

Regeneration patterns in *Naineris aurantiaca* (Müller, 1858) (Annelida, Orbiniidae)

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ABSTRACT

Regeneration is a widespread ability in annelids, and each species and developmental stage may present differences in healing and regeneration processes. Some species can completely regenerate the posterior or anterior region or both. Among the orbiniids, regeneration studies are scarce. In *Naineris aurantiaca* (Müller, 1858), first species of *Naineris* described in Brazil, studies about the species' biology and regeneration capabilities are absent. Aiming to observe the regeneration capabilities of *N. aurantiaca* and its abnormalities, we sampled the specimens from Lagoa da Sereia Beach, Mel Island, Brazil, among algae tufts and between the mussel's shells. The experimental design consisted of three different amputations: i) at the end of the thorax—creating treatment AM (anterior and medium) and P (posterior) —; ii) at the chaetiger where branchiae first appear—treatment A (anterior) and MP (medium and posterior) —; iii), and combined—treatment A (anterior), treatment M (medium) and P (posterior). Analysis showed that time (weeks) and treatments (A, AM, M, MP, P) affected survivorship and other features such as length and width. Treatments AM and A had higher mortality rates than P and MP, showing robust anterior regeneration. Treatment M had no survivors. Mean size and width were higher in treatment MP. Remarkable and robust head regeneration and inability to regenerate the gut and the branchial segments suggests that food absorption and gas exchange are key functions in this species.

Keywords: Morphological variation, Polychaete, Body reconstruction, Worm, Regeneration experiment

INTRODUCTION

Regeneration of large body sections is widespread in the animal kingdom (Brockes and Kumar, 2008; Bely, 2010) but limited to some lineages of Deuterostomia, Lophotrochozoa, Urochordata and Craniata (Bely and Nyberg,

2010). This property is particularly relevant in annelids, which exhibit a broad range of regeneration patterns, including anterior and posterior regeneration (Hyman, 1940; Herlant-Meewis, 1964; Bely, 2006; Álvarez-Campos et al., 2023). According to Bely and Nyberg (2010), whole-body regeneration occurs in an ecological context related to natural disturbances and predators. Abiotic factors are a potential cause of injury in invertebrates, developing an important role in propitiating the regeneration process in early evolution (Bely and Nyberg,

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2010). Moreover, animals in Annelida can present adaptive characteristics to the environment, especially those living in high-energy habitats (Glasby et al., 2021).

While posterior regeneration in annelids appears frequent, anterior regeneration is less common (Bely, 2006; Zoran, 2010). Regeneration capabilities are diversified within the same family (e.g., Syllidae) or show the same patterns within subclasses (i.e., Sedentaria and Errantia). Among Errantia species, anterior regeneration was described in Syllidae and Onuphidae, but is rare in other complex jaw-bearers of Nereididae, Dorvilleidae and Eunicidae. It is also present in more basal families of Annelida, such as Chaetopteridae, Amphinomidae, and some Oweniidae (Özpolat and Bely, 2016). Regeneration studies using sedentary annelids as models, such as Arenicolidae, Capitellidae, and Ophellidae, showed their incapability to regenerate anterior segments (Bely, 2006). However, it is widespread in Sabellidae, Spionidae, Clitellata, Cirratulidae, Maldanidae, and Serpulidae (Bely et al., 2014).

In orbiniids (Sedentaria), the regeneration abilities of their members are little known. *Phylo foetida* can regenerate posterior segments (Probst, 1931), and *Proscoloplos cygnochaetus* can regenerate both anterior and posterior segments (Meyer et al., 2007). Morphological observations suggest that other species (i.e., *Leodamas dubius*, *Leitoscoloplos bilobatus*, *Naineris grubei*) can regenerate at least posterior segments (Gravier, 1908; Zhadan et al., 2015; Zhadan, 2020).

Since rocky shores are often exposed to wave energy without sediment, few species of burrowing annelids are expected to survive under such turbulent conditions. Among the orbiniid species that live in mud or sand, they were shown to use their broader head, which fluctuates in width over a burrowing cycle, to decrease backward slipping in the sand or use internal body expansions to pack sand grains, another mechanism to prevent burrow collapse (Francoeur and Dorgan, 2014). Intriguingly, recent sampling along Brazilian exposed rocky shores revealed evidence of

the remarkable abundance and regeneration capabilities of the orbiniid *Naineris aurantiaca* (Müller, 1858), which is associated with the algal tufts of *Caulerpa fastigiata*. *Naineris aurantiaca* was first described for Santa Catarina Island as *Theodisca aurantiaca* and re-recorded from Rio de Janeiro to Santa Catarina (Álvarez et al., 2019), being one of the most abundant annelids on exposed rocky shores (Álvarez, 2019). The species possesses a rounded prostomium (Figure 1A) and two distinct body regions: the thorax, with parapodia laterally displaced, and the abdomen, with parapodia dorsally shifted (Figure 1A-C). They also have dorsal branchiae (Figure 1A-C), cylindrical ciliated structures for oxygen uptake, starting from a variable number of thoracic segments (9-17) (Álvarez et al., 2019), continuing posteriorly.

This study describes the regeneration patterns of *N. aurantiaca*, including anterior, posterior, and bidirectional regeneration, as a potential evolutionary pathway to survival in such a turbulent environment. We tested the hypothesis that the species can independently regenerate the anterior and posterior parts of the animal after amputation. Since there are few annelids used as biological models, our results represent a promising biological model for investigating regeneration in the annelids to address questions regarding the taxonomic, morphological descriptions, environmental stress, and how the functional burrowing strategies may couple to different scenarios of decapitation in rocky shores.

METHODS

SAMPLING AND ACCLIMATIZATION IN THE LABORATORY

Approximately 80 adult individuals (~5.45 cm long) of *Naineris aurantiaca* were collected from rocky shores around Lagoa da Sereia Beach, Mel Island (-25.573386°, -48.316551°; Figure 2), Paraná, Brazil, during low tide on 9 Dec 2022. We scrapped algal tufts of *Caulerpa fastigiata* and the sediment retained among rhizoids. After sieving, the animals were removed from the sediment at the Marine Ecology laboratory of the Center for Marine Studies, Federal University of Paraná.

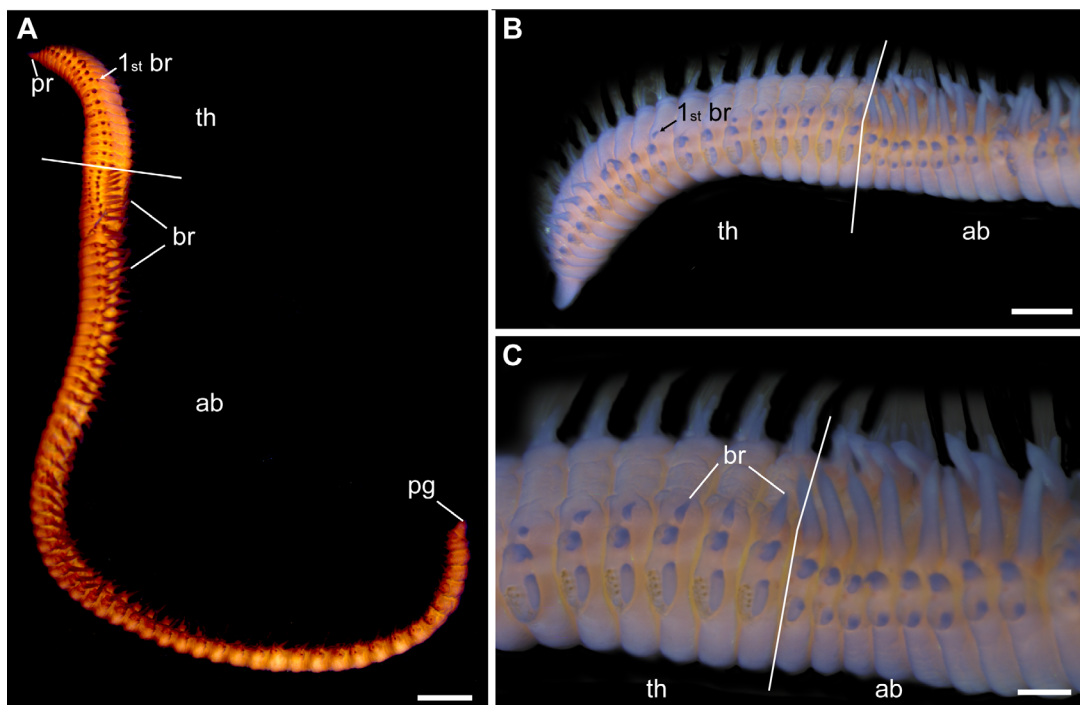


Figure 1. *Naineris aurantiaca* from Mel Island (Shirlastain stained). A – Complete specimen. B – Anterior end of the same specimen. C – Transition between thorax and abdomen. (ab, abdomen; br, branchia; pg, pygidium; pr, prostomium; th, thorax. Scales - A = 1 mm, B = 0.5 mm, C = 0.2 mm).

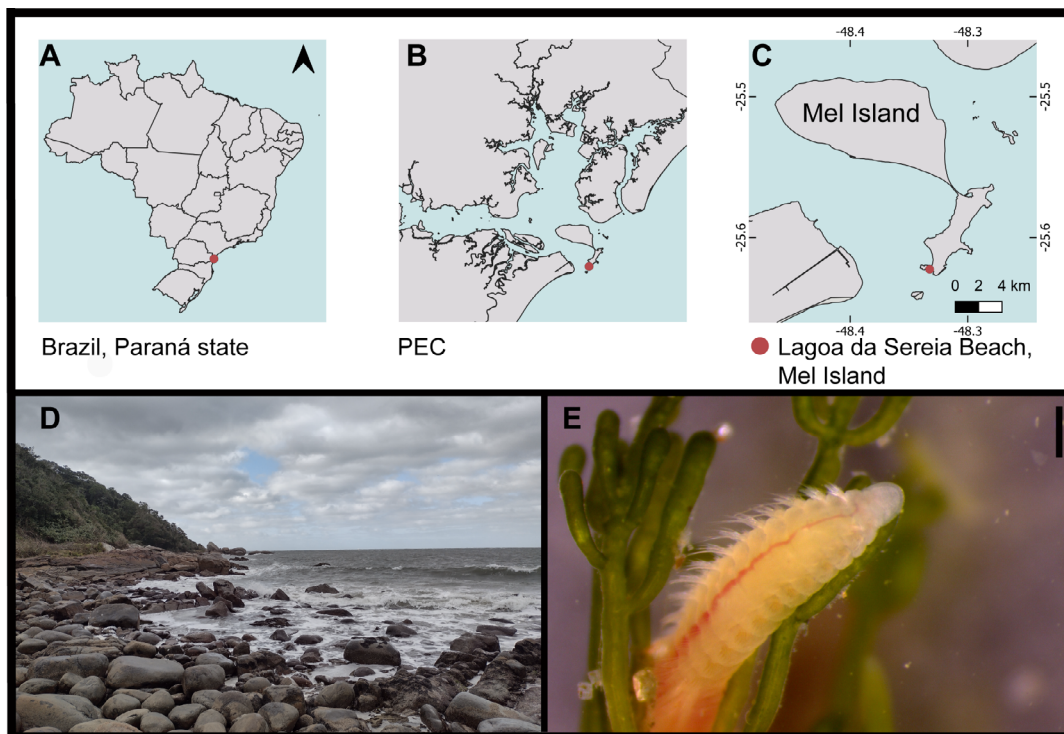


Figure 2. Sampling location of *N. aurantiaca* at Mel Island (red dot). A – Map of Brazil. B - Map of the Paranaguá Estuarine Complex (PEC). C – Map of Mel Island. D - Rocky shores at Lagoa da Sereia Beach. E - Live specimen of *N. aurantiaca* associated with algal tufts of *Caulerpa fastigiata*. (Scale - C = 1 mm).

In the laboratory, 20 animals were randomly distributed in Beckers (600mL) containing 0.22- μ m-filtered seawater (200mL) from the CPP-CEM/CAMAR facilities obtained in the vicinity of the sampling location and maintained under constant aeration for acclimatization for three days. During acclimatization, they were subjected to a 12:12 h photoperiod (light: dark) regime, 25°C temperature, and salinity 30, simulating the environmental conditions at the time of collection.

EXPERIMENTAL DESIGN AND REGENERATION TEST

The effects of amputations on *N. aurantiaca* were evaluated, considering amputation region and time as factors (Figure 3).

The animals were anesthetized with 8% MgCl₂ in seawater before each amputation. Amputation procedures lasted 3 days until all animals were amputated. It was performed using an ophthalmic bistoury at the end of the thorax (21 spms), at the chaetiger where branchiae first appear (18 spms), and combined (20 spms) (Figure 3), resulting in four levels of fragments labeled as A (anterior), P (posterior), M (middle), AM (anterior+middle), and MP (middle+posterior) of which derived 38, 41, 20, 21 and 18 fragments, respectively. Treatment control was not anesthetized; it was used for survival during the experiment and consisted of the entire worms.

Each fragment was kept separately in 6-Well-Plates filled with seawater. In each of them, 10 mL of filtered water and 2 mL of *Tetraselmis* sp. cells were added for feeding. Water ($\frac{2}{3}$) was replaced twice a week during the first week of the experiment. Then, it was changed every two weeks. The animals were fed with *Tetraselmis* sp. at the same time as water replenishment. All experimental units were maintained under a 12:12h (light: dark) photoperiod and monitored daily for visual assessment of health status and survival of the organisms. Dead animals were counted and removed at each exposure time (2, 5, and 7 weeks).

Morphological changes were photographed at weeks 2, 5, and 7 with a Canon EOS REBEL T7 camera adapted to a Zeiss stereo microscope.

The length and width of regenerated segments were measured from the pictures using ImageJ 1.46r software.

STATISTICAL ANALYSIS

With a binomial error distribution, the generalized linear mixed model (GLMM) method was applied to assess the statistical significance of survivorship. After defining the best distribution family, the structure of fixed effects was defined for the weeks (2nd, 5th, and 7th), plate (1 to 24), fragments (A+M, P, A, M+P), and the interactions among them. Fragment M was excluded from the analysis because only few fragments showed regeneration capability in the first week and survived to below 50%. The entire selection process was based on the significance of the Akaike Information Criterion (AIC) terms. For significant terms ($p < 0,05$), post hoc comparisons were made using the package “lsmean” and “lme4” with adjust “tukey”. All statistical and graphical analyses were performed using the R language (version 4.3.1; R Development Core Team, 2021). An exploratory analysis was used to determine the mean length and width growth values. The data were filtered to select only fragments with measurement values from week 0 to week 5. Means and standard deviations were calculated for each situation—width and growth—of each fragment, and the results were presented and visualized through column charts. To assess the survivorship of each fragment, we counted the survivors at weeks 0, 2, 5 and 7 after exposure. All drawings and plates were made in Adobe Photoshop C6 version 24.1.1. and Illustrator C56 version 27.2.

RESULTS

To investigate if the presence of specific structures limits anterior and posterior regeneration processes, we performed a series of amputation procedures that resulted in fragments lacking a combination of different structures to be restored: A, P, M, AM, and MP (Figure 3). We recorded their survivorship, and regenerated structures and measured the regenerated parts of the animals.

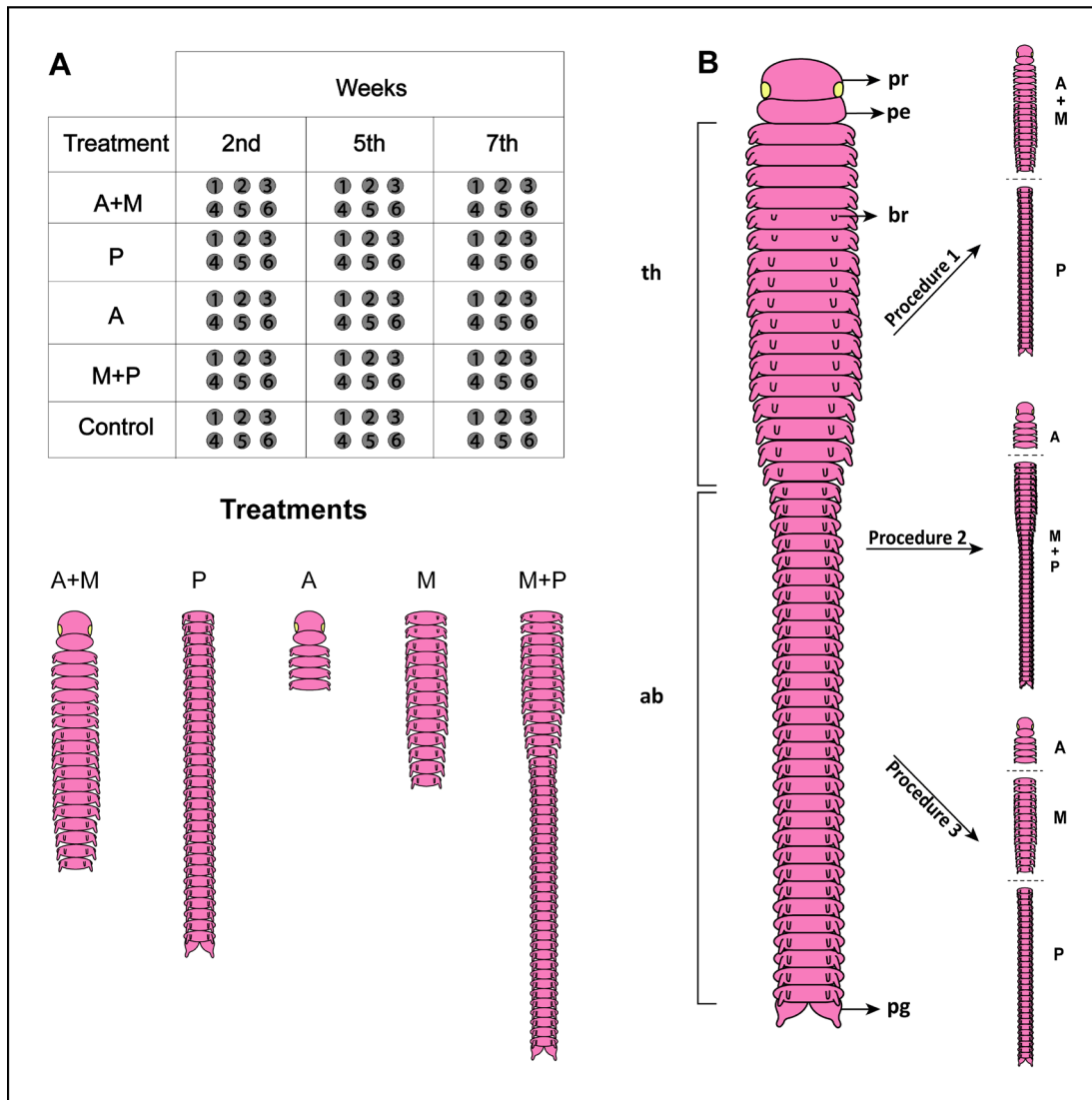


Figure 3. Experimental design. A - Representations of the three experimental treatments of this study and experimental design. B - Schematic drawings of an adult specimen of *N. aurantiaca* and amputation procedures (A, anterior fragment; ab, abdomen; br, branchia; M, mid-body fragment; P, posterior fragment; pe, peristomium; pg, pygidium; pr, prostomium).

SURVIVORSHIP

Regeneration of some fragments was interrupted because of mortality detected during the test. Mortality episodes affected all treatments at seven weeks of regeneration, including fragments A, AM, MP, and P (up to 100%, 95%, 72%, and 73% of individuals, respectively), and intact controls (up to 92% at seven weeks). Even though we didn't use fragment M because of the survivorship below 50% in the first week, we highlighted the bidirectional regeneration in fragment M for *N. aurantiaca*.

The survival of the fragments varied according to the different regions, mainly the fragments corresponding to region A during the first two weeks; survival rates were much higher in the other fragments ($p < 0.0001$) (Figure 4). Furthermore, differences between treatments were significant between fragments MP-A ($p=0.02$) and P-A ($p=0.01$) in Tukey Contrasts, showing that, in general, survivorship was lower in A and A+M fragments. Time (week) did not influence survivorship (GLMM, $df=3$, $p=0.08$; Table 1), nor did the interaction between treatment and time (GLMM, $df=9$, $p=0.71$; Table 1).

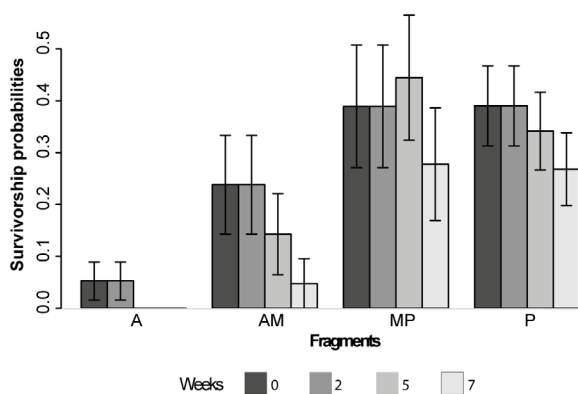


Figure 4. Survivorship probabilities of fragments at weeks 0-7 (anterior - A, anterior+middle – AM, middle+posterior – MP, posterior – P)

Table 1. Generalized linear mixed model with the Treatments (anterior fragment - A, posterior - P, anterior+middle - AM, middle+posterior - MP) and Weeks (0, 2, 5, and 7 weeks) of this study.

	df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)
Null			471	492.70	
Treatment	3	72.885	468	419.82	1.029e-15***
Week	3	6.758	465	413.06	0.08001.
Treatment: Week	3	6.208	456	406.85	0.71898

AMPUTATIONS

Amputation procedures were standardized for all individuals and were not necessarily performed at the same chaetiger number. Procedure 1 was performed just between the first abdominal and last thoracic segment, procedure 2 one chaetiger before branchiae start, and procedure 3 included both procedures 1 and 2 simultaneously. The three procedures resulted in treatments with prostomium and anterior segments without branchiae (A), treatment with all thoracic segments (A + M), treatment with branchiae and without abdomen and the most anterior abbranchiate segments (M), treatment with all abdominal segments (P), and treatment with all branchiate segments and (M+P). As a result of the different procedures conducted, we obtained 38 fragments of treatment A, 20 of M, 41 of P, 21 of A+M, and 18 of M+P.

MORPHOGENESIS

Animals regenerate, but their abilities differ depending on the fragment. *Naineris aurantiaca* regenerates the anterior part at 26.8% (fragment P) and 27.77% (fragment MP) until week seven, while the posterior part only regenerates at 4.76% (fragment AM). The MP and P fragments showed a higher regeneration capability than fragments A and AM. Fragment M was capable of regenerating both parts almost simultaneously but was excluded from the statistical analysis due to missing data.

Anterior. Two weeks after amputation, posterior regeneration was represented by a blastema, but there were no signs of new segments (Figure 5A). In the fifth week, the regenerated segments were the same size as the regular segments but still whitish. Seven weeks after injury, the new segments recovered their normal morphology but were still whitish.

Anterior + middle. Two weeks after amputation, the regenerated region had increased in length, not in width, and it was possible to distinguish fully developed pygidium and anal cirri (Figure 5B). During the following weeks, segments rapidly increased in width. Seven weeks after injury, segments were whitish but morphologically like the untreated segments.

Posterior. Two weeks after amputation, the regenerated anterior end was fully elongated, with both the prostomium and peristomium fully developed, and feeding behavior was observed (Figure 5D). Afterward, the regenerated segments increased but were still narrower than the regular segments. In the fifth week of regeneration, the segments grew wider. Based on our observations, seven weeks after injury, the chaetigers were morphologically similar to the untreated segments, but whitish.

Middle + posterior. Two weeks after amputation, there was clear evidence of anterior regeneration with discernible prostomium and peristomium. All lost segments grew again, but it was impossible to determine whether they grew simultaneously or in successive steps (Figure 5C). In the fifth week of regeneration, the segments grew wider. During the following days, the new chaetigers enlarged, maintaining a white coloration.

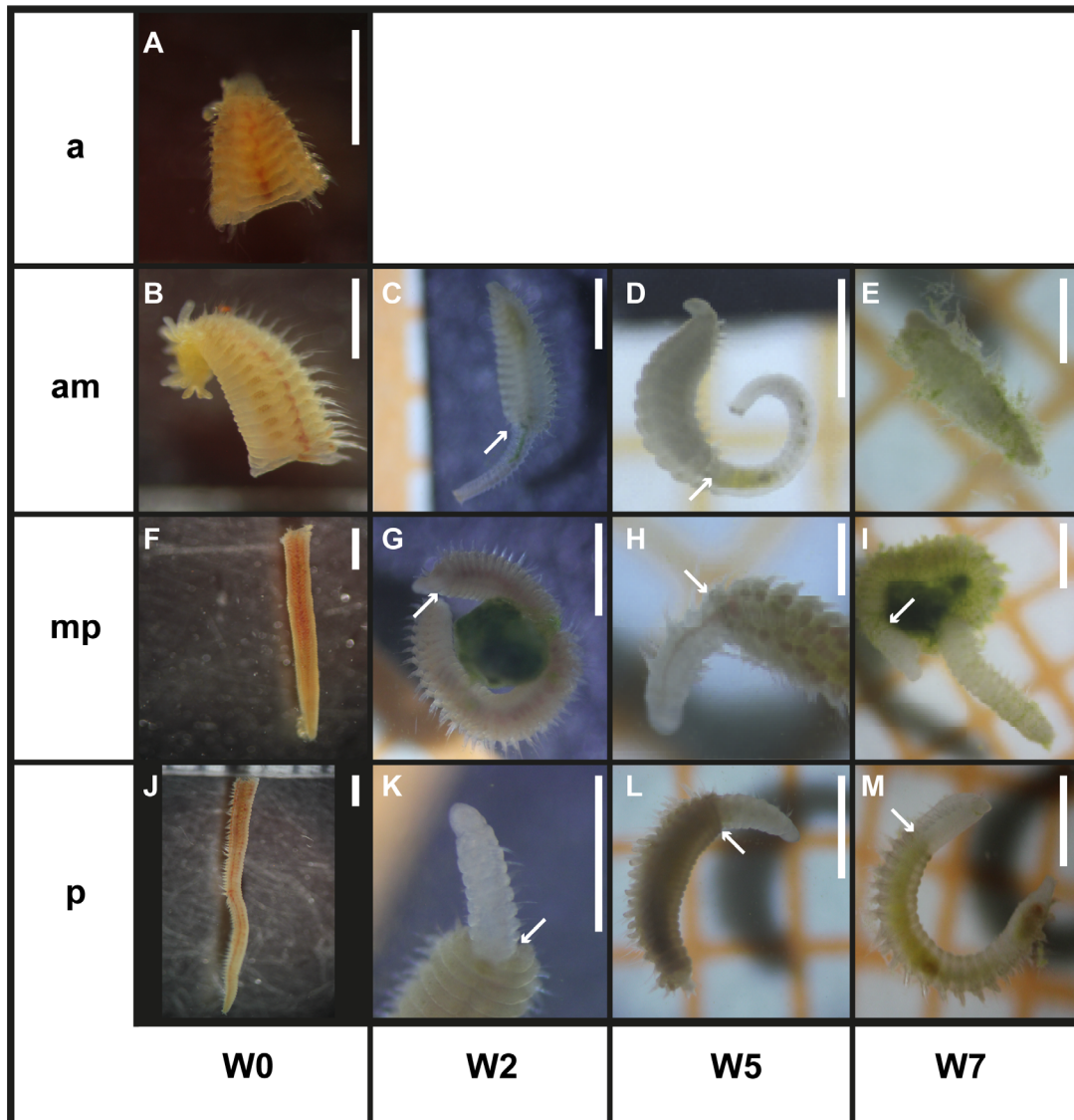


Figure 5. Light microscopy pictures of the regenerating *Naineris aurantiaca*. Posterior regeneration. A: fragment a, B-E: fragment am. Anterior regeneration. F-I: fragment mp, J-M: fragment p. Scale bar A-G, I-M = 1 mm, H = 0.5 mm. Arrows point to the limit between uncut and newly regenerated segments, except for panel E, because the fragment was decomposed. Orange lines out of focus are from the millimeter paper used as a scale.

MEASUREMENTS DURING REGENERATION

Mean growth of surviving fragments is shown in Figure 6. The mean width of fragment P at the beginning of the experiment was 0.827 mm, whereas fragment MP had a mean width of 0.693 mm. The width of P fragment ranged from 0.827 to 0.455 mm in the first week, while the MP fragment from 0.693 mm to 0.386 mm. The mean width of fragment P between weeks 2 and 3 was 0.0785

mm, whereas fragment MP had a mean width of 0.0883 mm. In the first week, fragment P had a mean length of 5.968 mm (range of 8.848 mm and 3.568 mm), while MP had a mean length of 5.472 mm (range of 7.097 mm and 4.560 mm). The mean lengths of AM, MP, and P fragments were 0.033, 0.596, and 0.605 mm, respectively. Fragment A was excluded from the analysis because of the limited availability of data.

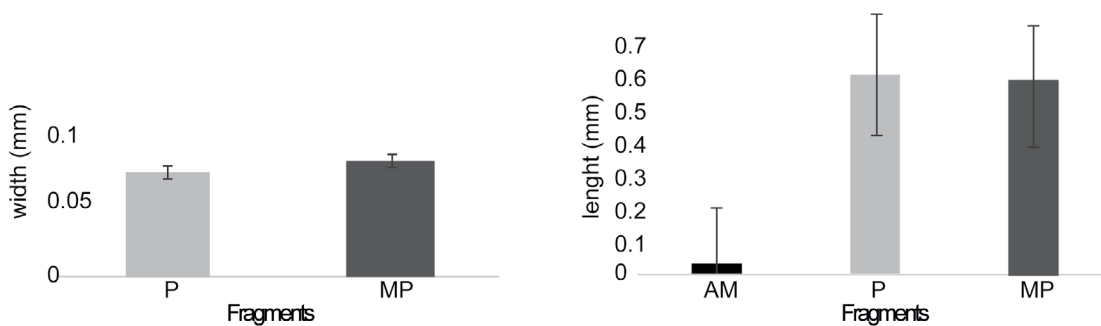


Figure 6. Mean growth of surviving fragments, width (left), length (right).

DISCUSSION

Naineris aurantiaca worms showed high anterior regeneration capability during the culture period. However, survivorship of the anterior fragments was drastically lower than the other treatments. Regeneration of the species was comparable to what has been considered an excellent model for investigating regeneration in annelids, such as *Alitta virens* (Kozin and Kostyuchenko, 2015), *Capitella teleta* (Seaver, 2022), *Enchytraeus japonensis* (Tadokoro et al., 2006), *Eurythoe complanata* (Yáñez-Rivera and Méndez, 2014), *Lamellibranchia satsuma* (Miyamoto et al., 2014), *Platynereis dumerillii* (Schenkelaars and Gazave, 2021), *Pristina leidyi* (Özpolat et al., 2016), *Timarete cf. punctata* (Weidhase et al., 2015) and *Typosyllis antoni* (Weidhase et al., 2016). Therefore, in the present experimental study, regenerative capacity may be representative of a process exclusive to some body regions and is associated with the energy reserve and post-traumatic regeneration capacity of the annelid region, which occurs through the active proliferation of undifferentiated cells that can regenerate mesodermal derivatives (coelomic epithelium, gonads, blood vessels, and metanephridia) and ectodermal derivatives (nervous system and integuments) (Nikanorova et al., 2020).

Regeneration of *N. aurantiaca* occurs in the anterior and posterior segments, but the process seems to be limited by the restoration of vital structures, such as gut and branchiae. In many syllids, anterior regeneration is limited by restoration of the proventricle, which plays a crucial role in stolonization and sexual determination (Ribeiro et al., 2018). In onuphids, regeneration is limited

by the specific number of chaetigers at which the amputation occurs (Schoeman and Simon, 2023). Among sedentary annelids, *C. teleta* outgoing in regenerating posterior segments without feeding and a brain (Seaver and de Jong, 2021). In *N. aurantiaca*, regeneration was interrupted in anterior fragments (A) and was insignificant compared to posterior fragments. The posterior fragments lack a mouth and are much smaller than the anterior fragments but have more branchiate segments than the anterior fragments. Oxygen tension is a limiting factor during the regeneration process of annelids (Anderson, 1956). Low oxygen tension retards the regeneration of *Tubifex tubifex*, whereas too high values may be lethal (Anderson, 1956). Considering that abdominal branchiae of *N. aurantiaca* are much longer than those of the anterior fragments, differences in mortality may also be explained by the reduction in gas exchange ability in abranchiate segments.

Furthermore, slow body growth may indicate low energy efficiency in the production of reconstructed tissues (Nikanorova et al., 2020). In other sedentary annelids, posterior regeneration and survivorship of the resultant fragments are determined by the migration of multipotent progenitor cell (MPC) clusters (de Jong and Seaver, 2017), which are limited to a specific region of the body (chaetigers 6-7 in *Capitella teleta*). It is necessary to perform an exploratory study to determine whether these migrating cells are also present in the regeneration process of orbiniids. The cephalic segments containing only the pharynx, which has an ectodermal origin, cannot regenerate the posterior segments, which include the gut of the endodermal nature (Berrill, 1952).

Although the P region is deprived of the thorax and mouth apparatus, its regeneration was highly efficient. The P fragments preserve the posterior growth zone, a pluri-/multi- potential zone placed immediately anterior to the pygidium, which may display long-term self-renewal activities and serve as stem cells (Özpolat and Bely, 2016; Planques et al., 2019; Kostyuchenko and Kozin, 2021). As in P fragments, the MP region had low mortality and was completely regenerated after seven weeks of exposure. We hypothesize that the permanence of the abdominal body structures and part of the thoracic region ensured sufficient energy reserves for constructing the mouth apparatus, allowing the animal to regenerate entirely after a few weeks. This may indicate that the posterior region has a significant energy reserve to supply the animal during the regeneration process of the entire thorax and mouth apparatus.

Naineris aurantiaca can perform bidirectional regeneration. Although we did not focus on M fragments, we observed a few fragments regenerating segments in both directions. Bidirectional regeneration has been described for other annelids, and is outstanding in earthworms (Cho et al., 2009). For example, in bait onuphids, this ability is limited by the number of segments removed (Schoeman and Simon, 2023), and it is believed that the recovery of the specimens is completely dependent of the presence of branchiae (Schoeman and Simon, 2023). Notably, the bidirectional regeneration of *N. aurantiaca* is a novelty among orbiniids and may indicate its plasticity behavior to burrowing and survive in a turbulent and unpredictable environmental as exposed rocky shores (Francoeur and Dorgan, 2014) *Proscoloplos cygnochaetus* proved to regenerate both anterior and posterior segments successfully (Meyer et al., 2007). However, as the authors did not perform any cut like as procedure 3, it is impossible to compare the performances of both species. The rationale for cutting in Meyer et al. (2007) differs from that of our study. As in *N. aurantiaca* there is a clear distinction between thorax and abdomen, we cut the specimens between both body regions to compare both body regions. In the case of *P. cygnochaetus*, there is no distinction thorax-abdomen, then Meyer et

al. (2007) cut the specimens just in the middle, obtaining fragments of equal length. Additionally, Meyer et al. (2007) approach was biased towards taxonomy, focusing on regeneration of structures with valuable taxonomic usage, such as branchiae and hook appearance. In contrast, our experiment was purely exploratory. Further experimental studies on *N. aurantiaca* and other orbiniids removing fragments of the body at different levels may help improve our understanding of the regeneration capabilities of the family.

The high regeneration ability of *Naineris aurantiaca* makes it a good candidate for studies on evolutionary developmental biology (EVO-Devo). Many syllids, capitellids, and nereidids are good models for EVO-Devo because they are easy to handle and have good regeneration capabilities. Besides being an easily sampled species, *N. aurantiaca* is abundant on rocky shores and has adequate size for experimental manipulation. The reason these animals inhabit and survive in a turbulent environment exposed to wave activities and tidal effects could be related to their successful regeneration capacity which acts as an adaptive mechanism sustained by this species to survive under such conditions.

A transcriptomic approach may help detect the differential mechanisms involved in posterior and anterior regeneration in *N. aurantiaca*. Recently, Ribeiro et al. (2019) found substantial discrepancies in gene expression between anterior and posterior regeneration of two closely related syllid species. Transcriptomic expression involved in the regeneration process has not yet been explored in orbiniids. Despite a transcriptomic approach beyond the scope of this study, understanding the driving mechanisms of the regeneration process may better explain the results obtained here.

CONCLUSION

This experiment demonstrated the capacity of regeneration of anterior and posterior fragments on *N. aurantiaca*, with complete reconstruction of the anterior body part, a fact not universal in Annelida. The successful regeneration of the head, while presenting difficulties in regenerating the gut

and branchial segments, could be related to food absorption and gas exchange in this species.

The findings of this study are highly relevant to the EVO-Devo field and enrich the acknowledgments of this species and genus. Therefore, new experiments on *N. aurantiaca* regeneration are necessary to better understand the physiological and biochemical processes that can be crucial in rebuilding their body parts.

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AUTHOR CONTRIBUTIONS

R.A.: Conceptualization; Investigation; Methodology; Formal Analysis; Writing – original draft; Writing – review & editing.

E.P.: Conceptualization; Investigation; Methodology; Formal Analysis; Writing – review & editing.

G.V.: Investigation Formal Analysis; Writing – review & editing.

M.D.D.: Conceptualization; Formal Analysis; Supervision; Resources; Project Administration; Funding Acquisition; Writing – review & editing.

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