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The influence of photosymbiosis in *Cassiopea xamachana* regenerative success

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Abstract
The regenerative capacity of Scyphozoans (Phylum Cnidaria) has been relatively understudied. The model organism *Cassiopea xamachana* hosts photosynthetic dinoflagellate symbionts in the host's motile amoebocyte cells. A handful of studies have reported regeneration in the polyps of *C. xamachana*, but the mechanisms underlying regeneration have not been fully explored. Despite undergoing drastic developmental changes when symbiotic, the effect of symbiont presence and species on host regeneration has never been explored. *C. xamachana* polyps were decapitated when aposymbiotic, and symbiotic with both a homologous and a heterologous symbiont species. Regeneration and asexual budding were observed, and EdU labeling was performed to observe patterns of cell proliferation in regenerating polyps. The presence of symbionts increased likelihood to regenerate, yet symbiont species did not affect success of regeneration. No blastema or dividing cells were observed, implying cell proliferation is not the primary mechanism behind regeneration in polyps of *C. xamachana*.

Keywords Scyphozoa · Wound healing · Upside-down jellyfish · Cell proliferation · Regeneration · Symbiodiniaceae

1 Introduction

Cnidarians represent one of the most basal metazoan lineages equipped with fascinating regenerative abilities (Gold and Jacobs 2013). Unifying principles of regeneration across cnidarian taxa are missing due to their diverse life histories (D'ambra 2021). Most regeneration studies have focused on hydrozoans, in particular the notable model organisms *Hydra* (reviewed by Vogg et al. 2019*)* and *Hydractinia* (Plickert et al. 2012). Hydrozoan "stem cells" are called I-cells (i.e., interstitial stem cells). They are found between ectodermal cells and can produce neurons, cnidocytes, gland cells, and germ cells (Juliano et al. 2014; Siebert et al. 2019; Gahan et al. 2016; Rebscher et al. 2008). Non-hydrozoan cnidarians seem to lack I-cells but possess amoebocytes (Tucker et al. 2011; Chapman 1999). Amoebocytes are involved in immunity through phagocytosis during wound healing (Tucker et al. 2011; Mydlarz et al. 2008) and play a role in endosymbiosis in photosymbiotic cnidarians (Fitt and Trench 1983). In some cnidarian models, such as *Hydractinia echinata*, head amputation (but not stolon amputation) is followed by blastema formation which at first involves stretching of epithelial cells and is then followed by cell proliferation (Bradshaw et al. 2015). In contrast, *Hydra* head regeneration can occur through cell reorganization. Cell proliferation only accompanies the head regeneration process (Cummings and Bode 1984, Govindasamy et al. 2014).

Studies on scyphozoan regenerative ability have predominantly been morphology-based, using both symbiotic (*Cassiopea* spp.) and non-symbiotic jellyfish (*Aurelia aurita* and *Chrysaora quinquecirrha*). These scyphozoans have been reported to regenerate epithelial tissue and repair wounds without the presence of I-cells as well as restore functional morphology without cell proliferation during ephyra (i.e., juvenile medusa) symmetrization (Black and Riley 1985; Curtis and Cowden 1972,1974; Steinberg 1963; Abrams et al. 2015). Considering the lack of I-cells and potential role of amoebocytes in both wound healing and symbiosis, studying the regenerative process in a symbiotic scyphozoan can help disentangle the different processes taking place.

Cassiopea xamachana, also known as the upside-down jellyfish, is a tropical scyphozoan commonly found in

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shallow water habitats such as mangroves, lagoons, and seagrass meadows. They are among the symbiotic scyphozoans that associate with photosynthetic dinoflagellates from the family Symbiodiniaceae. The jellyfish provides a sheltered habitat rich in nitrogen and phosphorus catabolites to the microalgae that, in turn, translocate organic carbon products (e.g., sugars) to their host (Mortillaro et al. 2009). *C. xamachana* is an emerging model organism due to its easy husbandry, the ability to culture its entire life cycle (Fig. 1) in the lab, and its developmentally symbiotic lifestyle (Ohdera et al. 2018, Medina et al. 2021). *C. xamachana* polyps are unable to develop into adults and complete their life cycle without the successful establishment of photosymbiosis with Symbiodiniaceae (Hofmann et al. 1996).

A few studies have examined regeneration across different stages along *C. xamachana* life cycle. Neumann et al. (1979) found that planulae can reassume a smaller larval shape or a stalkless polyp from aboral or oral fragments, respectively. Curtis and Cowden (1972, 1974) tried different types of wounds on polyps showing that 1) polyps could regenerate a missing tentacle or part of the hypostome, 2) stalks without heads were able to regenerate completely, while 3) heads with no stalk could only produce buds. These studies suggest that *Cassiopea* initializes the quick regeneration of body parts that guarantee a function (i.e., mouth, tentacles, and hypostome), followed by the slower reacquisition of their radial symmetry (Curtis and Cowden 1972). Decapitated heads, despite not regrowing a new stalk, continued to produce planuloid buds from the point between the calyx and the stalk suggesting that energy allocation in *Cassiopea* centers around function and reproduction (Niina et al. 2015). Although adult medusae have less-studied regenerative ability, they are known to regenerate sense organs (rhopalia) (Cary 1916). Gamero-Mora et al. (2019) and Ostendarp et al. (2022) showed that the regeneration of an entire medusae can take place as a consequence of an injury in the bell, suggesting pluripotentiality. However, like in other scyphozoans, there has been limited evidence of stem cells in *Cassiopea*, except for the planula larvae where I-cells were found in the endoderm (Martin and Chia 1982).

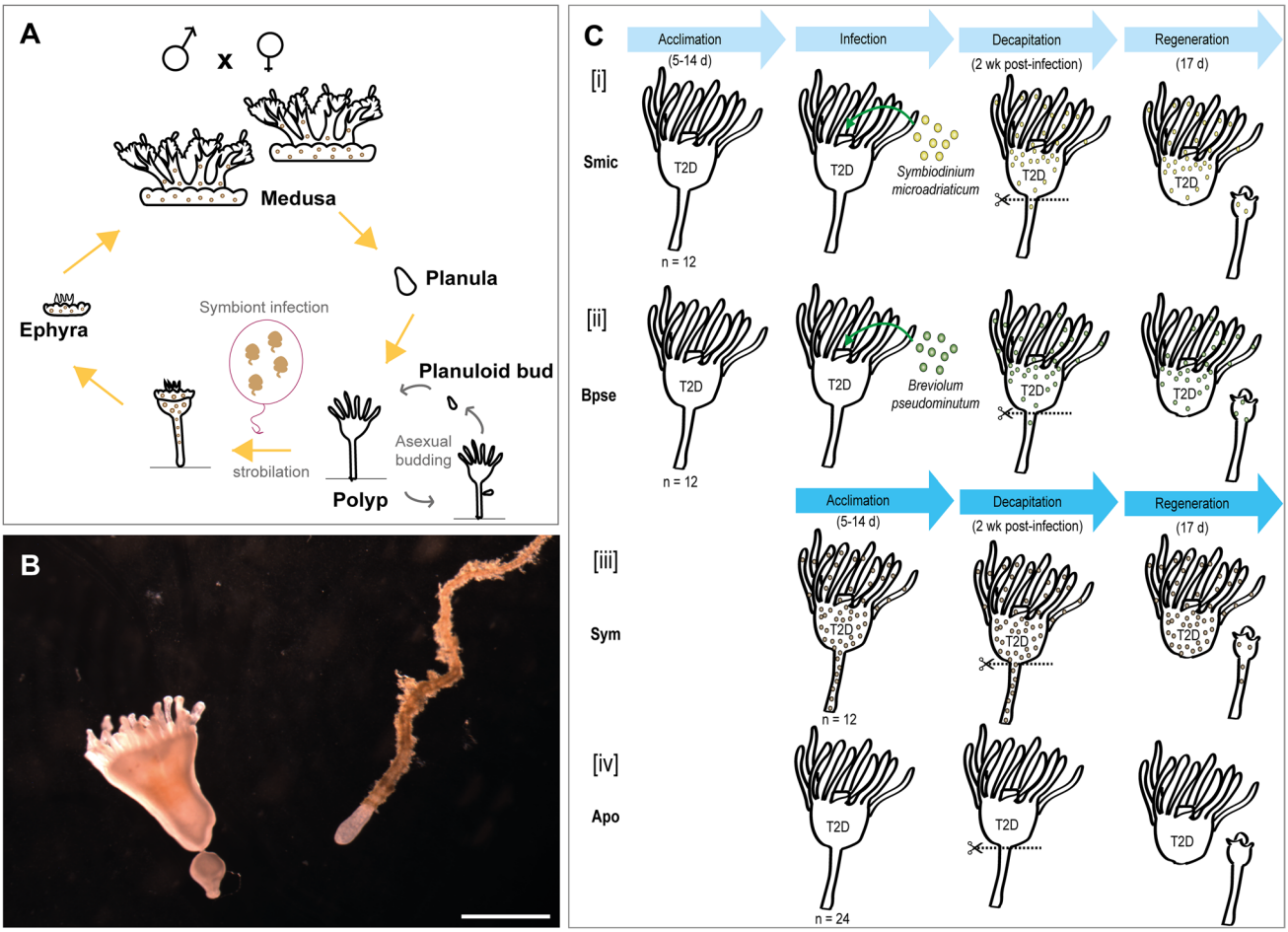

Fig. 1 Study design. A) Life cycle of *Cassiopea xamachana*: alternation of sexual (medusa) and asexual phases (polyp). Polyps reproduce asexually through budding and/or strobilation only after symbiont acquisition. B) Example of the performed decapitations and polyp bud production from the polyp decapitated head. C) Experimental design

Here, we followed decapitated aposymbiotic and symbiotic polyps with the aim of understanding how the presence of distinct Symbiodiniaceae species in symbiotic *Cassiopea* can influence the regenerative process. Observations of the bud production and localization/proliferation of dinoflagellates were carried out with the hypothesis that symbionts play an important role in facilitating host regenerative processes, providing additional sources of energy.

2 Material and methods

2.1 Experimental setup

We used aposymbiotic *C. xamachana* polyps (clonal line T2D) from the Medina Lab polyp collection (Pennsylvania State University) (referred to as *Apo*). We established symbiosis with specific dinoflagellate species (i.e., *Symbiodinium microadriaticum* and *Breviolum pseudominutum*) kept in culture. Our symbiotic *C. xamachana* polyps (referred to as *Sym* hereafter) possessed unknown symbiont species and came from other Medina Lab aquaria. *Sym* polyps underwent DNA extraction (n = 3) and sequencing for the LSU/28S gene following the protocols in LaJeunesse et al. (2018) with the primers: 28S-forward (5'-CCCGCTGAATTTAAGCATATAAGTAAGCGG-3′) and 28S-reverse (5'-GTTAGACTCCTTGGTCCGTGTTTCAAGA-3′). The sequences were compared against the Symbiodiniaceae LSU sequences in LaJeunesse et al. (2018) for species identification using Geneious Prime 2023.1 (https://www.geneious.com).

We acclimated polyps for 5–14 days to a 12:12 light/dark cycle at approximately 150 µmol/m^2/s, and day/night temperatures of 27.5 °C and 25.5 °C, respectively, in artificial seawater (Instant Ocean) 35 ppt. Polyps were fed daily with *Artemia franciscana.* Water changes were done triweekly.

2.2 Regeneration assays

For our regeneration assays we used four different treatments: [i] T2D polyps infected with *S. microadriaticum* strain CassKB8 (*Smic*, n = 12), [ii] T2D polyps infected with *Breviolum pseudominutum* strain rt-147 (*Bpse,* n = 12), [iii] *Sym* polyps (n = 12), and [iv] aposymbiotic T2D polyps as control (Apo n = 24: 12 used as *Smic* and *Bpse* control and 12 used for *Sym* control). *Smic* and *Bpse* polyps were infected with a concentration of ~150,000 cells/mL of respective Symbiodiniaceae symbionts two weeks before decapitation. The decapitation consisted of a wound made between the calyx and the stalk using a thin razor blade (Fig. 1a) two weeks post infection. Both parts (stalk and head) were kept in the same well of 6-well culture plates (Sigma-Aldritch). They were fed daily, and water (35 ppt) changes were done triweekly.

2.3 Imaging and observation

We took daily images of the decapitated polyps in each treatment on a Leica MZ16 F fluorescence stereo microscope. We counted daily 1) fully detached buds for each polyp, 2) survival and 3) regeneration of the cut stalks and heads. These observations proceeded for 17 days after decapitation. We used a separate group of polyps (n = 3 per treatment) for confocal microscope imaging by a 780 NLO confocal microscope (Carl Zeiss AG), 4 times during the 17 days for each trial and treatment. These confocal images were used for counting symbiont numbers in Day 1 (post-decapitation) and Day 11 polyps, for all symbiont treatments using the Cell Counter plug-in in ImageJ (https://imagej.nih.gov/ij/). Symbiotic microalgae were observed at a wavelength of approximately 650–700 nm.

2.4 EdU labelling

We performed EdU (5-ethynyl-2′-deoxyuridine) labeling of regenerating polyps (n = 2 per timepoint per treatment, separate group) using the Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit (Life Technologies), according to the manufacturer's protocol with the following adjustments: polyps were incubated for 30 min at 50 µM EdU concentration, and polyps were fixed in 3.7% paraformaldehyde in PBS instead of formaldehyde. Polyps were labeled and fixed at pre-incision, immediately after decapitation, 6 h after, 8 days after, and 17 days after. Images were taken on an Olympus BX61 Microscope at the Penn State Huck Microscopy Center.

2.5 Statistical analysis

We ran statistical analysis on R (R Core Team 2013) using the "stats", "rstatix", and "RVAideMemoire" packages. We used Fisher's Exact Test to compare status of stalks (i.e., regenerated, dead, dying, or no change) with symbiont species and symbiotic state (i.e., symbiotic or aposymbiotic). We used a one-way ANOVA (analysis of variance) to compare number of buds produced across symbiont treatments. Plots were generated using the "ggplot2" package.

3 Results

3.1 DNA extraction and polyp regeneration observation

Sym polyps' LSU sequences matched to *Symbiodidium microadriaticum* (Fig. S1).

Decapitated polyp stalks regenerated a head, but heads did not regenerate the stalk. The status of the stalk was

tracked and recorded for budding observation (Fig. 2, S2, 4); confocal microscopy imaging (Fig. 3, S3), and EdU labeling (Fig. 5, S4), as described in 'Materials and Methods'. We observed regeneration in all treatments. In most cases, first signs of regeneration appeared at Day 4 and were completed by Day 14 (Fig. 2). Some stalks did not regenerate and died, characterized by the loss of tissue and algal overgrowth (Fig. S2). None of the decapitated heads regenerated stalks, although the ones with stalk tissue left on the calyx post-incision could potentially be mistaken for regeneration. In heads with precise incisions between the calyx and stalks (ex. Figure 2), no stalk regenerated compared to polyps with stalk tissue remaining on the head (Fig. 2, red arrow). Although decapitated heads did not show evidence of stalk regeneration during our experiment, they still produce planuloid buds (Fig. 2) as usual, and symbiotic heads were able to strobilate (Fig. S2).

Morphologically, we observed no evident influence of the dinoflagellate symbionts on stalk regeneration (e.g., head shape and tentacles number; Fig. 3). Counts of symbionts in polyps showed *Sym* polyps had a much higher initial starting concentration of symbionts in decapitated stalks (42/polyp) than the other symbiont treatments, though all symbiotic polyps increased in symbiont abundance by Day 17 (Table S1). However, in stalks with fewer symbionts initially, no dinoflagellates were seen migrating to the point of incision or noticeably proliferating within the regenerating head. For example, at Day 7, *Smic* and *Bpse* polyps still had a limited number of symbionts although most tissues had regenerated. Contrary to symbionts in *Smic* and *Bpse* polyps, symbionts in *Sym* polyps were located at the apex of the cut stalk at Day 1, and more symbionts were present during regeneration (as seen in Fig. 3).

4 Bud counts

We counted the number of buds produced by decapitated polyp heads across all treatments (i, ii, iii, and iv) to assess differences in regeneration (Fig. 4 A,B). Both symbiont species and symbiotic state of the host contributed to significant differences in bud count between treatments (one-way ANOVA $p = 2.716e\text{-}07$ and $p = 0.002188$, respectively; Table S2). *Sym* polyp heads produced significantly more buds than those from other treatments (88 in total), while *Smic* polyps produced the fewest detached buds overall (19 in total; Fig. 4A). Apo polyps produced a total of 23 buds compared to the symbiotic polyps (Fig. 4).

Stalk status was classified as: regenerated (head regrown), no change (without signs of disintegrated or regeneration), dying (degrading tissue), or dead (disintegrated and covered in algae). There was a significant difference in symbiotic state and final status of stalk (Fisher's Exact Test, $p = 0.01365$; Table S2). *Sym* stalks had more regeneration and yielded fewer dead polyps than the predicted expected values, whereas Apo polyps resulted in more dead polyps and fewer regenerated polyps than expected (Fig. 4C, D). This was confirmed with a Fisher's Exact Test on only *Sym* polyps compared to Apo polyps ($p = 0.002059$; Table S2, S3), and the post-hoc test showed the results of dead polyps against regenerated polyps was driving the significance. When all symbiotic polyps were grouped (*Bpse, Smic,* and *Sym*), there was a significant difference between aposymbiotic and symbiotic polyps for living versus dead polyps (grouping no change and regenerated numbers compared to dead and dying number) (Fisher's Exact Test, $p = 0.02358$) and for regenerated versus non-regenerated polyps

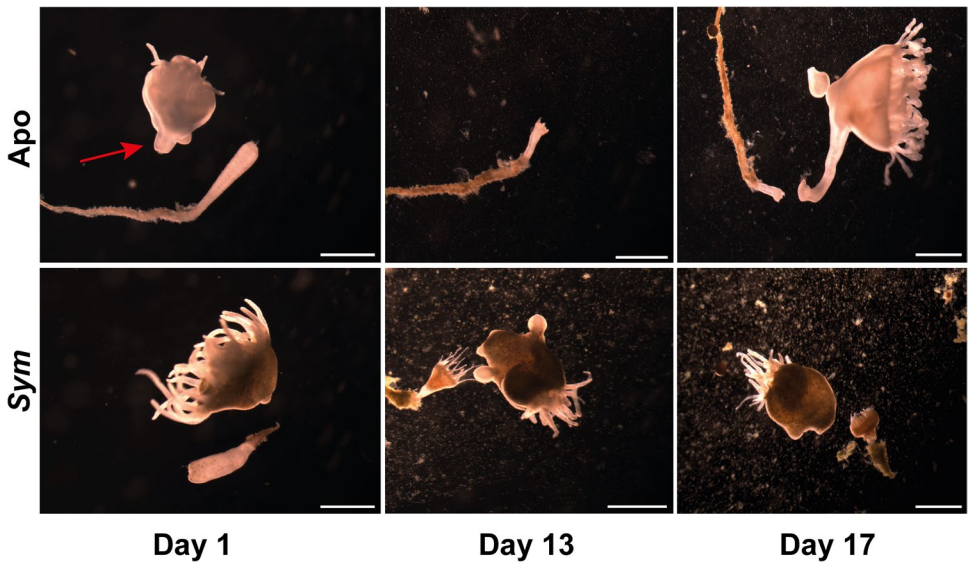

Fig. 2 Time series of an aposymbiotic polyp regenerating (top) and a *Sym* polyp regenerating. The red arrow indicates an unprecise incision leaving residual stalk tissue on the calyx. Scale bars = 1 mm

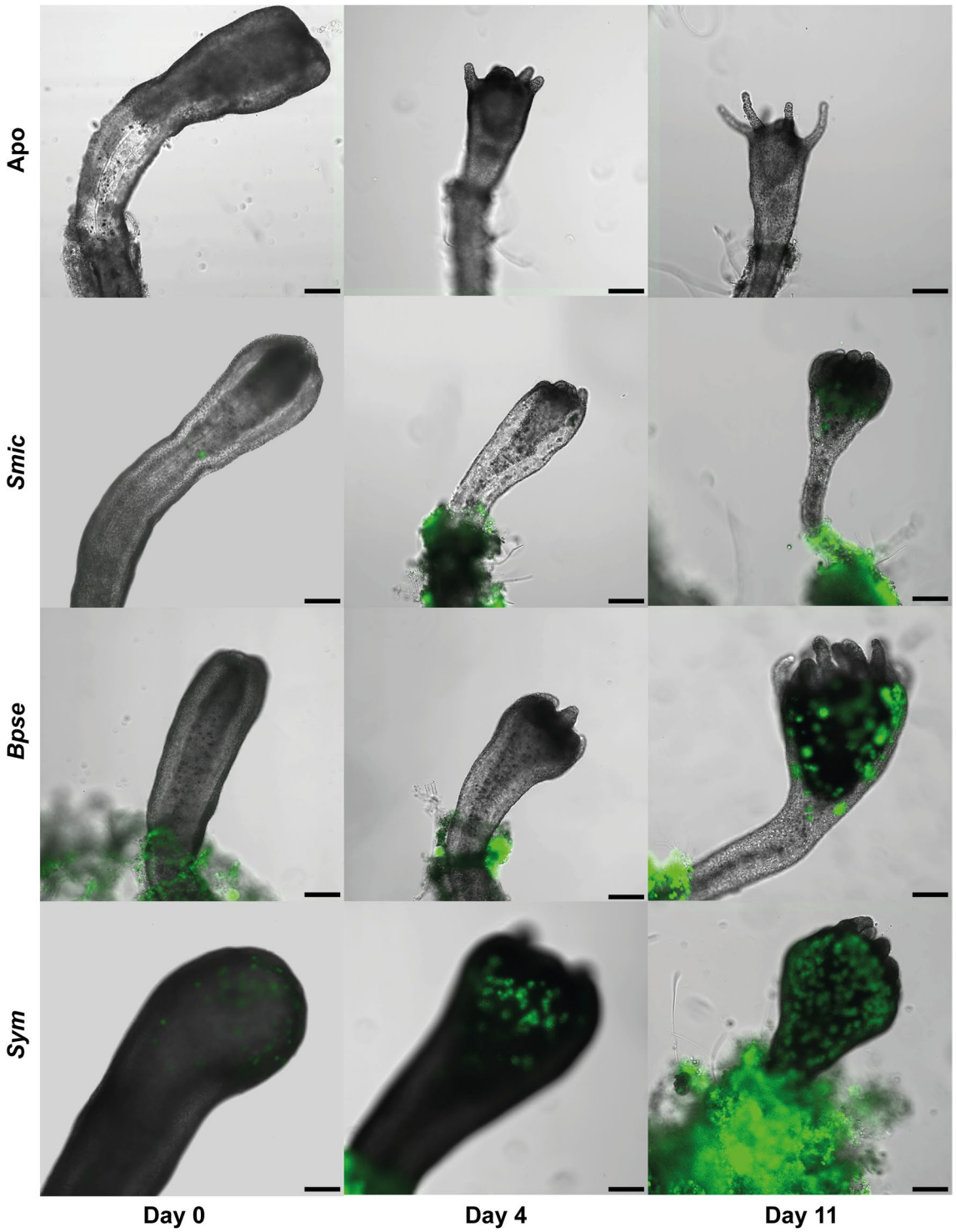

Fig. 3 Confocal imaging micrographs of a time series of polyp regeneration. Both Symbiodiniaceae and non-symbiotic brown algae from the aquaria, are visible in green. Treatments are listed on the left and days post-decapitation at the bottom. Scale bars represent 100 µm

(grouped no change, dead, and dying) (Fisher's Exact Test, $p = 0.03728$). There was no significant difference (ANOVA, $p = 0.116959$) between stalk status and experiment (i.e., polyps used for bud counts, confocal imaging, or EdU labeling), or between stalk status, treatment, and experiment (ANOVA, $p = 0.9822$).

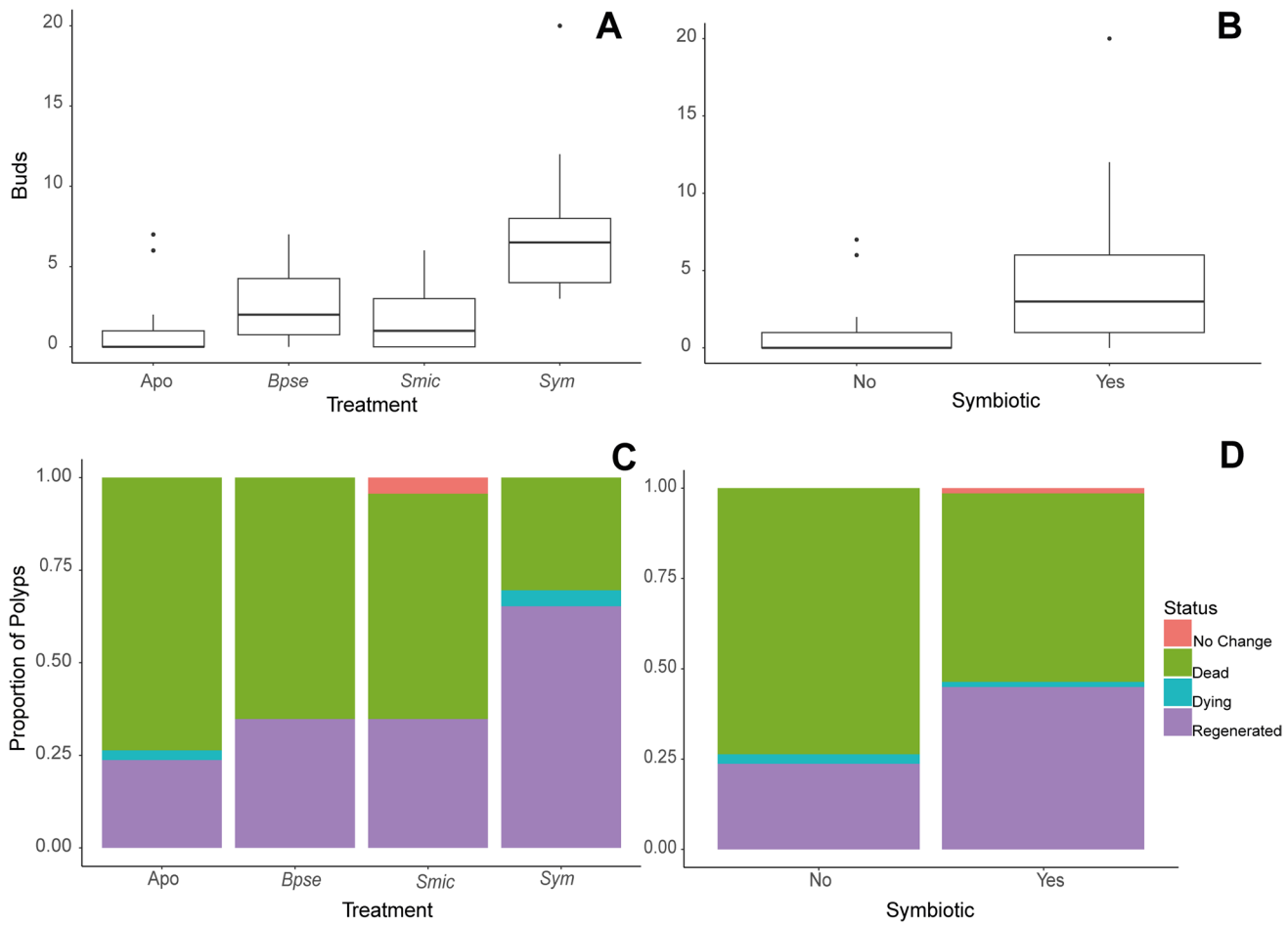

Fig. 4 Bud counts and stalk status. Stalk status categorized as: regenerated, dead, dying, and no change stalks. A) Box plot distribution of bud production grouped by treatment. Number of buds on the x-axis. B) Box plot distribution of bud production grouped by symbiotic status. Dead stalks lost visible tissue and were overgrown by algae. C) Stacked bar plot of proportion of stalk status grouped by treatment. D) Stacked bar plot of proportion of stalk status grouped by symbiotic status

4.1 EdU

Regenerating polyps were labeled with both EdU and the nuclear Hoescht staining, according to the kit manufacturer's protocol. No difference in number or location of EdU-labeled cells was observed between algal symbiont or time point (Fig. 5,4S); no decapitated *Sym* polyps were included in this assay because they had all died after day 8 due to algal overgrowth in the holding container.

5 Discussion

Overall, our results show a higher bud production for symbiotic polyps and significant difference in symbiotic state and final status of stalk, with higher mortality for aposymbiotic polyps. We found no correlation between localized proliferating cells and host regeneration in any aposymbiotic or symbiotic polyp, suggesting regeneration in *Cassiopea xamachana* occurs through a different mechanism.

Consistent with previous studies (Curtis and Cowden 1972,1974; Niina et al. 2015), heads removed from stalks never regenerated a new stalk. However, some heads had a piece of stalk tissue left at the bottom of the calyx due to inaccuracies in the dissection (Fig. 2). The stalk tissue left on these heads was able to grow and elongate into a full stalk, simulating regeneration. As polyp stalks continuously grow across a polyp lifetime (unpublished, Sharp, V., Ohdera, A., Medina, M.), the remaining stalk tissue gave the illusion of regrowth. On specimens where the stalk was removed completely from the head, there was no stalk regrowth but only bud formation, suggesting that bud formation and polyp stalk regeneration are regulated by distinct mechanisms. These results suggest that polyp heads either lack the capability for regeneration of stalks or prefer to allocate their energy in reproduction.

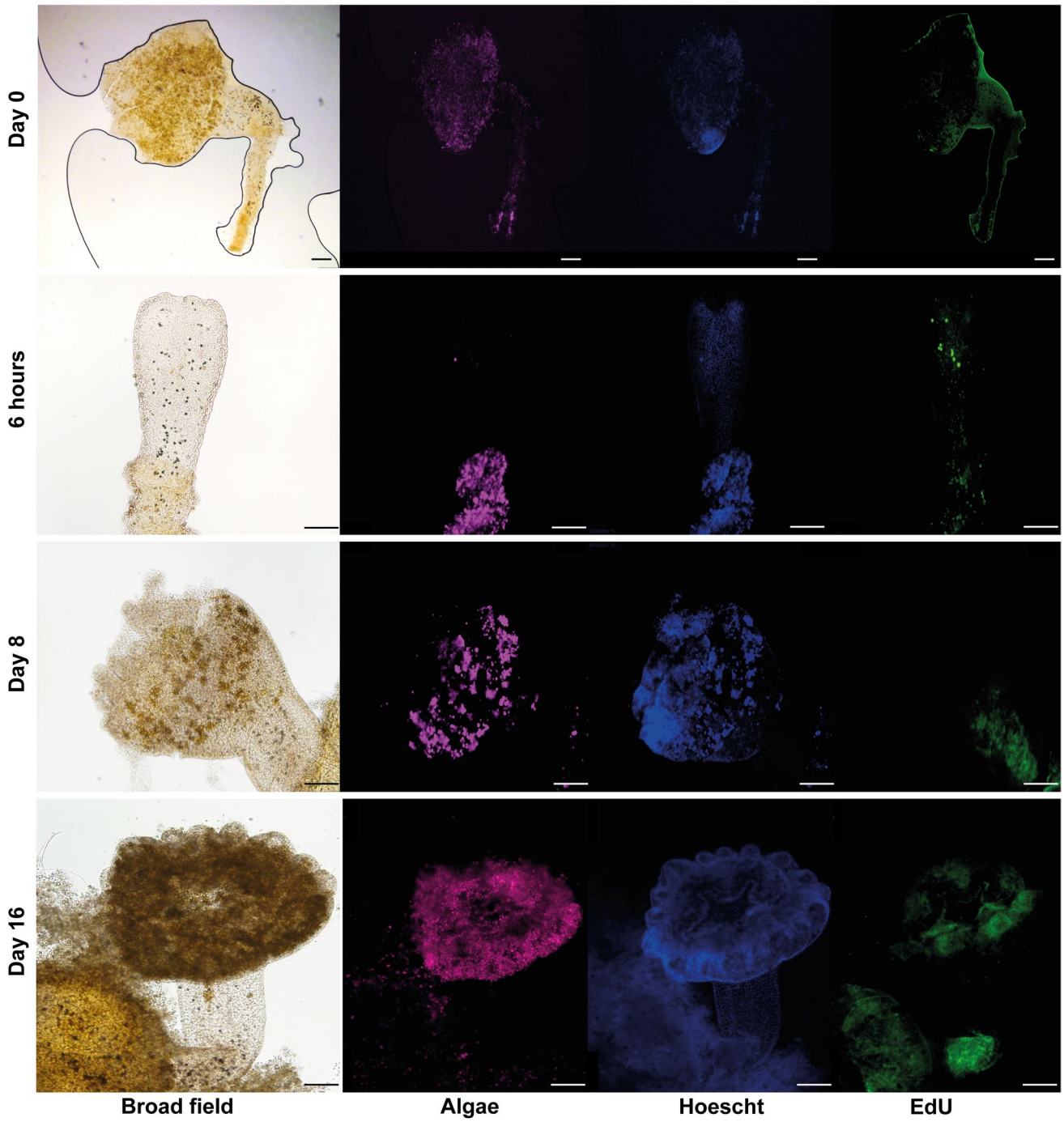

Fig. 5 EdU-labeled *Bpse* polyps. Time post-decapitation listed on left, where Day 0 indicates before decapitation. Each row represents a single polyp split into broad field, ~650 nm, ~200 nm, and 488 nm fluorescent wavelengths from left to right. Scale bars represent 200 µm

Decapitated *Sym* polyps exhibited more regeneration than polyps from any other treatment and a higher bud production. Bud generation is caused by cell division along the perradial sites of the polyp calyx (Hofmann and Gottlieb 1991). Hofmann found that cell division occurred mid-polyp, which then morphs into the cilia-covered buds. In the three symbiotic treatments (*Smic, Bpse*, and *Sym*), polyp heads also produced more buds after decapitation than those of aposymbiotic polyps (Fig. 4A,B) suggesting that the photosymbiotic algae provide an excess of organic carbon compounds, and therefore energy to the host, allowing for faster production of new cells.

There were more algal cells in *Sym* polyps than in *Smic* and *Bpse* polyps, which had fewer algal cells (Fig. 3). The greater abundance of algal cells present in *Sym* stalks after decapitation may have contributed to successful regeneration by providing more photosynthetic energy that could not be obtained from the other symbiotic treatments or from aposymbiotic stalks. *Sym* polyps originated from an aquarium tank that had been previously inoculated with Symbiodiniaceae approximately 8 months before, and genotyping matched its symbiont species to *Symbiodinium microadriaticum.* The significantly different results in regeneration success and bud production between *Smic* and *Sym* polyps suggests the driving influence on these fitness phenotypes is relative degree of symbiosis. As *Cassiopea* progresses in its symbiosis, from initial exposure to symbionts, there is a steady positive proliferation rate of symbionts within host tissue (Colley and Trench 1985; Newkirk et al. 2018), which follows the differential abundance patterns we see in our polyps at the beginning and end of our experiment (Table S1). Along with this, the regeneration potential of *Smic* polyps, hosting the native homologous symbionts of *C. xamachana* adults (*S. microadriaticum*), was not different from that of *Bpse* polyps (hosting the heterologous symbiont *B. pseudominutum*), suggesting that symbiont species may not affect the host's ability to regenerate and reproduce as much as abundance of symbionts in the host. However, it remains to be formally tested whether a greater initial abundance of symbionts can improve regeneration capability of *C. xamachana* polyps.

Algal colonization of newly regenerated tissue took approximately 11–16 days, as shown by the confocal (Fig. 3) and EdU images (Fig. 5). The exception was *Sym* polyps, which were fully colonized before the cut. This suggests that, while symbionts may support regeneration, algal cell colonization is an independent process. As symbionts are almost exclusively hosted within amoebocytes that form from digestive cells upon establishment of symbiosis (Colley and Trench 1985), this suggests phtosymbiont-harboring amoebocytes did not travel to the point of incision during regeneration.

There was no increase in EdU-labeled cells between intact polyps and any of the timepoints after decapitation (Fig. 5), suggesting that cell proliferation is not an important process during regeneration compared to what is seen in other cnidarian models such as *Hydractinia echinata* and *Nematostella vectensis* (Hydrozoa) (Bradshaw et al. 2015; DuBuc et al. 2014). In fact, in intact *H. echinata* polyps, cell proliferation visualized through EdU was restricted in the lower part of the polyp body. However, 24 h after head amputation, some cells (mainly I-cells) had migrated to the wound point and after 48 h proliferating cells were concentrated where the new head was forming (Bradshaw et al. 2015).

In our study, little cell proliferation was seen at the point of injury six hours after decapitation; capturing what happens at the start of wound healing before regeneration. The lack of evidence for cell proliferation during wound healing could be due to: (i) it occurred before or after the 6 h timepoint, (ii) our imaging failed to capture cell division, or (iii) it is driven by a mechanism other than cell proliferation in *C. xamachana*. Nonetheless, the observation of numerous EdU-labeled cells in buds as also observed by Khabibulina and Starunov (2021); and strobilating heads (Fig. 5) indicates that the employed staining protocol successfully labeled proliferating cells. Once regeneration was completed (Day 16, Fig. 5), cell proliferation was detected at the base of the stalk, indicating that normal growth patterns had resumed. The lack of increase in polyp cell proliferation at the point of injury suggests thus that tissue regeneration in *C. xamachana* is not the result of localized cell division, but of cell migration, similar to regeneration in *Hydra* (Vogg et al. 2019). In contrast, in *N. vectensis*, wound healing and regeneration seems to be two separated processes, where the onset of cell proliferation represents the transition to regeneration (DuBuc et al. 2014). Clearly within Cnidaria, regeneration is complex and shows fluctuations in mechanisms across the model organisms most widely examined. *C. xamachana* regeneration could be linked to currently undescribed stem cells, or amoebocytes that may play a role in this process. Further study on the nature of amoebocytes, and the development of an accurate way to identify them in situ, is needed to explore the mechanisms behind *C. xamachana* regeneration.

Overall, our findings do not support localized cell division as the mechanism behind regeneration in *C. xamachana*. However, our data suggest that the presence of symbionts affects mortality and the speed of the regeneration process. Lastly, our results demonstrate that *C. xamachana* can be a valuable system to study symbiosis-driven tissue regeneration, particularly in light of the current interest in the impacts of microbial interactions on tissue regeneration of multicellular hosts.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13199-023-00920-0.

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Author contributions Marta Mammone, Victoria Sharp and Mónica Medina contributed to conceptualization of the presented study. Marta

Mammone and Victoria Sharp carried out the research and analysis. Michael Hewitt performed the DNA extractions, and Victoria Sharp did the PCR reaction and sequence analysis. Marta Mammone and Victoria Sharp wrote the first draft, and all authors commented and edited the following versions. Mónica Medina provided resources and supervision. All authors read and approved the final manuscript.

Declarations

Conflicts of interests the authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval all applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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