



OPEN Physalia gonodendra are not yet sexually mature when released

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The blue bottle genus *Physalia* is one of the well-known siphonophore belonging to the Cnidaria, Hydrozoa. *Physalia* is also known as a ferocious predator, occasionally stinging and fatally wounding humans, but key details of its life cycle and reproductive biology are unclear. *Physalia* have separate sexes, and sexual reproduction occurs through the release of complex structures called gonodendra that contain many gonophores that will release either eggs or sperm. It is not known how mature the gonophores are when the gonodendra are released. In this study, we aim to characterize germ cell maturation by conducting histological, cytological, and gene expression analyses of the gonodendron of *Physalia utriculus* from Japan. We found a layered structure of the gonophore, consistent with other studies; however, gametes were not found even in gonophores that were within the released gonodendra. Moreover, haploid cells were not detected by flow cytometry. Analysis of the expression of putative germ cell marker and meiosis related genes showed high expression in the gonophore. These results strongly suggest that germ cells do not mature until after gonodendra are released. These findings provide valuable insights into the reproductive ecology and life cycle of *Physalia*.

Keywords Gonophore, Germ cell, Flow cytometry, Gene expression, Histology

The genus *Physalia*, commonly known as the blue bottle or Portuguese Man O' War is one of the most well-known siphonophores (Cnidaria, Hydrozoa, Cystonectae). *Physalia* is a member of Cystonectae, a small clade that is sister group to other siphonophore, and has many unique attributes specific to its life at the surface of the ocean^{1–3}. *Physalia* is pleustonic animal that float on the surface of seawater and is widely distributed in oceans globally, including the Atlantic Ocean, Pacific Ocean, and Indian Ocean^{4–11}. *Physalia* is a ferocious predator^{12–14}, with occasional reports of stinging humans to deaths¹⁵, making its ecology of great interest. However, much of its lifecycle and reproductive ecology are poorly understood, including where and how it reproduces, develops, and drifts to the coastlines.

Members of the order Siphonophorae, to which *Physalia* belongs, possess a complex life cycle, in which individual bodies (i.e., zooids), with specialized morphology and function, form highly sophisticated colonies. The morphology of individual zooids is so specialized that they function much like “organs”, and the entire colony behaves like a single organism, such that the entire colony is sometimes described as a “superorganism”^{1,16,17}. The zooids within a colony are generated through asexual budding, while new colonies are reproduced sexually^{1,6,18–20}. In some species of *Physalia* although juveniles have been described^{3,21}, its life cycle is not fully elucidated, and sexual reproduction is poorly characterized.

In siphonophores, sexual reproduction occurs via gonophores. These zooids are reduced medusae that bear gametes but lack structures for other functions such as feeding. In cystonects, including *Physalia* (Fig. 1), the gonophores are born in complex branching structures known as gonodendra that contain other zooid types as well³. *Physalia* gonodendra grow from the main colony while it floats at the surface, but then break away and sink before gamete release and fertilization (Supplementary Figure S1; Table 1). Because sexual reproduction occurs away from the mature colony, many features of sexual reproduction in *Physalia* are relatively inaccessible to study and there are many critical factors that remain unknown. This includes basic facts about whether gametes are generated before or after gonodendra are released, how much time elapses between gonodendron release and spawning, how spawning is coordinated in space and time, and whether eggs are released from gonophores before fertilization.

Like other species of Cystonectae, *Physalia* is dioecious. This means that sexes are separate and each *Physalia* produces all male gonophores or all female gonophores. This is the ancestral state in siphonophores, with

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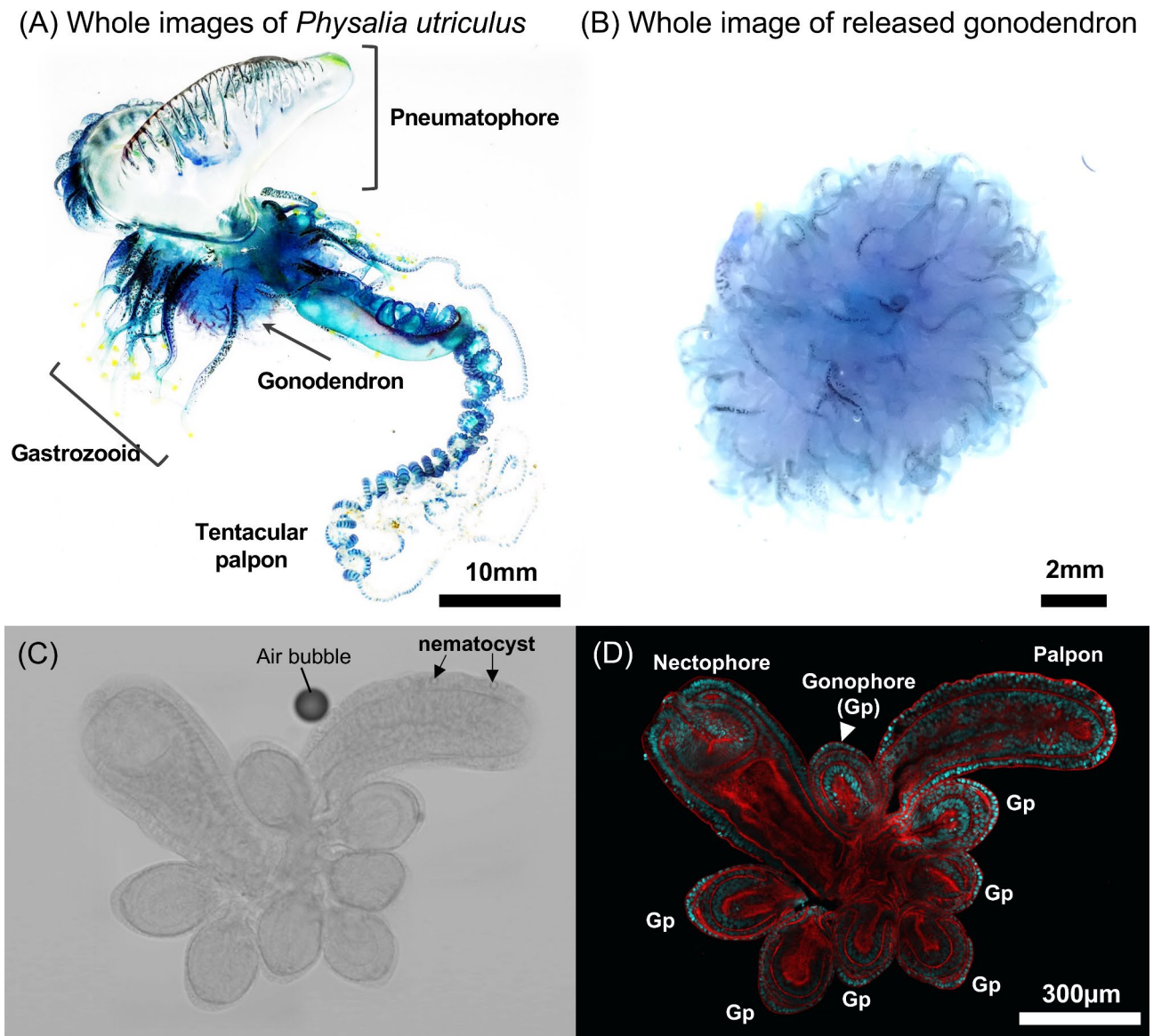


Fig. 1. Whole images of *Physalia utriculus* and anatomical structures of gonodendron. Whole image of *P. utriculus* (A). In this species, pneumatophore: gas filled float, gastrozoid: feeding zooid, tentacular poalpon: tentacles attached to foraging zooid, gonodendron: putative reproductive structures. Whole image of gonodendron which released from the colony (B). Fine anatomical structures of gonodendron. nectophore: medusa like structures which involved in locomotion, palpon: digestive zooid, gonophore: putative gonad (C,D). Bright field images (C) and fluorescent images (D). DAPI signals targeting host nuclear DNA (blue), and fluorescent phalloidin signals targeting muscular actin filaments (red), respectively.

Date	Sites	Observers	Latitude, longitude	References
December 28, 2018	Wollongong NSW, Australia	Lou De Beuzeville	-34.593209, 150.900495	https://www.inaturalist.org/photos/29791487
December 19, 2019	Port Macquarie-Hastings - Pt B, New South Wales, Australia	Thomas Mesaglio	-31.622383, 152.834835	https://www.inaturalist.org/photos/59465554
June 29, 2022	Miura, Kanagawa, Japan	Kohtsuka Hisanori	35.159252, 139.611907	This study (Fig. S3A)
November 11, 2023	Miura, Kanagawa, Japan	Kohei Oguchi	35.159252, 139.611907	This study (Fig. S3B)
December 10, 2023	South Pacific Ocean, New Plymouth City Ward, Taranaki, NZ	paritutu_beach_comber	-39.063856, 174.019687	https://www.inaturalist.org/photos/340267386

Table 1. Observed examples of released gonodendron.

subsequent shifts to monoecy (colonies that produce both male gonophores and female gonophores) in some siphonophore groups^{2,3}.

However, previous work has characterized the gross morphology of gonodendra and gonophores^{4,11,16,22,23}. These studies confirm that, as in other siphonophores, the sex of *Physalia* can be established based on gonophore morphology. Male gonophores have a conspicuous centrally located spadix²². In female gonophores, the spadix takes up nearly the entire gonophore, so is not apparent as a distinct internal structure²². However, gametogenesis and gametes remain poorly described, and little is known about sexual maturation.

In this study, in addition to the histological observation of gonodendra, we characterized germ cells of *Physalia utriculus* from the coast of Sagami Bay in Kanagawa prefecture, Japan based on the diagnosis of the nuclear phase using flow cytometry, and further clarified their localization by gene expression analysis. Genes encoding *vasa* and *piwi* are widely conserved in metazoans and are markedly expressed in germ and stem cells²⁴. In hydrozoans, these genes are expressed not only in germ cells, but also in stem cells called i-cells, which are widely distributed in epithelial cells^{25,26}. Particularly in *Nanomia bijuga*, a species of siphonophore, these genes are strongly localized in both female and male gonophores²⁷, and were used as markers for putative germ cells in this study. Moreover, to characterize stage of germ cell development, expression level of conserved meiosis related genes *Dmc1*, *Mnd1*, *Msh4/5* and *Sycp3*^{30,31,32,33} was focused. Additionally, *Agalma okenii* (Physonectae) was used to ensure technical validity in the diagnosis of karyotypic phases by flow cytometry. Species of the genus *Agalma* form hermaphroditic colonies, with distinct sexes of gonophore^{2,28}, and cells in the process of gametogenesis were easily detectable.

Results and discussion

Morphological and histological observations of a gonodendron in *Physalia utriculus*

Morphological and histological observations were performed on gonodendra to examine presence of gametes and germ cells. We observed an ovoid gonophore, a pouch-like digestive zooid as the palpon, and a nectophore (Fig. 1C, D), consistent with other studies^{3,4,22}. Histological observation using confocal laser-scanning microscope (CLSM) revealed that the palpon has a simple two-layered structure, whereas the gonophore has at least three layers (Fig. 1D) as shown in previous studies²². The apical region of the nectophore was complex and intricate (Fig. 1D). Some of the collected samples showed release of gonodendron within a day immediately after collection (Supplementary Table S1). Among the five released gonodendra samples, the minimum size of gonophore was 151 μm , the maximum was 1037 μm , and the average was $599 \pm 188 \mu\text{m}$ (Supplementary Figure S2).

Detailed histological structures were observed in various sizes of gonophores (Fig. 2). The gonophore was approximately oval in shape, extending in the direction of the apical axis as it developed (Fig. 2). Gonophores with diameters of 200 μm and 250 μm contained an outer epithelial cell layer, an inner gastrovascular cavity, and gastrodermic cells (Fig. 2A, B, C). A distinct two or three layers of cells was present between epithelial and gastrodermic cells, referred to as the germ cells in other studies^{4,11,16,22,23}. Gonophores with major axis diameters of 450 μm had a three-layered structure, consisting of epidermal epithelial cells, putative germ cells, and gastrodermic cells, but had developed a gastrovascular cavity and lumen (Fig. 2D). Gonophores with major axis diameters of 800 μm had a thickened gastrodermic cell layer and a structure with an intricate lumen (Fig. 2E). In all gonophores studied, we observed no variation in the morphology of putative germ cell nuclei, as well as no oocytes or spermatozoa.

Nuclear phase diagnosis by flow cytometry

Since morphologically distinct gametes were not identified in histological surveys (Fig. 2), a diagnosis of the nuclear phase was performed using flow cytometry to clarify whether haploid cells (gametes) were actually absent. In *P. utriculus*, somatic cells derived from the pneumatophore, gastrozooid, and tentacular palpon showed a large peak in cell count at ~ 20 of the BUV395 signal and a smaller peak at ~ 40 (Fig. 3A–C). We considered cells at the large and small peaks to be diploid (2C) and tetraploid (4C), respectively, in which DNA had been replicated for cell division. In addition, only two peaks were detected in the gonodendron (Fig. 3D). Haploid cells (1C) should be detected as a peak at approximately one-half of the 2C signal. Therefore, no haploid cells were detected in the gonodendron of *P. utriculus* in this study. Interestingly, more tetraploid cells were detected in gonodendron than in other parts (Fig. 3). Considering that no distinct meiotic pairing chromosome or dividing cells were observed (Figs. 2 and 3), gonophores might be in the early stages of meiosis.

To ensure technical validity and to confirm these results, the nuclear phase of each part was analyzed using *A. okenii*, in which species gametes and germ cells are clearly present (Supplementary Figure S5). In *A. okenii*, two peaks (diploid and tetraploid cells) were detected in the tentacle, and feeding zooid: gastrozooid (Supplementary Figure S5B, C), whereas three distinct peaks (diploid, tetraploid, and haploid cells) were detected in the female-gonozooid and male-gonozooid (Supplementary Figure S5D, E). This result indicates that the experimental system accurately detects nuclear ploidy in these species.

Gene expression analysis for putative germ cell markers and meiosis related genes

Both histological observations and nuclear phase diagnostics were unable to detect gamete, however, tetraploid cells were more frequently detected in gonodendra. We therefore sought to characterize earlier stage germ cells in the gonophores. We performed gene expression analysis of putative germ cell markers (*piwi*, *vasa-1*, and *vasa-2*) and meiosis related genes (*Dmc1*, *Mnd1*, *Msh4/5* and *Sycp1*) (Fig. 4). Real-time qPCR analysis showed significantly higher expression of *piwi*, *vasa-1*, and *vasa-2* in the gonophore than other structures (Fig. 4A). Detailed gene expression localization visualized by whole-mount in situ hybridization (WISH) revealed clear *piwi*, *vasa-1*, and *vasa-2* signals in the gonophore, whereas no signals were detected in the nectophore, palpon, etc. (Supplementary Figure S3). These genes were expressed at a relatively superficial level of the gonophore

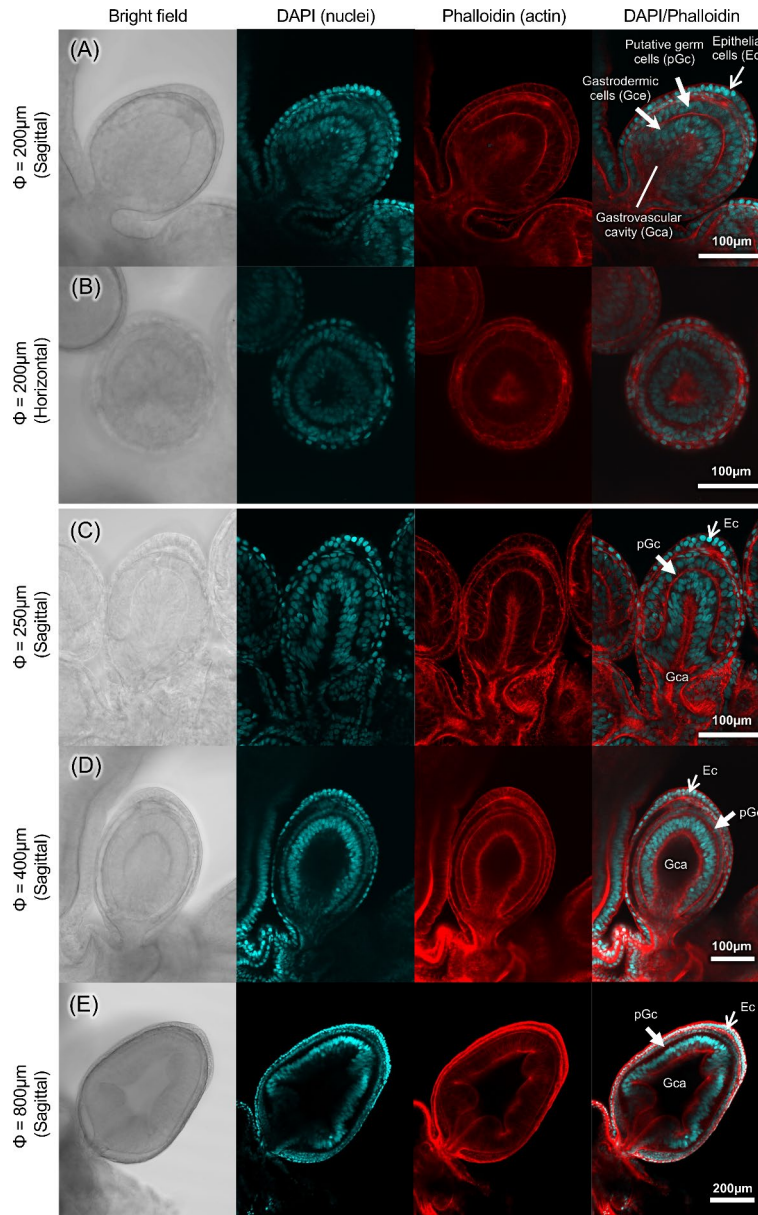
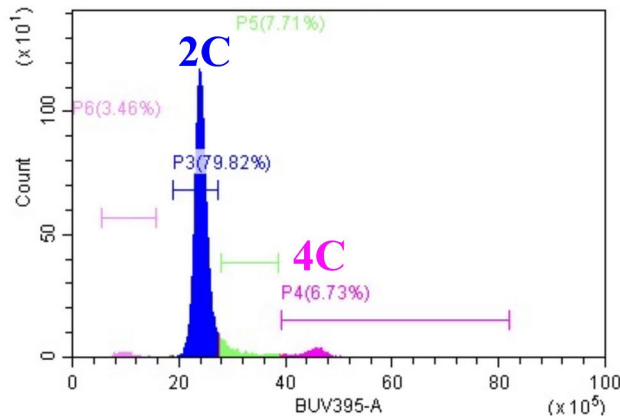


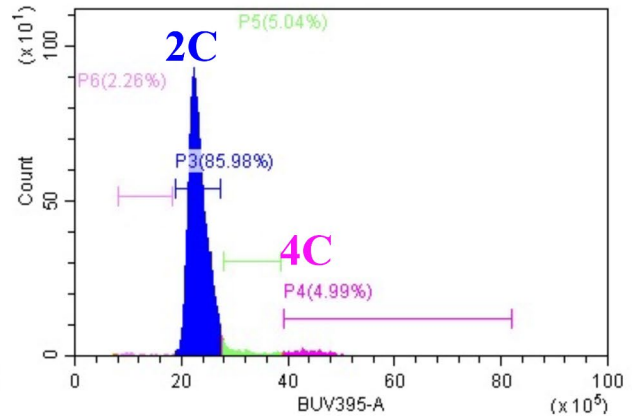
Fig. 2. Histology of gonophore in various developmental stages of *Physalia utriculus*. Sagittal section of a gonophore with a major axis diameter of 200 μm (A). Horizontal section of a gonophore with a major axis diameter of 200 μm (B). Sagittal section of a gonophore with a major axis diameter of 250 μm (C), 400 μm (D) and 800 μm (E). From left to right: brightfield, DAPI fluorescence image, phalloidin fluorescence image, and DAPI/phalloidin stacked image. DAPI signals targeting host nuclear DNA (blue), and fluorescent phalloidin signals targeting muscular actin filaments (red), respectively. Epithelial cells (Ec), gastrovascular cavity (Gca), gastrodermic cells (Gce) and putative germ cells (pGc) were seen in every developmental stages.

(Supplementary Figure S3), confirming that the cell layer near the surface is the germ cell layer. Both real-time qPCR and WISH results strongly suggest that the putative germline marker genes are expressed in the gonophore inside the gonodendron, and the germ cells are distributed in the gonophores (Fig. 4, Supplementary Figure S3). Additionally, high expression of meiosis-related genes was observed only in gonophores (Fig. 4B). These genes were highly conserved in eukaryota, involved in interhomolog recombination, stable heteroduplex DNA formation after double strand break and expressed in the early stages of prophase I^{29–32}. It was therefore considered that the germ cells were in prophase I stage and that development of germ cells might be arrested at this stage until the gonodendron were released.

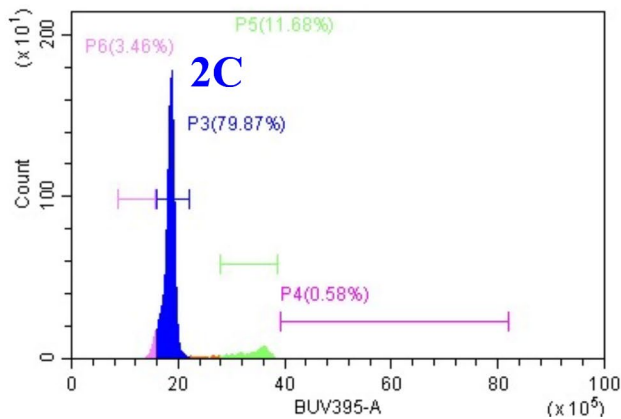
(A) Pneumatophore



(B) Gastrozoid



(C) Tentacular palpon



(D) Gonodendron

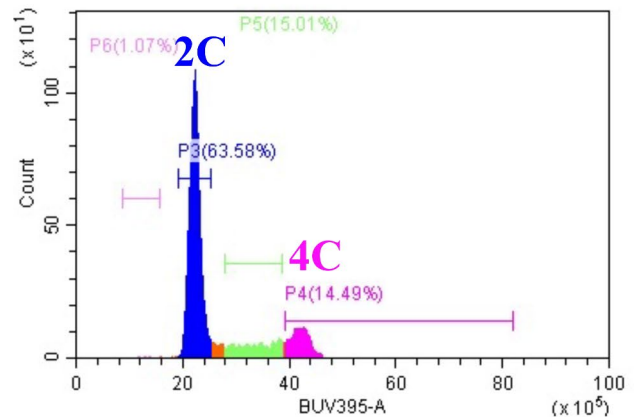


Fig. 3. Diagnosis of karyotypes in the several structures of *Physalia utriculus*. Pneumatophore (A), gastrozoid (B), tentacular palpon (C) and gonodendron (D). Diploid (2C) and tetraploid (4C) cells were seen in every structure, while monoplod cells (1C) were not detected.

Conclusion

Where and how do they reproduce? — insights into the reproductive ecology and life cycle

In this study, (i) we observed no morphologically distinct gametes in the gonodendron and gonophore (Figs. 1 and 2), (ii) we observed no haploid cells (gametes) in the gonodendron (Fig. 3), and (iii) we did find putative germ cell marker genes expressed in the gonophore (Figs. 4 and 5), (iv) we revealed germ cells were in prophase I stage of meiosis.

These findings indicate that all the gonodendra observed in this study were not yet sexually mature. These results are consistent with the histological characteristics of other study⁴, suggesting that sexual maturation of gonophores occurs after gonodendron release.

All of the samples examined in this study were either washed ashore or collected in the intertidal zone of reefs along the coast of Sagami Bay, Japan. Because most of the samples drifted ashore after typhoons or strong winds blowing from the south and only a few cases of immature populations have been reported, gonodendra do not develop in shallow waters, at least not in the Sagami Bay area. The origin of this population is unclear, but because *Physalia* are often found in tropical and subtropical oceans³³, they may reproduce near the equator and drift to the waters around Japan on the wind and waves. The internal structures of the gonodendron are complex and consists of various parts (Fig. 1). The functions of these parts are largely unknown. The palpon contains cnidocytes (Fig. 1C), the nectophore is open to the outside (Fig. 1D), and the gastrovascular system is well-developed and connected to the gonophore (Figs. 1 and 2). Our work here sets the stage for describing the subsequent steps in *Physalia* reproduction after the gonodendra are released. Many questions remain about how long it takes them to sexually mature, what depths they sink to, if gamete release is coordinated to maximize fertilization success, and whether spawning occurs all at once or is a gradual process.

Methods

Sample collection and morphological observation

Samples of *Physalia utriculus* and *Agalma okenii* were collected by plastic bags from the rocky shores and beaches at Kanagawa prefecture, Japan, in 2020–2024 (Supplementary Table S1). Specimens were immediately dissected

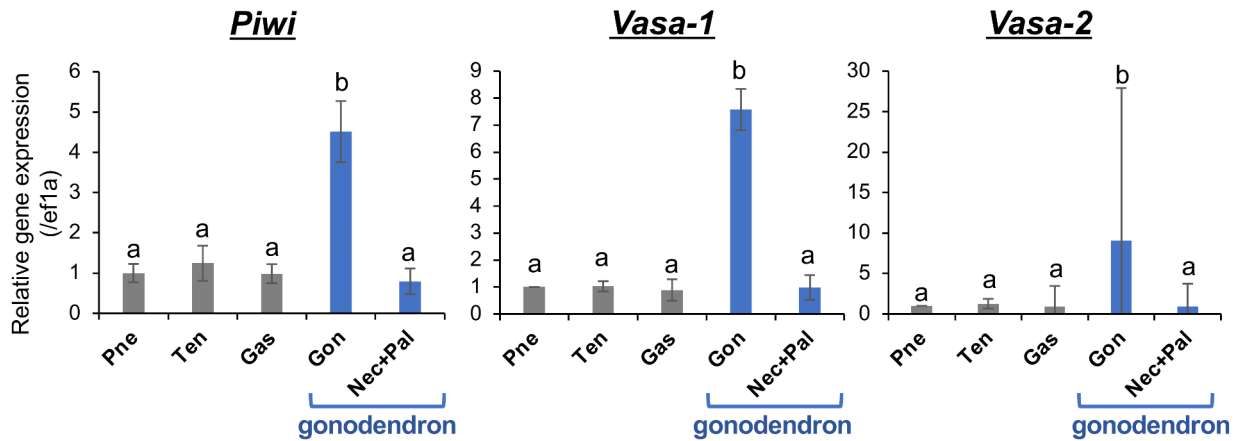
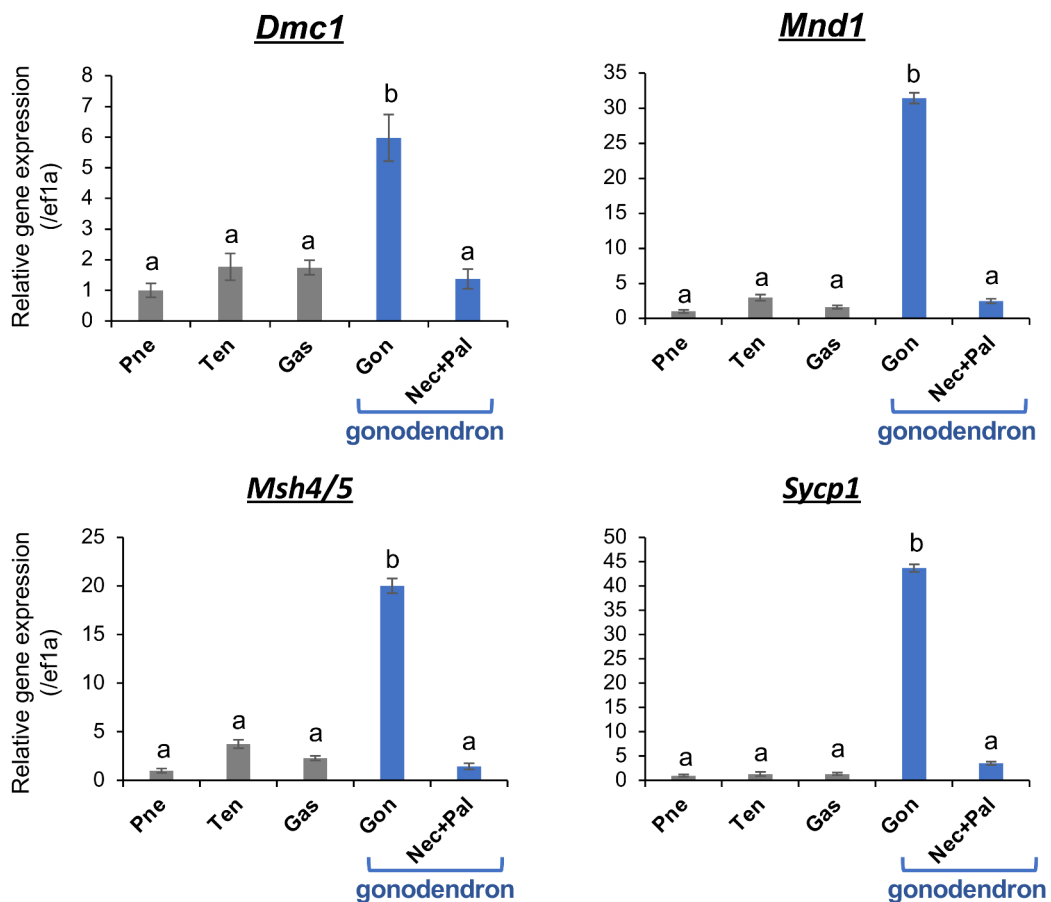
(A) Putative germ cell marker genes**(B) Meiosis related genes**

Fig. 4. Gene expression profiles using real-time qPCR of three putative germ cell marker genes (*piwi*, *vasa-1* and *vasa-2*) (A) and four meiosis related genes (*Dmc1*, *Mnd1*, *Msh4/5* and *Sycp3*) (B) in several structures of *P. utriculus*. Relative expression levels of targeting genes (mean \pm S.D., $n=4$) were calibrated using an internal control (*ef1a*) (A). Different letters shown above the bars indicate significant differences among categories (Tukey's test, $p < 0.05$). Pneumatophore (Pne), tentacular palpon (Ten), gastrozooid (Gas), gonophore (Gon) and other structure in gonodendron i.e., nectophore and palpon (Nec+Pal).

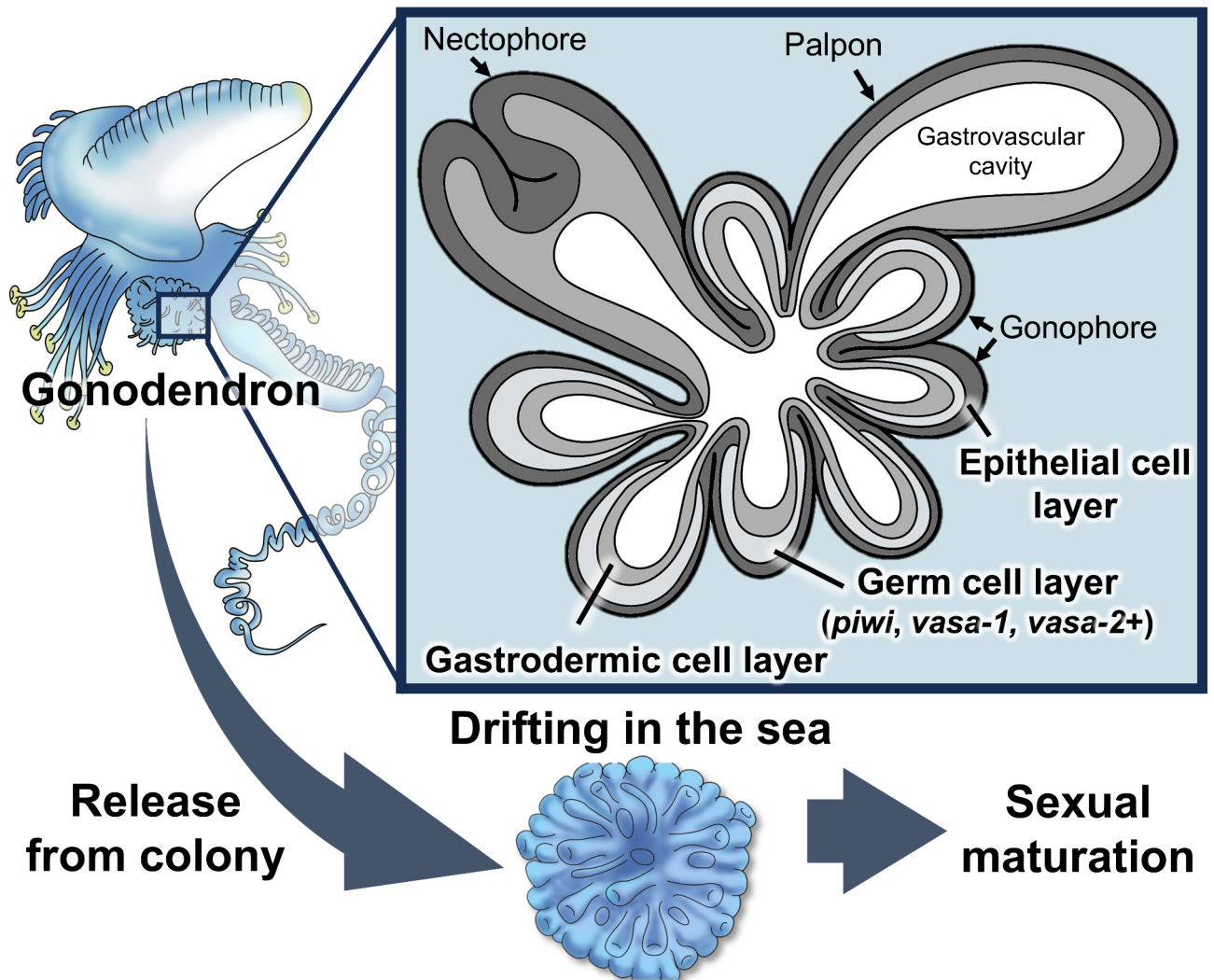


Fig. 5. Schematic diagram showing the localization of germ cells and hypothetical sexual maturation processes in *Physalia*. Whole anatomy of *Physalia* and detailed histological structures of gonodendra.

using fine tweezers under a stereomicroscope SZX16 (Olympus, Tokyo, Japan) and fixed in 4% paraformaldehyde (PFA). Among the collected *Physalia* samples, especially those that released gonodendra (Supplementary Figure S1; Table 1), the long axis diameter of the gonophore was measured to determine the degree of maturity.

Histological observation by confocal laser-scanning microscopy

To investigate the histological features, the gonodendron was observed using a confocal laser-scanning microscopy (CLSM). Gonodendrons were dissected out from 10 specimens, fixed in PFA in filtered seawater for > 3 h, and washed for 15 min with 0.3% Triton-X 100 in 1× phosphate-buffered saline (PBST) at least three times. The nuclei (DNA) and cytoskeleton (F-actin) were stained with 40,6-diamidino-2-phenylindole (DAPI) (2 µg/mL; Sigma, St. Louis, MO, USA) and rhodamine-phalloidin (1:40; Invitrogen, Paisley, UK), respectively, for 1 h at room temperature. Then, the samples were washed twice in PBST, mounted in 50% glycerol in PBS, and observed under the CLSM (FV3000; Olympus, Japan).

Cytological examination by flow cytometry

To detect gametes and haploid germ cells, the nuclear phases composing the gonodendron were diagnosed using flow cytometry (*P. utriculus*: $n = 10$, *A. okenii*: $n = 3$). Using the DNA content of diploid somatic cells derived from several parts of *P. utriculus* and *A. okenii*, which have obvious developed gonads (Fig. 4; Supplementary Figure S5), nuclear DNA content and ploidy were evaluated. Briefly, several parts were homogenized with the buffer of a Single Cell Isolation Kit (Invitrogen Biotechnologies, Plymouth, MN, USA), stained with DAPI, and fixed in 4% PFA. Nuclear DNA was quantified using a CytoFLEX flow cytometer and monitoring CytExpert software (Beckman Coulter, CA, USA).

RNA extraction and cDNA synthesis

To identify the location of germ cells, the expression of putative germ cell marker genes was analyzed. Several parts, including the pneumatophore, tentacular palpon, gastrozoid, gonophore, and other structure in gonodendron (i.e., nectophore and palpon) (Fig. 1), were dissected from four specimens. Total RNA was extracted using ISOGEN (NIPPON GENE, Tokyo, Japan) and treated with DNaseI (Thermo Fisher Scientific, Waltham, MA, USA). For each sample, 200 ng of total RNA was reverse transcribed with a HighCapacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

Gene identification

For gene expression analysis, orthologs of candidate germ cell marker genes were searched in the transcriptome database of *Physalia* (Transcriptome Shotgun Assembly of NCBI: accession numbers: SRX5780675-SRX5780684; SRX5780715- SRX5780720)³⁴. Protein sequences of candidate genes (*Piwi*: AHI50300.1; *Vasa-1*: AHI50304.1; *Vasa-2*: AHI50305.1; *Mnd1*: NP_115493.1; *Dmcl1*: NP_008999.2; *Msh4/5*: NP_002431.2; *Sycp3*: NP_001171419.1) were used as queries for tBLASTn searches against the *P. physalis* dataset. The obtained nucleotide and amino acid sequences in *P. physalis* were aligned with orthologous sequences in several metazoan lineages with ClustalW. To verify the orthology of candidate genes, phylogenetic analyses were performed with MEGA-X³⁵ (Supplementary Figure S3, S4). Best-fit evolutionary models were determined according to Bayesian information criterion and bootstrap support values were estimated using 1,000 runs. Based on the obtained sequences, primers for real-time quantitative PCR (qPCR) and probes for in situ hybridization were designed using Primer3 software³⁶ (Supplementary Table S2).

Gene expression analysis by real-time qPCR

Real-time qPCR was performed using SYBR Green Master Mix with the sequence detection system ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA), using the relative standard curve method. RefFinder software³⁷ used to analyze gene expression level of glyceraldehyde 3-phosphate dehydrogenase (*gapdh*), 18 S rRNA, ribosomal protein S9 (*rps9*), and elongation factor 1 alpha (*ef1a*) to identify suitable reference genes for quantification. Finally, *ef1a* was chosen as the best reference gene. Relative gene expression was measured compared with the reference gene *ef1a*. For statistical analysis, Tukey's multiple comparisons test ($n = 4$; $p < 0.05$) was performed after one-way ANOVA ($p < 0.05$), using R 4.2.2 (<https://www.r-project.org>).

Whole-mount in situ hybridization

To examine the detailed localization of *piwi*, *vasa-1*, and *vasa-2*, whole-mount in situ hybridization (WISH)²⁷ was performed on the gonodendron of *P. utriculus*. First, the target genes were amplified by specific primers with T7 and SP6 sequences (Supplementary Table 2). DIG-labeled sense and antisense RNA probes were prepared by in vitro transcription using a DIG Labeling Kit (Roche Diagnostics, Mannheim, Germany). The gonodendron was dissected and fixed with 4% PFA/PBS. The samples were treated sequentially with PBST for 10 min (three times), 10 µg/mL proteinase K (20 min), and 4% PFA/PBS (20 min), then the samples were prehybridized for 1 h at 60 °C in a hybridization buffer [5× SSC, 50% formamide, 0.1% Tween 20, 5× Denhardt's solution (Nacalai tesque, Kyoto, Japan), 750 µg/mL Salmon sperm DNA (Roche Diagnostics, Mannheim, Germany)], samples were hybridized with the digoxigenin-labeled RNA probes at 60 °C for approximately 24 h.

To visualize signals, hybridized samples were rinsed two times in buffer I (50% formamide, 2× SSC with 0.1% SDS) for 60 min, washed in buffer II (2× SSC with 0.1% SDS), incubated in blocking buffer (1% blocking reagent in buffer II) for 60 min, and then incubated with the anti-DIG-AP conjugate (Roche Diagnostics, Mannheim, Germany) (500× dilution) in buffer II for 60 min. The samples were washed two times with PBST for 15 min, and the hybridized probe was detected by incubating with BM-purple (Roche Diagnostics, Mannheim, Germany) solution for 2 h at room temperature. The reaction was stopped with 4% PFA/PBS. The samples were washed with PBST, mounted with methanol solution, and images were captured using a stereomicroscope SZX16 (Olympus, Tokyo, Japan) connected to a DP26 CCD camera (Olympus, Tokyo, Japan).

Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files.

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Author contributions

K.O. and C.W.D. conceptualized and designed the study. K.O. performed histological, cytological and gene expression analysis. K.O., G.Y., and H.K., performed field sampling and morphological observations. All authors wrote the manuscript and approved the final version of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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