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SCIENTIFIC NOTE

Production of clone polyps of the model organism *Exaiptasia diaphana* (Rapp, 1829)

Producción de pólipos clonales del organismo modelo *Exaiptasia diaphana* (Rapp, 1829)

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RESUMEN

Antecedentes. La anémona marina *Exaiptasia diaphana* (Orden Actiniaria) es un organismo modelo ideal para estudiar procesos biológicos, fisiológicos e inmunitarios en corales (Orden Scleractinia), debido a su estrecha relación filética y sus rasgos compartidos. *E. diaphana* tiene una distribución amplia en las zonas tropicales costeras. Esta especie es fácil de mantener en acuarios bajo diversas condiciones experimentales, ya que se reproduce asexualmente y puede transformarse en aposimbiótica. Sin embargo, los métodos de propagación son diversos, lo que dificulta la comparación directa de resultados. Un protocolo estandarizado de propagación de *E. diaphana* puede contribuir a mejorar el entendimiento de su biología. **Objetivo.** Determinar el método más rápido de producción de pólipos clonales en condiciones controladas. **Resultados.** En el tratamiento de micro laceración, el 50% del tejido remanente dio lugar a un nuevo pólipo clonal, mientras que cada anémona amputada formó dos pólipos con tentáculos y pie desarrollados. Los pólipos clonales amputados desarrollaron tentáculos a partir del tercer día, mientras que los pies del grupo control y el tejido remanente de la micro laceración desarrollaron tentáculos a partir del sexto día. El grupo control liberó naturalmente cinco pólipos con tentáculos bien desarrollados en los diez días del experimento. **Conclusión.** La amputación transversal fue el método más rápido para obtener pólipos clonales desarrollados, por lo que se propone como método estándar para la propagación artificial eficiente de pólipos clonales del organismo modelo *E. diaphana*.

Palabras clave: *Aiptasia*, laceración pedal, organismo modelo, pólipos clonales.

ABSTRACT

Background. The sea anemone *Exaiptasia diaphana* (Order Actiniaria) is an ideal model organism to study diverse biological, physiological, and immune processes in corals (Order Scleractinia) due to its close phyletic relationship and shared traits. *E. diaphana* is widely distributed along the world's tropical coastal areas. This species is easy to grow in aquariums under diverse experimental conditions since reproduces asexually and can be rendered aposymbiotic. However, there are a variety of methods to propagate them, making difficult comparisons of results. A standardized propagation protocol for *E. diaphana* can also contribute to improving the understanding of its biology. **Goal.** Determine the most rapid method of clonal production in controlled conditions. **Results.** In the micro-laceration treatment, 50% of the remnant tissue gave rise to a new clonal polyp, while all the amputated anemones resulted in two polyps with tentacles and a pedal disc. Amputated clonal polyps developed their tentacles from the third day, being this treatment the most rapid compared with the control group and the micro-laceration treatment. In both cases, the tentacles started to develop from the sixth day of the experiment. The control group naturally released five clonal polyps with tentacles in the ten-day experiment **Conclusion.** Transversal amputation was the most rapid method to obtain developed clonal polyps. We, therefore, propose transversal amputation as a standard method for the efficient artificial propagation of the clonal polyps of the model organism *E. diaphana*.

Keywords: *Aiptasia*, clonal polyps, model organism, pedal laceration.

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Model organisms are species extensively studied to understand specific biological processes. The interest in understanding the symbiotic association between cnidarians and dinoflagellate algae is at the foundation of the reef system (Yellowless *et al*., 2008), where reef corals inhabit waters with a low concentration of nutrients and yet develop into a vastly biodiverse and productive ecosystem. However, the breakdown of this symbiotic association due to global warming and pollution is endangering the whole ecosystem (Hoegh-Guldberg *et al*., 2007).

Coral reefs are a good example where model organisms have helped advance the understanding of the mechanisms that initiate, maintain, and propagate the symbiotic association between cnidarians and dinoflagellate algae. The sea anemone *Exaiptasia diaphana* was identified as a model organism for corals (Weis *et al*., 2008; Baumgarten et al., 2015; Dungan et al., 2020), due to the symbiosis with dinoflagellate algae, as those found in corals (Thornhill *et al.,* 2013; LaJeunesse *et al*., 2018). Further, *E. diaphana* represents an experimentally tractable model organism that grows rapidly by asexual reproduction under aquarium conditions. This species can also live without symbiotic algae, therefore providing an opportunity to evaluate the role of the symbionts (Weis *et al*., 2008; Voolstra, 2013).

Asexual reproduction in sessile cnidarians like sea anemones is a natural strategy to both, rapidly colonize new substrates, and to increase the presence of successful genotypes within the local environment (Hambleton *et al.,* 2014). Since *E. diaphana* is a model organism, producing enough biological material is needed to answer experimental questions.

Despite the common use of *E. diaphana* in several laboratories (e.g., Cook *et al*., 1998; Sunagawa *et al*., 2009; Costa-Leal *et al*., 2012; Grawunder *et al*., 2015; Presnell *et al*., 2022), and even the increasing amount of publications about *E. diaphana* (either as *Exaiptasia diaphana* or as the unaccepted name, *Exaiptasia pallida*), the information on the biology of the species is not sufficient. This anemone is contrastingly considered a model organism by scientists but a plague by aquarists. However, a controlled propagation technique is needed that considers the decrease in propagation rates when anemones occur at high density (Costa-Leal *et al*., 2012). Here, we aimed to determine the most rapid method of clonal production in terms of the number of anemones, under controlled conditions.

Husbandry. We collected 48 anemones (3 to 6 mm in pedal disc diameter) from the Reef Systems Academic Unit tanks where they were introduced one year ago. After the collection, anemones were relocated into a plant chamber with filtered seawater (0.22 µm), at 26°C, in 12/12 hrs light/dark conditions, at 13 µmol photon/m^2/s, pH = 8.1, and a salinity of 35 PSU. Then, anemones were acclimatized for ten days in glass crystallizers with daily water replacements and fed three times a week with *Artemia* sp. nauplii. Next, 16 anemones per treatment were placed in three different six-well plates (one anemone per well) with 10 mL of filtered seawater (Fig. 1a). The anemones were then acclimatized in the wells for five days before the start of the experiments. We selected anemones in apparently healthy conditions, including extended column and tentacles, without a tissue colour change (no paleness nor tainting).

Experimental design. After the acclimatization, 16 anemones per treatment (48 in total) were subjected to amputation, relocation, or left as controls. Observations were carried out each day for ten days under the microscope; during the experimental phase, feeding was discontinued. In the transversal amputation, 16 anemones were submerged in a sterile petri dish with filtered seawater. After two hours, when the tentacles and column were completely extended, we transversely amputated each anemone approximately at the rows of cinclides (i. e. minute specialized pores in the mid-column), using a sterile scalpel, following van der Burg *et al.* (2020) (Fig. 1b). Anemones were daily assessed for the generation of the mouth and tentacles.

Micro-laceration by relocation treatment was addressed to determine whether the extraction of the anemones by hand would produce new polyps. The anemones (n = 16) were cut with a sterilized spatula by the pedal disc leaving at least one piece of remnant tissue in the well (< 2 mm approximately). The remnant tissue was monitored daily under a microscope, checking for the formation of tentacles (Fig. 1b).

In the control group, anemones were checked daily for the generation of new clonal buds per anemone and recording the days that the new buds took to generate the tentacles (Fig. 1b).

To evaluate the differences between the treatments, a chi-square test was applied due to the nominal nature of the data. The statistical significance was established at $p < 0.05$. The statistical analysis was performed in R v.4.1.1.

The amputated anemones developed tentacles from day three (Fig. 2a). Meanwhile, the micro-laceration remnant tissue and the buds released by the control group developed tentacles from the sixth day.

Furthermore, results indicated that, after the micro-laceration, 10 of the 20 remnant tissues (50%) developed tentacles, forming new clonal polyps, suggesting that not all remnant tissue shows the capacity to generate a new clone during the experimental time (Fig. 2b). The chi-square test applied showed that there were significant differences between the groups (X-squared = 5.871; df = 2; p-value = 0.05311).

These results contradict the common knowledge that when detaching an anemone from life-rock, it can always grow new anemones (reviewed in grey literature elsewhere). Apparently, the capacity for the generation of new clones after the micro-laceration depends on the size of the remnant tissue and the size of the detached anemone. According to Cary (1911), and Presnell *et al*., (2022), after micro-laceration the generated clones can be explained by the natural bud dispersion of the anemones; bud dispersion occurs when several pieces of remnant tissue, attached to the pedal disc, are released normally when the adult polyps travel across the water column. Bud dispersion could better explain the rapid capacity of adult polyps to generate new clones, instead of the micro-laceration by relocation or by detachment by hand.

The amputation treatment was the most rapid generating two well-developed clonal anemones from the same pedal disc size. Moreover, acontia, a threadlike tissue with abundant stinging cells located in the gastrovascular cavity, was one of the first inner structures that were conserved in both parts from the first day (data not shown). This is consistent with the report by Lam *et al*., (2017) who mentioned that acontia serves as an important mechanism against predator attacks. Further, acontia is the last inner structure the anemone loses after a pathogenic infection before dead (pers. obs.).

Finally, over the ten days of our experiment, the 16 anemones of the control group released 20 clonal buds naturally, but just 5 of the buds

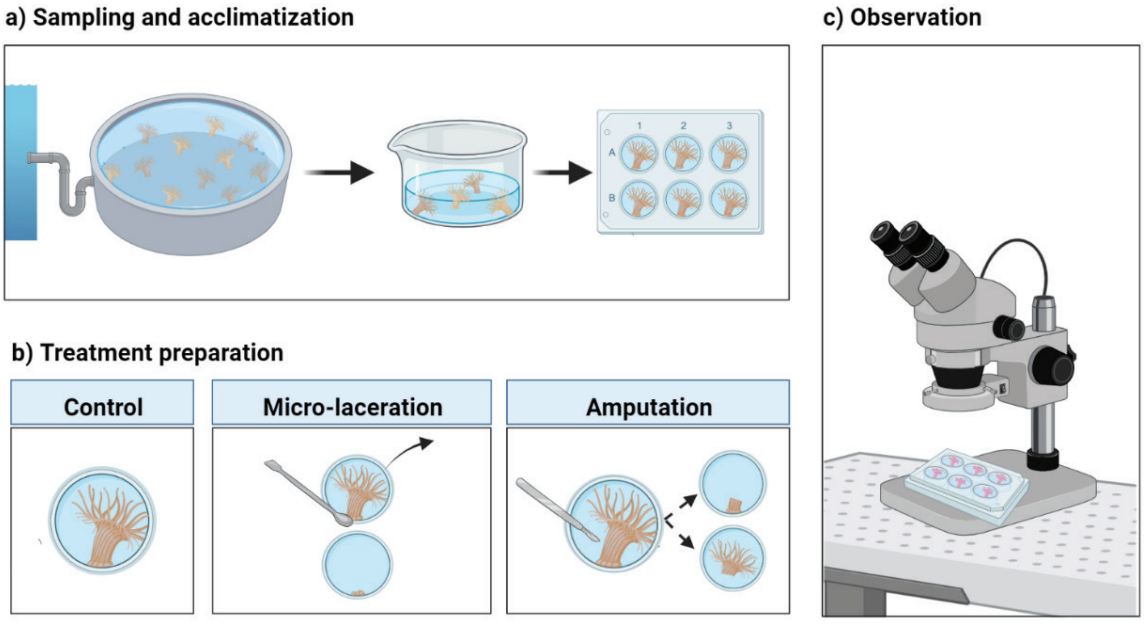

Figure 1. Experimental design. a) *Exaiptasia diaphana* anemones were sampled from the tank that is under natural conditions and relocated under laboratory conditions, and acclimatized, first in a crystallizer and then each anemone in one well of a six-well plate; b) The treatment preparation was conducted as follows: micro-laceration by relocation (n = 16), transversal amputation (TA) (n = 16) and control (n = 16). In the micro-laceration, by relocation (MRL) at least one piece of tissue was left after a cut at the pedal disc. The transversal amputation (TA) was made at the rows of cinclides. Control anemones were not manipulated. All plates were incubated in a growth chamber for 10 days, recording the regeneration of macrostructures in time (days) using a microscope.

The authors, JRO y PT, express that the figures presented here are from our authorship.

(25%) developed tentacles, forming a new clonal polyp (Fig. 2b). Conversely, Grawunder *et al*., (2015), reported a rate of clonal production between 1.7 and 8 lacerates per week. Our results could be influenced by the size of the anemones which were smaller in our experiments (3 to 6 mm oral disc diam., compared to >7 mm in Grawunder *et al*. (2015). Further, it was suggested that the early phase of development for natural pedal lacerates (remnant tissue), relies on stored nutrients (Presnell *et al*., 2022), necessarily influenced by size.

The success of amputation as the most rapid clonal treatment in contrast with remnant tissue, is probably because of the size of the amputated fragments and remnant tissues. Two features characterize modular organisms. The first is that the organisms are provided by at least one stem cell lineage during their whole life (Jackson & Coates, 1986). That feature allows them to completely regenerate themselves. The second feature is that the higher the size, the better the chances of surviving and regenerating (Jackson & Coates, 1986). Therefore, since the remnant tissues are smaller than the amputated fragments of the polyp, then the amputated fragments could be most successful due to their size.

Finally, we did not consider different temperatures in our study, which has a positive effect on asexual reproduction in *E. diaphana*. It has been shown that summer temperatures increase pedal laceration tenfold (Schlesinger *et al.,* 2010), suggesting that the metabolism under warmer and longer days can have some regulation in the process. However, we maintained the anemones under illuminated conditions and at a constant temperature of 26°C.

We conclude that transversal amputation is the most rapid treatment to generate new clonal polyps. By transversal amputation, two adult clonal polyps from a single parent were produced, and after three days, the apical and basal parts showed tentacles as a new polyp. Here, we propose transversal amputation as an alternative protocol for the rapid, artificial propagation of asexual clones that we hope will be useful in laboratories where this anemone is under study. Such method will allow for rapid propagation of biological material in a controlled way, independent of the natural response of the anemones to the culture conditions, that is, to the development of pedal lacerates, remnant tissues, or buds.

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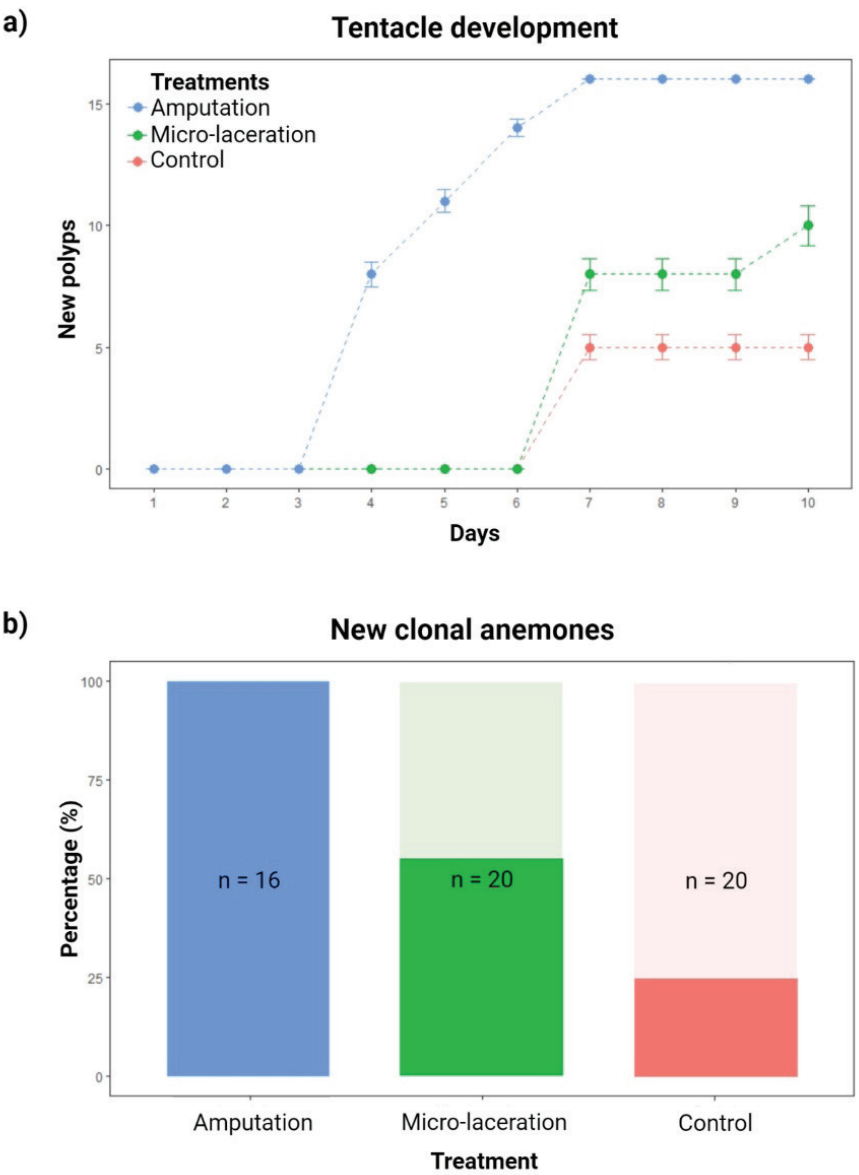

Figure 2. Development of clonal polyps. a) Tentacle development of the polyps in the two treatments and control group (n = 16) during the ten-day experiment. The bars represent the standard deviation. b) The percentage of clonal buds that showed tentacles during the experiment. The two parts of each anemone in the amputation treatment (in blue, n=16) developed tentacles (100%). Micro-laceration treatment (in green, n=16) left 20 tissue pieces in the wall of the well of which ten (50%) developed tentacles. The control group (in red, n=16) released 20 buds of which five (25%) developed tentacles.

Figure 2 was made in R version 4.1.1 and both images were edited with BioRender.

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