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Establishing bilateral symmetry in hydrozoan planula larvae, a review of siphonophore early development

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Abstract:

Siphonophores are colonial hydrozoans, characterized by directional asymmetric colony organization and unparalleled zooid functional specialization. Recent genomic studies have offered an evolutionary perspective on how this morphological complexity arose, but characterization of symmetry breaking in siphonophores is still largely missing. The advent of culturing methods of siphonophores has opened the possibility to investigate in detail their larval development. Here, bringing together historical data on early development with new immunohistochemical data, we review the diversity of developmental trajectories that leads to the formation of bilaterally symmetric planula larva across the siphonophore phylogeny. We then discuss siphonophore development within the broader, cnidarian context to highlight open questions and set the framework for future work on siphonophores.

Introduction

Siphonophores are a clade of hydrozoans found from the ocean surface to the deep sea that are especially known for their highly integrated, functionally specialized colonial development (Totton 1965; Mapstone 2014). Siphonophores are widespread and abundant predators in pelagic ecosystems, playing critical trophic roles in the pelagic 'jelly web' (Choy et al. 2017; Damian-Serrano et al. 2021; Hetherington et al. 2022; Boosten et al. 2023). Unlike other gelatinous zooplankton, siphonophores are typically extremely fragile and are difficult to collect intact using plankton nets, and must be collected by remotely operated vehicle, scuba diving, or from the surface in locations with upwellings where they can be found close to the coast (Dunn

et al. 2005). One such location is Villefranche-sur-Mer, where significant progress describing embryonic development in many siphonophore species was made by Claude and Danielle Carré (Carré and Carré 1991, 1993).

Siphonophores are colonial organisms, with each of the bodies within the colony (called zooids) connected to a central stem with a shared gastrovascular cavity. These zooids are considered to be homologous to free-living polyps and medusae, but are physically integrated such that they do not function individually but rather as a colonial 'super-organism' (Mackie 1963; Totton 1965). Each of these zooids is functionally specialized for particular tasks, including feeding, swimming, and reproducing (for a glossary of specialized siphonophore terms, including the names of each of these zooids and their function, see table 1). Siphonophores have traditionally been divided into three main groups based on colony morphology: Cystonectae, Physonectae, and Calycothorae (Totton 1965). Cystonectae, a monophyletic group including the famous Portuguese man of war *Physalia physalis*, are sister to the clade Codonophora, which includes all other siphonophores (Figure 1A) (Dunn et al. 2005; Munro et al. 2018). The Codonophora comprises the clade Calycothorae nested within the paraphyletic 'Physonectae' (Figure 1A) (Dunn et al. 2005; Munro et al. 2018).

In recent years, significant progress has been made in describing the body plan and development of the 'adult' polygastric colony (=bearing multiple feeding polyps, called gastrozooids) at molecular and morphological levels (Dunn 2005; Haddock et al. 2005; Dunn and Wagner 2006; Siebert et al. 2013, 2015). Within cystonects, zooids arise as single buds along the stem (Dunn and Wagner 2006), although *P. physalis* has a more complex colony organization, resulting from the development of a body plan adapted to life at the ocean surface (Totton 1960; Munro et al. 2019). Within Codonophora, most zooids arise from one or two growth zones, starting with a pro-bud that subdivides to give rise to different zooid types (Dunn and Wagner 2006). As the stem elongates, these zooids continue to develop and are carried down along the stem; other zooids can arise later along the stem, for example, additional bracts and palpons grow within already differentiated cormidia (Siebert et al. 2013). As a result of pro-bud subdivision, zooids are found in reiterating units, called cormidia, along the stem (Dunn and Wagner 2006). Siphonophore polygastric colonies are linear and are typically oriented vertically along an anterior-posterior axis, reflecting the typical direction of locomotion (Figure 1B,C,D, but note *P. physalis* A-P axis is oriented horizontally, see Munro et al. 2019). The anterior pole of the polygastric colony corresponds to the aboral pole of the larva (Haddock et al. 2005). The secondary, dorso-ventral axis is always present, with the growth zone, pro-buds, and cormidia on the ventral side (Figure 1C,D). Additional left-right asymmetries have been documented across siphonophore taxa (Dunn 2005; Dunn and Wagner 2006).

Siphonophores also reproduce sexually to generate new polygastric colonies. The gametes develop within specialized zooids, the gonophores, and are spawned in the water column. The environmental and molecular factors triggering gamete maturation and release are still to be identified in siphonophores, even though dark-light transitions are likely to play major roles in many species, as observed in other hydrozoans (Quiroga Artigas et al. 2018; Takeda et al. 2018). Depending on the species, siphonophores are either monoecious (egg or sperm bearing

gonophores are both found on the same colony) or dioecious (colonies have either egg or sperm bearing gonophores but not both) (Totton 1965). After fertilization, embryonic development gives rise to a planula larva that develops directly into the colonial form. As a result of this unique development, siphonophore planulae are unusual as compared with other hydrozoan larvae in that they exhibit bilateral symmetry. Here, we review current knowledge of colonial body plan establishment during embryonic and larval development in siphonophores, and highlight several key open questions.

Major transitions in the siphonophore life cycle

While early and late stages of development have been described in detail in several codonophoran species, no information exists on cystonect embryogenesis and very little on later developmental stages (Figure 1B,C,D), all based on fixed material (Okada 1932, 1935; Munro et al. 2019). We will thus primarily focus in this review on codonophoran development. In all codonophoran species studied so far, a series of unipolar and equal cleavages, followed by gastrulation by primary delamination give rise to a typical cnidarian planula larva. Spatial organization of the planula endoderm leads to the emergence of the ventral endoderm, a future site of zooid budding. The developmental trajectories of Calyphorae and 'physonects' diverge as soon as budding starts, primarily due to the absence of a gas-filled float (the pneumatophore) in calyphorans. The budding gives rise to either calyconula (in Calyphorae) or siphonula (in 'physonects' and cystonects) larvae. The chronology and sequence of budding appear to be species-specific, though in the majority of Calyphorae it starts with the nectophore, while in siphonula larva the pneumatophore tends to arise first (Figure 1C,D). The early budding is often accompanied by shedding of larval zooids (larval zooids differ in morphology from the same-type zooid in the polygastric colony). Simultaneously, the embryonic body differentiates into the protozooid (primary gastrozooid or feeding body); the vitelline reserves are used so that the gastric cavity hollows, the endodermal gland cells differentiate at the oral pole of the protozooid, the aboral portion of the protozooid swells and differentiates into nematogenic tissue, and the tentacle buds between the protozooid and nectophore through ecto-endodermic evagination. The appearance of the subsequent zooids, primarily the additional cormidial units, each bearing a single gastrozooid, mark the transition into the polygastric colony. The time needed to reach this stage depends on the water temperature (Carré and Carré 1991; Sherlock and Robison 2000), though total siphonophore life-span is unknown.

Gametes and fertilization

Siphonophore eggs are relatively large (e.g., 180 μm in mediterranean *Nanomia bijuga* Carré 1969, 500 μm in *Lensia conoidea* Carré 1967) and transparent; they undergo meiotic maturation prior to spawning, and as in other cnidarians both polar bodies are extruded prior to fertilization (Carré and Carré 1993). Fully grown eggs are polarised with the germinal vesicle displaced to one pole, and following the meiotic divisions an extracellular structure called the cupule that overlays the polar bodies – this is the region of the egg where fertilization will take place (Carré and Sardet 1981). The presence of cupule has been identified in only a few species across the siphonophore phylogeny (Carré and Sardet 1981), with no information on cystonects. The cupule forms early during oocyte maturation, probably through exocytosis (Carré and Sardet

1981). It is highly chemoattractive to spermatozoa in a species-specific manner (it contains a peptide that modifies the curvature of the sperm trajectories, Cosson et al. 1984), and is shed quickly from the egg after fertilization (Carré and Sardet 1981). Siphonophore spermatozoa have a typical cnidarian morphology, with a conical nucleus, a few mitochondria and a single flagellum (Carré and Sardet 1981). Their centriolar apparatus includes a striated rod extending anteriorly, which has been implicated in an acrosomal reaction during gamete fusion (Carré 1984).

Cleavages and gastrulation

Cleavages are unipolar and equal until the 32 cell stage (Freeman 1983; Carré and Carré 1993). At the 32 cell stage, the blastomeres consist of an outer cytoplasmic region containing the nucleus and organelles, and an inner cytoplasmic region containing the vitelline reserves (Carré and Carré 1993). At this point, divisions have been reported as superficial and partial, with furrows only penetrating the outer cytoplasmic region containing the nucleus (Carré and Carré 1993). The periblastula stage thus consists of an outer flat layer of cells surrounding an internal anucleated mass of vitelline vesicles (Carré and Carré 1993). Gastrulation occurs via primary delamination, where certain cells divide tangentially or obliquely relative to the surface of the embryo, resulting in a sheet of endodermal cells directly below the sheet of ectodermal cells (Freeman 1983; Carré and Carré 1993). During the transition to the planula larva stage, these endodermal cells - that are located in the region that is by convention called the ventral side of the planula - continue to divide in this region, while other endodermal cells migrate inwards between the vesicles of vitellus (Carré and Carré 1993). The precise site of gastrulation (if any) is not known, however Carré and Carré (1993) imply that gastrulation occurs on the ventral side (“certain endodermal cells stay in the place where they were formed, multiply, forming a layer that lines the ventral face of the larva”, translated from French), and our confocal data (Figure 2, Figure 3) also point towards an endodermal layer only on the ventral side. The site of origin of cells that migrated and are interspersed amongst the vitelline vesicles is unclear. Carré and Carré (1993) point out that the internal mass of vitellus with endodermal cells interspersed were referred to by many authors, including themselves in earlier papers, as the “primary” endoderm, while the ventral “thickening” of cells were referred to as the “secondary” endoderm. Given the unclear order of appearance of these two populations of endodermal cells, or the fate of the cells interspersed among the vitelline vesicles, we will not be using these terms in this review, and instead will refer primarily to the ‘ventral endoderm’ when discussing this thickened ventral layer that plays a critical role in establishing the larval body plan.

Diversity of larval trajectories

The ciliated planula larva is bilaterally symmetrical, it is elongated with an oral and aboral pole, and the endoderm consists of the ventral layer, as well as scattered cells amongst the vitelline vesicles (Figure 2, Figure 3) (Carré and Carré 1993). The planula stage is very short in duration (typically less than 24 hours Carré and Carré 1993), after which the first zooid buds or structures, such as tentacles, begin to form. Larval development has been described in detail in a number of species with variation between species in terms of budding sequence and development (Figure 4).

The budding sequence has been described for several 'physonect' siphonula larvae, and the first buds vary considerably in both budding location and identity. In *N. bijuga* the pneumatophore arises first, via invagination at the aboral pole of the larva (Figure 2A), subsequently the first larval tentacle buds on the ventral side (Figure 2B) (Carré 1969a). In *Halistemma rubrum*, the pneumatophore bud forms first on the dorsal side close to the aboral pole, simultaneously, the first nectophore bud forms on the ventral side close to the aboral pole (Carré 1971, Figure 4A). In *Cordagalma ordinatum* [referred to as *C. cordiformis*] (Carré 1973, Figure 4C) and in *Agalma elegans* (Fewkes 1885), however, the bud of the larval bract forms first, ventrally close to the aboral pole, then subsequently the pneumatophore forms via invagination on the dorsal side close to the aboral pole. The third bud gives rise to the nectophore, much like in *Physophora hydrostatica* (Haeckel 1869). Lastly, in *Forskalia edwardsii*, three buds arise simultaneously on the ventral side that will give rise to the pneumatophore, larval bract, and larval tentacle (Figure 4B, Carré 1967b). The pneumatophore continues to elongate, meanwhile in the middle and oral regions of the larva, a protozoid begins to differentiate (Figure 2C,D). At the base of the differentiated protozoid, a secondary tentacle forms on the opposite side of the first tentacle (Figure 2E). The narrowing at the level of the tentacle bulb (Figure 2B-D) will later stretch to give rise to the colony stem.

Within Calycophorae, the first bud to form on the planula body is almost uniformly the nectophore (e.g. *Muggiaea kochii* Carré and Carré 1991, *Sphaeronectes koellikeri* [referred to as *S. gracilis*] Carré 1969b), with just two described exceptions. In *Abylopsis tetragona*, both the nectophore and bract bud appear at the same time (Figure 3C, D; Carré 1967a), while in *Lilyopsis medusa* a conspicuous outgrowth starts to project from the planula body (Figure 4F, Carré and Carré 1969). The nectophore budding usually starts on the planula ventral side, although there are species in which nectophore bud appears on the aboral pole (e.g. *Sphaeronectes irregularis*, Figure 4G, Carré 1969b) or even close to the oral pole (e.g. *L. medusa*, Figure 4F, Carré and Carré 1969). Irrespectively, nectophore development always progresses analogously to hydrozoan medusae, via ectoderm delamination and formation of the entocodon. Below the nectophore bud, on the ventral side of the larva, the primary tentacle develops (e.g. in *L. conoidea*, Figure 4D, Carré 1967b). After that, the oral region of the planula differentiates to form the protozoid (Figure 3F), which will later develop a mouth opening (Figure 3G). The most notable deviation from this otherwise conserved trajectory is the fate of planula body in members of the two calycophoran families: Hippopodidae and Prayidae (Metschnikoff 1874; Carré and Carré 1969). In their larvae, after entocodon formation, an extensive deposition of mesoglea starts between the endoderm and both the planula ectoderm and nectophore bud ectoderm, so that the entirety of the embryonic body becomes enclosed within the nectophore and forms the somatocyst (Figure 4F).

Post-larval development

Later on, both siphonula and calyconula transition into the post-larva, which is typically characterized by a presence of fully differentiated zooids (Carré 1969a), though there is no clear definition of this stage. As the post-larva continues to grow, its stem elongates and the additional zooids are budded from the growth zone(s). When two growth zones are present, the

aboral one buds nectophores, while the other buds cormidia, consisting of all other zooid types. If only a single growth zone is present, it buds only cormidia.

Cormidia are budded on the same side as were the primary zooids of the planula, i.e., ventral, with the exception of *S. koellikeri* (Figure 4G), whose cormidia in the polygastric colony are budded from planula dorsal side. The nectophores, on the other hand, can be budded off either from the same side of the stem as the cormidia (e.g. in *F. edwardsii*, Carré 1967b) or the opposite (nectophores - dorsal, cormidia - ventral; *A. elegans*, Fewkes 1885).

As development progresses, some caducous larval zooids detach, while others are retained in the polygastric colony (see Pugh 2009). In calycophorans, the larval nectophores, for example, are typically retained within Sphaeronectidae (Carré 1969b) and Prayidae (Carré and Carré 1969), and replaced by one or more definitive ones in Diphyidae (Carré and Carré 1991, 1993), as evidenced by differences in the morphology of larval vs. definitive zooids (size, course of radial canals, ridge patterns on the exumbrella; Carré 1971). The larval bracts, on the other hand, are carried over during development in agalmatiid 'physonects' (Fewkes 1885; Carré 1973), and seem caducous in many other codonophorans (*F. edwardsii* Carré 1967b and *A. tetragona* Carré 1967a). The fate of larval zooids thus appears specific to particular phylogenetic groups.

Establishing the colony body plan

Polarity and axis specification

Fully grown siphonophore oocytes, as noted above, are polarised with a germinal vesicle displaced to one pole. After meiosis is complete, the female pronucleus remains positioned at the animal pole, immediately below the extracellular cupule and the two polar bodies. Carré and Carré (1993) noted that the female pronucleus itself is polarised, with digitations in the nuclear membrane towards the cell membrane along which most of the nuclear pores are located. Within the gonophore prior to oocyte release, the egg is oriented with the germinal vesicle pole facing the release opening (Carré and Carré 1993). The planula larvae are bilaterally symmetrical, with an oral pole that will give rise to the future protozooid, and an aboral pole which is defined in some species by the presence of a pneumatophore or nectophore (Figure 5). They also have a dorsal-ventral axis, with the ventral side characterised by the presence of a thickened layer of endoderm. It is along this side where the first zooids begin to bud.

Within the polygastric colonies, directional asymmetries have also been described during pro-bud subdivision. In *Bargmannia elongata* polygastric colonies, for example, gonozooids attach to the left, and there are two lateral left bracts and one right bract (Dunn 2005). The directionality of this displacement is consistent across individuals of the same species, and arises from asymmetric budding during pro-bud subdivision (Dunn 2005). Meanwhile *P. physalis* colonies are dimorphic and may be either left or right handed (this in turn determines sailing direction), with the attachment point of the tentacle of the tentacular palpon as the first indicator of this asymmetric budding pattern in fixed larval samples (Okada 1935; Totton 1960; Munro et al. 2019).

Experimental data on siphonophore embryos provide some intriguing insights into aboral-oral and dorsal-ventral axis specification and patterning. In a series of experiments conducted on the 'physonect' *Nanomia cara* and calycothoran *Muggiaea atlantica*, Freeman (1983) used chalk marks to demonstrate that the site of the first cleavage furrow marks the future oral region of the larva. In *N. cara*, oral-aboral specification is established prior to the 8-cell stage, while in *M. atlantica*, the aboral half is specified by the 8-cell stage but the oral half is capable of regulation (Freeman 1983). To trace the origin of bilateral symmetry in *M. atlantica* he drew a chalk ring around the egg along the cleavage furrow. Only a few cases (6 out of 30) were suitable for analysis and in all of these planulae one side of the chalk ring coincided with the side of the ventral endoderm (Freeman 1983). In cutting experiments separating dorsal and ventral halves in *M. atlantica* embryos, Freeman (1983) found that in all cases the ventral halves gave normal larvae, while for the dorsal halves the outcome depended on the stage at which the cutting experiments occurred. At 6-7 h of development, dorsal/ventral sides cannot be distinguished, however when cut at this time, both halves develop into normal larvae. If cutting occurred during early stages (around 15-17 h) the ventral endoderm formed on the dorsal side opposite the cut site, meanwhile if it occurred at later stages (22-24 h and 30-36 h) the ventral endoderm took longer to form and budding was disrupted. Taken together, these data suggest that asymmetries in the egg that are set up before first cleavage direct the development of both the oral-aboral and dorsal-ventral axes. The first cleavage furrow has been shown to initiate at or close to the site of polar body formation in other hydrozoans, including *Clytia gregaria* [referred to as *Phialidium gregarium*] and *Hydractinia echinata* (Freeman and Miller 1982), implying that the region of the egg with the cupule corresponds to the site of the first cleavage furrow and therefore will give rise to the oral pole of the larva (Figure 5). No cellular feature defining either of the dorsal or ventral poles in the egg has yet to be identified. Interestingly, based on the descriptions of Carré & Carré (1993), the initiation site of gastrulation in the embryo may correspond to the future ventral side.

Pneumatophore and nectophore budding

The transition to siphonula involves the budding of pneumatophore, always found at the aboral pole of the polygastric colony. The gas-filled float development starts from invagination of a few ectodermal cells (Figure 6B.1) at a species-specific position (see 'Diversity of larval trajectories' section above), which then push inside onto the endoderm (Figure 6B.2). After a few days, the roughly circular bud formed by the ectodermal cells, arranged around a primary cavity (Figure 6B.3) is completely enclosed within the planula body by the overlying ectoderm. At this stage, the ectodermal secretion of chitin within the cavity begins and cells located basally in the bud divide to give rise to the aeriform ectoderm (Figure 6B.4), which later proliferate to cover the entirety of the primary cavity (Figure 6B.5). Such internal reorganization of the bud leads to the accumulation of the chitin in a single layer covering the aeriform tissue, which starts to produce gas. Subsequently, the bud, which now defines the aboral/anterior pole (Figure 5), is being pushed out of the planula body outline, together with the associated layer of endoderm (Figure 6B.5). The pneumatophore bud is thus covered by the ectoderm and two-layered endoderm while itself is made up of two layers of ectoderm, separated by chitin (Figure 6B.6). In some species, the additional folding of the endodermal layer gives rise to a few longitudinal cavities (8

in *N. bijuga* Carré 1969a, 6 in *H. rubrum* Carré 1971) stretching from the tip to the base of pneumatophore which remain in contact with gastrovascular cavity.

The development of codonophoran swimming bells, the nectophores, parallels that of medusae in other hydrozoans. The nectophore budding usually starts on the ventral side of the planula, although it may begin elsewhere (Figure 4). The budding begins with the delamination of the ectoderm (Figure 6A.1), with the newly appearing cell mass pushing back on to the ventral endoderm (Figure 6A.2). As a result of the continued cell proliferation within that hemispherical cell mass it begins to separate from the overlying ectoderm (Figure 6A.3), up until formation of the typical medusa nodule, the entocodon (Figure 6A.4). The entocodon, doubled exteriorly by the ventral endoderm, grows considerably, acquiring more spherical shape and hollowing internally; it then starts to project externally from the planula body, with its long axis (ostium - tip of the nectophore) perpendicular to the planula oral-aboral axis (Figure 6A.5). The nectophore bud enlarges asymmetrically, with growth more pronounced at the aboral pole, leading to the repositioning of the nectophore, so that its long axis becomes parallel to the oral-aboral axis of planula body (Figure 6A.6). Simultaneously, the ventral endoderm, lying between the outer ectoderm and the entocodon ectoderm, begins to hollow, giving rise to the gastrovascular canals (radial and ring) and the somatocyst; additionally, the nectophore musculature starts to differentiate at roughly the same time (Figure 6A.6). Finally, an orifice opens at the site of contact between the entocodon and the planula ectoderm, marking the stage at which the nectophore becomes functional.

The pneumatophore is not traditionally considered to be a zooid, contrary to nectophores, as it arises once during embryonic development, like the protozooid, but unlike non-larval zooids which largely arise from the growth zones in the polygastric colony. However, it is made of different tissue types, has complex anatomy and performs a small number of specialized functions that are critical to the colony. The superficial resemblance of pneumatophore and nectophore early buds have fueled attempts at homologizing these two structures (Metschnikoff 1874; Chun 1897), while others refute their claims (Garstang 1946). Histological analyses of siphonophore embryos have provided arguments for both sides of the debate (see e.g., Carré and Carré 1993), although they predominantly point to a lack of homology between pneumatophore and nectophore (Mackie et al. 1988). Pneumatophore budding site varies between species, is not restricted to the aboral pole of planula, and can even bud from the same position as nectophores (Figure 4). However, the pneumatophore aeriform ectoderm, postulated by Metschnikoff (1874) as homologous to the medusoid manubrium, arises through proliferation of ectoderm (Figure 6B.5), and not through ecto-endodermal evagination as the manubrium does in other medusozoans (note that siphonophore nectophores do not have a manubrium, Carré 1967b). Although the pneumatophore primary cavity can be interpreted as homologous to nectophore subumbrella cavity (Figure 6A.4/A.5, Figure 6B.3/B.4), it is later replaced by the definitive cavity arising internally, within the chitin-delimited zone (Figure 6B.6, Carré 1967b). Lastly, nectophore radial canals arise through cavitation of the endoderm, while pneumatophore cavities are separated from the gastrovascular cavity through the endodermal folding (Carré 1969a).

Establishing growth zones

Budding in siphonophores is highly restricted spatially, with zooids arising from either one (Calycophorae) or two growth zones ('physonectae'; Dunn and Wagner 2006). The growth zones are sites of high cell proliferation, and interstitial stem cells (i-cells) are found in the ectoderm and endoderm of the horn (anterior structure in the growth zone where pro-buds arise) (Siebert et al. 2015). The developmental origin of either growth zone during larval development has not been described in detail, however, it seems likely that the primary growth zone originates from the planula ventral endoderm. The subsequent spatial separation into two growth zones in 'physonectae' occurs during the siphonula stage, when stem elongation moves them apart (Carré 1969a). In calycophorans, however, the calyconula body elongates only orally, with the embryonic body between protozoid and larval nectophore remaining competent to bud both the cormidia and definitive nectophores. Even when mature, the stem of certain calycophorans retains the ability to replace damaged or lost nectophore(s) (Carré 1979), hence suggesting that they either also possess two, albeit unseparated, growth zones, or that their single growth zone retains its larval budding plasticity.

Larval zooids bud differently from zooids in the polygastric colony that arise from growth zones. During larval development, the body of the planula differentiates into the protozoid and other zooids are budded individually from the ventral endoderm. Meanwhile, once growth zones are established, budding in polygastric colony growth zones occur via pro-bud subdivision, i.e. a single bud gives rise to the whole cormidium (Carré and Carré 1993; Dunn and Wagner 2006). This transition occurs early in the larval development, in *Forskalia edwardsii* for example, the young siphonula still without any nectophores, starts to bud cormidia from the zone between protozoid and larval bracts (Carré 1967b). Little is known about the patterning and specification of either larval zooids or zooids arising by pro-bud subdivision, or indeed whether these two processes are similar.

Molecular perspectives on the early development of siphonophores

Siphonophores exhibit unparalleled zooid functional specialization and have highly organized polygastric colonies where zooids are arranged in an ontogenetic series from anterior to posterior in repeating species-specific units of zooids. It is no surprise therefore that siphonophore embryonic development is also unusual as compared with other cnidarian species. The work of Danielle and Claude Carré, along with Gary Freeman and others, have provided an excellent foundation for our understanding of early siphonophore development, however many open questions remain that also have implications for our broader understanding of cnidarian development and body plan specification.

The molecular players underlying axis specification and symmetry breaking in siphonophores, setting up the oral-aboral and dorsal-ventral axes during early development, as well as directional asymmetry of the cormidia, have yet to be investigated. Recent work on other hydrozoan species, notably *Clytia hemisphaerica* (Momose and Houlston 2007; Momose et al. 2008), *Dynamena pumila* (Vetrova et al. 2022), *Hydractinia echinata* (Duffy et al. 2010; Kraus et al. 2014), or on the anthozoan sea anemone *Nematostella vectensis* (e.g., Lebedeva et al. 2021) all showed that the Wnt- β -catenin signaling pathway plays a major and conserved role in

setting up the oral-aboral axis during embryogenesis. Activation of this pathway at the animal pole of early cleaving *Clytia* embryos, through deposition of Wnt ligand and receptors on the animal and vegetal sides of the egg, leads to the activation of a set of transcription factors and signaling molecules setting up the oral pole (Momose and Houliston 2007; Momose et al. 2008). Considering that the animal pole also gives rise to the oral pole in siphonophores (Freeman 1983), it is likely that the Wnt- β -catenin signaling plays a similar role in specifying the primary body axis in siphonophore embryos as it does in other cnidarians. The primary axis of siphonophore planulae is also evident in their oriented swimming toward the aboral pole. This process is driven by beating of cilia covering the entire planula ectoderm (Carré 1967, Carré 1969). A Frizzled-dependent Planar Cell Polarity pathway was shown to control this process in *Clytia* embryos (Momose et al. 2012), and is likely similarly involved in siphonophores.

The molecular factors establishing the secondary dorsal-ventral axis in siphonophore planulae are also to be uncovered, notably those responsible for the second symmetry breaking before gastrulation and those responsible for the accumulation of endodermal cells on the ventral side and activation of the first zooid budding, leading to the formation of a bilateral planula larva. Besides siphonophores, bilateral symmetry in cnidarian embryos is only evident in anthozoan larvae, in the folding of their inner cavity (Genikhovich and Technau 2017). The BMP pathway was shown to play a major role in the formation of this secondary axis in the sea anemone *N. vectensis* (Genikhovich and Technau 2017). Early budding of zooids on the ventral side of siphonophore planulae bears resemblance to the asymmetric budding of polyp or stolon observed in most colonial hydrozoans, suggesting that similar patterning mechanisms may be involved (e.g. Hobmayer et al. 2000; Bagaeva et al. 2019; Sanders et al. 2020). Polyp budding in *Hydra* was notably shown to involve a non-BMP member of the TGF- β family (Watanabe et al. 2014). Expression studies of Wnt, BMP and other TGF- β signaling components as well as conserved downstream targets (e.g. Brachyury, Schwaiger et al. 2022) in several siphonophore species would be particularly helpful to start uncovering mechanisms of symmetry breaking and axis specification in siphonophores. Considering the great variability in the order and position of the different zooids budded during early development it is entirely possible that different factors are involved in secondary axis formation in the different siphonophore clades.

A peculiarity of siphonophore colonies is that they display only one or two growth and budding zones in very precise locations, as compared to most other colonial hydrozoans in which growth budding zones are widely and less precisely distributed within the colony. It was hypothesized that the spatial restriction of i-cells only in mature zooids and growth zones could play a role in the reduced developmental plasticity of most parts of the colony (Siebert et al. 2015). It is currently unknown how these zones are defined during colony formation, which mechanisms precisely define their number and location and how interstitial stem cells are restricted to these zones. Detailed cellular and molecular descriptions of the development of the growth zone in several siphonophores, coupled with single cell sequencing approaches, would provide important insights. Involvement of the Notch and FGF signaling pathways in the formation and maintenance of growth zones in siphonophores should also be investigated as they play major role in the regulation of budding or tentacle morphogenesis in several hydrozoans (Münder et al. 2010; Lange et al. 2014; Gahan et al. 2017; Hou et al. 2021).

RNA sequencing approaches during development could also highlight genes involved in the specification and patterning of different zooids during budding, as well as the pneumatophore, and give insights into their homology within Siphonophora, and with the medusa and polyp zooids of other hydrozoans. Considering the difficulty of getting a large number of individuals of the different stages, future development of spatial transcriptomic approaches could be particularly interesting in this regard.

The next challenge is bringing siphonophores and their larvae into the lab. There is optimism for this, as recent advancements in culturing are opening up possibilities to maintain fragile siphonophore species more easily (Patry et al. 2020). With the increasing availability of genomic resources for siphonophore species, including zooid-specific transcriptomes of polygastric colonies (Siebert et al. 2011; Munro et al. 2022), and the recent advancement of morphological and molecular techniques applicable to hard-to-get wild planktonic organisms, the moment is ripe for a renewed understanding of siphonophore development integrating morphological, cellular and molecular levels.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Figures and tables

Table 1. Glossary of siphonophore terms

Term	Definition
Bract	Gelatinous zooid, likely required for buoyancy, defense
Calyconula	Larval stage in calycophorans (see 'Siphonula' below), variously used to describe the stage when the first zooids start to bud, or when the protozooid is functional
Cormidium	Reiterating sequence of zooids found along the stem
Gastrozooid	Feeding zooid, considered homologous to polyps, typically bears a tentacle to catch prey
Gonozooid	Reproductive zooid, polyp, that bears gonophores (reproductive zooids, medusae). Contain eggs or sperm.
Medusa	One of two main adult body forms in Cnidaria, typically planktonic and swimming e.g. "jellyfish" lifecycle stage in <i>Aurelia aurita</i> , <i>Clytia hemisphaerica</i>
Nectophore	Swimming zooid, considered homologous to medusae
Palpon	Digestive/defensive zooid, considered homologous to polyps
Polygastric colony	'Adult' colony - poly (many) gastric (gastrozooids)
Polyp	One of two main adult body forms in Cnidaria, typically sessile and benthic with tentacles surrounding a mouth
Pneumatophore	Gas filled float, typically at the anterior of the polygastric colony.
Protozooid	First gastrozooid (see above) that forms during larval development.
Siphonula	Larval stage in 'physonects' and cystonects (see 'Calyconula' above), variously used to describe the stage when the first zooids start to bud, or when the protozooid is functional.
Somatocyst	Endodermal diverticulum functioning as a colony lipid/nutrient reservoir
Stem	Central stem/stalk to which all zooids are attached.
Zooids	Bodies/units that are functionally specialized to different tasks and are evolutionarily homologous to free living

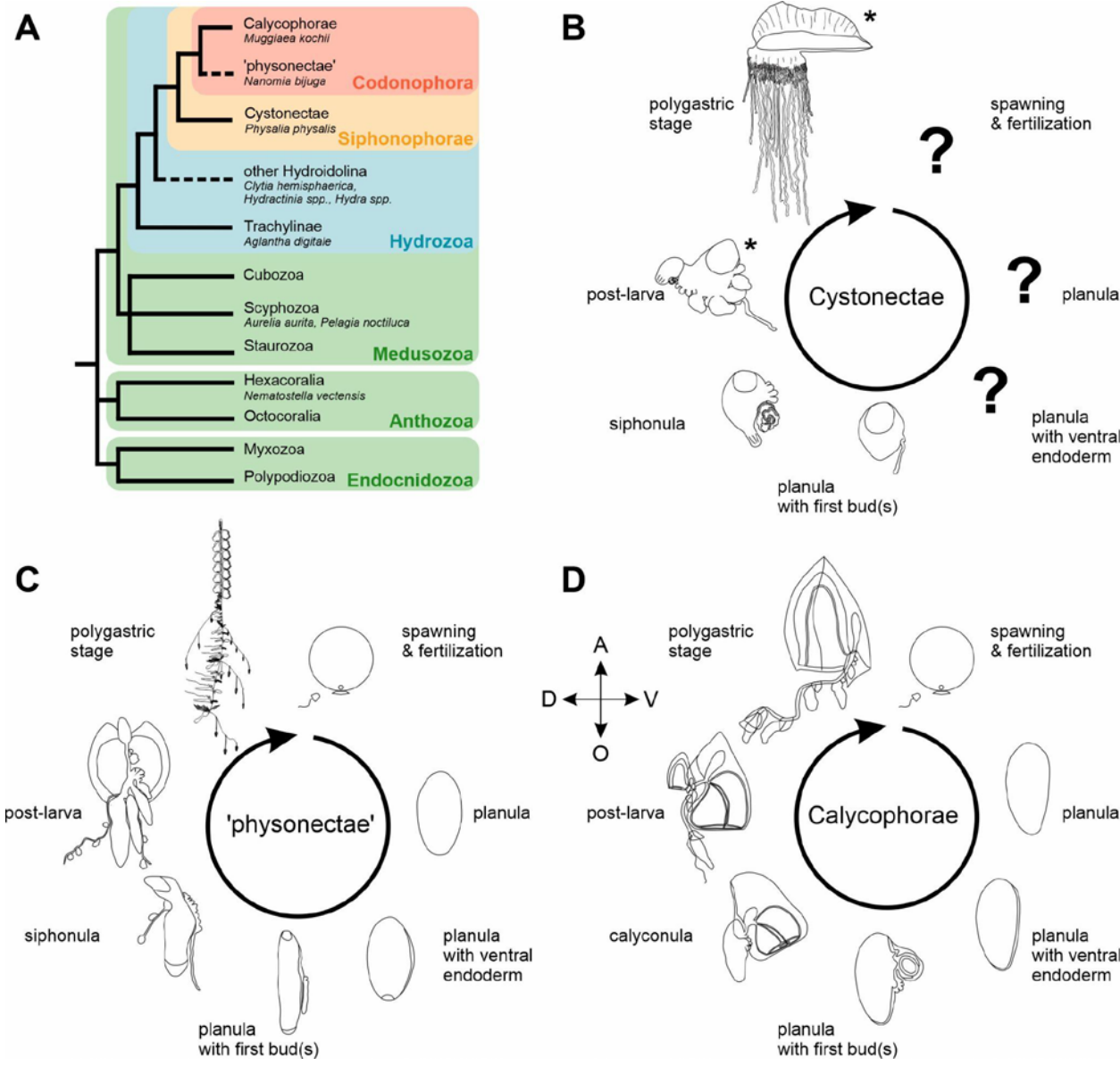


Figure 1. Diversity of siphonophore life cycles. A. Phylogenetic relationships between main Cnidarian lineages (based on Chang et al. 2015; Zapata et al. 2015; Munro et al. 2018). B-D. Schematic representation of siphonophore life cycle: B. *Physalia physalis* cystonect (after Munro et al. 2019); C. *Nanomia bijuga* 'physonect' (redrawn based on Carré 1969a, except for polygastric colony - diagram by Freya Goetz http://commons.wikimedia.org/wiki/File:Nanomia_bijuga_whole_animal_and_growth_zones.svg); D. *Muggiaea* spp. calycophoran (redrawn after: polygastric colony (Carré and Carré 1991); post-larvae (Chun 1897); other stages after (Russell 1938). Orientation shown in D, aboral (A, up) - oral (O, down) and dorsal (D, left) - ventral (V, right), except for *P. physalis* life cycle stages marked with an asterisk (for reference see Munro et al. 2019). Not drawn to scale. Question marks represent probable life cycle stages that were so far not documented.

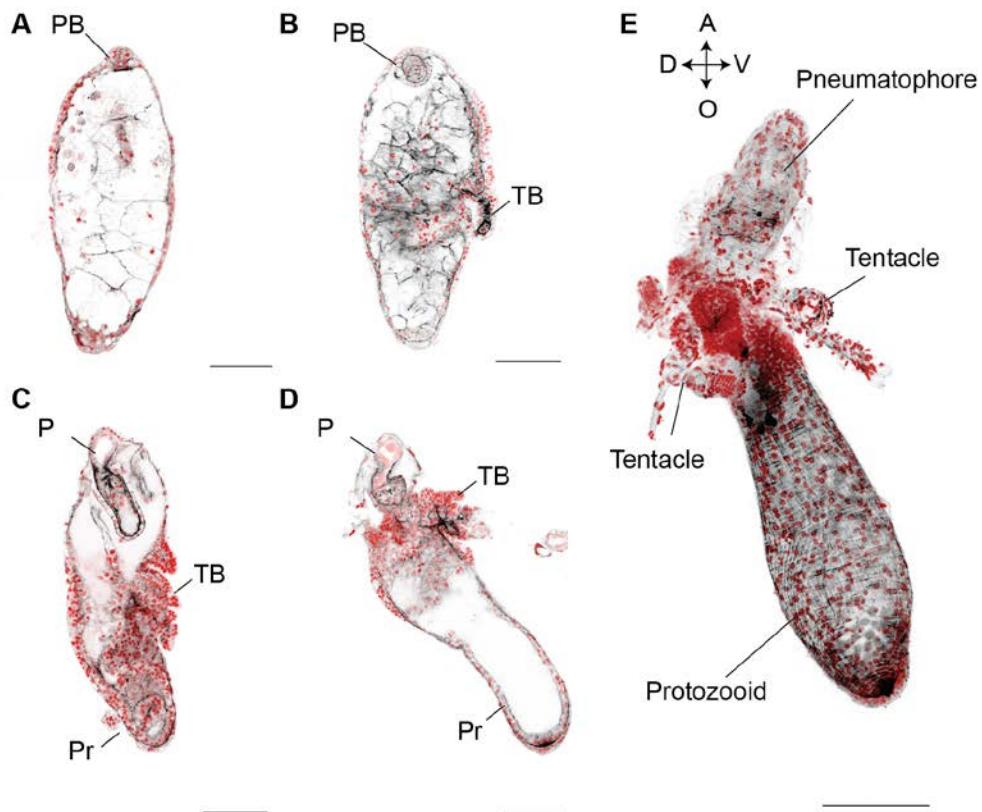


Figure 2. Larval development of *Nanomia bijuga* starting from the planula larva. Nuclei were stained with Hoechst dye (red) and F-actin was stained with phalloidin (black). A-D single confocal plane. E, Maximum intensity projection of multiple confocal planes. Orientation shown in D, aboral (A, up) - oral (O, down) and dorsal (D, left) - ventral (V, right). Scale bar is 100 μ m. Abbreviations: PB- pneumatophore bud, TB - tentacle bud, Pn - pneumatophore, T - tentacle, Pr - protozoid. Immunostaining methods included as a supplementary file.

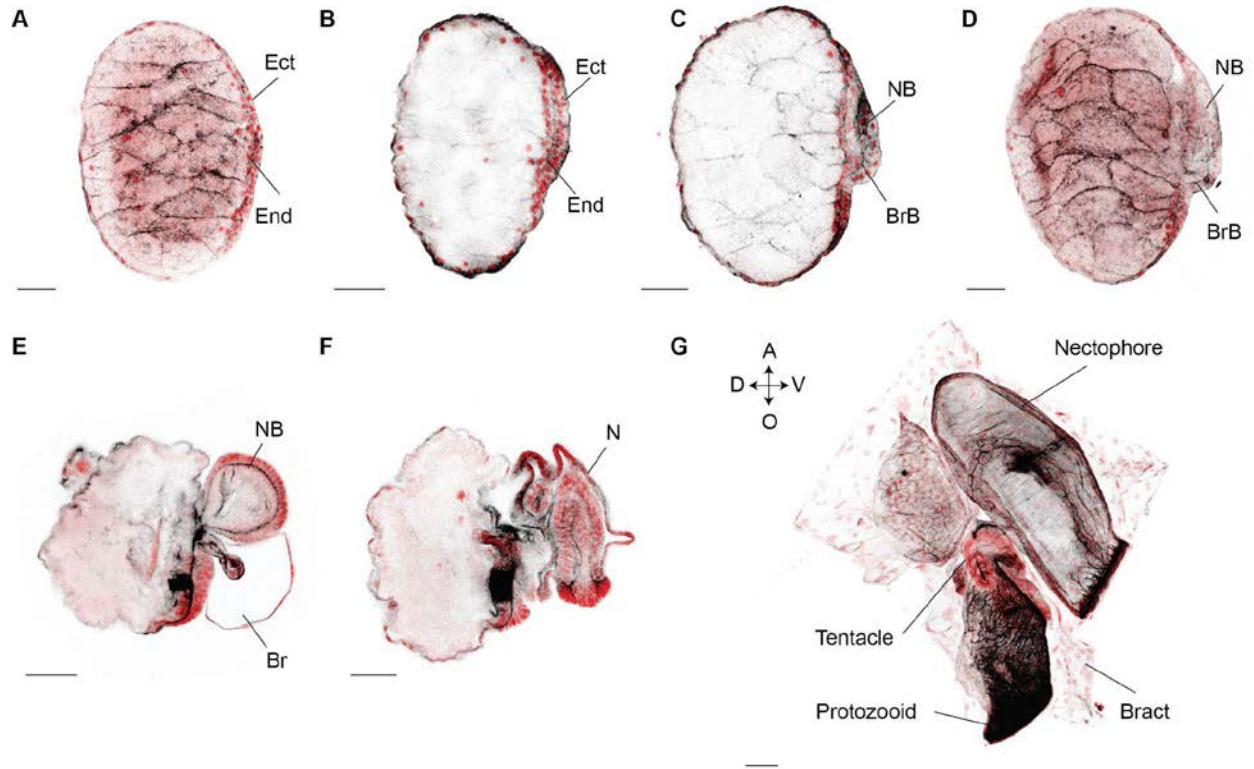


Figure 3. Larval development of *Abylopsis tetragona* starting from the planula larva. Nuclei were stained with Hoechst dye (red) and F-actin was stained with phalloidin (black). A-F single confocal plane (note, in F, bract is out of plane). G, Maximum intensity projection of multiple confocal planes. Orientation shown in G, aboral (A, up) - oral (O, down) and dorsal (D, left) - ventral (V, right). Scale bar is 100 μ m. Abbreviations: Ect - Ectoderm, End - Endoderm, NB - nectophore bud, BrB - bract bud, N - nectophore, Br - Bract. Immunostaining methods included as a supplementary file.

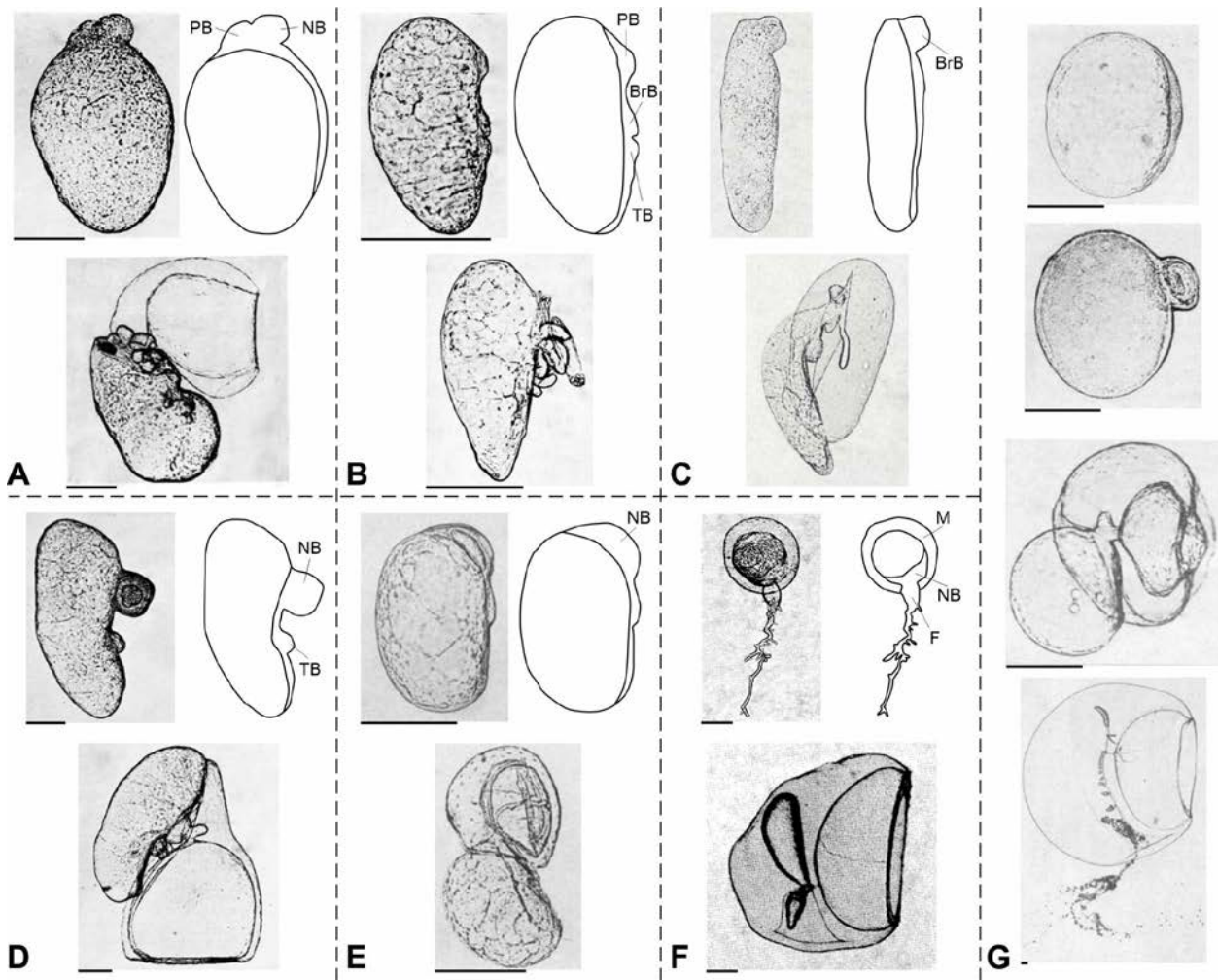


Figure 4. Developmental trajectories across codonophoran siphonophores; ‘physonects’: A. *Halistemma rubrum*, B. *Forskalia edwardsii*, C. *Cordagalma ordinatum* and Calycophorae: D. *Lensia conoidea*, E. *Sphaeronectes irregularis*, F. *Lilyopsis medusa*, G. *Sphaeronectes koellikeri*. In sections A-F, the top row contains a photograph of an actual young planula (left) and a schematic (right) with an approximation of the delimitation of the ventral endoderm, while the bottom row shows a photograph of a later stage of the development. Section G shows development from the planula (top) to the polygastric colony (bottom). Photographs were reproduced from Carré 1967b (B. and D.), 1969b (E. and G.), 1971 (A.), 1973 (C.); Carré and Carré 1969 (F.) with permission from the publishers of Cahiers de biologie marine and Contributions to Zoology. All pictures are oriented so that: top-aboral, bottom-oral, left-dorsal, right-ventral. Labels as in Figures 2-3, plus M- mesoglea, F- filament. Scale bar is 250 μm .

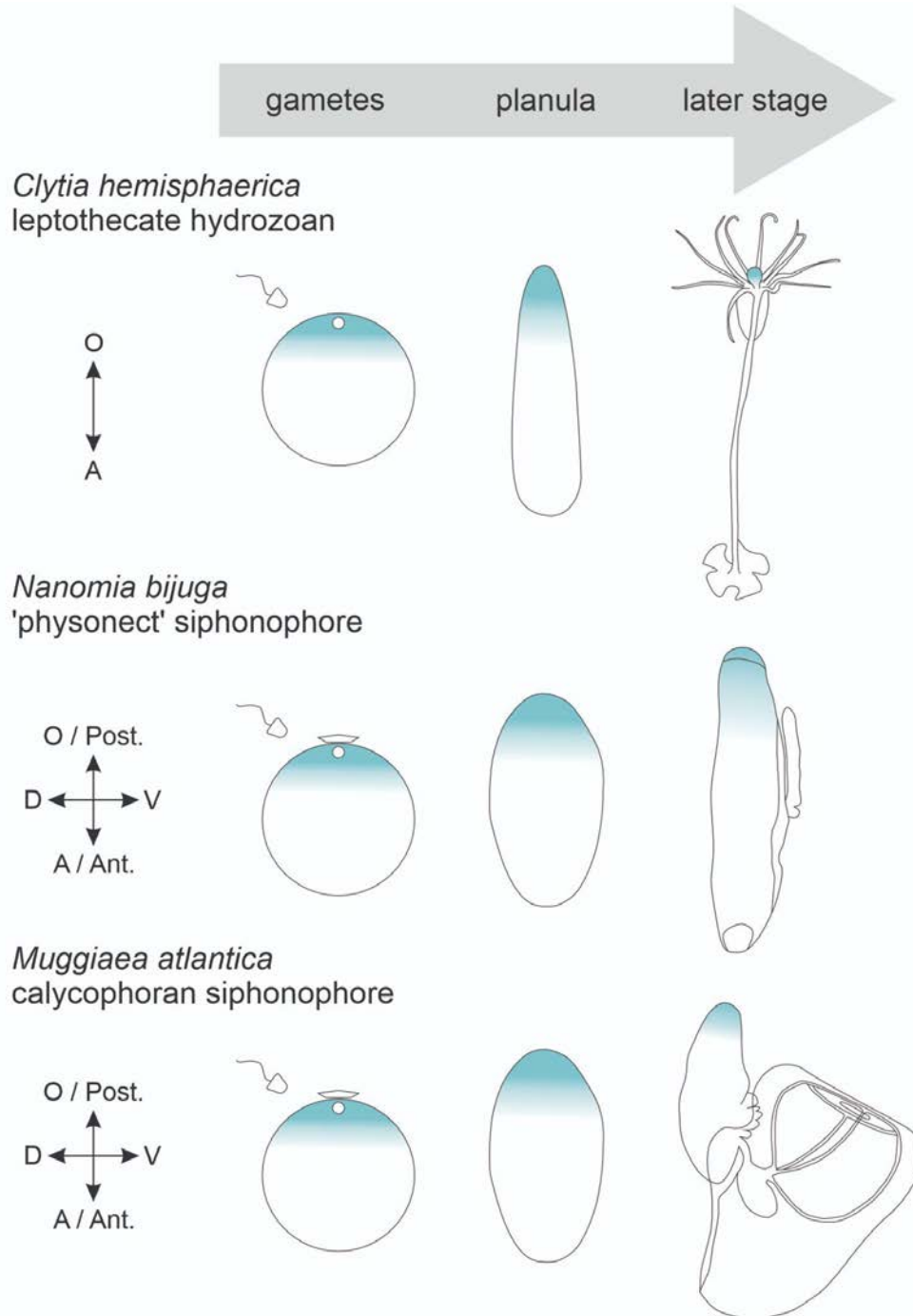


Figure 5. Comparison of body axes in developing siphonophores (*Nanomia bijuga* and *Muggiaea atlantica*, mid and bottom row, respectively) and a leptothecate hydrozoan (*Clytia hemisphaerica*, top row). Blue shading marks the oral pole. Abbreviations: A - aboral, Ant. - anterior, O - oral, Post. - Posterior, D - dorsal, V - ventral. Not drawn to scale. *Clytia* drawings after Munro et al. 2023; *Nanomia* and *Muggiaea* larvae redrawn from Russell 1938; Carré 1969a, respectively.

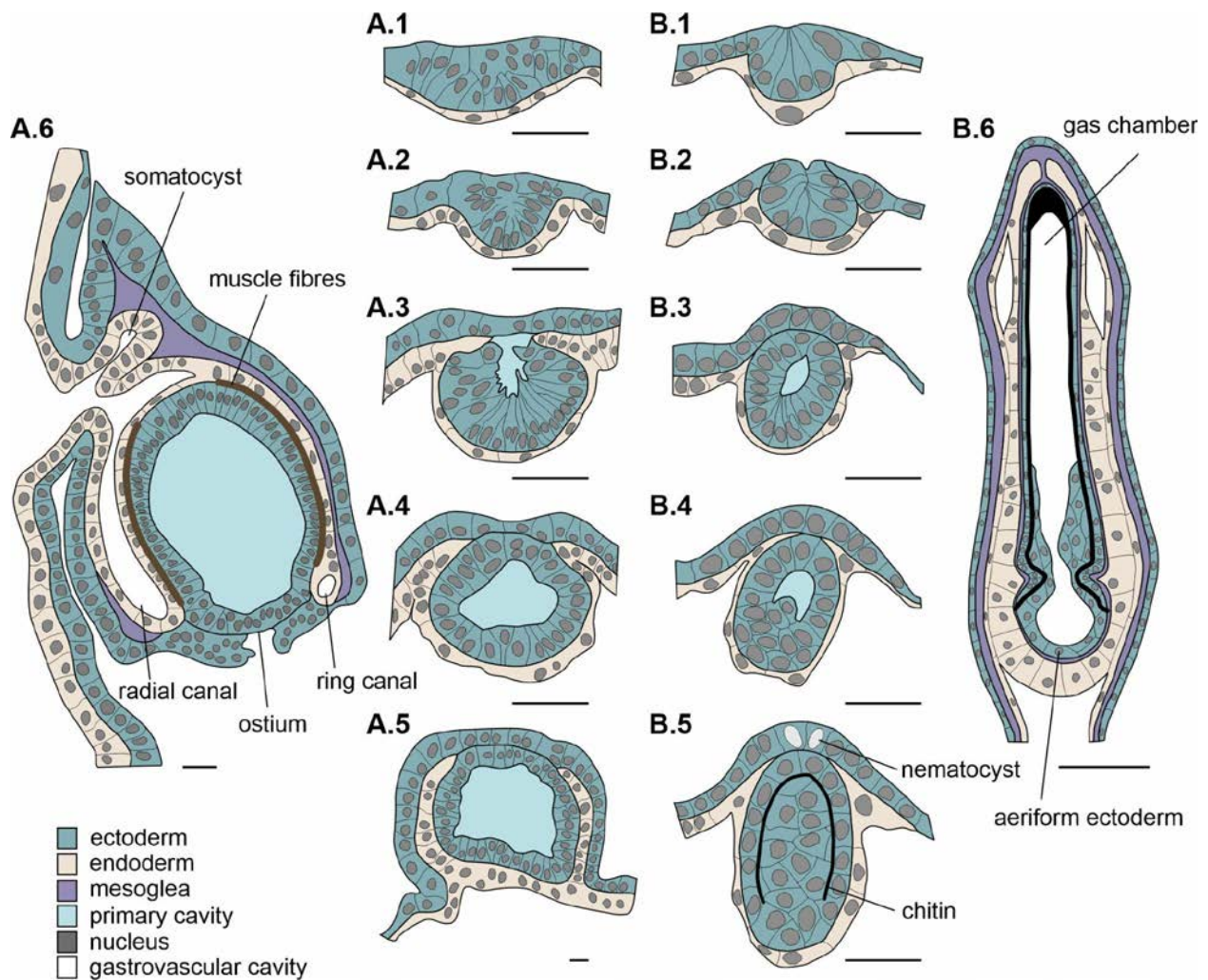


Figure 6. Comparison of budding process between *Lensia conoidea* nectophore (A.1-6) and *Forskalia edwardsii* pneumatophore (B.1-6). Color-coding has been used to distinguish germ layers (ectoderm teal, endoderm beige), mesoglea (violet), primary ectodermal cavity (light blue) and nuclei (dark grey). Thick black solid line represents a layer of chitin, while the thick dark brown solid line corresponds to muscle fibers. Redrawn after Carré 1967b. Scale bar is 20 μ m.