

Regenerative Capacity of the Upside-down Jellyfish *Cassiopea xamachana*

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This study provides the first observation that umbrellar tissue can lead to the formation of virtually all body structures in jellyfish of the order Rhizostomeae. The regeneration process was observed in two specimens of the upside-down jellyfish *Cassiopea xamachana* Bigelow, 1892, one housed at the Vienna Zoo, Austria and the other in a laboratory at the University of São Paulo, Brazil. The process was triggered by an injury and ended with the formation of two new sets of body structures. Our observation offers evidence that *C. xamachana* has a hidden regenerative capacity exceeding that previously recorded.

Key words: Bell tissue, Medusa, Scyphozoa, Regeneration, Wound healing.

BACKGROUND

Evidence suggests that regeneration is a common trait in metazoans, from sponges to chordates (Bely and Nyberg 2010). Nevertheless, the regeneration potential of early-diverging metazoans clearly differs from those of more recently diverged eumetazoan phyla. The difference lies in how this ability is controlled in the adult individual (Sánchez-Alvarado 2000). Among cnidarians, regeneration has been widely studied in *Hydra* (Cnidaria, Hydrozoa), an animal capable of regenerating a whole polyp from a small amount of tissue or even from dissociated cells (Holstein et al. 2003). Studies using *Hydra* as a model have provided information on the role of stem cells in hydrozoan regeneration. These studies also helped to lay the foundation for understanding tissue organization and differentiation during regeneration in the animal kingdom (Gold and Jacobs 2013). However, even within cnidarian model organisms, some basic questions about

regeneration remain unanswered.

Although not all cnidarians share the same regeneration mechanisms, some non-hydrozoan taxa have regenerative capacities comparable to those of *Hydra* (Gold and Jacobs 2013). In scyphozoans, regeneration has been observed at different levels according to the life-cycle stage. On one hand, regeneration of complete organisms from a piece of tissue is usually observed in earlier stages. So, isolated polyp epithelial tissue reassumes the polyp form in taxa such as *Aurelia*, *Cassiopea* and *Chrysaora* (Steinberg 1963; Curtis and Cowden 1974; Black and Riley 1985). In stages with a higher degree of cellular differentiation, such as ephyra, reorganization of pre-existing structures and regeneration have also been observed (da Silveira et al. 2002; Jarms 2010; Abrams et al. 2015; Allen et al. 2016). Regeneration at the medusa stage, however, has mostly been reported to occur to reconstruct lost structures (Zeleny 1907; Mills 1993; Stierwald et al. 2004; Stamatis et al. 2018).

The vast majority of studies in the field of regeneration conclude that, as organization and cell differentiation proceed, more regulatory checkpoints appear to control pluripotentiality (Sánchez-Alvarado 2000). Therefore, it is expected that, in the medusa stage, pieces of tissue would not regenerate into new organisms as occurs with polyps. Nevertheless, the use of *in vitro* experiments has demonstrated that some differentiated jellyfish cells preserve their ability to alter cellular commitment, which makes it possible for them to create new cell types (Schmid 1992; Piraino et al. 1996).

These considerations make it clear that regeneration in scyphozoan jellyfishes has been underestimated, owing to limited reports of wound healing or regeneration. Although many aspects of the biology and physiology of the upside-down jellyfish, *Cassiopea xamachana* Bigelow, 1892, are known (Ohdera et al. 2018), here we report a previously undescribed regeneration pattern following umbrellar injury, which led to the formation of new sets of jellyfish body structures.

MATERIALS AND METHODS

Morphological observations

Regeneration was observed in two specimens:

Specimen #1: a 6-month-old jellyfish (male) that arose from a polyp culture (originally collected at Imbé, Rio Grande do Sul, Brazil) in August 2006, in our laboratory in São Paulo, Brazil. Once it was detected, in February 2007, the regeneration process was monitored for the next 6 months, until August 2007. **Specimen #2:** a 17-month-old jellyfish (male) that arose from a polyp culture (originally collected in Florida, USA) in March 2014, at the Vienna Zoo, Austria. The regeneration process was monitored from the time that it was detected, in July 2015, until February 2016, after which no further progress was observed.

Specimen #1 was kept in a 160 L (40 cm × 20 cm × 20 cm) aquarium with sand on the bottom, together with a few other individuals derived from the same polyp culture. This tank had a protein skimmer, and evaporated water was replaced every other day. Water temperature was 23–25°C and salinity 35 PSU. The tank was located near a window to receive natural light. The specimen was fed freshly hatched brine shrimp. **Specimen #2** was kept in a 220 L (90 cm × 39 cm × 65 cm) aquarium with no sand on the bottom, together with 50 other individuals derived from the same polyp culture. The *Cassiopea* tank was connected to the Vienna Zoo's large tropical reef tank system, which has

a volume of about 100,000 L, with 2.5% of the water changed daily. Water temperature was maintained at 25°C and salinity at 35 PSU. A fatty-acid enrichment emulsion (Selco) was used to enrich freshly hatched brine shrimp to feed the jellyfish; the photoperiod was 12h light/12h dark, using 14000K- 400W HQI lighting.

After no further morphological changes were detected, the specimens were fixed. **Specimen #1** was fixed in 4% formaldehyde solution in seawater in August 2007. Since this specimen was eventually lost, no morphological observations could be made. Other individuals from the same polyp culture that originated **specimen #1** were fixed in 96% ethanol and sequenced. **Specimen #2** was preserved in February 2016; a piece of the umbrella was fixed in 96% ethanol and the rest of the animal in 4% formaldehyde solution in seawater. **Specimen #2** was deposited at the Zoology Museum of the University of São Paulo (MZUSP 8403, GenBank: MN539723). While it remained alive in the Vienna Zoo, **specimen #2** was photographed, and the photographs were used as the basis for further observations. These observations were confirmed by inspection of the preserved specimen under a stereomicroscope, at the Laboratory for Cnidarian Studies and Cultivation, University of São Paulo, Brazil.

Molecular data

DNA was isolated from bell tissue using a protocol based on ammonium acetate (Fetzner 1999). From each specimen, we amplified a ~700-bp fragment of the mitochondrial Cytochrome *c* Oxidase I gene (*COI*) using “FishF1” and “med-cox1” primers (Lawley et al. 2016; Ward et al. 2005; for other primers used successfully to amplify *COI* from *Cassiopea*, see Rizman-Idid et al. 2016). A conventional PCR program was run: 3 min at 95°C for initial denaturation, followed by 35 cycles of amplification (denaturation at 95°C for 30 s, annealing at 54°C for 40 s, and extension at 72°C for 50 s) and a final extension for 7 min at 72°C. The PCR products were observed after electrophoresis in 2% TBE agarose gel (stained with Biotium GelRed). PCR products were purified using Agencourt AmPure XP. The sequencing reaction was carried out with the BigDye Terminator V.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc). DNA was precipitated with 3 M sodium acetate/ethanol and sequenced bidirectionally on an ABI 3730 at the Biosciences Institute, Botany Department, University of São Paulo (USP). The resulting ABI files were assembled and trimmed using Geneious 6.1.8 (Biomatters Ltd.).

For the sole purpose of identifying the species, a phylogenetic analysis was performed using (a) the newly generated sequences, (b) the sequences and

species hypotheses by Morandini et al. (2017), and (c) the sequence with the GenBank accession number JN700936.1 (Kayal et al. 2012). DNA sequences were aligned using the L-INS-i method with MAFFT v7.271 and other default parameters (Kato and Standley 2013). Aligned regions were trimmed at the 5' and 3' ends, based on options for a less-stringent selection, using Gblocks v0.91b (Castresana 2000; Talavera and Castresana 2007). The best-fit substitution model selection and the phylogenetic analysis by maximum likelihood were conducted via IQ-TREE multicore v1.6.10 (Nguyen et al. 2015; Kalyaanamoorthy et al. 2017). The clade stability was evaluated with two parametric (aBAYES, aLRT) and two non-parametric methods (standard bootstrap, SH-aLRT).

RESULTS

Using a phylogenetic approach, both specimens (GenBank: MN602311, MN539723) were identified as *Cassiopea xamachana* (*sensu* Kayal et al. 2012; identification updated on 15 Oct 2018). They cluster with a Panamanian specimen (GenBank: JN700936.1), forming a clade that is sister to *C. andromeda* (Fig. 1). Since this study does not focus on systematics or phylogeny, these aspects are not discussed further here.

According to our observations, in specimen #1, after an injury to the umbrellar margin the wound started to regenerate, first forming bell tissues and then new mouth arms. Eventually it was possible to recognize two individuals connected by the umbrella (Fig. 2).

In the case of specimen #2, before the regeneration

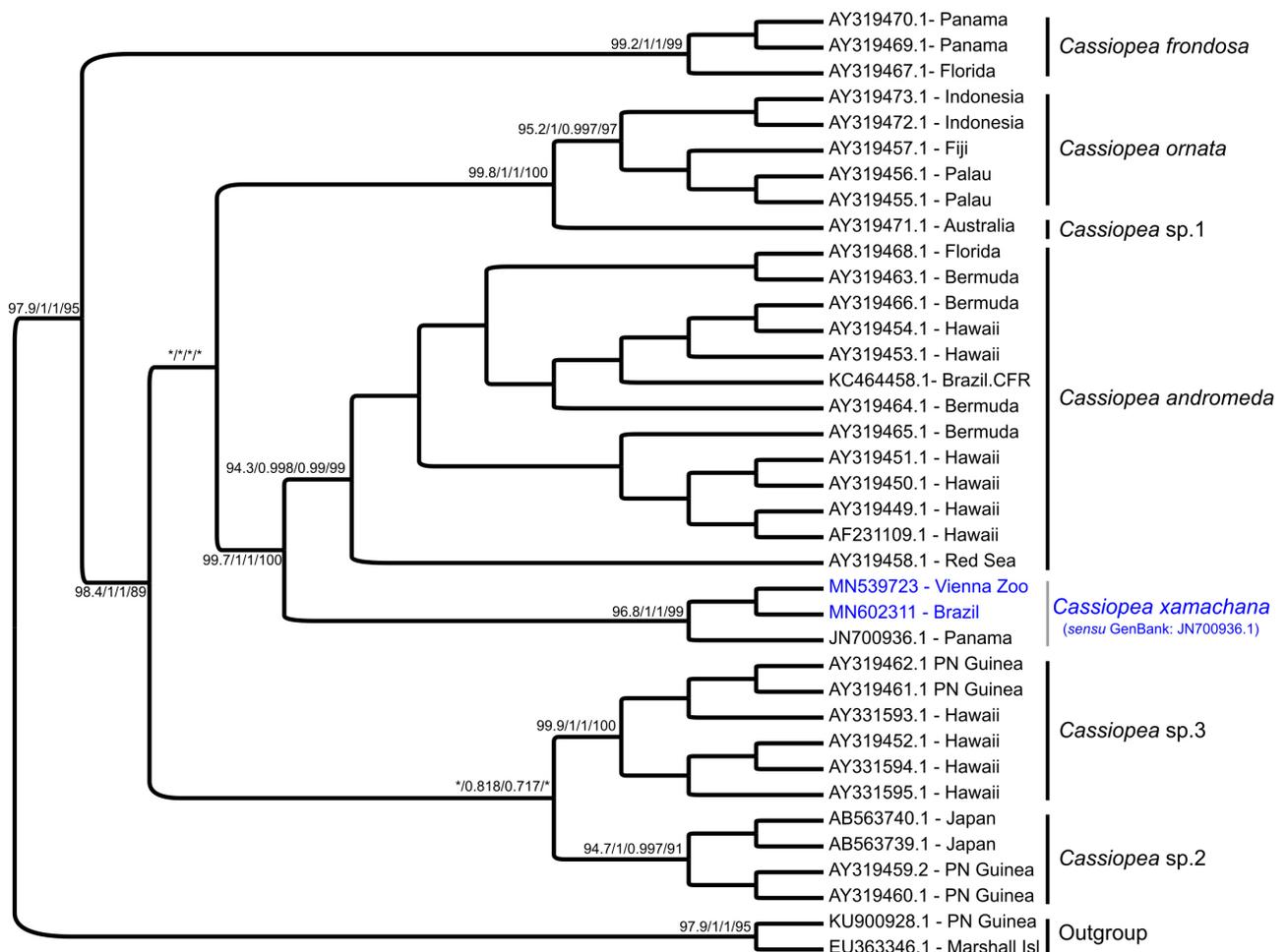


Fig. 1. Cladogram representation of the maximum likelihood tree for *Cassiopea* (lnL = -3620.5074) under the GTR+F+I+G4 model (chosen according to AICc), highlighting the identification of the newly sequenced organisms and their phylogenetic position (species identifications were based on the species hypothesis by Morandini et al. (2017) and Kayal et al. (2012; GenBank: JN700936.1—identification updated on 15 Oct 2018). Support values are shown on branches (as in figure order: SH-aLRT (%) / parametric aLRT support / aBayes support / bootstrap support (%)); * = less than 0.7, 70).

process started, the jellyfish had a bell diameter of 12 cm; 14 faint white exumbrellar marks; thick oral disk; 27 radial canals; 14 rhopalia; four rounded subgenital ostia protected by the edge of the oral disk; eight oral arms, less than the bell radius in length; and greenish-brown oral appendages of different sizes, club- and ribbon-shaped, distributed over the oral surface. According to our observations, the regeneration process was triggered by an injury to the umbrella. The wound healing started with tissue regeneration but did not end with the closure of the wound. The process generated new tissue that led to the initial formation of two secondary gastrovascular cavities and radial canals. Eventually, it was possible to recognize three sets of structures in the regenerated individual, the first, original one (ST-1), and two that developed later (ST-2 and ST-3). Hence, tissue regeneration and reorganization formed anastomosing vessels, bell tissue, digitata, oral arms, oral appendages, radial canals, subgenital ostia and wandering cells (white streaks; *sensu* Gohar and Eisawy 1960) (Figs. 3A-E and 4A, C). ST-1, ST-2, and ST-3 have a canal system composed of canals extending directly to the bell margin and anastomosing networks, but in ST-2 and ST-3 in smaller numbers and without a symmetrical pattern of distribution. In ST-3, the least-developed specimen, it was possible to observe a connection with ST-1 through anastomosing networks and radial canals. ST-2 was connected to ST-1 only by a network of anastomosing canals derived from the angles formed by the apical branches of the radial canals (Fig. 4A).

Interestingly, in both cases (ST-2 and ST-3) there was no formation of the sense organs (rhopalia), but the rhopalial niche did form (Fig. 4C).

DISCUSSION

Upside-down jellyfish are recognized for their high regenerative capacity (Stockard 1908). At the planula stage, aboral and oral fragments can reassume reduced planula or stalkless polyp organizations respectively (Neumann 1979). Similarly, aboral fragments of the polyp regenerate a whole polyp, whereas the oral surface of the polyp produces only head structures (Curtis and Cowden 1972 1974). Even the medusa stage of *Cassiopea*, which usually shows less regenerative potential, is capable of regenerating a considerable number of missing structures, even complex ones such as functional sense organs (Cary 1916). The cellular and molecular mechanisms underlying regeneration in *Cassiopea* are mostly unknown. Nonetheless, there is no evidence of significant differences in telomerase activity in somatic tissues of the polyp and medusa stages (Ojimi et al. 2009). Also, it is known that planulae, free-swimming buds, and polyps produce regulators of regeneration (Curtis and Cowden 1974; Neumann 1977 1979).

Our observations on specimens #1 and #2 (after 6 and 7 months, respectively) showed no signs of fission, indicating that we documented true regeneration and not an unusual mode of asexual reproduction. Our



Fig. 2. General view of a new set of regenerated structures of *Cassiopea xamachana* (specimen #1) from Rio Grande do Sul, Brazil.

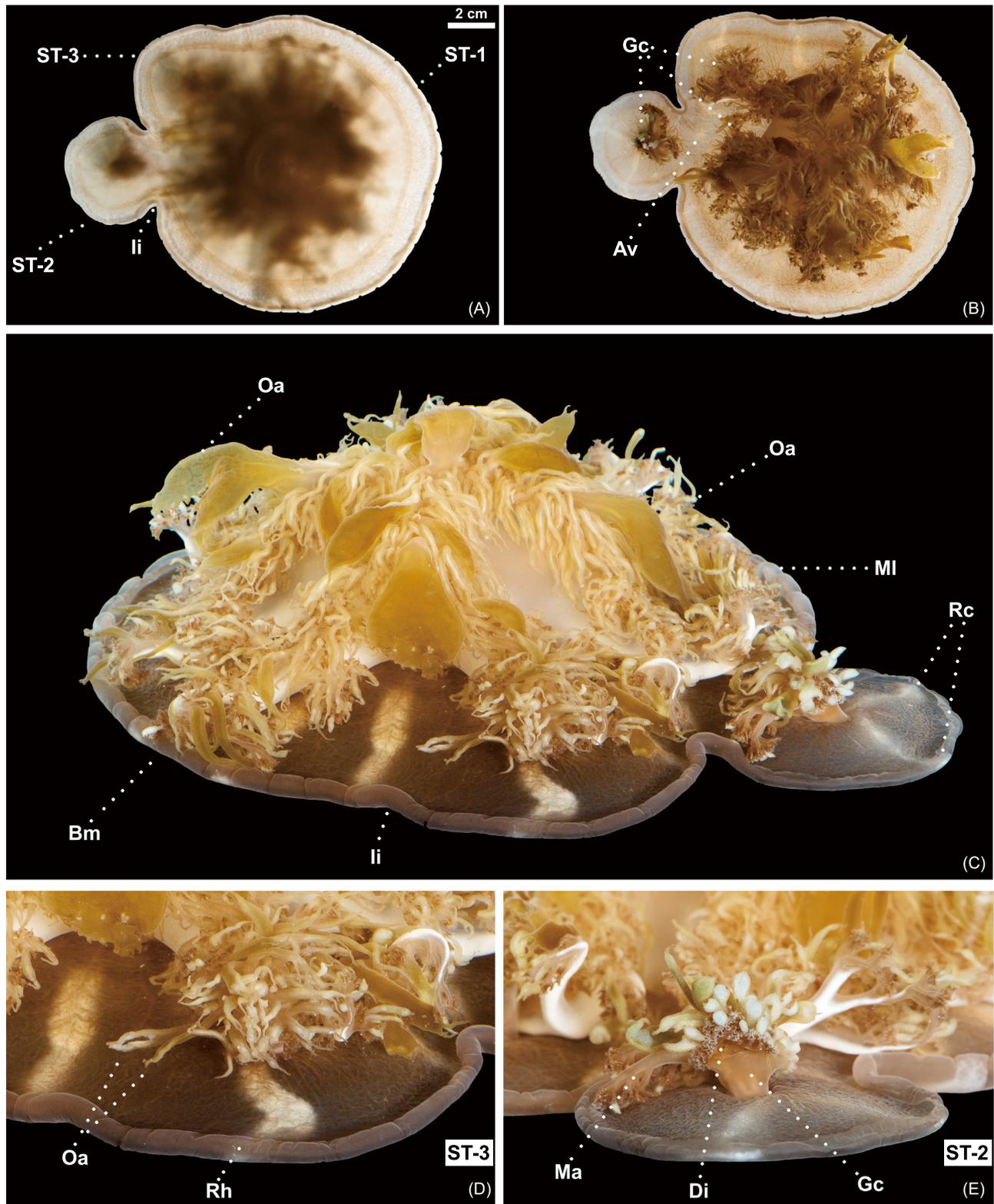


Fig. 3. *Cassiopea xamachana* (specimen #2) regenerated, photographed seven months after the appearance of the injury. Aboral (A), oral (B) and lateral (C) views of specimen #2 (ST1-3), and close-ups of the two sets of generated individuals: ST-2 (E) and ST-3 (D). Abbreviations: Av: anastomosing vessels; Bm: bell margin; Di: digitata; Gc: gastrovascular cavity; li: Inward invagination; Ma: Mouth arm; MI: marginal lappet; Oa: oral appendage; Rc: radial canal; Rh: rhopaliar niche; ST-1 original specimen; ST-2 and ST-3: generated individuals.

data provide evidence that the jellyfish *C. xamachana* has hidden regenerative capacities that exceed those previously recorded for the genus, being able to regenerate virtually any structure from umbrellar tissue, as *Aurelia* sp.1 and *Chrysaora hysoscella* are also able to do (Hadži 1909; He et al. 2015). Previously, Russell (1953) and Stretch and King (1980) reported a somewhat similar condition in the medusa stage of the hydrozoans *Aequorea macrodactyla*, *Clytia hemisphaerica* and *Gastroblasta raffaelei*, which suggests that, even when there is divergence in stem cell dynamics in Hydrozoa and Scyphozoa, they share morphogenic abilities concerning regeneration (Gold and Jacobs 2013). Nevertheless, the phenomena shown in the above-mentioned hydromedusae are clearly different from the one reported here. While all the observations include the development of new sets of structures, the production of new structures in *A. macrodactyla*, *C. hemisphaerica* and *G. raffaelei* was followed by asexual reproduction (schizogony followed by longitudinal splitting, or direct fission).

The canal system of jellyfish is associated with functions in the circulation of food particles by contractions of the umbrella and peristaltic contractions of the subumbrellar membranes (Hamner et al. 1995). Among the members of Scyphozoa, Rhizostomeae and Ulmaridae possess the most complex network of canals (Dawson and Hamner 2009), with the number of anastomoses increasing as the bell diameter increases (Lee et al. 2008). In taxa such as *Aurelia* and *Cassiopea*, functional differentiation of the canals has been observed. Some canals transport fluids in only one direction, and others have bidirectional flow via the generation of ciliary currents (Agassiz 1862; Russell 1970). Even when ST-2 and ST-3 did not have a complete number of canals, and those canals present do not show a symmetrical pattern of organization (the rhopaliar canals in individuals with 16 rhopalia are in the adradial, interradial, and perradial positions, and the inter-rhopaliar in the sub-radial position), it was possible to observe radial canals containing circulating fluids. Thus, we expect that the circulation of fluids

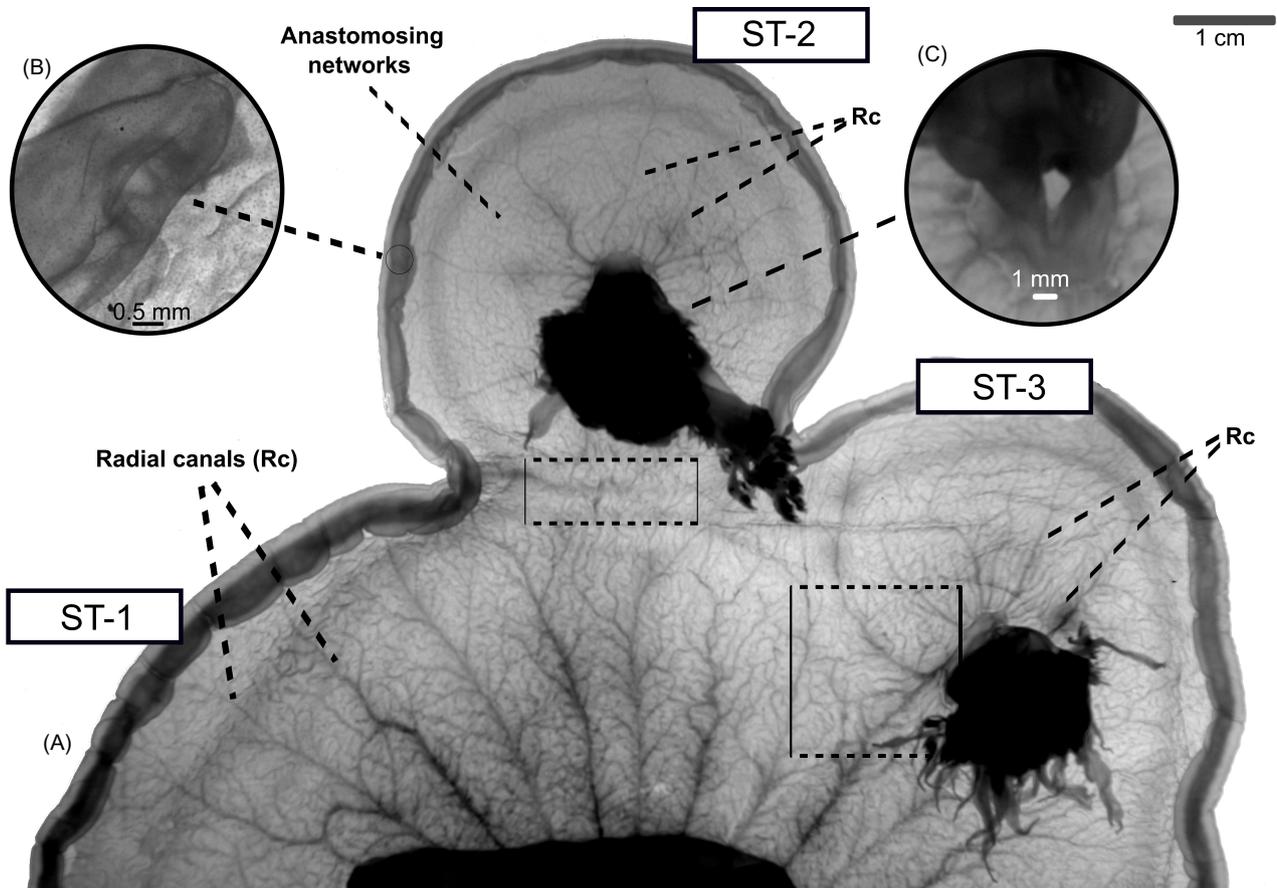


Fig. 4. Specimen #2 of *Cassiopea xamachana*. (A) Gastrovascular system, indicating some of the radial canals and anastomoses. In the square selection, observe the connection between ST-1 and ST-3 through anastomosing networks and radial canals. In the rectangular selection, ST-2 is connected to ST-1 by a network of anastomosing canals. (B) Rhopaliar niche without rhopodium; (C) a detailed view of the subgenital ostium of ST-2. Note: the gastrovascular system was injected with dye to highlight connections.

in the canals of ST-2 and ST-3 enable them to survive independently of the larger specimen (ST-1). The canal system is hypothesized to enable mass occurrence of jellyfish because it allows medusae to grow to a large size, consequently increasing their feeding ability and fecundity (Dawson and Hamner 2009). Having not observed the individuals separating, it may be more beneficial to remain connected in a “single organism” than to attempt to survive independently without a completely developed canal system.

The initiation of the regeneration process observed here, with an injury to the bell, lends support to the hypothesis that the umbrella tissue possesses special abilities with respect to pluripotentiality. Schmid and Reber-Müller (1995) observed that the umbrellar tissue of *Podocoryne carnea* containing striated-muscle cells exposed to different culture conditions can transdifferentiate into new cell types that can then regenerate feeding and sexual structures. On the other hand, Piraino et al. (1996) found that *Turritopsis* medusae can reverse their life cycle only when cells of the exumbrellar epidermis and gastrovascular system are present. Martin and Chia (1982) found that the planula of *Cassiopea* contains interstitial cells in the endoderm, which have been recognized as stem cells in Hydrozoa. However, in the scyphistoma of *Cassiopea*, only ameboid cells have been reported to have an equivalent function (Curtis and Cowden 1974). No studies have yet focused on the cellular cues and molecular inductors or inhibitors that trigger or halt proliferation, differentiation or transdifferentiation in the upside-down jellyfish.

CONCLUSIONS

Although in the last few years there have been considerable advances in the knowledge of genetic factors that regulate regeneration in hydrozoans (Leclère et al. 2016), there is still a long way to go to understand this process among the Rhopaliophora. The occurrence of regeneration at the level recorded here in *Cassiopea* jellyfish raises some intriguing questions, such as why is the medusa regeneration regulated in such a way that it is not observed at the level recorded here? Or, what are the factors that stimulate pluripotentiality and then regeneration? Understanding the developmental aspects in these non-bilaterian lineages could provide information for the establishment of an evolutionary hypothesis for the rest of the animal kingdom (Lanna 2015). The use of appropriate experimental designs, manipulative techniques to regulate gene expression, and advanced microscopy techniques will be invaluable tools for understanding regeneration in Scyphozoa.

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Authors' contributions: Edgar Gamero-Mora: authored the first version of the manuscript, prepared figures, reviewed drafts, and approved the final version. Roland Halbauer: cultured specimen 2, reviewed drafts of the manuscript, and approved the final version. Vanessa Bartsch: cultured specimen 2, reviewed drafts of the manuscript, and approved the final version. Sérgio N. Stampar: cultured specimen 1, reviewed drafts of the manuscript, and approved the final version. André C. Morandini: authored the first version of the manuscript, reviewed drafts, and approved the final version.

Competing interests: EGM, RH, VB, SNS and ACM declare that they have no conflict of interest.

Availability of data and materials: The two partial sequences of Mitochondrial Cox1 are accessible via GenBank accession numbers MN602311 and MN539723.

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Ethics approval consent to participate: Not applicable.

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