Title

Stem cells in a colonial animal with localized growth zones

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Summary

Siphonophores (Hydrozoa) have unparalleled colony-level complexity, precision of organization, and functional specialization between zooids (i.e., the units that make up colonies). Previous work has shown that, unlike other colonial animals, most growth in siphonophores is restricted to one or two well-defined growth zones that are the sites of both elongation and zooid budding. To understand this unique growth at the cellular level, we characterize the distribution of interstitial stem cells (i-cells) in the siphonophore *Nanomia bijuga*. Within the colony we find that i-cells are present at the tips of the growth zones, at well-defined sites where new zooid buds will arise, and in the youngest zooid buds. As each zooid matures, i-cells become progressively restricted to specific regions until they are mostly absent from the oldest zooids. We find no evidence of the migratory i-cells that have been observed in colonial cnidarian relatives. The restriction of i-cells to particular developing structures and sites of growth suggest a plant-like model of growth for siphonophores, where the growth zones function much like meristems. This spatial restriction of stem cells could also explain the precision of colony-level organization in siphonophores as a consequence of restricted growth potential.

Highlights

- Siphonophore stem cells are largely restricted to growth zones and developing zooids
- No evidence for migratory capacities of these stem cells
- The growth zones of siphonophores function much like the meristems of plants
- Restriction of stem cells may play a major role in facilitating the precision of siphonophore growth but also lead to reduced developmental plasticity

Results

Colonial animals provide a unique opportunity to investigate general questions about the evolution of development, and to better understand development beyond embryogenesis [1-3]. Animal colonies arise when asexual reproduction is not followed by physical separation [4]. This results in many genetically identical multicellular bodies, known as “zooids”, that are attached and physiologically integrated to form a colony. Each zooid is homologous to a solitary free-living animal, but lives as part of the colony. The complex lifecycles of colonial animals require multiple developmental processes - the embryological development of the zooid that founds the colony, the asexual development of subsequent zooids, and the colony-level development that regulates larger-scale colony formation including zooid placement [3].

Siphonophores are pelagic colonial hydrozoans (Cnidaria). Among colonial animals, they have both the highest degree of functional specialization between zooids and the most precise and complex colony-level organization [3]. In most siphonophore species, zooids are arranged in intricate repeating patterns along a linear stem (Figure 1). Each colony has one or two main growth zones (depending on the species) where stem elongation takes place and new zooids arise by budding [5]. This budding process has been described at a gross scale for several species [6-8]. The youngest zooids are closest to the growth zone and the oldest are furthest from it, providing complete ontogenetic sequences of zooid development within a colony. This greatly facilitates developmental studies.

Nothing is known, however, about the cellular dynamics of siphonophore colony growth. Describing the distribution of their stem cells is a prerequisite to understanding these cellular dynamics. Stem cells were first described in hydrozoans [9], where they have come to be referred to as interstitial cells (i-cells) since they are located within interstices between epithelial cells. Among colonial hydrozoans, i-cells have been studied in the greatest detail in *Hydractinia echinata* and *Clytia hemisphaerica* [10-12]. In *Hydractinia*, they give rise to all cell types (including epithelial cells). These i-cells migrate throughout the colony and facilitate growth at different sites [2, 10]. Hydrozoan i-cells have a distinct round or spindle shape, an enlarged nucleus, and chromatin that is less dense than that of other cells [13], which makes them conspicuous in micrographs. They also have characteristic gene expression [12, 14-16].

Here we describe the i-cells of a siphonophore for the first time. We accomplished this through histological and gene expression analyses of the siphonophore *Nanomia bijuga* (Figure 1). These observations allow us to answer fundamental questions about colony-level development in siphonophores.

Our gene expression analyses are based on *in situ* mRNA hybridization o[2, 12, 15, 16] - *nanos-1*, *nanos-2*, *PL10*, *piwi* and *vasa-1*. Broadly-sampled phylogenetic analyses indicate that the sequences we identified in *Nanomia bijuga* are orthologs of these genes (Figures S2A-C). Negative controls with sense probes were performed for *in situ* hybridizations of all genes in all zooids, and none were positive (Figures S4-S8).

I-cells are present in the siphosomal growth zone

The siphosomal growth zone produces most zooids in *Nanomia bijuga* (Figure 1A,1B). The general structure of the *N. bijuga* siphosomal growth zone, as well as its budding process, has previously been described [7]. The zooids are arranged in repeating groups, known as

cormidia. The budding sequence that produces cormidia and the zooid arrangement within them are highly organized (Figure 1A, [7]). The siphosomal growth zone has a protrusion at its anterior end - the horn (labeled h in Figure 1B). Pro-buds form at the tip of the horn and then subdivide into zooid buds as they mature and are carried to the posterior. These buds give rise to five different zooid types - gastrozooids (feeding polyps), palpons (polyps with function in circulation, defense, sensing and digestion), bracts (defense), and female and male gonophores (gamete production).

All examined genes were expressed at the tip of the siphosomal horn and in all buds and young zooids within the siphosomal growth zone (Figures 2A-B, S4C, S5C, S6C, S7C, S8C). Expression was strongest in an inner cell layer within the horn (Figure 2B). Semi-thin sections and TEM analysis confirmed the presence of two types of cells within the ectoderm of the siphosomal horn, epithelial cells and i-cells (Figures 2C-E). Within the horn, undifferentiated cells with i-cell morphology could also be found in the endoderm. In the analyzed sections the mesoglea within the horn appeared discontinuous suggesting migratory activity of i-cells between ectoderm and endoderm (Figure 2D). In the endoderm of young zooid buds, however, only cells without i-cell characteristics were observed. Their nuclei were located close to the mesoglea (Figure 2F). In the ectoderm of young zooids both epithelial and i-cells were distinguishable (Figure 2C,2F).

I-cells populations become spatially restricted during ontogenesis and are largely absent from mature zooids

The distal portion of the pro-bud gives rise to the gastrozooid – the feeding zooid (Figures 1B 2B,3). Young gastrozooid buds had strong expression of all marker-genes (Figures 3A, S5C, S6C, S7C, S8C). The basigaster, a specialized region of nematocyst formation [5], was evident in young gastrozooid buds as a thickening of the proximal ectoderm (Figure 3B). In the course of basigaster development, expression of all examined genes, except *nanos-2* (Figure 3F), became restricted to deep basigaster ectoderm (Figures 3B-E,S6C-D,S7C,S7E,S8C,S8E-F). As gastrozooids matured, expression of all the examined genes, again except *nanos-2,* decreased until it was no longer detectable (Figure 3H,S6G,S7E-F,S8F-G). In some cases expression was observed in the basigaster region of one gastrozooid but expression was absent from the next older one (S8F). *nanos-2* expression persisted in the basigaster region of gastrozooids of all ontogenetic stages (Figures 3F,S5E,S5G-H). This finding was consistent with previous studies that indicated a *nanos-2* function in nematocyst formation [12, 17]. Within the basigaster *nanos-2* seemed colocalized to the same region as minicollagen (see [18]), which is know to be involved in capsule formation [19]. Though *vasa-1, PL10*, *nanos-1* and *piwi* transcripts were not detected in basigasters of mature gastrozooids (Figures 3H,S6G,S7F,S8G), undifferentiated cells were still found along the mesoglea (Figure 3G) indicating the presence of a determined progenitor cell population which gives rise to nematocysts but has lost interstitial stem-cell transcriptional signatures. Forming capsules could be observed in the outer layers of the basigaster (Figure 3G).

Each gastrozooid has a single tentacle. The tentacle has side branches, known as tentilla, which bear packages of nematocysts at their terminal ends (Figure 1A). Marker-gene expression could be found at the tentacle bases in all cases throughout all ontogenetic stages of

gastrozooids (Figures 3B,3D-F,3H,S5H-I,S6G,S7F,S8G). The expression domains, however, differed between marker-genes. Whereas *nanos-2* expression was restricted to the very proximal end of the tentacle and very early tentilla buds (Figure 3F,S5H-I), signal for the other four genes persisted in later developmental stages of the outgrowing tentilla (Figures 3D-E, S3E,S6G,S7F,S8G). Marker-genes were not expressed in the mature tentilla.

The gene *vasa-1* was expressed in the same regions of the young gastrozooids as *PL10*, *nanos-1* and *piwi*. In addition, it was expressed in both the ectoderm and endoderm of the tips of the young gastrozooids (Figures 2A,3B-E) whereas the exact source of the signal could not be determined.

Anterior to each gastrozooid, a series of buds develop into palpons – zooids thought to have a function in circulation of gastrovascular content, digestion, defense and sensing [20], Figure 1B). Like gastrozooids, each palpon has a single tentacle (Figure 1A), which is known as a palpacle. The palpacle is, in contrast to the gastrozooid tentacle, unbranched and nematocysts can be found along its entire length. Analogously to gastrozooids, strong expression was detected for all marker-genes in young palpons within the growth zone, but expression disappeared from later developmental stages (Figures 2A-B). Expression was absent from mature palpons (Figures 2A,S3A,S6I,S7H,S8I), except for *nanos-2*, which remained expressed in a small domain at the proximal end of the palpon (Figure S3C). Unlike in gastrozooids, this *nanos-2* expression domain did not extend around the entire zooid but was restricted to a small patch close to the palpacle base. Semi-thin sections indicated this patch as a site of nematogenesis (Figure S3D), suggesting that it is equivalent to the basigaster of gastrozooids. These similarities between gastrozooids and palpons were consistent with the hypothesis that palpons are derived gastrozooids that lost the ability to feed, i.e. they lack a mouth opening [5]. Expression of all marker-genes was found at the proximal end of the palpacle (Figures S3A-C,S4G,S5J,S6I,S7H,S8I,S8K). Undifferentiated cells with i-cell morphology were present within palpacle bases (Figure S3D). Additional palpons may be added at the anterior end of the growing cormidium. We found small buds with marker-gene expression anteriorly from the youngest primary palpon (Figure S3E-G). Regular gonodendra form laterally of these secondary palpons.

Bracts are protective zooids of scale-like morphology, which can be found laterally along the siphosomal stem and associated with palpons (Figure 1B, [5, 7]). Analogously to gastrozooids and palpons, marker-gene expression was found in early developing bract buds (Figure S3B) but was absent from older bracts once the typical bract morphology became obvious (Figure 2A).

I-cells and germ line cells in sexual zooids

A colony of *Nanomia bijuga* produces gametes of both sexes. Gametes are produced by gonophores, each of which is male or female. These gonophores are arranged into groups called gonodendra [5]. Gonodendra develop laterally at the base of the palpon peduncle with different sexes on opposite sides of the palpon and sexes alternating sides from palpon to palpon (Figures 1A,4A). Distinct round clusters of cells with strong marker-gene expression were visible at the base of the young palpons close to the growth zone (Figures 1B,4B,S5M). These are the sites where the gonodendra will form in more mature cormidia and these clusters

may consist of primordial germ cells. Early round cell clusters of male and female gonodendra were indistinguishable from each other.

Female gonodendron formation has been described previously [21]. Female gonodendra start to form as small buds protruding at the base of the palpon peduncle. Germ cells develop in between endoderm and ectoderm. Each gonophore within the female gonodendron contains a single egg. The egg is enclosed by a thin ectodermal layer within the developing female gonophore. Two lateral canals form from endodermal epithelial cells within the gonophore. The mature gonophore is attached to the blind-ending central stalk of the gonodendron by a delicate peduncle. There are up to two female gonodendra per palpon (Figures 1A,4A).

In situ hybridization revealed expression of all five marker-genes in a helical pattern in the mature female gonodendron. This pattern corresponds to a previously unobserved helical morphological organization (Figures 4,S5G,S5M-P,S6K,S7J,S8M). Marker gene expression was strong and homogenous in the early forming gonodendra buds (Figure 4B,S5M). Buds started to spiral early in development and a stronger signal was observed on the outer side of the helix (Figure 4C-D,S5N-O), indicating the region of gonophore formation. This pattern persisted during the first turns until the gonodendron appeared as a dense grape-like structure. At this stage all marker-genes were strongly expressed in all gonophores along the gonodendron and the helical organization was not apparent. Helical organization became obvious again in later ontogenetic stages (Figures 4E-F,S5G,S5P,S6K,S7J,S8M) when mature gonophores became identifiable. During gonophore maturation marker-gene expression decreased and was not detectable in mature gonophores (Figure 4F). The presence of signal in a helical pattern along the gonodendron indicated that new gonophores were produced along one side of the entire twisted stalk of the gonodendron.

The male gonodendron starts with the formation of a primary gonophore, which is cone shaped (Figure 4A). Secondary and tertiary gonophores bud off the delicate peduncle of the primary gonophore (Figure 4G). The male gonophore is an elongated structure with a massive population of germ cells amplifying and maturing in between endoderm and ectoderm. All marker-genes were strongly expressed in young and medium-sized gonophores but signal intensity was lower or absent in gonophores close to or at maturity (Figure 4H,S5Q,S6M,S7L,S8N). The absence of graded signals along the proximal-distal axis indicated that sperm maturation took place along the entire gonophore.

Nectosomal growth zone has a similar structure as the siphosomal growth zone

Nanomia bijuga, like most other siphonophore species, has a nectosomal growth zone near the anterior end that produces the swimming zooids, called nectophores, which propel the whole colony through the water. All five genes were strongly expressed in the nectosomal growth zone at the tip of the horn, in nectophore buds, and in young developing nectophores (Figures 5A-D,S7A). *In situ* hybridization and histological sections indicated the presence of i-cells in the thickened region of the nectosomal stem, the horn of the growth zone and young nectophore buds (Figures 5B,5D-E). In case of *vasa-1,* the transcript was the longest detected along the ridges of the nectophores (Figure 5A). Older nectophores were free of marker-gene transcripts (Figures 5B-C). In contrast to the other marker-genes, *nanos-2* expression was restricted to the very youngest nectophore buds (Figure 5C). In addition, in the stem subtending

the growth zone the transcript was detected on the nectosomal stem in a salt and pepper pattern (Figures 5C). Sections revealed developing nematocysts in this region of the stem (Figure 5F). Undifferentiated cells with interstitial cell morphology were identified in the ectoderm of developing nectophores (Figures 5F-G).

Discussion

We found that interstitial stem cells, as identified by morphology and the expression of five canonical stem cell genes, are restricted to distinct, well-defined locations in a siphonophore colony. These locations are the tips of the growth zones, specific sites on the stem where new palpons and gonodendra will arise at the anterior end of each cormidium, young zooid buds, and particular locations within maturing zooids. The expression of these stem cell genes within maturing zooids becomes progressively more restricted until it disappears from most regions in the oldest zooids. Expression persists the longest in gametogenic and cnidogenic regions and in regions where cell proliferation and differentiation is known to occur [5] such as tentacle and palpacle bases. The expression domains of the analyzed genes were, however, not identical indicating that genes are differentially expressed in particular cell lineages. The differences were most obvious in case of *vasa-1* and *nanos-2*.

Since the original discovery of stem cells in Hydrozoans [9], they have been characterized in a wide diversity of animal species and are a major focus of many current research programs. Further work has revealed that there is even diversity within Hydrozoa in the potency of i-cells. In the freshwater polyp *Hydra*, i-cells are pluripotent but cannot give rise to epithelia [22-24]. In contrast, the i-cells of the marine colonial hydrozoan *Hydractinia* can give rise to all cell types including epithelia [2, 11]. The potency of the siphonophore i-cell remains to be elucidated. In the present study, cells with interstitial cell characteristics could be identified predominantly interspersed in between ectodermal cells, which is consistent with observations made in *Hydra* [13].

This study did not address the embryological origin of stem cells in *Nanomia bijuga*, which may still be similar to that of other colonial hydrozoans [12] despite the radical differences in the distribution of stem cells in the mature colonies. Likewise, this study does not differentiate between several possible origins for the i-cells that give rise to palpons and gonodendra at the anterior end of each cormidium. These cells could arise by transdifferentiation, migration that we failed to find evidence for, or be relict populations of interstitial stem cells deposited early in cormidial development.

In combination with previous descriptions of the general structure of siphonophore growth zones [6, 7, 20], these findings suggest a novel model of growth in siphonophores that is unique among colonial animals. Hydrozoan relatives of siphonophores have i-cells that can migrate to multiple points of growth and differentiation [2, 11]. We could not find hints for such migratory capacities in case of the siphonophore i-cell, i.e. no marker-gene expression could be detected along the stem of the colony or at locations within mature zooids. Instead, the siphonophore *Nanomia bijuga* has two major proliferating populations of i-cells at the tips of its two growth zones. These are self-renewing, and deposit i-cells in the young zooid buds as they form. These i-cells then proliferate, differentiate and stop expressing i-cell markers as the zooids mature and are carried away from the growth zones by the elongating stem. In many respects,

this mode of growth is similar to that of land plants - the growth zones of siphonophores act much as plant meristems do. They are the primary sites of proliferating pluripotent cells, which differentiate as the structures they create are carried away from the point of growth.

This extreme restriction of sites of zooid formation in siphonophores, accompanied by the indicated lack of migrating i-cells, may be the mechanism by which siphonophores have realized such complex and precise colony-level development and organization. In other species of colonial animals, where stem cells can migrate, growth and differentiation can occur at many different points. This leads to great plasticity in growth, and different colonies of the same species do not have the exact same organization of zooids relative to each other. They also have fewer zooid types than siphonophores do. The restriction of proliferating pluripotent cells to particular sites in *N. bijuga* might reduce plasticity, enabling far more precise growth, and also explain previous observations of reduced regenerative capacities in siphonophores relative to other colonial animals [25].

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Figure 1

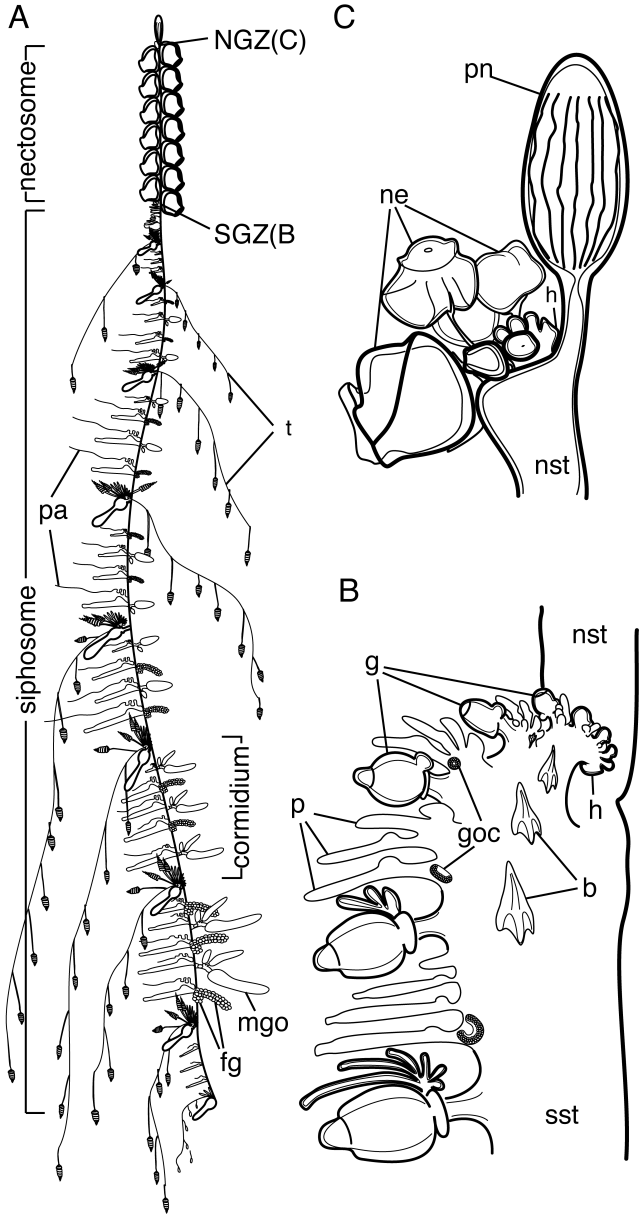

Figure 1. Schematic of *Nanomia bijuga*. Anterior is towards the top of the illustrations. (A) Colony stage of the life cycle. For clarity reasons, protective bracts were not pictured and gonodendra of only one sex are shown per palpon in older parts of the colony. Approximate length of the illustrated colony was about 15cm. The side of zooid attachment within the siphosome is defined as the ventral side of the stem [26]. The complete life cycle of *N. bijuga* is shown in Figure S1. (B) Siphosomal growth zone and anterior part of the siphosome. i-cell clusters (goc) at sites of gonodendra formation are set aside early in the growth zone. Gonodendra mature in older cormidia further to the posterior (see A). Only gonodendra cell clusters accompanying the oldest most posterior palpons are visualized within the growth zone. (C) Nectosomal growth zone with gas filled floating organ, the pneumatophore, at the top. b: bract; fg: female gonodendron; g: gastrozooid; h: horn; mgo: male gonophore; ne: young nectophores; NGZ: nectosomal growth zone; nst: nectosomal stem; p: palpon; pa: palpacle, goc: gonodendron cell cluster; pn: pneumatophore; SGZ: siphosomal growth zone, sst: siphosomal stem; t: tentacle. Figure modified from [27].

Figure 2

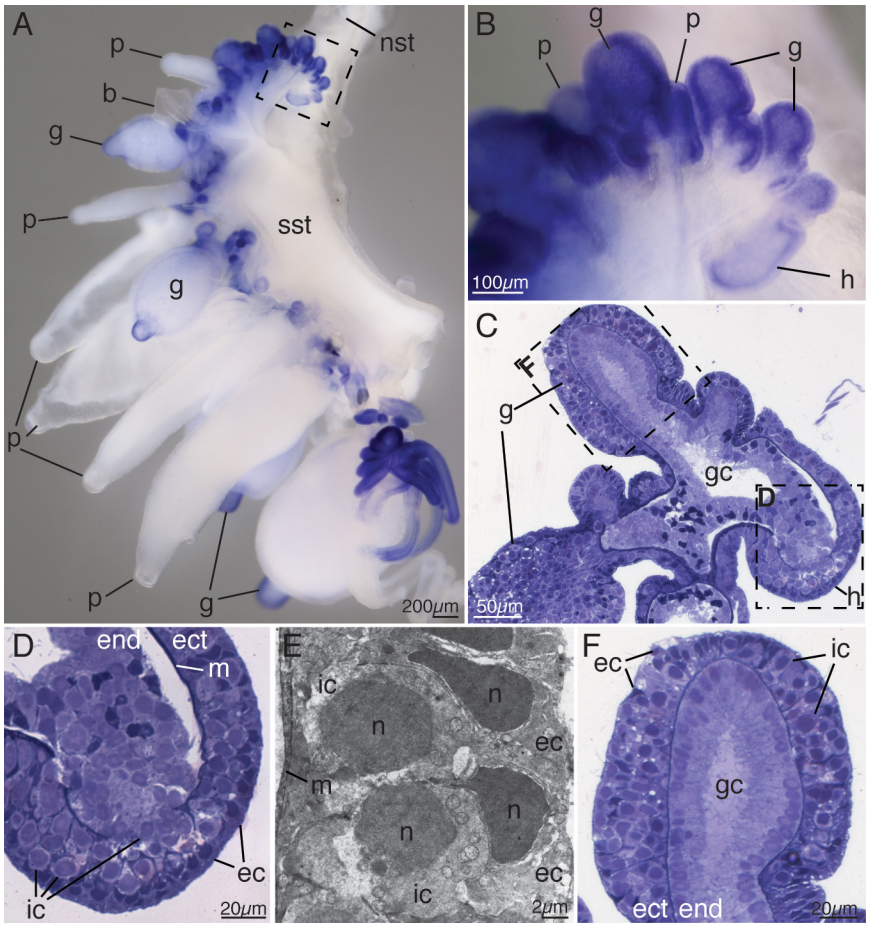

Figure 2. Siphosomal growth zone. (A) Anterior part of the siphosome, *vasa-1* transcript. Lateral view. Anterior is up, ventral to the left. (B) Close-up growth zone from box in A. (C). Semi-thin longitudinal section of the tip of the siphosomal growth zone stained with toluidin blue. (D) Siphosomal horn, close-up of box in C. (E) Transmission electron micrograph of the ectoderm of the siphosomal horn. Interstitial cells reside in between epithelial muscle cells of the ectoderm. (F) Tip of youngest gastrozooid, close-up of box in C. b: bract; ec: epithelial cell; ect: ectoderm; end: endoderm; g: gastrozooid; gc: gastric cavity; h: horn of the growth zone; ic: interstitial cell; m: mesoglea; n: nucleus; nst: nectosomal stem; p: palpon; sst: siphosomal stem.

Figure 3

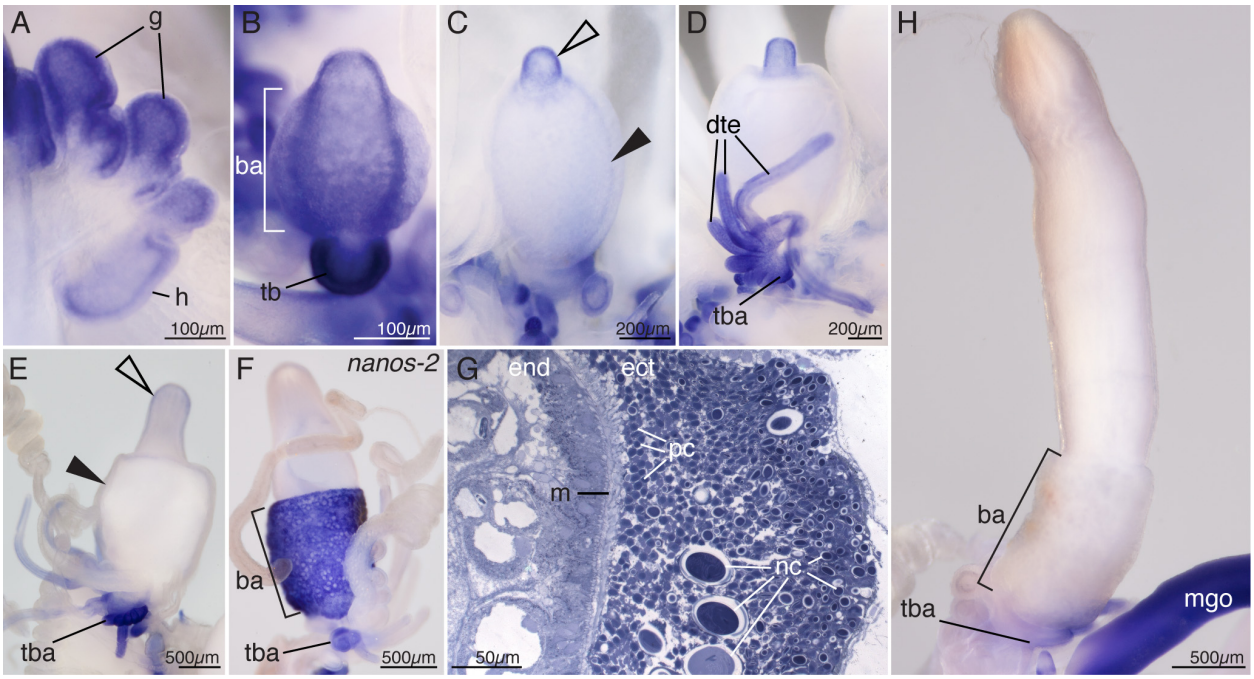

Figure 3. Gastrozooid development. (A-E, H) Ontogenetic series of gastrozooids, *vasa-1* transcript. Distal is up. (A) Young gastrozooid buds close to the siphosomal horn. (B) Young gastrozooid with strong *vasa-1* expression in the developing tentacle bud. Within the basigaster region transcript could be found predominantly in deeper tissue layers. Anterior view. (C) Slightly older gastrozooid with *vasa-1* expression in the gastrozooid tip (empty arrowhead) and faint signal in the basigaster region (filled arrowhead). Posterior view. (D) Early stage of tentacle formation with developing tentilla branching off the tentacle. Anterior view. (E) *vasa-1* transcript disappears from maturing gastrozooid within the developing tip (empty arrowhead) and from the basigaster region (filled arrowhead) but remains present in tentacle bases and developing tentilla. Lateral view, anterior to the left. (F) *nanos-2* expression in the basigaster region and the tentacle base of a gastrozooid. Lateral view, anterior to the right. (G) Semi-thin longitudinal section of a mature gastrozooid basigaster, stained with toluidin blue. Undifferentiated cells can be found along the mesoglea in ectodermal tissue. (H) Mature gastrozooid, *vasa-1* expression absent. Lateral view, anterior is to the right. ba: basigaster; dte: developing tentilla; ect: ectoderm; end: endoderm; g: gastrozooid; h: horn; m: mesoglea; mgo: male gonophore; nc: developing nematocysts; pc: putative nematocyte progenitor cells, tb: tentacle bud; tba: tentacle base.

Figure 4

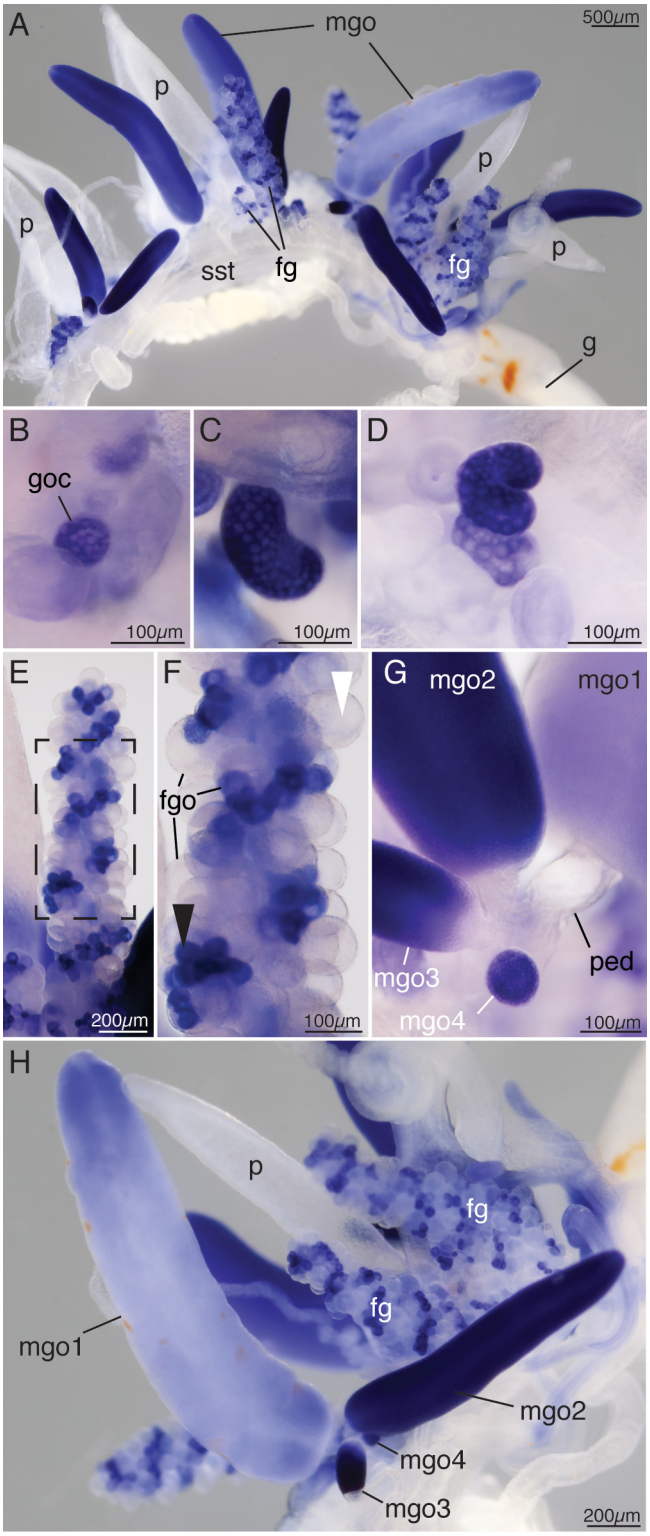

Figure 4. Gonodendra development, *vasa-1* transcript. (A) Mature cormidium, including male and female gonodendra. Anterior is to the left of the pane, ventral to the top of the pane. (B-E) Ontogenetic series of developing female gonodendra. (B) Cell cluster with *vasa-1* expression at the site of gonodendron formation at the base of a palpon. (C) Developing bean-shaped female gonodendron. (D) Developing female gonodendron starting to spiral. (E) Mature female gonodendron with developing gonophores with marker gene expression and mature gonophores with marker gene expression absent. (F) Close-up of female gonodendra (boxed area in E) with developing (black arrowhead) and mature gonophores (white arrowhead). Distal is up. (G) Close-up of the base of a male gonodendron. Later gonophores bud off the peduncle of the primary gonophore. The primary male gonophore (mgo1) is visible to the right. (H) Male gonodendron with an ontogenetic series of male gonophores, labeled mgo1-4 from oldest to youngest. *vasa-1* transcript abundance decreased as the male gonophore matured. fg: female gonodendron; fgo: female gonophore; g: gastrozooid; goc: gonodendron cell cluster; mgo: male gonophore; mgo1: oldest male gonophore; p: palpon; ped: peduncle; sst: siphosomal stem.

Figure 5

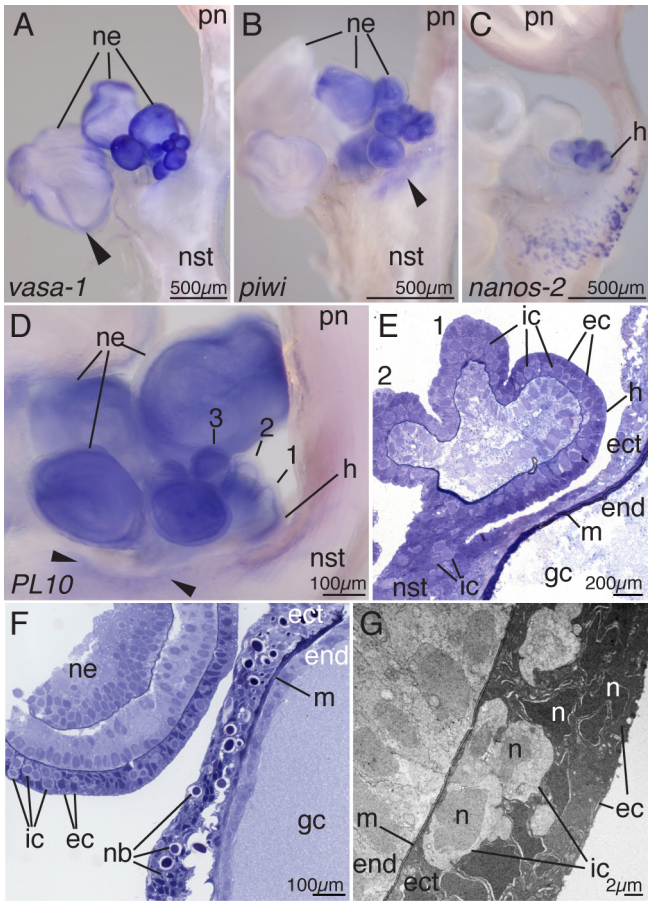

Figure 5. Nectosomal growth zone. Anterior is up in all figures. (A) *vasa-1* transcript. Transcription was longest detectable along the nectophore ridges (arrowhead). (B) *piwi* transcript. Marker gene expression was observed within the protruding nectosomal bulge (arrowhead), young buds and developing nectophores. (C) *nanos-2* expression in the nectosomal horn and young developing buds. Signal on the nectosomal stem indicates sites of nematogenesis. (D) *PL10* transcript present in the nectosomal horn, youngest buds (1-3), young developing nectophores and within the protruding nectosomal bulge (arrowheads). (E) Semi-thin longitudinal section of early nectophore buds and the horn, stained with toluidin blue. Interstitial cells could be identified in the protruding bulge of the nectosomal stem, the horn and young developing buds (1-2). (F) Semi-thin longitudinal section in the region of the nectosomal horn showing nematogenesis in the ectoderm of the nectosomal stem subtending the growth zone and interstitial stem cells in the ectoderm of a developing young nectophore. (G) Transmission electron micrograph showing interstitial stem cells in in the interstices of the epithelial muscle cells within the ectoderm of a young nectophore. ec: epithelial cell; ect: ectoderm; end: endoderm; gc; gastric cavity; h: horn of the growth zone; ic: interstitial cell; m: mesoglea; n: nucleus; nb: nematoblasts; ne: nectophore; nst: nectosomal stem; pn: pneumatophore.

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