

Proliferation activity in the polyps of *Cassiopea xamachana*: where the planuloid buds grow

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Abstract

Polyps of the Cassiopeidae family possess a unique type of asexual reproduction by producing free-swimming buds — planuloids. The process of planuloid development and transformation to polyp has been described earlier, however, the source of tissue formation is still poorly studied. Using the method of EdU incorporation we have analyzed DNA synthesis activity during planuloid formation and growth in *Cassiopea xamachana*. We revealed the active proliferation zone at the early stage of bud formation. This zone continued to function during planuloid growth, providing the formation of polyp structures, and preserved in polyp calyx after metamorphosis. Its proliferation activity varied at different growth stages, whereas the localization remained relatively the same.

Keywords: cnidaria, budding, development, polyp, scyphozoa, proliferation, asexual reproduction

Introduction

Asexual reproduction is widespread in Cnidaria (Fautin, 2002). Polyps can reproduce by various ways, including fragmentation, longitudinal or transversal division and budding (Boero et al., 2002; Bocharova and Kozevich, 2011; Schiariti et al., 2014). The process of lateral budding is the most studied, primarily in *Hydra* sp. (Galliot, 2012). However, little is known about other types of budding, found in different cnidarian groups. Particularly in Scyphozoa, reproduction by budding plays a significant role in jellyfish bloom formation, therefore investigation of the ecological features of this process is the object of intense attention (Arai, 2008; Lucas et al., 2012). However, morphological aspects of bud formation and development are often left out.

The budding type descriptions in Scyphozoa were mostly done in life cycle studies. It was shown that scyphozoan polyps have several ways to propagate. Many scyphozoan species reproduce by lateral budding, for example, semaestomeans *Aurelia aurita* (Spangenberg, 1965), *Phacellophora camtschatica* (Widmer, 2006) and rhizostomean *Catostylus mosaicus* (Pitt, 2000). During lateral budding, a new polyp almost completely forms at the calyx of the parent individual and preserves this anatomical connection for long time. Stolonial budding is much less common and is described only in a few species, such as *Aurelia aurita* (Gilchrist, 1937), *Sanderia malayensis* (Adler and Jarms, 2009), *Rhizostoma pulmo* (Fuentes et al., 2011). In this case buds develop at body processes — stolons — which are usually used for polyp fixation or moving. Numerous scyphozoan polyps produce resting buds — podocysts (Arai, 2008). Podocysts are formed from the polyp stalk tissues, covered by thick cuticle (Chapman, 1968; Ikeda et al., 2011). Finally, longitudinal division has also been observed in some species

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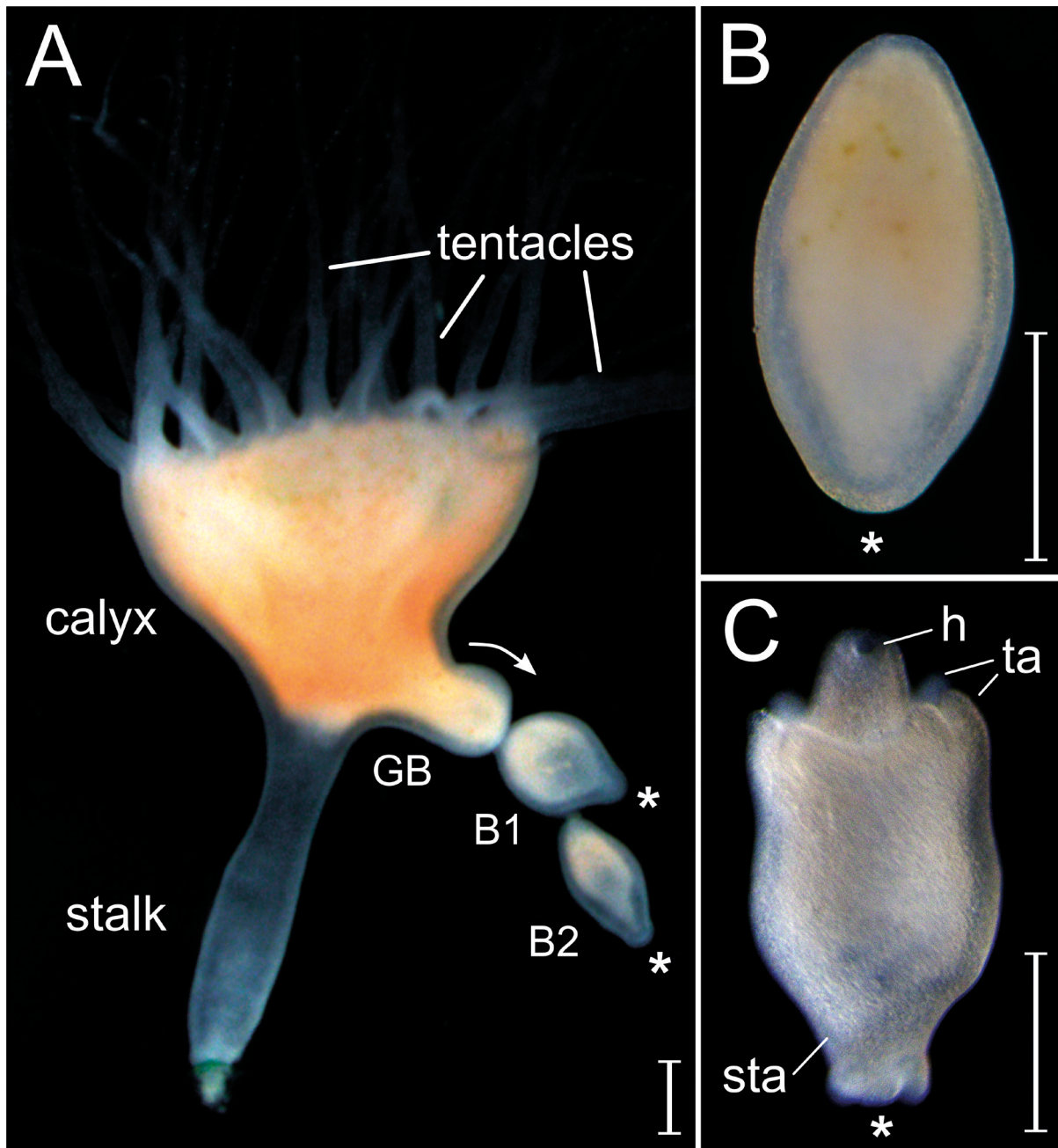


Fig. 1. Gross morphology of the polyp and planuloids of *C. xamachana*.

A — polyp during the budding process with one growing bud and two planuloid buds in chain, B — planuloid just after separation, C — late planuloid with organ anlagen. Abbreviations: B1, B2 — planuloid buds, GB — growing bud, h — hypostome anlage, sta — stalk anlage, ta — tentacle anlage. The functionally anterior end of the planuloid is marked with an asterisk; the arrow shows the direction of bud growth. Scale bar — 500 μ m.

(Pitt, 2000; Widmer, 2006, 2008). In all these cases newly formed polyps stay close to the parent individual, which limits the population spread.

Among these budding types, planuloid budding stands out because the forming buds are motile. It was observed in several species of the rhizostomean suborder Kolpophorae: *Cephea cephea* (Sugiura, 1966), *Cotylorhiza tuberculata* (Kikinger, 1992), *Mastigas papua* (Sugiura, 1963); and order Coronatae: *Nausithoe aurea* (da Silveira and Morandini, 1997) and *Nausithoe plan-*

ulophora (Werner, 1971). The budding process is well studied in polyps of the Cassiopeidae family (Rhizostomeae: Kolpophorae) using the methods of histology (Bigelow, 1900), scanning (Van Lieshout and Martin, 1992) and transmission electron microscopy (Hofmann and Gottlieb, 1991). The first stage of bud growth is similar to that of lateral budding. A rounded evagination forms at the base of the polyp calyx in one or several sites simultaneously. The evagination quickly grows and forms a ciliated lemon-shaped bud called a planuloid (Fig. 1A).

The planuloid detaches and swims away, wherein the distal end of the growing bud becomes the functionally anterior end of the planuloid (Fig. 1B). Often a new bud starts to grow before the separation of the previous planuloid. In this way a chain of up to 5–6 planuloids can form at one budding site (Hofmann et al., 1978; our observations). During the swimming period the planuloid forms tentacles and hypostome anlagen at the posterior end (Fig. 1C). Soon it attaches with the anterior end and transforms to a young polyp (Thieme and Hofmann, 2003).

It was repeatedly shown that both tissue layers are involved in the early stages of bud development (Bigelow, 1900; Hofmann et al., 1978; Van Lieshout and Martin, 1992). Besides the migration of epidermal cells into the bud, evagination was traced using vital markers (Hofmann and Gottlieb, 1991). However, no cell division was observed in the growing bud or planuloid, and it is still unclear where the proliferation zones are located. To find out the cell source of planuloid formation and growth we have studied the dynamics of DNA synthesis during the budding process in *Cassiopea xamachana* Bigelow, 1892.

Material and methods

A culture of *C. xamachana* polyps was maintained in artificial sea water with 35‰ salinity at room temperature in plastic culture tanks. Polyps were regularly fed on the nauplii of *Artemia*. In laboratory conditions, planuloids formed constantly without any induction.

Polyps with growing buds and planuloids at different developmental stages were collected in small plastic dishes and incubated for 1–1.5 hours at room temperature in a 50- μ M solution of 5-ethynyl-2'-deoxyuridine (EdU) in filtered artificial sea water. Longer incubation time led to excess labeling. After incubation the specimens were washed 3 times for 10 minutes in filtered sea water, then anesthetized in 7.5% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ until complete musculature relaxation, and fixed in 4% paraformaldehyde on 1X Phosphate-buffered saline (PBS) at room temperature for 1–2 hours. After fixation, specimens were washed in PBS with 1% Tween-20 (PBT) 3 times for 15 minutes.

All specimens were labeled with fluorescent Cy3 dye following the standard protocol (Shunatova and Borisenko, 2020). After staining, specimens were washed in PBS 3 times for 15 minutes, additionally labeled with DAPI, and mounted in Mowiol 4–88 (Wurm et al., 2010). The confocal stacks were acquired with a Leica TCS SP5 Laser Scanning Confocal Microscope, and analyzed using Fiji (Schindelin et al., 2012).

Calculation of the labeled nuclei was done for 4 specimens at every stage of the planuloid development. A set of sagittal confocal slices of the whole speci-

men 10–15 μm thick were used for each calculation. Thus, only two tissue layers were included in counting and not the whole animal. In the case of polyps, every sample was divided in areas of $100 \times 100 \mu\text{m}^2$, in which labeled nuclei were counted; in the case of planuloids, the labeled nuclei were counted in the whole specimens and then recalculated for $100 \times 100 \mu\text{m}^2$ areas. The value of labeled nuclei in these areas was averaged. In every case the range of extreme values is shown, taking into account the standard deviation, calculated in Microsoft Office Excel.

Results

We determined several zones of active DNA synthesis at different stages of budding growth and planuloid development in *C. xamachana*. In a polyp with growing buds, two zones of EdU incorporation were observed (Fig. 2A). The first one was situated at the large region of polyp calyx from its base to the oral disk. The labeled nuclei were distributed evenly. Solitary labeled nuclei were found in tentacles and stalk nearby the calyx. The second zone was located throughout the bud evagination from the base to the distal end, except the small area at the tip (Fig. 2B). The EdU signals in the evagination were located very densely, so that individual nuclei were sometimes indistinguishable. The number of labeled nuclei in the evagination area was about 120–140 per $100 \times 100 \mu\text{m}^2$. The EdU incorporation in the bud evagination was notably higher than in the calyx. However, we never observed any changes in labeling rate near the bud evagination. At this area as well as in the calyx, the number of labeled nuclei varied from 45 to 65 per $100 \times 100 \mu\text{m}^2$.

In almost formed planuloids the number of labeled nuclei slightly decreased to 30–50 per $100 \times 100 \mu\text{m}^2$. It was especially noticeable in planuloid chains (Fig. 2C). In this case the most abundant signal was still observed in the evagination, whereas in each subsequent planuloid in the chain it was much weaker. The location of the EdU labeling zone remained the same: the strongest level of label incorporation was detected near the planuloid base. The number of labeled nuclei insignificantly decreased to the distal end, without any signal at the tip.

In swimming planuloids we observed the same pattern of EdU labeling (Fig. 3A, B). The rate of incorporation varied between different specimens from 40 to 60 labeled nuclei per $100 \times 100 \mu\text{m}^2$, but the character of label distribution remained constant. The number of labeled nuclei at the posterior end of the planuloid was about 3.2–3.4 times higher than at the anterior end: about 34–41 and 8–14 per $100 \times 100 \mu\text{m}^2$, respectively. During further development, the planuloids become elongated and start to form tentacle and hypostome anlagen at the posterior end, as well as stalk anlagen at the

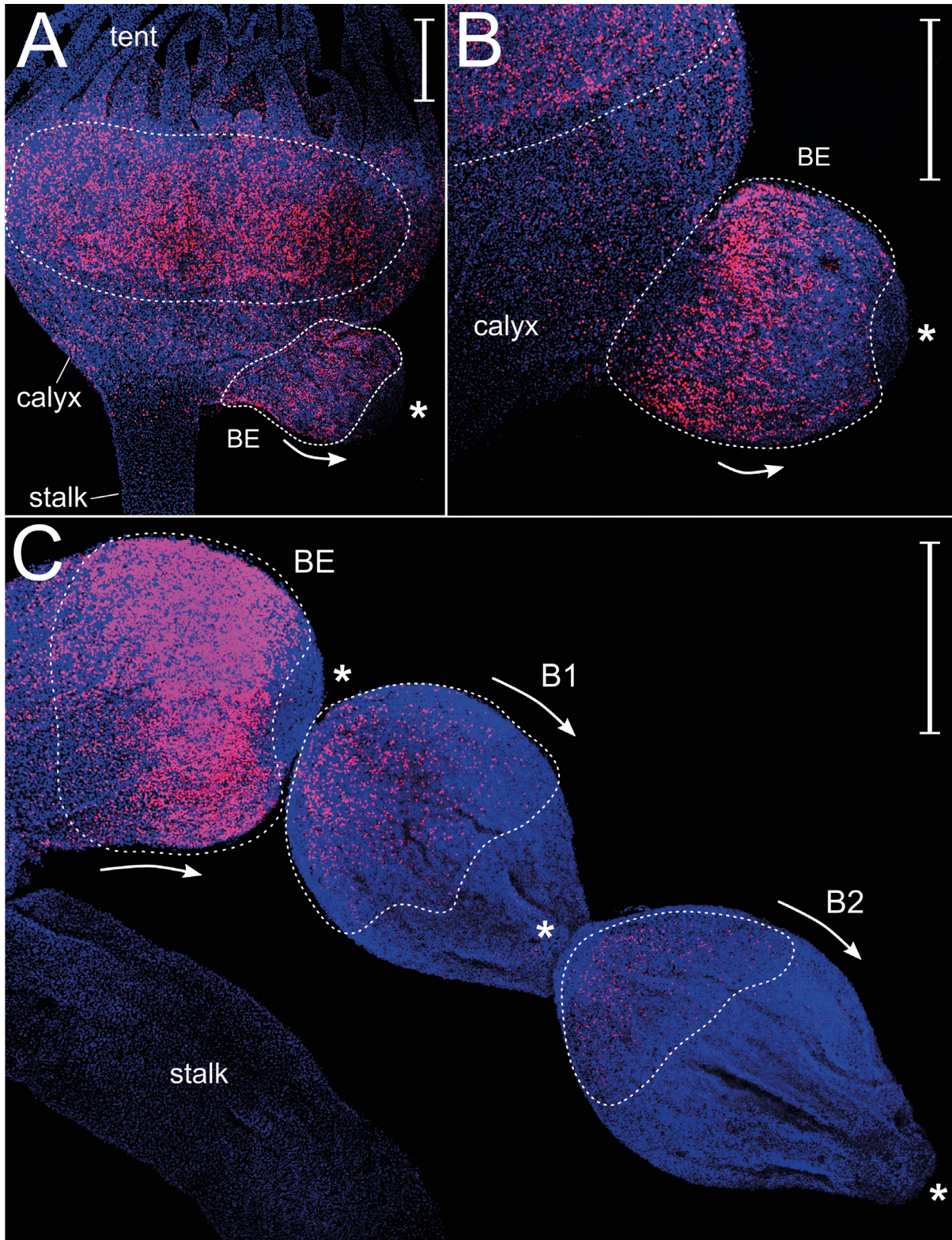


Fig. 2. Polyp and growing buds of *C. xamachana*, labeled with EdU (pink) and DAPI (blue).

A — polyp with growing bud evagination, B — bud evagination, C — bud evagination and two planuloid buds in chain. Abbreviations: B1, B2 — planuloid buds, BE — bud evagination, tent — tentacles. The functionally anterior end of the planuloid is labeled with an asterisk; arrows show the direction of bud growth; the dotted line marks the border of labeling zone. Scale bar — 100 μm .

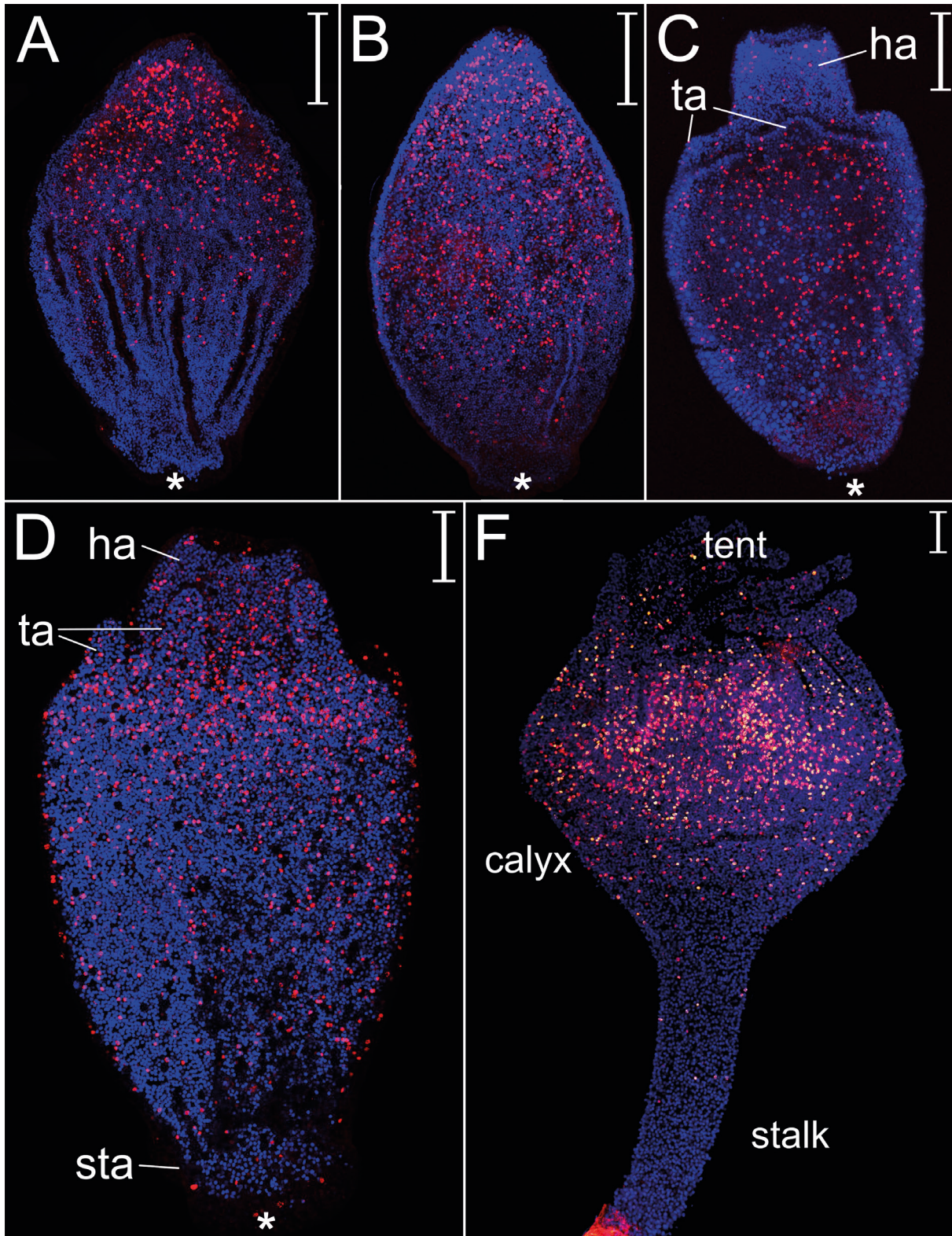


Fig. 3. Planuloid development stages and young polyp of *C. xamachana*, labeled with EdU (pink and red) and DAPI (blue). A — planuloid, just after separation, B — planuloid, one day after separation, C — late planuloid, D — planuloid before settlement, F — young polyp. Abbreviations: ha — hypostome anlage, sta — stalk anlage, ta — tentacle anlage, tent — tentacles. The functionally anterior end of the planuloid is labeled with an asterisk. Scale bar — 100 μ m.

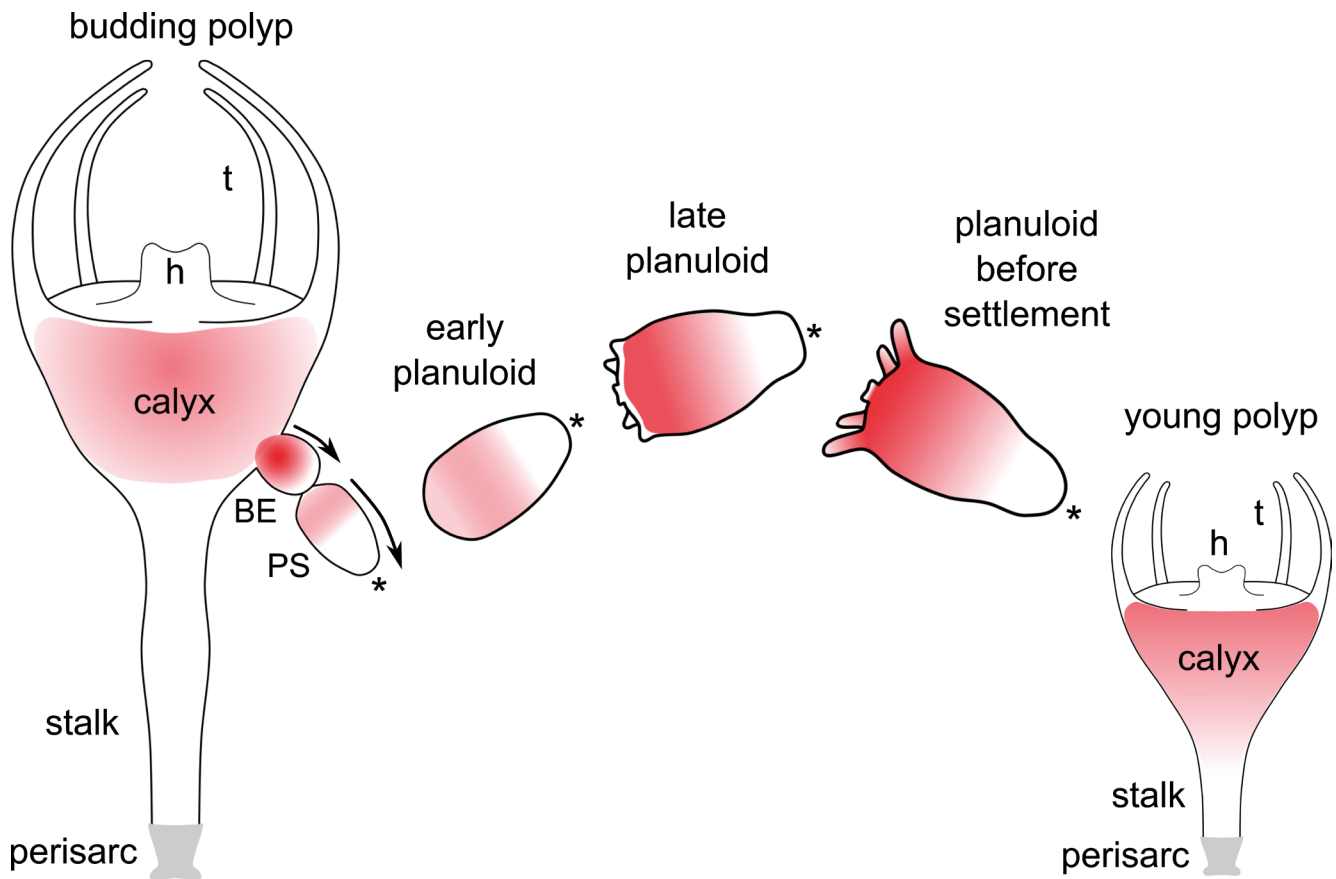


Fig. 4. Scheme of proliferation zone activity at the different stages of planuloid budding in *C. xamachana*.

Abbreviations: BE — bud evagination, h — hypostome, PS — planuloid before separation, t — tentacles. The functionally anterior end of the planuloid is labeled with an asterisk; the arrow shows the direction of bud growth. Relative dimensions are not kept.

anterior end (Fig. 3C, D). In this moment the EdU labeling zone occupied the whole body. However, the incorporation rate was the strongest at the area of the tentacle anlagen — about 38–42 labeled nuclei per $100 \times 100 \mu\text{m}^2$. Fewer labeled nuclei were observed at the tip of the anterior end, where they had not been present before. In total, the number of labeled nuclei at the anterior end of the late planuloid was about 10–14 per $100 \times 100 \mu\text{m}^2$.

After settlement, the planuloid transforms into a young polyp with differentiated calyx, stalk, and tentacles (Fig. 3F). In the young polyp the EdU labeling zone was located at the calyx. The number of labeled nuclei was still high — about 30–40 per $100 \times 100 \mu\text{m}^2$ — and was the largest at the tentacles base — about 42–52 per $100 \times 100 \mu\text{m}^2$. Individual labeled cells were also observed in the tentacles and stalk.

Discussion

In polyps and planuloids of *C. xamachana*, EdU labeling occurs in several regions during bud growth and planuloid development. This indicates active DNA synthesis, suggesting the presence of functioning proliferation zones during budding and formation of the new polyp.

The general scheme of proliferation activity in *C. xamachana* polyp is presented in Figure 4.

The earliest stages of budding are usually poorly distinguishable in polyps, since there are no obvious morphological features of future bud anlage. However, it was shown in previous studies that the primary cell source for bud formation is parent polyp tissues. Epidermal and gastrodermal cells can migrate to the budding site and possibly their proliferation provides the bud protrusion forming (Hofmann and Gottlieb, 1991; Hofmann et al., 2003). Subsequently, the active proliferation zone forms in the growing bud. The EdU labeling rate in this region is much higher than in any other part of the polyp body. Besides, the labeling level of calyx tissues does not increase near the budding site. Possibly at this stage the growth of the planuloid is carried out by cell divisions precisely in the bud evagination.

The same process of cell migration to the bud anlage and proliferation activity in the bud protrusion was also observed during lateral budding in *Hydra* sp. (Otto and Campbell, 1977; Holstein et al., 1991). However, in this case cell proliferation is less important for bud growth than cell movement (Clarkson and Wolpert, 1967). Proliferation activity was also shown during the

lateral budding of *Aurelia aurita*, but the labeling level in buds was only slightly higher than in polyp tissues (Balcer and Black, 1991). Although the initial stages of bud anlage formation might be similar in planuloid budding of *C. xamachana* and lateral budding of other studied species, further steps of bud development differ.

Proliferation activity decreases up to the moment of complete planuloid formation. In the brief period after separation, planuloids do not grow but start to differentiate their own musculature (Khabibulina and Starunov, 2019). Shortly thereafter the new growth phase begins, and proliferation activity increases again. The highest labeling level is observed in the hypostome and tentacle anlagen at the posterior end of the planuloid. The forming cells are not necessary immediately involved in tissue composition. They probably accumulate for growth acceleration after settlement.

Interestingly, the anterior end of the planuloid remains unlabeled from the start of bud formation to the late planuloid stage. At this end mostly glandular cells, used for substrate attachment, are located (Van Lieshout and Martin, 1992). Since these cells differentiate early, no proliferation activity occurs in the anterior region until the beginning of stalk formation and settlement. However, even at this stage only a few labeled nuclei are observed.

After settlement the anterior end of the planuloid becomes the oral end of a new polyp. The zone of active DNA synthesis in a young polyp continues to function and expands as the calyx grows, but never involves the tentacles and stalk, where only a few individual labeled nuclei are found. The further growth of tentacles and stalk during the polyp life is apparently provided by cell sources at their bases.

The calyx active proliferation zone is preserved in budding polyps, though the rate of cell divisions in it decreases. Apparently this zone proceeds during the whole life of a *C. xamachana* polyp. It may act as the cell source not only for budding and body growth, but also for regeneration (Curtis and Cowden, 1974) or jellyfish production by strobilation (Hofmann et al., 2003; Helm, 2018).

Thus, the *C. xamachana* polyp has one active proliferation zone, which forms at the early budding stage and remains active throughout the polyp's life. Although the activity of this zone changes depending on the growth phase, its relative localization in the body is the same in the growing bud, early and late planuloid, and then in the polyp.

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