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Research Article

Regeneration in a Neotropical land planarian (Platyhelminthes, Tricladida)

Piter Kehoma Boll[1] , Ilana Rossi[1,2] , Silvana Vargas do Amaral[1] , Ana Maria Leal-Zanchet[1]

1 Instituto de Pesquisa de Planárias and Programa de Pós-Graduação em Biologia, Universidade do Vale do Rio dos Sinos (UNISINOS), São Leopoldo, RS, Brazil
2 Instituto Federal de Educação, Ciência e Tecnologia do Rio Grande do Sul, Campus Vacaria, Vacaria, RS, Brazil
Corresponding author: Piter Kehoma Boll (piterkeo@gmail.com)

Abstract

Planarians are known for their ability to regenerate missing body parts. However, little is known about the regeneration ability of land planarians, especially regarding Neotropical species. Herein, we investigated the regeneration in the Neotropical land planarian *Luteostriata abundans*. Specimens were cut in two at different points along the body and monitored for 50 days. Larger and anterior pieces survived more than smaller posterior pieces. Anterior pieces that retained the pharynx continued to feed normally as intact animals, while posterior pieces that retained the pharynx lost its function temporarily. The growth rate was similar amongst all pieces across 50 days. Anterior mouthless pieces regenerated the pharynx and mouth significantly faster than posterior mouthless pieces. After 50 days, the relative position of the mouth along the body reached values close to intact animals in all regenerating pieces. In general, anterior pieces showed higher survival and regenerated faster than posterior fragments, which agrees with observations with other planarian species. However, surviving posterior pieces were able to retain the proportions of intact animals as well. Our results suggest that *L. abundans* has a good regenerative capacity similar to many freshwater planarians.

Key words: Geoplanidae, Geoplaninae, pharynx, regenerative power, survival

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Introduction

Planarians (Tricladida) are flatworms known for their ability to regenerate missing body parts in response to almost any kind of physical injury. This is especially true for many freshwater species, which have been studied intensively during the last decades (Elliott and Sánchez Alvarado 2013). A population of pluripotent cells in the planarian parenchyma, the neoblasts, gives them this amazing capacity (Wagner et al. 2011).

Various reproductive strategies and a great variation in longevity can be found amongst planarians. Amongst freshwater species, some are known to alternate between sexual and asexual modes of reproduction across the year, others use a sexual or asexual strategy in different populations and others rely solely on asexual reproduction (Vila-Farré and Rink 2018).

Usually, asexual populations show an increased capacity for regeneration because they rely on fission and regeneration to reproduce and, therefore,

have a larger proportion of neoblasts in their bodies when compared to sexual populations (Baguñà and Romero 1981; Baguñà et al. 1999). Exclusively sexual populations, on the other hand, may have a limited or almost non-existent capacity to regenerate missing parts (Ivankovic et al. 2019) either because of a species-specific limitation (Brøndsted 1969) or because the presence of sexually mature organs inhibits spontaneous fission, although it does not block regeneration (Kobayashi and Hoshi 2002). In many species, the regenerative capacity declines towards the posterior end, especially regarding the capacity to regenerate a head. Some species cannot regenerate a head from pieces behind the pharynx (Reddien and Sánchez Alvarado 2004). However, this gradient in regenerative capacity does not seem to be related to the neoblasts. On the contrary, it seems to be caused by the differentiated tissues and the clues they provide for neoblasts to restore missing parts (Reddien and Sánchez Alvarado 2004) since the distribution of neoblasts is relatively uniform along the anteroposterior axis of the body (Newmark and Sánchez Alvarado 2000; Orii et al. 2005).

Land planarians (Geoplanidae) are the sister group of freshwater planarians of the family Dugesiidae (Sluys et al. 2009), which include the most common models of planarian regeneration (Reddien and Sánchez Alvarado 2004). However, the regenerative capacity of land planarians is not so well known, although Darwin (1844) already observed their capacity to regenerate missing parts when cut in half. Some land planarians have asexual reproduction by fission as their main mode of reproduction. This is the case with some invasive species like *Bipalium kewense* (Bipaliinae) and *Dolichoplana striata* (Rhynchodeminae) and is likely the reason for their success in invading new ecosystems (Winsor 1983; Alvarez and de Almeida 2007; Brown et al. 2022). Other species, such as *Endeavouria septemlineata* (Rhynchodeminae), use autotomy to escape predators and, therefore, likely rely on a good regenerative capacity as well (Boll et al. 2015). However, only a few experiments studying regeneration in land planarians exist, most of them dealing with asexually reproducing species of the genus *Bipalium*, which seem to be almost as good as freshwater planarians in their ability to regenerate (Morgan 1900; Shirasawa and Makino 1978, 1979, 1983, 1984, 1987, 1988; Makino and Shirasawa 1985). Spontaneous asexual reproduction has never been observed in Neotropical land planarians (Geoplaninae) and no hypothesis seems to have been presented to explain the lack of this feature. Not even the successfully invasive species *Obama nungara* (Lago-Barcia et al. 2018; Negrete et al. 2020) has been shown to reproduce asexually. They can regenerate wounds and lost parts (Froehlich 1955), but it is currently unknown whether they may restore a complete organism from small fragments.

To shed some light on this question, we examined the regenerative capacity of different pieces of *Luteostriata abundans*, a native land planarian in southern Brazil that is common in human-disturbed habitats, such as gardens and forest borders. Like other Neotropical land planarians, it seems to reproduce only sexually. Therefore, we expect its regeneration ability to be lower than that of asexually reproducing species. We hypothesise that larger and anterior pieces show increased survival and growth than smaller and posterior pieces, especially mouthless ones since posterior regions seem to have a limited regenerative capacity in many species. In addition, the absence of a mouth in small pieces

will force them to regenerate this organ before being able to ingest food. If the piece is too small, it may not be able to regenerate a mouth before depleting its resources by rearranging and consuming its own tissues.

Methods

We captured specimens of *Luteostriata abundans* in gardens, parks and forest borders in the cities of Ivoti, Montenegro, Novo Hamburgo and São Leopoldo, State of Rio Grande do Sul, Brazil. In the laboratory, they were kept in the dark in small plastic containers with moistened earth and log fragments at 18 to 20 °C. We chose this species because it is abundant in urban habitats and, therefore, it was easy to obtain an adequate number of specimens to conduct the experiments.

We used 35 animals in the study, of which nine were left intact (group I) and 26 were cut transversely into two pieces, an anterior and a posterior piece, using a razor blade. Three different cuts were performed in different animals, resulting in the following arrangement (Fig. 1):

- Eight animals cut before the region of the pharynx, resulting in an anterior end (group AE) and a posterior end with the mouth and gonopore (group MG-PE);
- Nine animals cut between the region of the pharynx and the gonopore, resulting in an anterior end with the mouth (group AE-M) and a posterior end with the gonopore (group G-PE);
- Nine animals cut after the gonopore, resulting in an anterior end with the mouth and gonopore (group AE-MG) and a posterior end (group PE).

We monitored the nine intact animals and the 52 pieces twice a week for 50 days, measuring their width and length at rest and while creeping. Each specimen received two neotropical woodlice (Philosciidae) as food after every measurement.
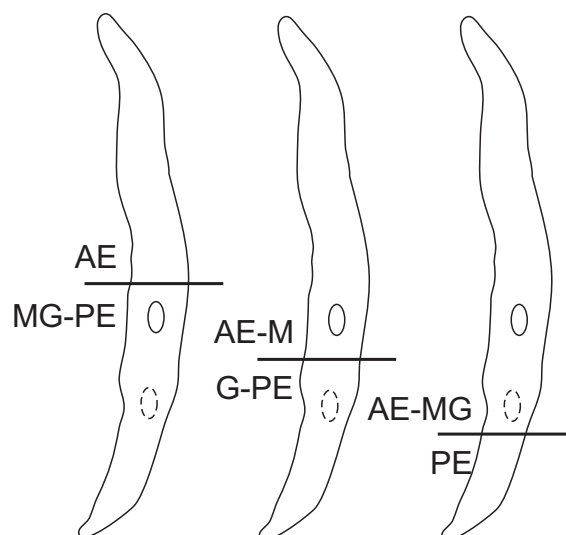

Figure 1. Representation of transversal cuts on specimens of *Luteostriata abundans* at different body regions. Horizontal lines represent the point at which a transversal cut was performed. Ellipsis with a continuous outline represents the location of the mouth and ellipsis with a dashed line represents the location of the gonopore. AE: anterior end; AE-M: anterior end plus mouth; AE-MG: anterior end plus mouth and gonopore; G-PE: posterior end plus gonopore; MG-PE: posterior end plus mouth and gonopore; PE: posterior end.

We calculated the size of the planarian as the elliptic area that it occupied while creeping, using the following formula to calculate the area of an ellipsis:

$$Ap = \frac{\pi * Lc * Wc}{4}$$

where Ap is the elliptic area occupied by the planarian, Lc is the planarian's length in millimetres while creeping and Wc is the planarian's maximum width in millimetres while creeping. Although this formula does not provide the exact area occupied by the animal, it is a reasonable approximation and keeps the relationship constant between different pieces.

We compared the initial size between groups using a Kruskal-Wallis test. Since the mouth in adult and intact individuals of *L. abundans* lies about 57% from the anterior end (data extracted from measuring specimens in the zoological collection of Instituto de Pesquisas de Planárias, UNISINOS), anterior pieces tend to be larger than posterior pieces.

We compared the survival of planarians in each treatment from Kaplan-Meier survival curves with a two-sided log-rank test. Additionally, we performed a Cox proportional hazards regression analysis using the initial size of the specimens as an additional variable with the treatment.

For pieces of each treatment having a mouth at the start of the experiment, we compared the time they took to eat for the first time after amputation from Kaplan-Meier survival curves with a two-sided log-rank test. Intact animals were used as a control. Since we only monitored the pieces twice a week, we considered the day that a woodlouse was eaten as the day immediately before the day in which we found the empty exoskeleton of the woodlouse.

For specimens that survived the 50 days of monitoring, we generated a scatter plot relating time in days and size and calculated the linear equation for the growth during the whole period. The slope of the line was used as the specimen's growth rate. We also calculated the size increase of each specimen 26 and 50 days after amputation by dividing their size on those days by their original size on day 1.

For pieces that lacked the pharynx and the mouth after amputation (AE, G-PE, PE), we counted the days until the pharynx and the mouth regenerated and the animal restarted to eat. For all pieces with a mouth, we measured twice a week the distance from the end where the animal was cut to the mouth.

To compare the growth and regeneration ability of each piece, we performed a series of Kruskal-Wallis and Mann-Whitney tests comparing the groups by: (1) the growth rate across the 50 days of monitoring, (2) the size increase of the animals after 26 and 50 days, (3) the number of days for the mouthless pieces to regenerate the mouth, (4) the relative distance from the mouth to the posterior end for anterior fragments (AE, AE-M, AE-MG) after 50 days and (5) the relative distance from the mouth to the anterior end for posterior fragments (MG-EP, G-EP, EP) after 50 days. We conducted all analyses in the programme IBM SPSS Statistics 20. Detailed data about each specimen and its measurements are presented in Suppl. material 1.

Since measuring the specimens twice a week already disturbed them considerably, we avoided exposing them to light for taking detailed photographs of the regenerative process, as this would disturb the animals further and likely have a negative effect on their recovery.

Results

Table 1 shows the size of all specimens at the start of the experiment and the mean size per group. Groups AE-M, AE-MG and I were formed by significantly larger pieces than groups G-PE and PE, while AE and MG-PE were not significantly different from any group (Kruskal-Wallis, $\chi^2(6) = 45.060$, $p < 0.001$) (Fig. 2). Planarians in the different groups had a significant difference in survival (log-rank test, $p = 0.008$). All specimens in group AE-MG survived 50 days, a significantly higher survival rate ($p < 0.05$) than other groups, except AE-M. On the other hand, only two specimens of group PE survived 50 days, a significantly lower survival rate ($p < 0.05$) than AE-M and AE-MG (Fig. 3). As a result, PE was excluded from all other analyses conducted with data from day 50. Cox

Table 1. Initial size (mm^2) of pieces and intact specimens of *Luteostriata abundans* used in the experiment. AE: anterior end; AE-M: anterior end plus mouth; AE-MG: anterior end plus mouth and gonopore; MG-PE: posterior end plus mouth and gonopore; G-PE: posterior end plus gonopore; PE: posterior end; I: intact animals; SD: standard deviation.

| Group	AE	AE-M	AE-MG	MG-PE	G-PE	PE	I
	43.98	94.25	73.83	34.56	15.71	15.71	80.11
	74.61	62.83	70.69	26.70	45.16	21.99	38.88
	56.55	96.21	74.61	50.27	40.84	17.28	89.54
	51.84	82.47	80.50	48.69	49.48	16.49	97.39
	96.21	58.12	103.67	54.98	31.42	21.99	131.95
	61.26	51.84	68.72	63.62	34.56	7.07	62.83
	42.41	64.80	80.50	34.56	26.70	18.85	117.81
	32.99	82.47	96.60	51.05	25.53	26.70	178.68
	–	86.39	86.39	–	33.38	17.28	65.97
Mean ± SD	57.48 ± 20.18	75.49 ± 16.33	81.73 ± 11.89	45.55 ± 12.39	33.64 ± 10.47	18.15 ± 5.43	95.91 ± 42.08
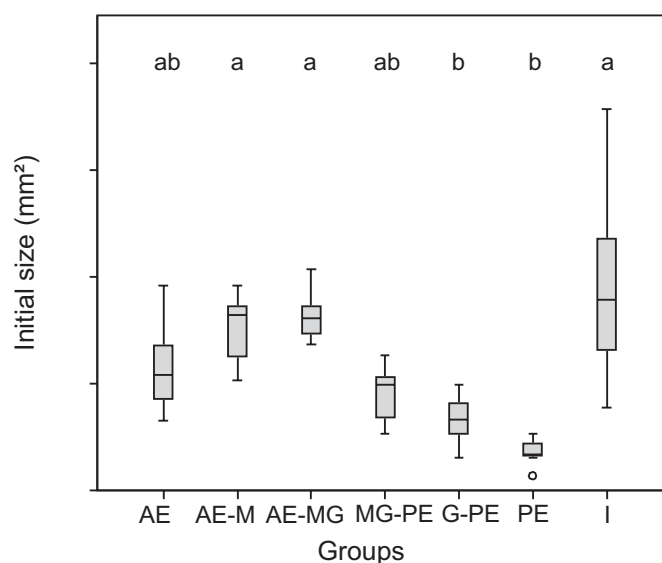
Figure 2. Boxplots showing the initial size of intact and regenerating specimens of *Luteostriata abundans*. AE: anterior end; AE-M: anterior end plus mouth; AE-MG: anterior end plus mouth and gonopore; MG-PE: posterior end plus mouth and gonopore; G-PE: posterior end plus gonopore; PE: posterior end; I: intact animals. Groups that do not share the same lowercase letters are significantly different. Circle indicates outlier.

regression (p = 0.008) explained survival by group (p = 0.036), but not by initial size (p = 0.070).

Pieces of the MG-PE group took significantly more time to eat for the first time after amputation than intact animals and pieces in the AE-M and AE-MG groups, whereas the latter three did not differ significantly from each other (log-rank test, p < 0.001) (Fig. 4).

The growth rate across 50 days of monitoring was not significantly different between the groups (Kruskal-Wallis, χ^2(5) = 3.807, p = 0.578) (Fig. 5).

The increase in size after 26 days was significantly different between the groups (Kruskal-Wallis, χ^2(6) = 12.748, p = 0.047). Group AE-MG increased significantly more than groups AE, MG-PE, G-PE and PE. Groups AE-M and I did not differ significantly from any group (Fig. 6A). After 50 days, however, the increase in size was not significantly different between groups anymore (Kruskal-Wallis, χ^2(5) = 6.941, p = 0.225) (Fig. 6B).
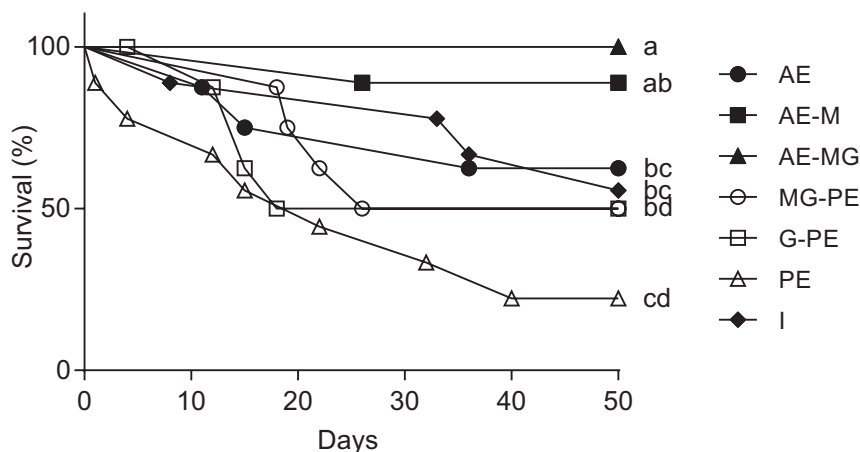

Figure 3. Kaplan-Meier survival curves for intact and regenerating specimens of *Luteostriata abundans*. AE: anterior end; AE-M: anterior end plus mouth; AE-MG: anterior end plus mouth and gonopore; MG-PE: posterior end plus mouth and gonopore; G-PE: posterior end plus gonopore; PE: posterior end; I: intact animals. Groups that do not share the same lowercase letters are significantly different.
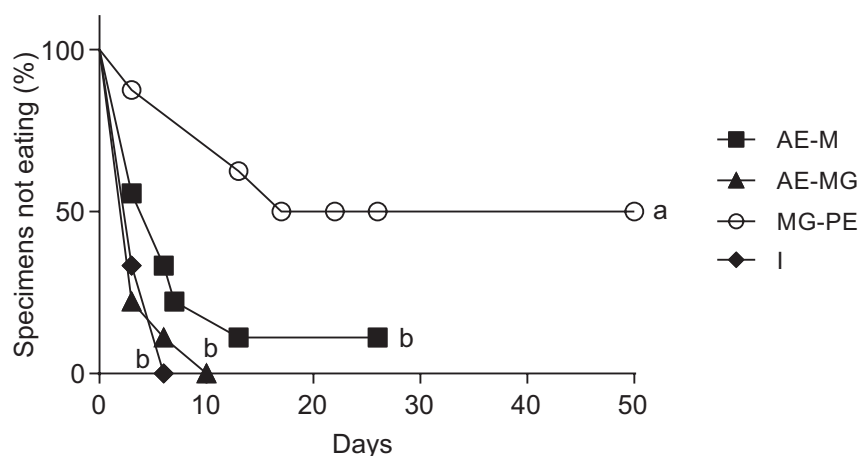

Figure 4. Kaplan-Meier survival curves comparing the time for intact and mouth-bearing regenerating specimens of *Luteostriata abundans* to eat for the first time since the start of the experiment. AE-M: anterior end plus mouth; AE-MG: anterior end plus mouth and gonopore; MG-PE: posterior end plus mouth and gonopore; I: intact animals. Groups that do not share the same lowercase letters are significantly different.
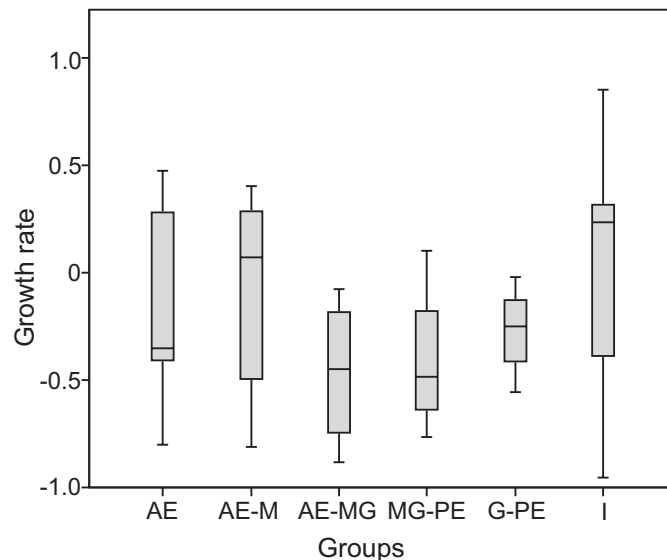
Figure 5. Boxplots showing the growth rate of intact and regenerating specimens of *Luteostriata abundans* over 50 days. AE: anterior end; AE-M: anterior end plus mouth; AE-MG: anterior end plus mouth and gonopore; MG-PE: posterior end plus mouth and gonopore; G-PE: posterior end plus gonopore; PE: posterior end; I: intact animals.

Figure 6. Boxplots showing the proportional increase in the size of intact and regenerating specimens of *Luteostriata abundans* after 26 (A) and 50 days (B). AE: anterior end; AE-M: anterior end plus mouth; AE-MG: anterior end plus mouth and gonopore; MG-PE: posterior end plus mouth and gonopore; G-PE: posterior end plus gonopore; P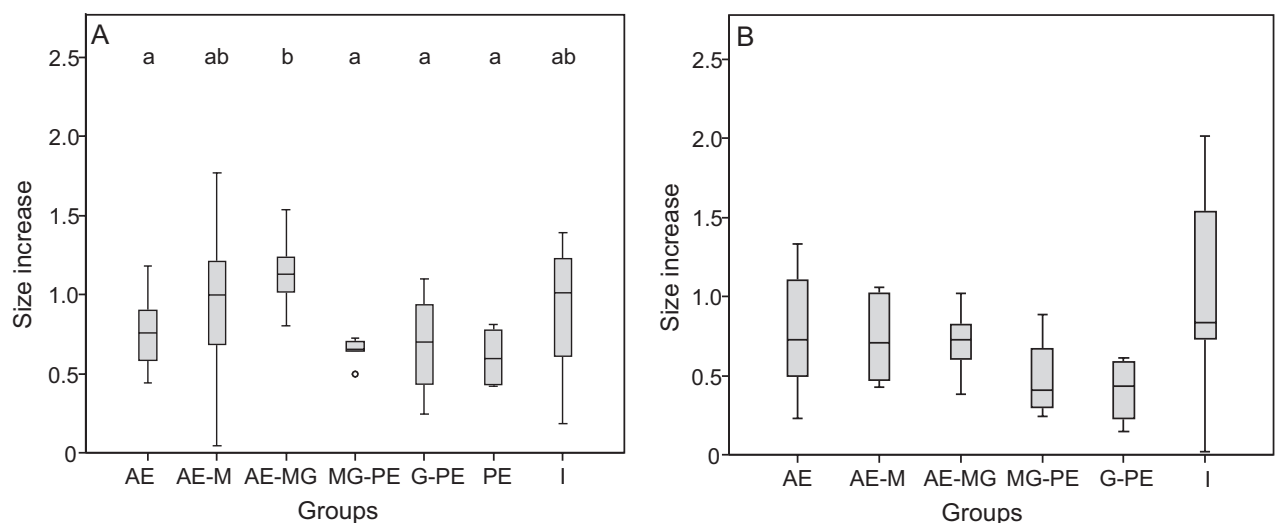E: posterior end; I: intact animals. Groups that do not share the same lowercase letters are significantly different from each other.

The time for regenerating the mouth and restarting eating was significantly different between the originally mouthless groups (Kruskal-Wallis, $\chi^2(2) = 10.964$, p = 0.004). It took longer for group G-PE to regenerate a mouth than for group AE (p = 0.005), while group PE was not significantly different from both (Fig. 7). Anterior fragments did not differ regarding the relative distance of the mouth to the posterior end after 50 days (Kruskal-Wallys, $\chi^2(2) = 0.347$, p = 0.841) (Fig. 8A) and neither did posterior fragments regarding the relative distance of the mouth to the anterior end after 50 days (Mann-Whitney, U = 10.000, p = 0.686) (Fig. 8B).
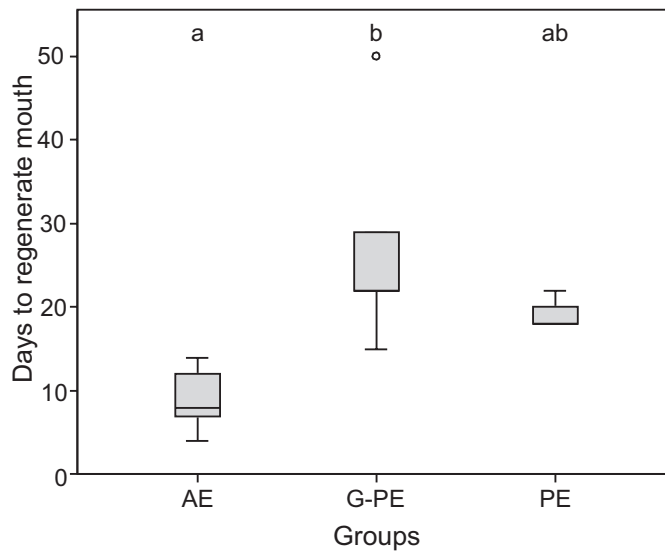
Figure 7. Boxplots showing the time in days that mouthless regenerating specimens of *Luteostriata abundans* took to regenerate the mouth. AE: anterior end; G-PE: posterior end plus gonopore; PE: posterior end. Groups that do not share the same lowercase letters are significantly different.
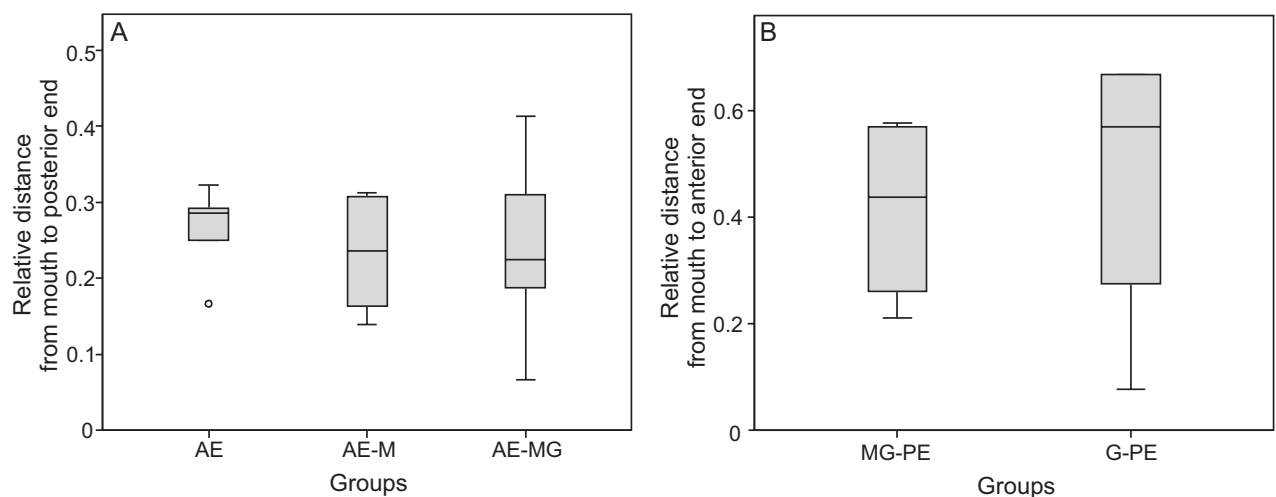

Figure 8. Boxplots showing the relative distance from the mouth to the regenerating end of anterior (A) and posterior (B) pieces of *Luteostriata abundans* after 50 days. AE: anterior end; AE-M: anterior end plus mouth; AE-MG: anterior end plus mouth and gonopore; MG-PE: posterior end plus mouth and gonopore; G-PE: posterior end plus gonopore.

Discussion

Regeneration in freshwater planarians has been studied for the past two centuries and approached from different morphological, physiological, molecular, behavioural and ecological perspectives, especially in more recent decades (Rink 2018). Amongst land planarians, only the genus *Bipalium* received increased attention on its regenerative capacity, especially regarding histological and behavioural aspects and only by two Japanese researchers in the 1970s and 1980s (Shirasawa and Makino 1979, 1985, 1988). These studies showed that species of *Bipalium* have a remarkable regeneration capacity similar to that of many freshwater species and can regenerate a whole organism from very small fragments. Our study is the second to investigate regeneration in

a Neotropical land planarian. A single previous study was conducted almost a century ago (Goetsch 1933) with a Chilean species, *Pseudogeoplana pulla*, in which specimens were cut into two, four or nine pieces and monitored for a few months. Herein, for a preliminary observation, we limited the experiments to only a single cut resulting in two pieces and monitored them for only 50 days. However, as we performed the cuts at different points along the body, we can compare some of our results to those of *P. pulla*, as shown below.

As Neotropical land planarians do not reproduce asexually and, therefore, are expected to have a smaller number of neoblasts (Baguñà and Romero 1981), we expected smaller pieces to have a lower regenerative capacity than larger ones, which was supported by our results. The group formed by the largest pieces (AE-MG) had the highest survival rate, while that with the smallest pieces (PE) had the lowest survival rate. Goetsch (1933) observed the same with *P. pulla*, where smaller pieces had a lower survival rate than larger ones. However, the statistical analyses suggest that not only size was responsible for the different survival rates, which could result from the relatively low survival of intact animals, an unexpected and unexplainable outcome. One possible explanation could be the now century-old hypothesis that regeneration triggers rejuvenation in flatworms, but more recent investigations did not support this (Mouton et al. 2018). Since all specimens were captured as adults in the wild, we cannot be sure whether age or other factors, such as place of origin, affected survival.

Besides size alone, we also expected posterior pieces to show a decreased capacity for regeneration, based on this general trend in many planarian groups (Reddien and Sánchez Alvarado 2004). Specimens formed by only the posteriormost piece, cut behind the copulatory apparatus, showed indeed the lowest survival rate, with only two surviving the 50 days of monitoring. However, as these were also the smallest pieces, we cannot confirm whether their low survival is due to their size, posterior position or both. Further studies with smaller fragments of the anterior end could help clarify this point. Goetsch (1933) suggested that the low survival rate of posterior pieces of *P. pulla* resulted from the small number of neoblasts in the posterior half since this region has much of its space occupied by complex organs, such as the pharynx and the copulatory apparatus. Therefore, there would be little room left for the parenchyma, which contains the neoblasts. However, more recent studies on neoblast distribution indicate that they are somehow uniformly distributed along the body, although they are concentrated on the dorsal part of the parenchyma, especially in three longitudinal rows running along the body (Orii et al. 2005).

Amongst the three pieces that kept the mouth and pharynx after amputation, only the two anterior fragments continued to ingest food normally soon after, feeding like normal intact planarians. Posterior pieces with a pharynx took a significantly longer time to eat, with half of them spending the 50 days of monitoring without ingesting food. Goetsch (1933) observed that posterior pieces of *P. pulla* that kept the pharynx lost this organ soon after amputation and built a new one, differently from anterior fragments with a pharynx, which kept the original one. However, in *L. abundans*, according to our observations, which were only external in live animals, the pharynx was retained in posterior pieces, although its function was lost and took more time to be restored than in other pharynx-bearing fragments. This agrees with observations on freshwater planarians of the families Dugesiidae and Planariidae, in which posterior

pieces do not shed the original pharynx, but its function takes a longer time to be recovered than in anterior fragments (Sheiman et al. 2010). This temporary loss of function in the pharynx of posterior fragments is likely the result of the loss of part of the nervous system, especially of the head ganglia, which seem responsible for controlling the pharynx function (Sheiman et al. 2010). In other flatworms, such as polyclads, the pharynx may retain its function after the amputation of the brain, but the animal's capacity to detect food is impaired (Schadt et al. 2021).

Although we did not find a significant difference in growth between the groups across the whole period, the group with the largest pieces (AE-MG), which had the highest survival rate, also showed the highest increase in size in the first 26 days. Since these pieces were the largest and needed to regenerate only a small posterior fragment lacking important organs (Sluys and Riutort 2018), it seems reasonable that they would have completed their regeneration in a few days and, afterwards, slow down their increase in size.

Anterior mouthless fragments (AE) regenerated a pharynx and mouth significantly faster than posterior fragments (with gonopore, G-PE). The pharynx is known as one of the few parts of a planarian's body that lacks neoblasts (Reddien and Sánchez Alvarado 2004; Orii et al. 2005). However, as neoblasts are the only cells capable of reproduction, they must participate in the regeneration of the pharynx as well. Studies on pharynx regeneration with freshwater planarians indicate that it is rebuilt, like other organs, with neoblasts, which migrate from other body regions following an injury (Kreshchenko 2009). According to the observations of Shirasawa and Makino (1991) with *Bipalium kewense*, cells of the intestinal wall also participate in the formation of the pharynx by undergoing dedifferentiation, but the possibility of dedifferentiation in planarians needs further investigation (Reddien and Sánchez Alvarado 2004). Observations with other species indicate that, although the pharynx seems to start regenerating sooner in posterior fragments, it becomes functional in a shorter time in anterior fragments (Kreshchenko 2009), which supports our observations. This is likely caused by the pharynx depending on the head ganglia to function properly (Sheiman et al. 2010).

The difference in the distance from the mouth to the posterior end between anterior pieces and to the anterior end between posterior pieces was not significantly different after 50 days. This suggests that they all approached the same level of regeneration by the end of the monitoring. In fact, the position of the mouth approached the expected position of intact animals in all fragments. This indicates that all fragments seem to have the same capacity to regenerate a whole organism, similarly to what occurs in many freshwater species in the family Dugesiidae (Reddien and Sánchez Alvarado 2004), the sister group of land planarians (Sluys et al. 2009). Species in the more distantly related families Planariidae and Dendrocoelidae (superfamily Planarioidea), on the other hand, usually have a more limited regenerative capacity (Ball et al. 1969; Brøndsted 1969), although some species are also as good as or even better at regenerating than dugesiid planarians (Sheiman et al. 2010). Therefore, the high regenerative capacity observed in *L. abundans* may be a shared trait of the superfamily Geoplanoidea (consisting of Dugesiidae plus land planarians) or a shared trait of the suborder Continenticola that decreased in some lineages.

Conclusion

Luteostriata abundans is a Neotropical land planarian with exclusive sexual reproduction, but our results indicate that it can regenerate a whole organism from both anterior and posterior fragments of different sizes, although small posterior fragments have a lower survival rate. Albeit preliminary, our findings suggest that Neotropical land planarians may have a regenerative capacity similar to asexually reproducing land planarians and dugesiid freshwater planarians. Further studies can be extended to other Neotropical species and explore the regenerative capacity of small pieces across an antero-posterior gradient, as well as examine the process through histological and molecular techniques.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

PKB conceived the study, conducted the experiments, analyzed the data and wrote the manuscript. IR and SVA conceived the study and conducted the experiments. AMLZ supervised the work, reviewed an early draft of the manuscript, and made suggestions on the analyses and discussion.

Author ORCIDs

Piter Kehoma Boll https://orcid.org/0000-0002-3029-0841
Ilana Rossi https://orcid.org/0000-0003-1525-9034
Silvana Vargas do Amaral https://orcid.org/0000-0001-9695-1393
Ana Maria Leal-Zanchet https://orcid.org/0000-0003-3927-5629

Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Specimens of *Luteostriata abundans* monitored for up to 50 days

Authors: Piter Kehoma Boll, Ilana Rossi, Silvana Vargas do Amaral, Ana Maria Leal-Zanchet
Data type: morphological
Explanation note: Data on the measurements of each specimen of *Luteostriata abundans* used in the regeneration experiment. Each specimen was monitored for up to 50 days and measured twice a week.
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