas Vi_II oocytes had an ooplasm full of yolk (large lipid droplets) and bacterial symbionts vertically transmitted (fig. 1*E*). A layer of collagen surrounded the oocyte and became thicker with development (fig. 1*C* and *E*). Among the mitochondrial clouds, an electron-dense material identified as nuage was present in Vi_II oocytes (fig. 1*F*).

### *Spermatogenesis*

All spermatic cysts were found in the same developmental stage (SP_I or SP_II) consistently within the same individual. Immature spermatic cysts (SP_I) had consistently fewer but bigger spermatic cells (fig. 2*A*), whereas mature spermatic cysts had a greater amount of dividing smaller spermatic cells (fig. 2*B*). SP_I spermatic cysts contained exclusively spermatogonia and primary spermatocytes (fig. 2*C*), whereas SP_II spermatic cysts were composed by gametic cells in mostly later developmental stages; secondary spermatocytes, spermatids, and spermatozoa, but also few spermatogonia and primary spermatocytes (fig. 2*D*). Along spermatogenesis, a decrease in size and condensation of the chromatin within

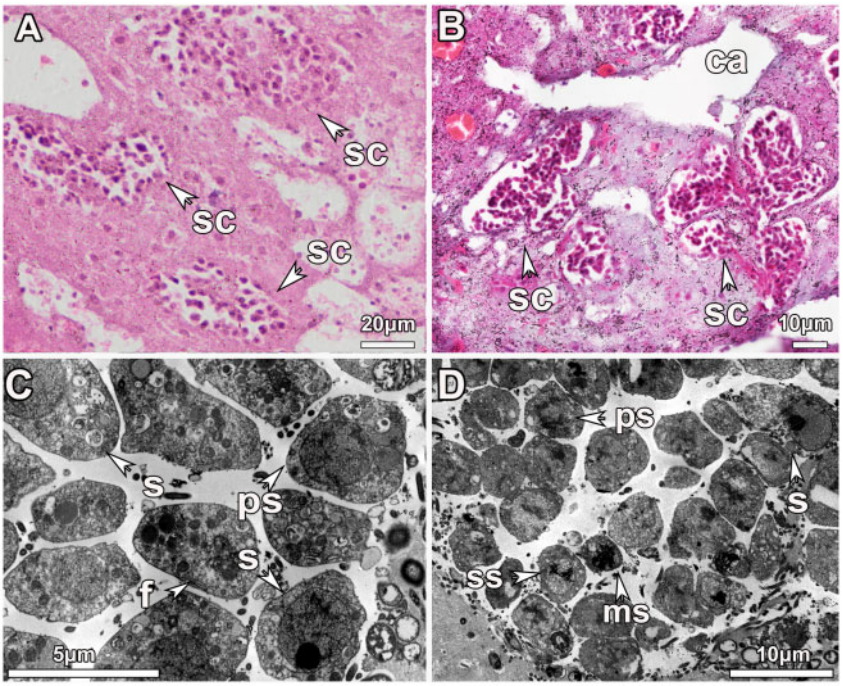


FIG. 2. Morphological features of spermatic cysts in sponge tissues of *Geodia* species. (*A*) Light microscopy image indicating spermatic cyst (sc) in developmental stage I (SP_I) in the mesohyl of *Geodia hentscheli*. (*B*) Light microscopy image depicting several spermatic cysts (sc) of developmental stage II (SP_II) accumulated around the canals (ca) in the tissue of *Geodia phlegraei*. (*C*) TEM micrograph of a SP_I spermatic cyst from *G. hentscheli* in which cells at the beginning of gametic development; spermatogonia (s) and primary spermatocytes (ps) were observed. Internalization of the flagellum (f) from the choanocyte occurs at the early stages. (*D*) TEM micrograph of a SP_II spermatic cyst from *G. phlegraei* with asynchronous development: spermatogonia (s) in the outer part of the cyst, and primary (ps) to secondary spermatocytes (ss) and mature spermatid/preliminary sperm (ms) in the inner part of the spermatic cyst.

the nucleus was observed (fig. 2*D*). Internalization of the choanocyte flagellum occurred at the early spermatic stages (fig. 2*C*) which then disappeared in the later stages (fig. 2*D*).

As a summary, PV oocytes were found in *G. hentscheli*, and *G. phlegraei*, Vi_I oocytes in *G. hentscheli*, *G. phlegraei* and *G. barretti*, Vi_II in *G. macandrewii* and Vi_III were observed only in *G. atlantica*. SP_I spermatic cysts (spermatogonia and spermatocytes) were observed in *G. hentscheli* and *G. phlegraei*, and SP_II (spermatogonia, spermatocytes, spermatids, and spermatozoa) were observed in all species, except for *G. hentscheli*.

## Transcriptomic and Proteomic Analysis

Using differential RNAseq, we studied the molecular toolkit upregulated during gametogenesis in all five *Geodia* species. The amount of raw and filtered reads as also the statistics and completeness metrics of the assembly can be found in supplementary table S1*A* and *B*, Supplementary Material online. Our lists of upregulated genes for oogenesis included genes upregulated when compared 1) with males and 2) with nonreproductive specimens; and upregulated genes for spermatogenesis included genes when compared 1) with females and 2) with nonreproductive specimens. In *G. hentscheli*, 6,107 genes were upregulated in female individuals and 10,491 in male tissues (supplementary tables S2 and S3*A*, Supplementary Material online). In *G. phlegraei*, 1,573 genes were upregulated in females and 4,604 genes in males (supplementary tables S2 and S3*B*, Supplementary Material online). In *G. barretti*, 7,625 and 9,243 genes were upregulated in female and in male tissues, respectively (supplementary tables S2 and S3*C*, Supplementary Material online). In *G. macandrewii*, 541 genes were upregulated in females and 965 genes in males (supplementary tables S2 and S3*D*, Supplementary Material online). Finally, in *G. atlantica*, 284 genes were upregulated in females when compared with specimens undergoing spermatogenesis and 107 genes were upregulated in male tissues when compared with female tissues, since we did not find any nonreproductive specimen (supplementary tables S2 and S3*E*, Supplementary Material online). Roughly between 12% and 69% of these differentially upregulated genes could be identified through BLAST using the Swissprot metazoan database (supplementary table S2, Supplementary Material online). Nonannotated genes could include both genes that are noncoding and others with currently unknown function, including genes that are sponge specific.

Despite studying all the five species, our discussion on oogenesis was essentially focused on the results obtained from *G. phlegraei* (PV), *G. barretti* (Vi_I), and *G. macandrewii* (Vi_II), whereas for spermatogenesis, we mainly discussed the results for *G. hentscheli* (SP_I) and *G. phlegraei* (SP_I and SP_II). The reasons for focusing on these species were 1) the higher amount of information obtained from their gene expression pattern and 2) the possibility to cover all the developmental stages along gametogenesis (table 1). Although *G. atlantica* provided an extra

**Table 1.** Collection Details of the Specimens Used from Each *Geodia* Species (*G. hentscheli*, *G. phlegraei*, *G. barretti*, *G. macandrewii*, and *G. atlantica*) and Used in the Transcriptomic and Proteomic Analysis.

| Species | Status | Code | Sampling Location | Date |
|---|---|---|---|---|
| *G. hentscheli* | Oogenesis | PV_1 | Schulz Bank | June 21, 2016 |
| | Oogenesis | PV_2 | Schulz Bank | June 21, 2016 |
| | Oogenesis | PV_3 | Schulz Bank | June 21, 2016 |
| | Oogenesis | Vi_I | Schulz Bank | June 21, 2016 |
| | Spermatogenesis | SP_I_1 | Schulz Bank | July 29, 2017 |
| | Spermatogenesis | SP_I_2 | Schulz Bank | July 29, 2017 |
| | No-reproductive | NR_1 | Schulz Bank | June 21, 2016 |
| | No-reproductive | NR_2 | Schulz Bank | July 29, 2017 |
| *G. phlegraei* | Oogenesis | PV | Tromsøflaket, Barents Sea | August 3, 2017 |
| | Oogenesis | Vi_I | Sula reef | July 23, 2017 |
| | Spermatogenesis | SP_I_1 | Sula reef | July 23, 2017 |
| | Spermatogenesis | SP_I_2* | Tromsøflaket, Barents Sea | August 3, 2017 |
| | Spermatogenesis | SP_II_1 | Tromsøflaket, Barents Sea | August 3, 2017 |
| | Spermatogenesis | SP_II_2 | Tromsøflaket, Barents Sea | August 3, 2017 |
| | No-reproductive | NR | Sula reef | July 23, 2017 |
| *G. barretti* | Oogenesis | Vi_I_1 | Sula reef | July 23, 2017 |
| | Oogenesis | Vi_I_2 | Tromsøflaket, Barents Sea | August 3, 2017 |
| | Spermatogenesis | SP_II | Tromsøflaket, Barents Sea | August 3, 2017 |
| | No-reproductive | NR_1 | Kosterfjord, West of Yttre Vantenholmen | May 4, 2016 |
| | No-reproductive | NR_2 | Kosterfjord, West of Yttre Vantenholmen | May 4, 2016 |
| | No-reproductive | NR_3 | Kosterfjord, West of Yttre Vantenholmen | May 4, 2016 |
| | *Only used for the assembly* | | Tromsøflaket, Barents Sea | August 3, 2017 |
| *G. macandrewii* | Oogenesis | Vi_II_1* | Skorpeodden, Korsfjord | September 8, 2016 |
| | Oogenesis | Vi_II_2 | Sula reef | July 23, 2017 |
| | Spermatogenesis | SP_II | Sula reef | July 23, 2017 |
| | No-reproductive | NR_1 | Tromsøflaket, Barents Sea | August 3, 2017 |
| | No-reproductive | NR_2 | Tromsøflaket, Barents Sea | August 3, 2017 |
| | No-reproductive | NR_3 | Tromsøflaket, Barents Sea | August 3, 2017 |
| *G. atlantica* | Oogenesis | Vi_III_1* | Skorpeodden, Korsfjord | September 8, 2016 |
| | Oogenesis | Vi_III_2 | Langenuen, Korsfjord | September 9, 2016 |
| | Spermatogenesis | SP_II* | Skorpeodden, Korsfjord | September 8, 2016 |
| | *Only used for the assembly* | | Skorpeodden, Korsfjord | September 8, 2016 |
| | *Only used for the assembly* | | Sula reef | July 23, 2017 |

Note.—The different color indicates the different reproductive status (pink: female; blue: male; gray: nonreproductive). The developmental stage of their gametogenesis is indicated. Asterisks indicate individuals that were used in the proteomic analysis.

developmental stage for oogenesis (Vi_III), this species was not extensively discussed in our transcriptomic analysis because of its very low gene expression patterns and lack of replicates. Transcriptomic analyses on female and male gametogenesis, including all five species, are provided in supplementary file SF1, Supplementary Material online.

We compared the upregulated genes in female and male individuals across species in order to verify if these species shared any ortholog genes related to gametogenesis. Regarding the upregulated genes in females, we found 29 shared ortholog clusters among *G. phlegraei* (PV, Vi_I), *G. barretti* (Vi_I), and *G. macandrewii* (Vi_II) with Gene Ontology (GO) functions that were related to phagocytosis, extracellular region, regulation of JNK cascade, Wnt signaling pathway, and positive regulation of ATPase activity (fig. 3*A* and supplementary table S4*A*, Supplementary Material online). Among the upregulated genes in males, we found 951 shared clusters between *G. hentscheli* (SP_I) and *G. phlegraei* (SP_I and SP_II) which were related, among others, to regulation of G2/M transition of mitotic cell cycle, spindle assembly, chromatin organization, sperm axoneme assembly, and spermatid development (fig. 3*B* and supplementary table S4*B*, Supplementary Material online). Unique ortholog clusters in *G. phlegraei* added the GO category spermatogonial cell division (supplementary table S4*C*, Supplementary Material online). Our analysis suggested that we could include the selected species in our analysis as they shared some gene clusters related to gametogenesis as explained in the following sections and also extract unique information from each of them.

In our proteomic analysis, 920 peptide sequences were identified for the *G. macandrewii* Vi_II female individual and 845 peptides for *G. atlantica* Vi_III female individual from which, 361 and 246 had an annotation (supplementary fig. S1*A* and table S5*A* and *B*, Supplementary Material online). From the annotated genes above, only 2.2–2.4% of the peptides were annotated for GO categories related to gametogenic processes (supplementary fig. S1*A* and table S5*A* and *B*, Supplementary Material online). In the *G. phlegraei* male (SP_I) sample, we isolated 1,244 peptides, and in *G. atlantica* male tissue (SP_II), we identified 811 peptides. From the annotated genes (530 and 256, respectively), only 1.9% and 2.1% had GO terms related to gametogenesis, respectively (supplementary fig. S1*B* and table S5*C* and *D*, Supplementary Material online).

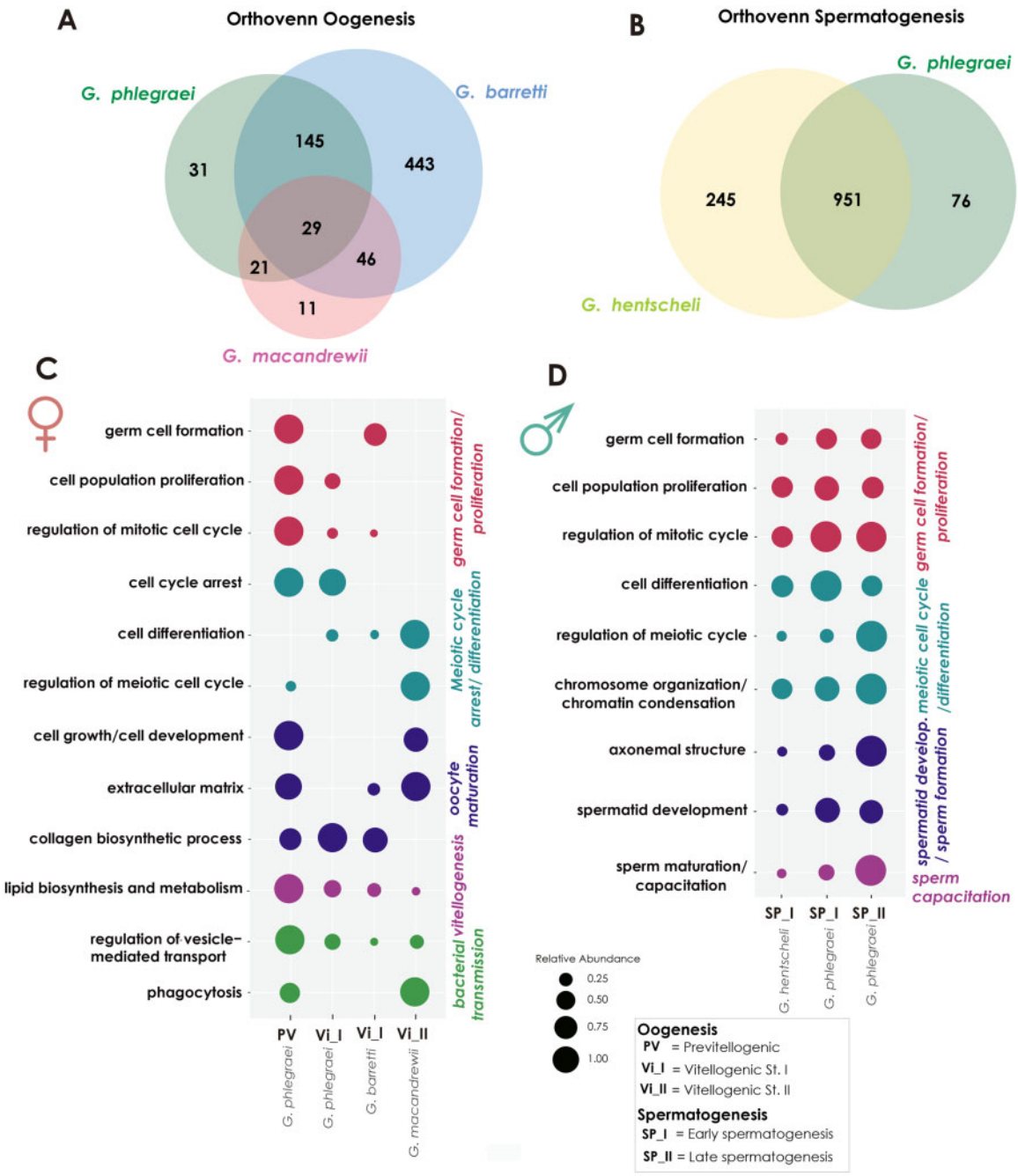


**Fig. 3.** Shared upregulated genes during gametogenesis and GO enrichment. Venn diagram depicting the shared ortholog genes which were upregulated during (*A*) oogenesis across the species *Geodia phlegraei*, *G. barretti*, and *G. macandrewii* and (*B*) spermatogenesis across the species *Geodia hentscheli* and *G. phlegraei*. (*C*) Bubble graph depicting selected GO-enriched categories related to oogenesis, extracted from the upregulated genes among female individuals with oocytes in different developmental stages, PV (*G. phlegraei*); Vi_I (*G. phlegraei*, *G. barretti*) and Vi_II (*G. macandrewii*). (*D*) Bubble graph depicting selected GO-enriched categories related to spermatogenesis, extracted from the upregulated genes among male individuals with spermatic cysts in SP_I (*G. hentscheli*, *G. phlegraei*) and SP_II developmental stages (*G. phlegraei*). GO enrichment analysis was conducted selecting a *P* value $\leq$0.05. Each color represents a different group of gene categories according to their function. The size of the circle in each case is relative to the expression level of each gene category. Each gene category was analysed seperately .

### *Initiation of Gametogenesis*

Gametogenesis in metazoans invariably involves an initial step of germline specification (e.g., Matova and Cooley 2001; White-Cooper and Bausek 2010; Saitou and Miyauchi 2016; McCarrey 2018). In our results, GO categories related to germ cell development, maintenance of pluripotency, primordial germ cell proliferation, and gamete generation in many animals were enriched either in females, males, or both (fig. 3C and *D* and supplementary tables S6, S7, and S8A, Supplementary Material online). Among the upregulated genes involved in primordial germ cell proliferation, we found *lin28*, *sushi domain-containing 2* (*susd2*), *tudor domain-*

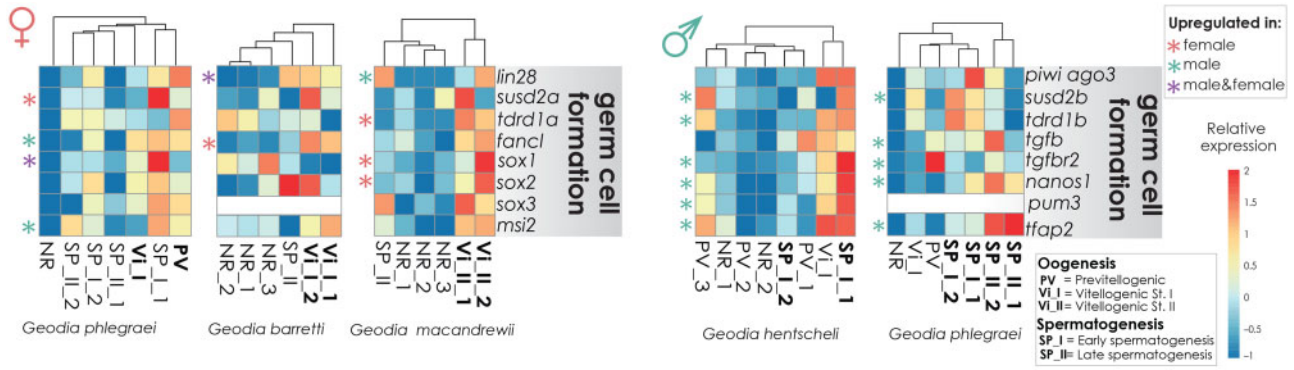


FIG. 4. Heatmaps depicting the expression level of genes related to germ cell formation during oogenesis and spermatogenesis in individuals of the studied species. The scale for relative expression values increases from blue to red. The asterisks indicate the upregulated genes, pink for upregulated genes found in females, green asterisk for upregulated genes found in males, and purple in both female and male specimens.

*containing 1* (*tdrd1*), *bone morphogenetic protein 6* (*bmp6*), and *E3 ubiquitin-ligase fancl*, all of which were previously discussed to be important for germ cell development (fig. 4 and supplementary tables S3 and S8*B*, Supplementary Material online) (Mahowald 2001; Zhao et al. 2005; West et al. 2009; Eguizabal et al. 2011; Sánchez and Smitz 2012). In addition, we have identified four transcription factors of the Sox family in our studied species (supplementary fig. S2, Supplementary Material online), belonging to the groups B, E/F, and C, and three of those were upregulated during early stages of gametogenesis in our studied species (fig. 4 and supplementary tables S3 and S8*B* and SF1, Supplementary Material online). Sox proteins play a pivotal role in sex determination, stem cell fate, differentiation, and development (Pangas and Rajkovic 2006). Demosponges have consistently three to four *Sox* genes, whereas seven were identified in the calcareous sponge *Sycon ciliatum* (Fortunato et al. 2012), maybe because of possible genome duplication (Fierro-Constaín et al. 2017; Renard et al. 2018). In our phylogenetic reconstruction of the Sox protein family evolution, all our *Geodia* sequences clustered with Sox sequences from other demosponges (*Amphimedon queenslandica* and *Ephydatia muelleri*) (supplementary fig. S2, Supplementary Material online). In *S. ciliatum*, SoxB and SoxC genes were highly expressed in oocytes and during embryogenesis, whereas *SoxF* genes were expressed in adult cells (Fortunato et al. 2012). In the ctenophore *Mnemiopsis leydi*, six *Sox* genes were identified, and their expression patterns were mostly restricted to adult cells in proliferative areas (Schnitzler et al. 2014). In our case, all three *Sox* genes seemed to have a widespread expression in both female and male individuals, but *Sox1* showed a more restricted expression pattern during early stages of gametogenesis, whereas *Sox2* and *3* (*SoxB* and *F*) could be less involved in germ cell differentiation (fig. 4 and supplementary SF1, Supplementary Material online). Whether these genes are related to sex determination is possible but not conclusive from our results.

Germ cell granules (nuage), which flag the germ cell nature in most organisms, have been previously described in sperm and oocytes of sponges (Lanna and Klautau 2010; Fierro-Constaín et al. 2017; Gonobobleva and Efremova 2017, Riesgo et al. 2018). They have also been discovered in archaeocytes (Isaeva and Akhmadieva 2011), reinforcing the idea that some stem cells in the adult sponge could potentially be predetermined for the germline. Here, we observed potential nuage in oocytes of *G. macandrewii* (fig. 1*F*). Many proteins are traditionally associated with nuage structures in animals, including members of the Vasa, Musashi, Argonaute/Piwi, TGF-beta receptors, Nanos, and Pumilio protein families, all of which have a role in the maintenance of the germline (Kobayashi et al. 1996; Cox et al. 1998; Shivdasani and Ingham 2003; Siddall et al. 2006; Seto et al. 2007; Lolicato et al. 2008; Funayama et al. 2010). We found *musashi homolog 2* (*msi2*), *piwi-ago3*, *transforming growth factor beta-1* (*tgfb1*), *TGF-beta receptor* (*tgfbr1*), *nanos 1*, and *pumilio* (*pum3*) and *transcription factor AP2* (*tfap2*) upregulated during gametogenesis in our studied species, especially during the early stages of spermatogenesis in males (fig. 4 and supplementary tables S3 and S8*B*, Supplementary Material online). Similarly, most of the abovementioned genes were also expressed in the adult multipotent cells and gametes of the calcareous sponge *S. ciliatum* (Leininger et al. 2014) and the homoscleromorph sponge *Oscarella lobularis* (Fierro-Constaín et al. 2017), indicating a potential role of those genes in transdifferentiation of somatic stem cells into germ cells in three out of the four Porifera classes. TFAP2 is fundamental in the cnidarian *Hydractinia symbiolongicarpus* to induce germ cell formation from adult stem cells (DuBuc et al. 2020). These indicate that some of the transcription factors and regulatory genes behind animal germ cell development, maintenance, and self-renewal during gametogenesis appeared very early in evolution and could be functioning in similar ways in Porifera and other Metazoa (e.g., White-Cooper and Bausek 2010).

When the expression of regulators maintaining an undifferentiated state is masked by the expression of regulators in favor of differentiation, a germ cell stops dividing mitotically and expresses genes triggering meiosis for further gamete development. In vertebrates and also some invertebrates, meiosis is stimulated by retinoic acid (RA) in both female and male gametogenesis (fig. 5). In male gonads, retinol is transported via retinol binding protein to the stimulated by retinoic acid (STRA) receptor to the Sertoli cell (Berry et al. 2013; Evans et al. 2014). RA synthesis occurs with the help of retinol dehydrogenase (RDH) and aldehyde dehydrogenase (ALDH) (Tong et al. 2013; Arnold et al. 2015). Once

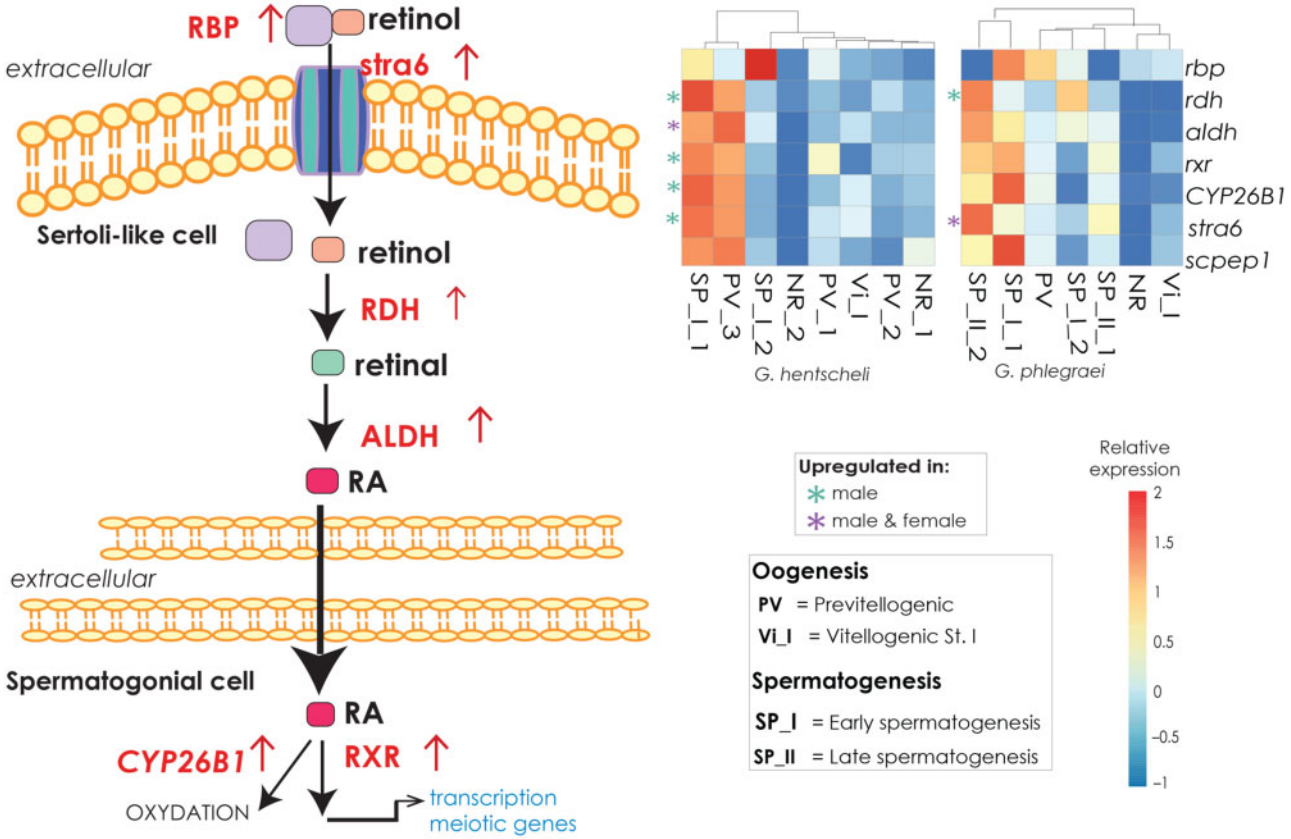


FIG. 5. The RA pathway. (*A*) Schematic representation of the RA pathway (modified from Griswold [2016]), which permits a balance between germ cell maintenance and activation of meiosis. Red arrows indicate upregulation of genes in our data. (*B*) Heatmap of depicting the expression level of the main genes in the RA pathway mainly focused on individuals with gametes in early stages among the studied species (*Geodia hentscheli* and *G. phlegraei*). The scale for relative expression values increases from blue to red. The asterisks indicate the upregulated genes in female and or male specimens; the color green was used for upregulated genes in male and purple when found in both female and male individuals. There was no gene exlusively upregulated in female individuals.

synthesized, RA is recognized by its receptors, RAR and RXR in germ cells and stimulates meiosis (Mark et al. 2015; Griswold 2016). The degradation of RA by the enzyme CYP26B1 in the Sertoli or germ cells prevents premature gamete development (Koubova et al. 2006; Feng et al. 2014). In mice, oogenesis is also triggered by STRA receptors, and the mechanism is highly similar to that occurring during spermatogenesis (Anderson et al. 2008). A similar control mechanism could have been already present in the last common ancestor of sponges and the rest of metazoans, since almost all the genes regulating RA synthesis mentioned above were upregulated mainly in males of *G. hentscheli*, and some of them in *G. phlegraei* (*rdh*, *aldh*, *stra6*, and *rxr*) (fig. 5 and supplementary tables S3*A* and *B* and S8*B*, Supplementary Material online). The *aldh* gene together with the gene Retinoid-inducible serine carboxypeptidase (*scpep1*), which is also part of the RA biosynthetic pathway, was translated into peptides (supplementary table S5*C* and *D*, Supplementary Material online). Regarding the female individuals, although there was a tendency of higher expression for some of these genes in individuals with PV oocytes, only two of those (*aldh and stra6*) were overexpressed in PV females (fig. 5 and supplementary table S3*A* and *B*, Supplementary Material online). Previous studies observed the presence of RA in sponges and its role in morphogenetic events and gemmulation, cell differentiation, and spiculogenesis (Biesalski et al. 1992; Imsiecke et al. 1994; Nikko et al. 2001; Wiens et al. 2003; Müller et al. 2011). Similarly, in our case, RA could induce either the differentiation of choanocytes to spermatogonia (germ cells) and/or the differentiation of spermatogonia to primary gametocytes and initiation of meiosis. Overall, RA pathway activation could be a candidate pathway for the exit of the cells from germ cell fate and initiation of meiosis also in Porifera (fig. 7).

### *Oogenesis*

*Meiosis and Oocyte Maturation.* The oogenesis in *Geodia* spp. was synchronous within individuals (fig. 1*B*), which means that once oogonia reached the point of cell cycle arrest and meiosis regulation, there was no further germ cell proliferation, and oogonia entered the phase of differentiation and maturation. Indeed, GO categories related to germline regulation were mainly present at the PV stages in oogenesis, indicating a role at the beginning of gamete formation, whereas those of meiosis and regulation of cell cycle arrest were enriched in later vitellogenic stages (fig. 3*C* and supplementary tables S6 and S8*A*, Supplementary Material online). Similarly, in the GO-enriched categories of our proteomic

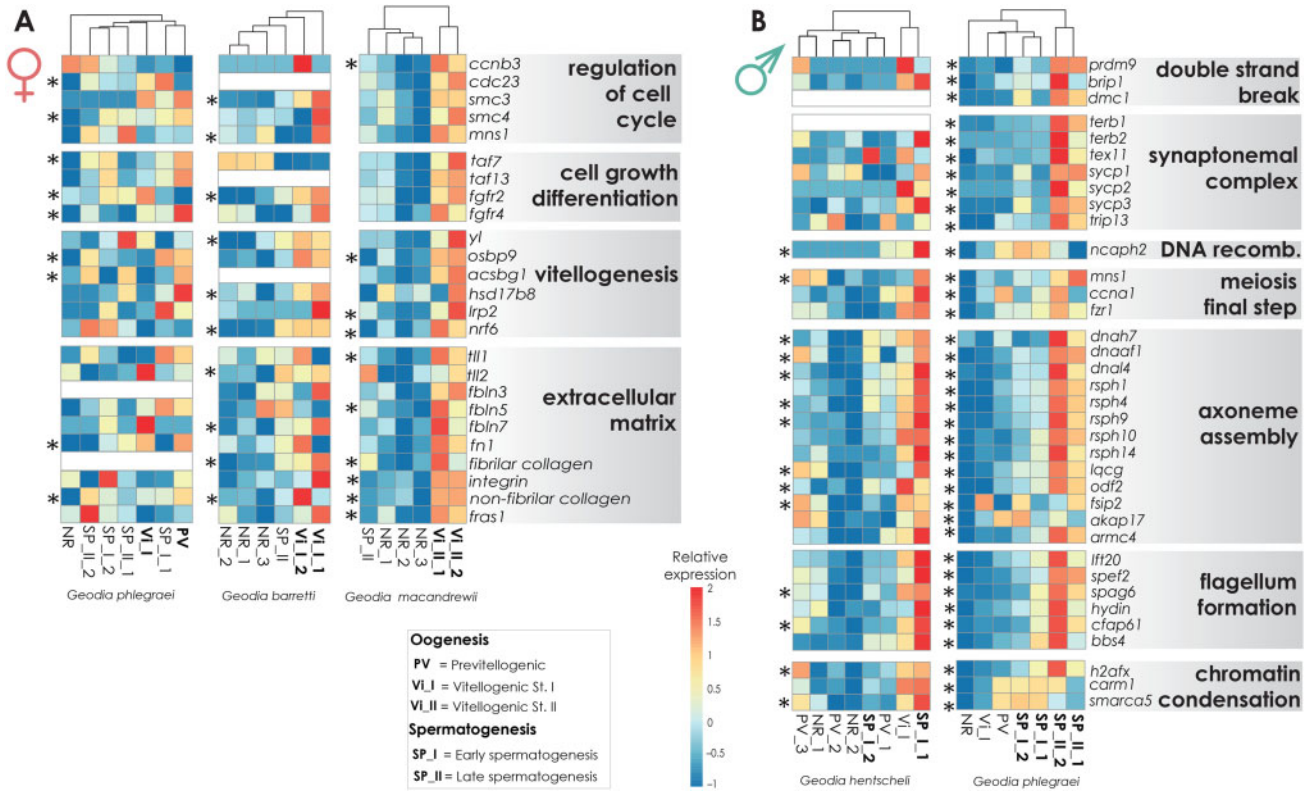


FIG. 6. (*A*) Heatmaps depicting the expression level of selected genes related to oogenesis, across individuals with oocytes in different developmental stages: PV, V_I, and V_II (in *Geodia phlegraei*, *G. barretti*, and *G. macandrewii*, respectively). (*B*) Heatmaps depicting the expression level of selected genes related to spermatogenesis in individuals with spermatid cysts in different developmental stages: SP_I (*Geodia hentscheli* and *G. phlegraei*) and SP_II (*G. phlegraei*). The genes are separated into few big categories according to their function. The scale for relative expression values increases from blue to red. The asterisks indicate the differentially expressed genes in female and male specimens, respectively.

data, "regulation of meiotic nuclear division" and "establishment of meiotic spindle localization," were enriched in later vitellogenic stages (Vi_II and Vi_III) (supplementary table S9*A* and *B*, Supplementary Material online).

The exact expression timing of the molecular complements for meiosis is largely unknown in invertebrates (Kishimoto 2018). There are two cell cycle arrests during meiosis in animals: one that occurs in prophase I (in oogenesis) and is regulated by the expression of *kinase cyclin B-associated Cdk1* (*cyclin B-Cdk1*), and a second arrest that occurs at variable moments across invertebrates but is invariably resumed by the interactions between oocytes and sperm during fertilization (Kishimoto 2018). Genes involved in chromosome stability during meiotic cell cycle arrest, such as *structural maintenance of chromosomes protein* (*smc*) (Bickel et al. 2010) and *meiosis-specific nuclear structural protein 1* (*mns1*), were upregulated in females with PV and Vi_I stages (fig. 6*A* and supplementary tables S3 and S8*B*, Supplementary Material online). Furthermore, classic genes regulating meiotic divisions were found differentially expressed in females of Vi_II stage (fig. 6*A* and supplementary tables S3 and S8*B*, Supplementary Material online), including several *G2 mitotic-specific cyclin B* genes (*ccnb3*), which are subunits of kinase cyclin B-associated proteins, important to facilitate progression through meiotic divisions in many organisms (Voronina et al. 2003; Dheilly et al. 2012; Peng et al. 2017; Kishimoto 2018). *Mos* genes, which are present from placozoans to mammals (Extavour 2009) and have a role in meiotic cell arrest in bilaterians and cnidaria (Amiel et al. 2009), were not found in our study, which corroborates that *mos* originated after sponges diverged from the metazoan stem (Extavour 2009). This suggests that cell cycle arrest is controlled by other genes in sponges.

Oocyte maturation is achieved in many animals during cell cycle arrest by the activation of several pathways (i.e., PI3K-AKT, FGF, and Mos-MAPK pathways) and transcription of growth factors (Thisse B and Thisse C 2005; Matus et al. 2007; Sugiura et al. 2007; Ornitz and Itoh 2015; Kishimoto 2018). In our study, females overexpressed genes encoding for transcription of growth factors important during oocyte maturation (Falender et al. 2005; Pangas and Rajkovic 2006; Voronina et al. 2007; Markholt et al. 2012; McGinnis et al. 2013), including *transcription initiation factor TFIID subunit gene* (*taf*) and *fibroblast growth factor receptor* (*fgfr*), which was also found in the proteomic data (fig. 6*A* and supplementary tables S3*B* and S5*B*, Supplementary Material online). So, in our data, we see signals for meiotic regulation and also meiotic arrest and maturation across all female samples (figs. 3*C*, 6*A*, and 7*A* and supplementary table S8, Supplementary Material online).

*Oocyte Growth and Vitellogenesis.* Oocyte growth involves a large increase in the volume and many shape modifications, but most importantly during this phase, yolk is accumulated in the oocyte to provide the necessary nutrients for the

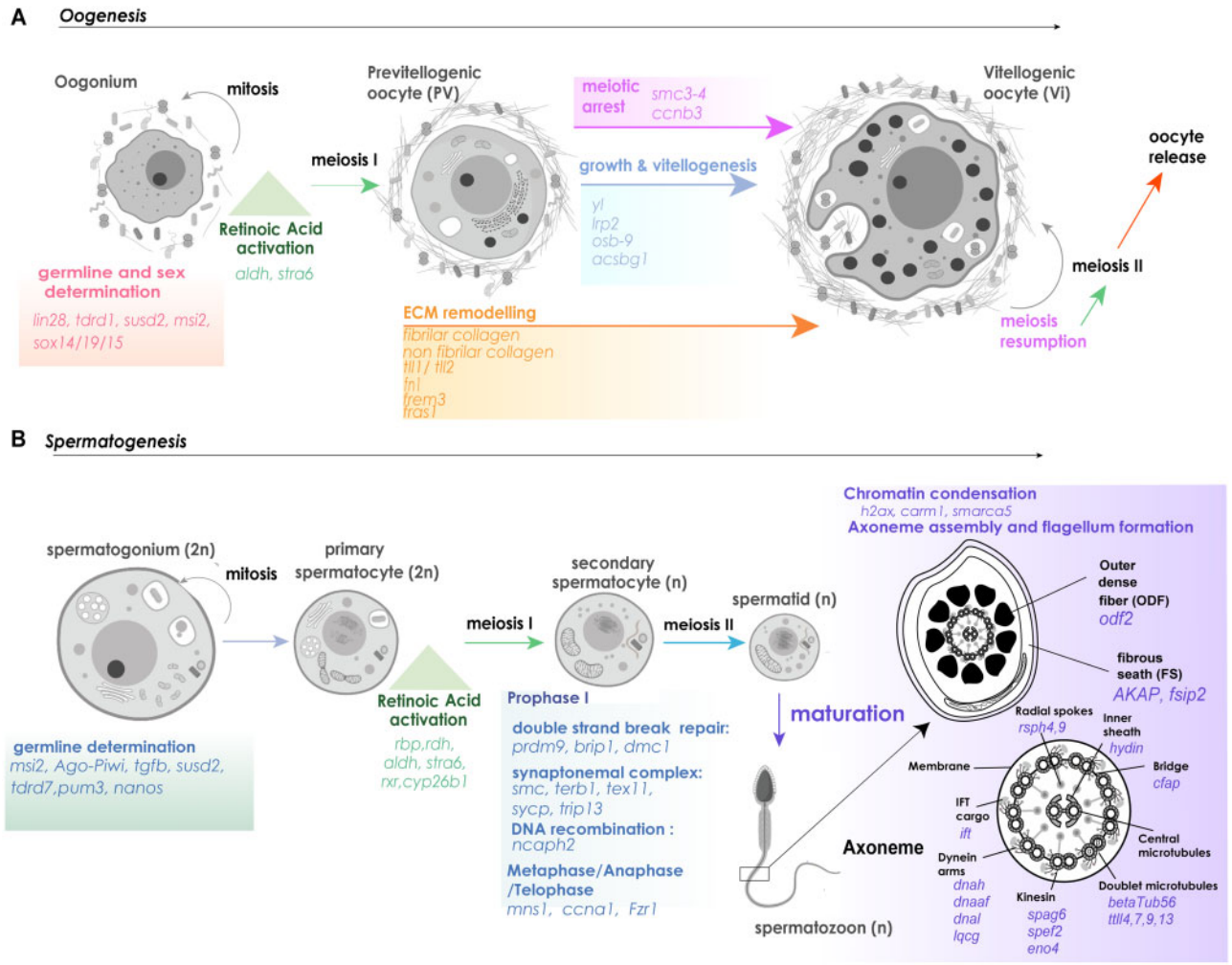


FIG. 7. A schematic representation of the molecular toolkits recruited and physiological changes during the progress of (*A*) oogenesis and (*B*) spermatogenesis in *Geodia* spp., based on our histological and transcriptomic results and the theoretical progress of gametogenesis in other Metazoa. The main overexpressed regulatory pathways and genes involved in each developmental stage and transition phase are reported.

embryo (Shikina et al. 2013; Reading et al. 2018). All sponges have lecithotrophic larvae, and therefore they entirely rely on the yolk that the oocyte (and then the embryo) contains to develop into a juvenile during the free-swimming stage and early settlement (Maldonado 2006). During the vitellogenesis of *Geodia* spp., nutrients mainly of lipidic origin are produced and packed by the Golgi apparatus within the ooplasm (fig. 1*E* and *F*). Although most of the yolk accumulation in the oocytes was observed at the stages Vi_II and Vi_III in our histological analysis (fig. 1*D*), GO categories for vitellogenesis started to be enriched at PV stages up to the vitellogenic stages I and II (fig. 3*C* and supplementary tables S6 and S8*A*, Supplementary Material online). Indeed, we identified many upregulated genes related to yolk formation in the female individuals which contained vitellogenic oocytes (figs. 6*A* and 7 and supplementary tables S3 and S8*B*, Supplementary Material online). Among the most important genes, we found the *putative vitellogenin receptor* (*yl*), *oxysterol binding protein* (*obp9*), and *long-chain fatty acid ligases* (*acsbg1*) (fig. 6*A* and supplementary tables S3 and S8*B*, Supplementary Material online). In vertebrates, vitellogenin, a main yolk component in all metazoans, is produced in the liver and is induced by the hormonal control of estradiol 17 (Polzonetti-Magni et al. 2004). Sponges do not have organs and hormonal production is poorly known, but homologs of vitellogenin proteins have been previously characterized in sponges (Riesgo et al. 2014). Furthermore, upregulation of *17-beta-hydroxysteroid dehydrogenase* genes (*hsd17b8 and hsd17b4*) could indicate an active role of those genes in fatty acid metabolism and/or in steroid hormone metabolism during oogenesis in sponges (Mindnich et al. 2004). Another vitellogenin receptor, whose expression level is high in vitellogenic oocytes of teleost fish (Reading et al. 2014), the *low-density liporeceptor-related 2* gene (*lrp2*), was upregulated in our analysis in the vitellogenic oocytes (fig. 6*A*). *HSD17B8* and *LRP2*, and also other genes related to lipid transport (e.g., *nrf-6*), are involved in fatty acid biosynthesis during oocyte maturation of the crab *Eriocheir sinensis* (Chen et al. 2019). Although we cannot speculate on the types of lipids used for yolk formation in our study species, several genes involved in the metabolism of long-chain fatty acids and phospholipids, including phosphatidylcholines, were upregulated

during the vitellogenic phase of the female gametes (supplementary table S3, Supplementary Material online). Indeed, fatty acids and phospholipids are abundant in *Geodia* spp. (Müller et al. 1987; Carballeira and Rodriguez 1991; Djerassi and Lam 1991; Genin et al. 2008). In our proteomic results, we found enriched GO categories, similar to those of transcriptomic data, related to phospholipid, glycolipid, sphingolipid, and glycosphingolipid metabolism in females with vitellogenic oocytes but not in male individuals (supplementary table S9*A* and *B*, Supplementary Material online), so this lipid diversity could be possibly linked to yolk formation, although other processes occurring in the female sponge tissue cannot be ruled out.

The role of the extracellular matrix (ECM) in cell growth, differentiation, migration, and maturation of female germ cells is well known (Hay 1991; Rodgers et al. 2003; Guzmán et al. 2014; Viana et al. 2018). Our data support the idea of an indispensable role of the ECM organization molecular machinery in the oocyte development from sponges to late branching metazoans (Morris 1993). In fact, our histological sections showed that gametic cells continuously changed shape, interacting with the surrounding mesohyl, and becoming massively enveloped by a collagenous sheath that increased in size as oogenesis progressed (fig. 1*C*–*F*). In all developmental stages, the GO categories of structural morphogenesis, ECM, and collagen production were largely enriched in our RNAseq data, indicating the importance of ECM reorganization during the whole process of oogenesis (figs. 3*C*, 6*A*, and 7*A* and supplementary tables S6 and S8*A*, Supplementary Material online). In addition, the GO categories "positive regulation of extracellular matrix disassembly" and "positive regulation of extracellular matrix organization" were enriched in the proteomic data set of both female individuals (supplementary fig. S1 and table S9*A* and *B*, Supplementary Material online). Among the upregulated genes related to the ECM assembly and reorganization, we found *tolloid* (*tll1*/*tll2*), *fibulin* (*fbln5*/*fbln7*), and *fibronectin* (*fn1*), which were significantly overexpressed in Vi_I and Vi_II females (fig. 6*A* and supplementary tables S3*C* and *D* and S8*B*, Supplementary Material online). These genes have been found to participate in cell growth and maturation in oogenesis of vertebrates (Asem et al. 1992). The most charismatic component of ECM across animals is collagen.

In our female sponges, we found overexpressed genes coding for both fibrillar and nonfibrillar collagens (fig. 6*A* and supplementary fig. S3 and tables S3 and S8*B*, Supplementary Material online). Overexpressed genes for fibrillar collagen were found to be from the same group as type I, V, and XI collagens of vertebrates (supplementary fig. S3, Supplementary Material online), but more closely related to collagen type V/XI than I–III, forming a clade with fibrillar collagens of cnidarians (Exposito et al. 1990, 2008, 2010). For nonfibrillar collagens, our sequences for spongin short-chain collagen in *Geodia* spp. clustered with those of other sponges (supplementary fig. S3, Supplementary Material online), in a separate clade, related to type IV collagens of vertebrates and invertebrates, including sponges (supplementary fig. S3, Supplementary Material online; Exposito et al. 1991; Aouacheria et al. 2006). Genes coding for both collagen families were overexpressed in the sponge *E. muelleri* during early development of asexual propagules (gemmules) (Exposito et al. 1990, 1991). Fibrillar collagen is the main component of the ECM and in vertebrates fibrillar collagen of types V and XI, together with fibronectins and integrins, initiates the collagen fibrillogenesis in ECM (Kadler et al. 2008). Although the nonfibrillar spongin short-chain collagen does not have an identified function yet (Aouacheria et al. 2006), it could be another component of ECM in sponges, or it could serve for the attachment of the propagules to the substrate (Exposito et al. 2002).

Finally, vesicles with bacterial symbionts were transported from the mesohyl and phagocytized but not digested by the oocyte (fig. 1*E*). These are the vertically transmitted bacterial symbionts, a transmission previously observed in *Geodia cydonium* (Sciscioli et al. 1994). GO categories of vesicle transport, phagocytosis, endocytosis, and bacteria recognition were enriched at transcriptomic and translational level (fig. 6*A* and supplementary fig. S1 and tables S6, S8*A*, and S9, Supplementary Material online). This vertical transmission is fundamental during oogenesis in many sponge species, reassuring inheritance of the microbial symbionts to the next generation (e.g., Taylor et al. 2007) and offering a possible advantage during juvenile settlement.

According to our data, oogenesis in Porifera develops within relatively few physiological phases, in which oogonia enter meiosis and meiotic cell cycle arrest, where maturation, growth, and vitellogenesis occur. In parallel, variations in structure morphogenesis of the oocytes take place to achieve round shape (fig. 7*A*). These are also the basic stages of oogenesis in other Metazoa (e.g., Telfer and McLaughlin 2007; Woodruff et al. 2018).

### *Spermatogenesis*

*Early Stages of Spermatogenesis.* During spermatogenesis, spermatogonia (male germ cells) become primary spermatocytes in which the first meiosis occurs, bringing substantial structural and physiological changes to the gamete cells. From primary to secondary spermatocytes, the number of chromosomes is reduced in half (2*n* to *n*) through the first round of meiosis, and then chromatids are separated during the second meiosis to produce spermatids. Then, spermatids experience a massive reduction in size before maturing into spermatozoa. In our study, we identified GO-enriched categories related to cell differentiation, meiotic cycle, and chromatin condensation in male individuals (fig. 3*D* and supplementary tables S7 and S8*A*, Supplementary Material online). Although the GO categories related to germ cell formation and proliferation, including "regulation of mitotic cycle," were similarly enriched in both developmental stages (SP_I and SP_II) (fig. 3*D*), "regulation of meiotic cycle" was more abundant in individuals with SP_II cysts (fig. 3*D*). This is something expected from our histological analysis, as males in SP_I stage had spermatic cysts at the beginning of their development, including mainly spermatogonia and primary spermatocytes, whereas in individuals with more mature spermatic cysts, within SP_II spermatic cysts there was

germ cell development in parallel with several other stages of sperm production.

During prophase I, homologous chromosomes get closer, the DNA double strands break, the synaptonemal complex is formed, and chromosome synapsis occurs (Haschek et al. 2010). In the pachytene phase, DNA recombination happens (allowed by the synaptonemal complex, a protein structure facilitating crossover of DNA strands), and finally during the diplotene/diakinesis phase, the synaptonemal complex disassembles, and the homologous chromosomes uncoil (Haschek et al. 2010). In particular, we found upregulated genes related to double strand breaks (e.g., *histone–lysine N-methyltransferase PRDM9* [*prdm9*], *fanconi anemia group J homolog* [*brip1*], and *meiotic recombination DMC1 LIM15* [*dmc1*]), formation of the synaptonemal complex (e.g., *telomere repeats-binding bouquet formation 1-2* [*terb1-2*], *testis-expressed protein 11* [*tex11*], *synaptonemal complex1-3* [*sycp1-3*], and *pachytene checkpoint 2 homolog* [*trip13*]), DNA recombination (e.g., condensin-2 complex subunit H2 [*ncaph2*]), and overall meiotic process (e.g., meiosis-specific nuclear structural 1, *mns1*; *cyclin-A1* [*ccna1*]; *fizzy-related homolog* [*fzr1*]) in males, mainly with SP_II spermatic cysts (figs. 6*B* and 7 and supplementary tables S3 and S8*B*, Supplementary Material online). Overall, most of the genes from the classic set of meiotic genes conserved in eukaryotes (Ramesh et al. 2005; Schurko and Logsdon 2008) were upregulated in males of *G. hentscheli* and *G. phlegraei* (*msh2,3,4,5,6*; *hop1,2*, *mlh1,3*; *rad50,51*; and *pms2*) (supplementary tables S3*A* and *B* and S8*B*, Supplementary Material online). This set of genes has been also identified in the genome of the protist *Giardia* and of the choanoflagellate *Monosiga ovata* (Carr et al. 2010) suggesting meiosis. In sponges, spermatogenesis involves two rounds of meiosis as in the rest of metazoans, and we hypothesize that this set of genes function in meiosis as in other eukaryotes. In addition, the protein tubulin beta-1 chain (BETATUB56D), which plays a role in centriole formation, myosin-9 (MYH9), which participates in spindle formation and positioning in meiotic cycle (Li and Yang 2016), and the inactive peptidyl-prolyl cis-trans isomerase (FKBP6), which allows the chromosome integrity and pairing during meiosis (Crackower et al. 2003) were isolated in the proteomic analysis (supplementary table S5, Supplementary Material online). From the above, we could say that molecular regulation of meiotic cell cycle seemed to be very conserved across metazoans.

*Late Stages of Spermatogenesis.* In most animals, during the transition from spermatid to spermatozoa, the axoneme of the flagellum is formed. This step is crucial for the maturation, capacitation, and mobility of spermatozoa (Inaba 2011). Although some sponges (e.g., *Aplysilla rosea*, *Halichondria (Halichondria) panicea*, and *Corticium candelabrum*) retain the flagellum during the transition from choanocytes to spermatogonia (Tuzet et al. 1970; Barthel and Detmer 1990; Bergquist 1996; Riesgo, Maldonado, et al. 2007), the most common pattern in sponges is that choanocytes get rid of their flagellum to become spermatogonia and undergo multiple mitoses, and in the final stages of spermatogenesis, spermatids make a flagellum de novo (Maldonado and Riesgo 2008; Ereskovsky 2010). In *Geodia* spp., the flagellum seems to be resorbed in spermatogonia and rebuilt at later stages (fig. 2*C* and *D*). The axoneme assembly is a well-conserved process across animal evolution. The most typical structure is the microtubule organization of nine outer doublet microtubules and two central singlet microtubules ($9 + 2$) in most eukaryotes (Manton 1953; Inaba 2003). Tubulins and particularly $\beta$-tubulins are structural proteins in the axonemal motif (Nielsen et al. 2001; White-Cooper et al. 2009) and the bending of the flagellum is regulated by the axonemal dyneins and the radial spokes, which are both attached to the doublet of microtubule (Porter and Johnson 1989; Smith and Yang 2004; Roberts et al. 2013). We found the complete axonemal toolkit upregulated in the male tissues of our *Geodia* spp. (figs. 3*D*, 6*B*, and 7 and supplementary tables S3, S7, and S8*B*, Supplementary Material online), including *tubulin beta chains*, the whole functional complement of dyneins (*dynein regulatory complex protein* [*Iqcg*], *dynein assembly factor axonemal* [*dnaaf*], *dynein heavy* [*dnah*], *dynein light* [*dnal*], and *radial spoke head* [*rsph*]) (figs. 6*B* and 7 and supplementary tables S3 and S8, Supplementary Material online). Other genes coding for proteins of the central apparatus including *tektin*, *hydin*, and *sperm-associated antigen 6*, *spag6* which participate in axonemal assembly and flagellum motility (Inaba 2011) were also overexpressed in male individuals of our study (figs. 6*B* and 7 and supplementary tables S3 and S8*B*, Supplementary Material online).

The main difference between the somatic flagella observed in animal cells and the sperm flagellum is the presence of Outer Dense Fibers (ODFs) and fibrous sheath (FS) (Kierszenbaum 2001; Inaba 2011), which are accessory structures that surround the axoneme to enhance the structural maintenance and motility of the flagellum (Fawcett 1975; Si and Okuno 1993). Although these molecules have been previously detected in sperm flagella of ascidians (Konno et al. 2010), echinoderms, and platyhelminths (White-Cooper et al. 2009), ODF genes may also have a role in centrosome function in other somatic cells (Ishikawa et al. 2005). We identified upregulated genes coding for ODF and FS in males with SP_I and SP_II spermatic cysts (fig. 6*B* and supplementary tables S3 and S8, Supplementary Material online), so we cannot prove that these genes participate only in the structure of the sperm flagellum, the production of centrosomes during meiosis (Inaba 2011), or the choanocyte flagellum. Although we did not recover any of the above genes in our proteomic analysis, we found other proteins making up enrichment of the GO categories of axonemal structure, which are related to axoneme formation (supplementary table S8, Supplementary Material online). Our findings of the complete axonemal toolkit activated during spermatogenesis, suggest that *Geodia* spp. generate their flagellum de novo in male gametes and agree with the conserved morphology and movement reported for the sperm flagellum in Porifera (e.g., Reiswig 1983). They are also in concordance with the description of the molecular machinery behind the axonemal structure of the mammalian sperm allowing the formation of competent spermatozoa.

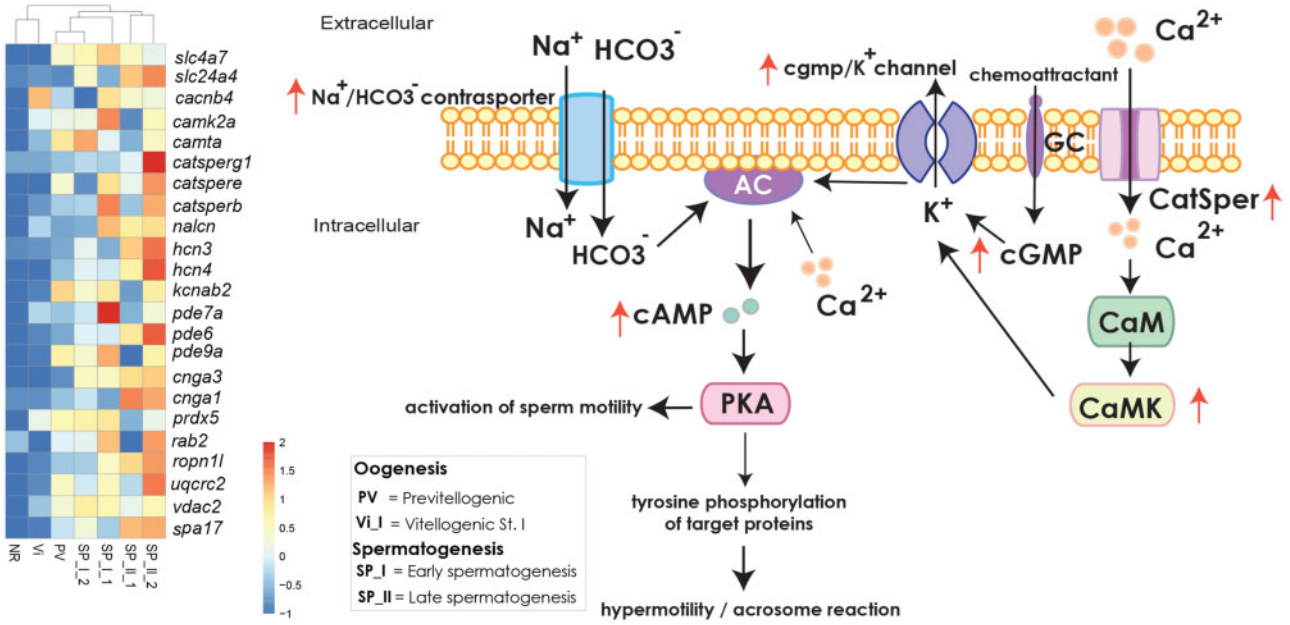


FIG. 8. Illustration of $Ca^{+2}$ driven regulation cascades related to sperm motility and capacitation. Bicarbonate, $HCO3^{-}$ is transported via a $Na^{+}$/$HCO3^{-}$ cotransporter and $Ca^{+2}$ via $Ca^{+2}$ channels including the sperm-specific $Ca^{+2}$ channel, CatSper. Those activate adenylyl cyclase (AC) which increases the levels of cAMP and leads to PKA activation. CaM-dependent increase of $Ca^{+2}$ as also activation of guanylyl cyclase receptor (GC) and further increase of cGMP into the cell also induce activation of AC and final activation of PKA. Activation of PKA causes flagellar movement. Further phosphorylation of PKA substrates leads to tyrosine phosphorylation and activation of all the final sperm capacitation steps (from sperm motility to axonemal reaction before fertilization) in animals. The illustration is a modified version according to Morisawa and Yoshida (2005), Ickowicz et al. (2012), and Rahman et al. (2017). The red arrows indicate the overexpression of the relevant genes in male specimens of the species *Geodia phlegraei* as it was the only one with several individuals containing SP_II spermatic cysts. The graph is accompanied by the expression level of the relevant genes in all the studied specimens of *G. phlegraei* and presented through a heatmap. All the genes indicated were upregulated in males of *G. phlegraei*. The expression level increases from blue to red color.

Another important process occurring during spermatogenesis is further DNA condensation. In our histological study, we observed chromatin condensation along the progress of spermatogenesis (fig. 2*D*), which is a widespread phenomenon across Porifera (e.g., Barthel and Detmer 1990; Riesgo et al. 2008; Lanna and Klautau 2010) and all the other metazoans (Haschek et al. 2010). Specialized histones are involved in this process, particularly H2AX, which replaces the regular histone H2A, and which has a higher affinity for DNA because of its higher arginine to lysine content (Fernandez-Capetillo et al. 2003). The overexpression of *h2ax* together with the *Histone–arginine methyltransferase gene* (*carm1*) in males (figs. 3*D* and 6*B* and supplementary tables S3, S7, and S8*B*, Supplementary Material online) suggests the presence of this high-DNA affinity histones in sponges and demonstrates a conserved mechanism of chromatin condensation across metazoans.

*Sperm Capacitation.* Sperm capacitation events, from marine invertebrates to mammals, include participation of ion channels ($Ca^{+2}$ and $Na^{+}$), increase of the pH, and hyperpolarization of the sperm membrane. The increased intracellular concentration of $Ca^{+2}$ and cyclic adenosine monophosphate (cAMP), through activation of soluble adenylyl cyclase, and further activation of protein kinase A (PKA) and tyrosine phosphorylation of downstream sperm proteins play a fundamental role in the capacitation events across eukaryotes (fig. 8) (Morisawa and Yoshida 2005; Visconti 2009; Yoshida and Yoshida 2011; Ickowicz et al. 2012). In echinoderms, membrane hyperpolarization is caused by an increase of intracellular cGMP concentration through the activation of GC receptor, and the further efflux of $K^{+}$ through cGMP/K channels. In ascidians, it is the $Ca^{+2}$ influx through a $Ca^{+2}$ channel (i.e., low voltage calcium channel), and further activation of calmodulin (CaM)-dependent pathway, which provokes membrane hyperpolarization and downstream motility events (fig. 8) (Yoshida et al. 1993, 1994; Izumi et al. 1999; Nomura et al. 2004).

In sponges, sperm capacitation has never been studied. However, we expect a basic mechanism of sperm capacitation, similar to that mentioned above, as a GO-enriched category related to sperm capacitation (fig. 3*D* and supplementary tables S7 and S8*A*, Supplementary Material online) and genes coding for enzymes and ion channels, part of the regulation cascades of sperm motility, were upregulated in male sponges of our study (*G. phlegraei*). Various types of calcium channels (*voltage-dependent L-type calcium channel* [*cacnb4*], *cation channels of sperm relative genes* [*catsperg*, *catspere*, *catsperb*]), sodium and potassium channels (*sodium hyperpolarization-activated cyclic nucleotide-gated channel* [*hcn3*, *hcn4*], *voltage-gated potassium channel* [*kbcab2*]), and cAMP- and cGMP-related channels (*cyclic nucleotide-gated cation channels* [*cng1,3*]) were overexpressed. Several enzymes involved in the metabolism of

both cAMP and cGMP were also upregulated, for example, *high affinity cAMP-specific 3, 5-cyclic phosphodiesterase* (*pde7a*), *specific 3, 5-cyclic phosphodiesterase* (*pde6*), and *high affinity cGMP-specific 3, 5-cyclic phosphodiesterase* (*pde9a*) (fig. 8 and supplementary tables S3*B* and S8*B*, Supplementary Material online). Finally, genes related to CaM-dependent pathway, such as *calcium calmodulin-dependent kinase* (*camk2a*) and *Calmodulin-binding transcription activator* (*camta1*), were upregulated in males, indicating that this pathway could also participate in sponge sperm motility. It is definitely difficult to prove, with the current analysis, that all these genes have a role in sperm capacitation in sponges as they have general functions and they could participate in many other physiological processes. However, compared with the marine invertebrates mentioned above and also other animals and plants, in which sperm are immotile or move very slowly inside the male tract (Wolters-Everhardt et al. 1986; Jonge and Barratt 2006) acquiring high motility when induced by female chemoattractants in their surrounding (e.g., sea water) or in the female reproductive tract (Austin 1952; Gervasi and Visconti 2016), the sperm of sponges move very rapidly from the very moment of their release (Hoppe and Reichert 1987; H. Reiswig, personal communication). This suggests that capacitation in terms of enhanced motility of sperm might occur before their release into the seawater and encounter with the eggs. Indeed, presence of upregulated genes in mature sperm (*G. phlegraei*, Sp_II) coding for proteins, enriched in ejaculated and capacitated sperm of vertebrates, such as *Ras-related Rab-2* (*rab2*), *peroxiredoxin-mitochondrial* (*prdx5*), and *ropporin-1* (*ropn11*) (Rahman et al. 2017) (fig. 8 and supplementary tables S3*B* and S8*B*, Supplementary Material online), suggests that sperm capacitation happens before release from the sponge tissue. In addition, the role of the mitochondrial cytochrome b-c1 complex subunit 1 (UQCR2) and voltage-dependent anion-selective channel 2, VDAC2, whose genes, *uqcr2* and *vdac2*, were also upregulated in our case (fig. 8 and supplementary table S3*B*, Supplementary Material online), are crucial regulators of the $Ca^{+2}$ influx mediating sperm capacitation (Kwon et al. 2013; Shukla et al. 2013; Rahman et al. 2014, 2017).

External chemoattractants have been found in many marine invertebrates but are not known from sponges. Given that the sponge sperm moves rapidly upon release, two scenarios are possible: 1) female chemoattractants could be already filtered by the male sponge, inducing sperm capacitation, and 2) last stages of sperm capacitation would be completed in a further stage since most modifications are posttranslational (Rahman et al. 2017) and therefore do not have changes in expression associated. Overall, it seems that already formed spermatozoa within mature spermatic cysts of *G. phlegraei* (SP_II) had expressed not only genes related to their maturation (a complete flagellum formation) but also molecular mechanisms responsible for the next and final step, their capacitation. In this sense, sponges seemed to have a similar mechanism regulating sperm capacitation in which ion channels and membrane hyperpolarization induce the enhanced sperm motility.

## Summary

In the animal kingdom, sponges are one of the first extant taxa in which female and male sex appeared (Sara 1974; Feuda et al. 2017). Here, we show that most physiological processes and molecular signals responsible for female and male gametogenesis in other metazoans are also present in Porifera, playing a role in the formation of gametes during the reproductive season. In both female and male gametogenesis, we observed upregulated genes related with the germline fate and self-renewal at the onset of the process. Production of RA could potentially regulate the cessation of germ cell multiplication and the entry in the meiotic cycle during the early stages of gametogenesis (fig. 7) also in Porifera as happens in other metazoans (Anderson et al. 2008; Mark et al. 2015), although functional studies are required to confirm this hypothesis.

Our data suggest that, at molecular level, spermatogenesis is more complex than oogenesis in sponges. During spermatogenesis, the progress from a spermatogonium to mature sperm requires the completeness of two meiotic cycles and several physiological, morphological, and functional transformations, whereas oocyte maturation occurs at meiotic arrest, without the necessity of radical cytological changes. On the other hand, many maternal gene transcripts are silenced and expressed later on during embryonic development (Chapman et al. 2014; Reading et al. 2018), which could also explain the consistent pattern of a lesser number of upregulated genes during oogenesis than spermatogenesis in our data.

During oogenesis, we did not observe any clear morphological structures associated with the meiotic process (synaptonemal complex, chromatin compaction), but we found upregulated genes involved in the regulation of both mitotic and meiotic cycles and meiotic arrest (fig. 7). What we did observe was radical changes in cell shape and size with parallel yolk formation, symbiont accumulation, and extracellular collagen formation, which are conserved processes of oocyte maturation in metazoans (except for symbiont acquisition) that occur during the meiotic arrest phase. In spermatogenesis, we observed both the morphological features and the molecular signals related to meiosis completion (e.g., synaptonemal complex, synapsis, and DNA recombination). Although the identity of these genes and proteins was mainly defined according to their function in studies on mice as model organism (Jung et al. 2019), meiosis is a well-conserved process across most of eukaryotes (Loidl 2016). Given the morphological evidence of meiotic processes in *Geodia* (fig. 2) and other sponge species (e.g., Barthel and Detmer 1990; Gaino et al. 2007; Abdo et al. 2008; Riesgo et al. 2008), we expect these genes to have an almost identical function from Porifera to Chordata. Furthermore, we identified conserved molecular signals associated with sperm cell modification after meiosis, including spermatid generation and sperm maturation. Noteworthy was the overexpression of the molecules regulating the axoneme construction in late spermatogenesis, which allows the formation of a successful sperm flagellum. Sperm flagella have the same typical structure as any flagellum but with some extra accessory structures

(ODF, FS) for motility optimization (Kierszenbaum 2001; Inaba 2011). Sponges have other flagellated cells (choanocytes), and some species transform the above cells into spermatozoa retaining the flagellum during the transformation (e.g., Tuzet et al. 1970; Barthel and Detmer 1990; Bergquist 1996; Riesgo, Maldonado, et al. 2007), but *Geodia* spp. generate the sperm flagellum de novo (figs. 2 and 6*B*). Interestingly, genes involved in sperm flagellum formation and sperm capacitation in mammals were recovered from our sponge transcriptomes and discussed here for first time, as many of those appeared overexpressed during spermatogenesis (fig. 7).

From cnidarians to chordates, many signaling pathways regulating the onset and correct progress of gametogenesis come from surrounding cells in the gonadal tissues, follicle cells, and Sertoli cells, for instance (e.g., Jørgensen and Lützen 1997; Kiger et al. 2000; Kuznetsov et al. 2001; Kalachev and Reunov 2005; Johnston et al. 2008). Although sponges lack gonads and specialized tissues involved in reproduction, our results show great conservation in the molecular programs regulating the gametogenesis, even in pathways activated in specialized gonadal tissues in other metazoans. This suggests that the main innovations regarding sexual reproduction along animal evolution are probably more related to the cellular specialization and task division than the molecular complements regulating the formation of gamete cells.

A caveat of our study is the large interindividual variability of the gametogenic process. Even though we tried to group individuals according to their similar developmental stage, we could not always obtain consistent intra-specific expression patterns across individuals at the same developmental stage (biological replicates). This could be related to several factors: 1) the individuals could be in slightly different developmental stages than the ones defined, given that it is difficult to distinguish a possible intermediate developmental stage based on morphology alone; 2) the individuals could have different physiological status that could potentially influence the gene expression pattern; and 3) variation of environmental conditions due to different collecting time and spatial scales could have also affected the physiological status. Finally, inter-specific variation is still possible within the same genus as each species could have adopted a slightly different strategy concerning the specific time of molecular regulation for each step of the gametogenesis. However, our study highlights that, overall, *Geodia* spp. have the same molecular mechanisms to control and conduct gametogenesis in a similar way as gametogenesis occurs in bilaterians. The great number of nonannotated genes in our analysis could also be sponge-specific genes with a specific function on gametogenesis in Porifera. Our study is the most exhaustive incursion into the evolution of the reproductive machinery of these early metazoans, but we only focused on the genus *Geodia* which is exclusively gonochoristic and oviparous. To further understand the evolution of the molecular toolkit for reproduction in sponges, it would be necessary to address the genomes and transcriptomes of species spanning the four classes and also include hermaphroditic and viviparous species. Indeed, several genes necessary for sex determination and yolk formation have been recovered from the transcriptomes of hermaphroditic but not from oviparous sponges (Riesgo et al. 2014). Understanding how the reproductive toolkits have been modulated during the course of sponge evolution will have profound implications on our understanding of the evolution of sexual reproduction in animals.

## Materials and Methods

### Sample Collection and Identification of Reproduction

Specimens from the species *G. hentscheli*, *G. phlegraei*, *G. barretti*, *G. macandrewii*, and *G. atlantica* were collected from expeditions supported by the SponGES project in the boreo-arctic Northeastern Atlantic in May and September 2016 (Swedish and Norwegian coast), July 2016, and July–August 2017 (Norwegian Sea and Greenland Sea) (table 1). Information about specimens' collection was archived in PANGAEA data repository (Koutsouveli et al. 2020). Three pieces of tissue were collected from all specimens and preserved 1) in RNAlater Stabilization Solution (Thermo Fisher Scientific) and 2) in 2.5% glutaraldehyde in 0.4 M Phosphate-Buffered Saline (PBS) for histological work. Samples were then stored at 4 °C for 1 day, and then samples in RNAlater were stored at −80 °C until further processing, whereas samples for light microscopy were kept at 4 °C.

### Histological Analysis

Tissue pieces collected for histological work were rinsed in a buffer (0.4 M PBS–0.6 M salt), and post fixed in osmium tetroxide 2% in 0.4 M PBS for 2 h. After that, a thorough rinse in distilled water was performed before incubating them overnight in hydrofluoric acid 4% to remove any silica remnant from their skeleton. Samples were then rinsed with distilled water several times and dehydrated in increased amounts of ethanol (50–70–96–100%). Once dehydrated, samples were further processed for light and transmission electron microscopy.

For light microscopy, samples were embedded in paraffin (60 °C melting temperature) overnight at ∼60 °C in an oven. Once the blocks were mounted, sections with thickness 5 $\mu$m were cut in a HM325 microtome (ThermoFisher Scientific). After deparaffining with xylene, sections were stained with a standard Harris' Hematoxylin and Eosin Staining Protocol and studied in Olympus microscope (BX43) with a UC50 camera. Specimens were examined for presence of oocytes or spermatocytes. The specimens with no-reproductive elements in their tissue were considered nonreproductive (table 1). Three fragments (5 $mm^3$ each fragment) from different parts of the sponge tissue were processed for light microscopy and more than 40 sections were obtained in different parts of each fragment to confirm the presence of gametes and the consistency in developmental stage across the body.

For transmission electron microscopy, samples were embedded in LRW resin according to the guidelines of the manufacturer (agar Scientific) overnight. Ultrathin sections of ∼60 nm were obtained with an Ultracut Reichert-Jung ultramicrotome, stained with 2% uranyl acetate/lead citrate (Reynolds 1963), and observed with a Hitachi Transmission Electron Microscope TEM (H-7650) at 80 kV.

## Transcriptomic Analysis

### *RNA Extraction and Library Preparation*

Twenty-two samples of five *Geodia* species were used for the transcriptomic analyses (table 1). Total RNA extraction was performed with a standard TRIzol Reagent (ThermoFisher Scientific) protocol, with an overnight incubation in isopropanol and two consecutive rinses in 100% ethanol of the RNA pellet. Further mRNA purification was done with the Dynabeads mRNA DIRECT kit (ThermoFisher Scientific), starting from the final stage of the protocol, "Elimination of rRNA contamination" and only slight modifications of time incubations. The quantity and overall quality of mRNA were assessed using NanoDrop 2000 (ThermoFisher Scientific) and Qubit RNA Assay kit (ThermoFisher Scientific). Then, cDNA libraries were constructed with either Truseq v2 or Scriptseq v2 kit (Illumina), according to the manufacturer's instructions, and ~50 ng of starting mRNA. For all purification steps, we used AMPure XP beads (Beckman Coulter). The cDNA quantity was assessed with a Qubit dsDNA HS Assay kit (ThermoFisher Scientific) and the quality of the libraries was checked with an Agilent Tapestation 2200 system (Agilent Technologies). The libraries were then pooled and further sequenced with an Illumina NextSeq 500 platform at the Natural History Museum of London sequencing facilities (Molecular Core Labs), using a paired-end read strategy (bp length: 2× 150 bp). The raw reads of all the 34 libraries were deposited at Sequence Read Archive with BioProject ID: PRJNA603347 and submission ID: SUB6821624.

### *Assembly and Annotation*

The quality control of the raw reads was conducted with FASTQC (Andrews 2010). The reads were cleaned with Trimmomatic (Bolger et al. 2014). Only the clean paired reads were subsequently used for the de novo assembly for each species with Trinity v2.4.2 (Grabherr et al. 2011). The transcript quantification was conducted using the alignment-based quantification method RSEM (Li and Dewey 2011), supported by the Trinity package, with the script align_and_estimate_abundance.pl. We tested the quality of the assembly by testing the percentage of properly paired-end reads that were aligned to the assembly with bowtie2 (Langmead and Salzberg 2012) and by calculating the transcriptome completeness with Benchmarking Universal Single-Copy Orthologs (Busco V2/3) against metazoan cassettes (Simão et al. 2015). Finally, we calculated the Nx length, GC content, and assembled bases with Trinity.

For the annotation, we used Diamond (Buchfink et al. 2015) against the selection of metazoan proteins in the *swissprot* database (Boeckmann et al. 2003) accessed in 2018 with a cutoff *e*-value of 1e-5. The sequences with blast hits were further annotated by Blast2GOPro (Conesa et al. 2005) to retrieve the functional information from the GO terms.

### *Differential Gene Expression Analysis*

We estimated the mapped read abundance using RSEM (Li and Dewey 2011) and then the differential gene expression (DE) analysis was conducted with edgeR (Robinson et al. 2010; McCarthy et al. 2012) with the following parameters: false discovery rate ≤0.01 and 2-fold change differences. We did pairwise comparisons between the two sexes (female vs. male) and female/male versus nonreproductive (NR) along the different species, taking into account that in some cases there were no replicates for some conditions (table 1). In these cases, we used dispersion 0.1 for the conduction of DE analysis with edgeR. The results regarding unreplicated analyses were taken with caution, and only discussed if they were similar to the results obtained in the replicated analyses of the rest of species. The terms "upregulated" and "overexpressed" genes in the Results and Discussion section (see above) refer to genes extracted with either of the two analyses. The difficulty for an intense sampling and appropriate replication for all the reproductive conditions in all species has to be considered, as the studied species come from deep-sea habitats with scarce access over the year. In order to understand how many orthologous upregulated genes were shared among the species during oogenesis and spermatogenesis, we used online platform OrthoVenn, uploading the translated peptide sequences of the upregulated genes in each condition for each species.

The GO terms associated with differentially expressed genes were used to obtain GO enrichment analysis by Fisher's exact test in Blast2GOPro platform (Conesa et al. 2005) for all the studied species with a threshold of *P* value 0.05. Genes upregulated from all the pairwise comparisons (for oogenesis: females vs. NR and vs. males, and for spermatogenesis: males vs. NR and vs. females) were used for the analysis in order to understand which genes and GO-enriched categories were expressed during the different developmental stages of oogenesis and spermatogenesis across the different species. The percentage of sequences contained in each GO term was extracted and used for the depiction of a bubble graph in R. In some cases, more than one GO term appeared enriched for the same process, so we joined the relevant categories to have a final summary percentage.

## Proteomic Analysis

In this study, we performed a complementary proteomic analysis to support our results derived from the transcriptomic data. We used two female and two male individuals of different species and different developmental stages: one female of *G. macandrewii* (Vi_II), one female *G. atlantica* (Vi_III), one male of *G. phlegraei* (SP_I), and one male of *G. atlantica* (SP_II). The protein extraction from freeze-dried samples was performed according to the protocol mentioned by Viarengo et al. (1997) and applied previously in sponges (Wanick et al. 2013). The protein extracts were concentrated in a stacking/resolving gel interface. The unseparated protein bands were visualized by Coomassie staining, excised, and cut for protein digestion (Moreno et al. 2014). The dried gel pieces were reswollen in 100 mM Tris–HCl pH 8, 10 mM $CaCl_2$ with 60 ng/$\mu$l trypsin or chymotrypsin at 5:1 protein enzyme (w/w) ratio. The tubes were kept in ice for 2 h and incubated at 37 °C (trypsin) or 25 °C (chymotrypsin) for 12 h. Digestion was desalted onto OMIX Pipette tips C18 (Agilent Technologies) until the mass spectrometric analysis.

Protein separation was performed with reverse phase-liquid chromatography RP-LC-MS/MS analysis (dynamic exclusion mode) in an Easy-nLC II system coupled to an ion trap LTQ-Orbitrap-Velos-Pro hybrid mass spectrometer (ThermoFisher Scientific). Peptides were eluted using a 180-min gradient (solvent A: 0.1% formic acid in water, solvent B: 0.1% formic acid, 80% acetonitrile in water). Peptide identification from raw data was carried out using PEAKS Studio 10 search engine (Bioinformatics Solutions Inc.) (Han et al. 2011; Zhang et al. 2012). Database search was performed against the transcriptome assembly for each species. False discovery rate for peptide spectrum matches was limited to 0.01. Only proteins with at least two distinct peptides identified from LC/MS/MS analyses were considered reliably identified.

### Phylogenetic Analyses

To understand the phylogenetic relationships among Sox genes, we screened for Sox genes in our transcriptomes of all *Geodia* spp. Three Sox genes were identified in all species, although one of them in *G. hentscheli* was too small to be included in the analysis. Ninety-three sequences of the high-mobility group domain of the Sox transcription factor (79 amino acids) that were used in Fortunato et al. (2012) and Schnitzler et al. (2014), as well as the Sox sequences from *E. muelleri* (Jager et al. 2006) were aligned with our 14 sequences retrieved from *Geodia* spp. using MAFFT v7 (Katoh and Standley 2013). The best evolutionary model was selected with ProtTest-HPC (Darriba et al. 2011) based on the Akaike Information criterion. A phylogenetic hypothesis was obtained with RAxML v8.1.22 (Stamatakis 2014) with LG-GAMMA as parameters of model evolution and 100 replicates for bootstrap sampling. The same pipeline was employed for fibrillar (types I, V, and XI) and nonfibrillar (type IV and spongin short-chain) collagens (separately) recovered as upregulated genes in our transcriptomes and sequences of these types of collagens retrieved from NCBI. For collagens, the full genes were used for the phylogenies. Accession numbers for the sequences used to construct the Sox phylogeny can be found in Fortunato et al. (2012) and Schnitzler et al. (2014), whereas those for fibrillar and nonfibrillar collagen are shown in the trees in supplementary figure S3, Supplementary Material online.

## Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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