Regeneration in a Neotropical land planarian (Platyhelminthes, Tricladida)

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1 **Regeneration in a Neotropical land planarian (Platyhelminthes: Tricladida)**
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12 **Abstract**
13 Planarians are known for their ability to regenerate missing body parts. However, little is known
14 about the regeneration ability of land planarians, especially regarding Neotropical species.
15 Herein, we investigated the regeneration in the Neotropical land planarian *Luteostriata*
16 *abundans*. Specimens were cut in two at different points along the body and monitored for 50
17 days. Larger and anterior pieces survived more than smaller posterior pieces. Anterior pieces
18 that retained the pharynx continued to feed normally as intact animals, while posterior pieces
19 that retained the pharynx lost its function temporarily. The growth rate was similar among all
20 pieces across 50 days. Anterior mouthless pieces regenerated the pharynx and mouth
21 significantly faster than posterior mouthless pieces. After 50 days, the relative position of the
22 mouth along the body reached values close to intact animals in all regenerating pieces. In
23 general, anterior pieces showed higher survival and regenerated faster than posterior fragments,
24 which agrees with observations with other planarian species. However, surviving posterior
25 pieces were able to retain the proportions of intact animals as well. Overall, our results suggest
26 that *L. abundans* has a good regenerative capacity similar to many freshwater planarians.
27

28 **Keywords:** Geoplanidae; Geoplaninae; pharynx; regenerative power; survival.
29

30 **Introduction**
31

32 Planarians (order Tricladida) are flatworms known for their ability to regenerate missing body
33 parts in response to almost any kind of physical injury. This is especially true for many
34 freshwater species, which have been studied intensively during the last decades (Elliott and
35 Sánchez Alvarado 2013). A population of pluripotent cells in the planarians' parenchyma, the
36 neoblasts, gives them this amazing capacity (Wagner et al. 2011).
37

38 Various reproductive strategies and a great variation in longevity can be found among
39 planarians. Among freshwater species, some are known to alternate between sexual and asexual
40 modes of reproduction across the year, others use a sexual or asexual strategy in different
41 populations, and others rely solely on asexual reproduction (Vila-Farré and Rink 2018).
42

43 Usually, asexual populations show an increased capacity for regeneration because they rely on
44 fission and regeneration to reproduce and, therefore, have a larger proportion of neoblasts in
45 their bodies when compared to sexual populations (Baguñà and Romero 1981, Baguñà et al.
46 1999). Exclusively sexual populations, on the other hand, may have a limited or almost non-
47 existent capacity to regenerate missing parts (Ivankovic et al. 2019) either because of a species-
48 specific limitation (Brøndsted 1969) or because the presence of sexually mature organs inhibits
49 spontaneous fission, although it does not block regeneration (Kobayashi and Hoshi 2002). In
50 many species, the regenerative capacity declines toward the posterior end, especially regarding

51 the capacity to regenerate a head. Some species cannot regenerate a head from pieces behind
52 the pharynx (Reddien and Sánchez Alvarado 2004). However, this gradient in regenerative
53 capacity does not seem to be related to the neoblasts. On the contrary, it seems to be caused by
54 the differentiated tissues and the clues they provide for neoblasts to restore missing parts
55 (Reddien and Sánchez Alvarado 2004) since the distribution of neoblasts is relatively uniform
56 along the anteroposterior axis of the body (Newmark and Sánchez Alvarado 2000, Orii et al.
57 2005).

58
59 Land planarians (family Geoplanidae) are the sister group of freshwater planarians of the family
60 Dugesiidae (Sluys et al. 2009), which include the most common models of planarian
61 regeneration (Reddien and Sánchez Alvarado 2004). The regenerative capacity of land
62 planarians, however, is not so well known, although Darwin (1844) already observed their
63 capacity to regenerate missing parts when cut in half. Some land planarians have asexual
64 reproduction by fission as their main mode of reproduction. This is the case with some invasive
65 species like *Bipalium kewense* (subfamily Bipaliinae) and *Dolichoplana striata* (subfamily
66 Rhynchodeminae) and is likely the reason for their success in invading new ecosystems (Winsor
67 1983, Alvarez and Almeida 2007, Brown et al. 2022). Other species, such as *Endeavouria*
68 *septemlineata* (Rhynchodeminae), use autotomy to escape predators and, therefore, likely rely
69 on a good regenerative capacity as well (Boll et al. 2015). However, only a few experiments
70 studying regeneration in land planarians exist, most of them dealing with asexually reproducing
71 species of the genus *Bipalium*, which, as expected, seem to be almost as good as freshwater
72 planarians in their ability to regenerate (Morgan 1900, Shirasawa and Makino 1978, 1979,
73 1983, 1984, 1987, 1988, Makino and Shirasawa 1985). Spontaneous asexual reproduction has
74 never been observed in Neotropical land planarians (subfamily Geoplaninae). They can
75 regenerate wounds and lost parts (Froehlich 1955), but it is currently unknown whether they
76 may restore a complete organism from small fragments.

77
78 To shed some light on this question, we examined the regenerative capacity of different pieces
79 of *Luteostriata abundans*, a native land planarian in southern Brazil that is common in human-
80 disturbed habitats, such as gardens and forest borders. Like other Neotropical land planarians,
81 it seems to reproduce only sexually. Therefore, we expect its regeneration ability to be lower
82 than that of asexually reproducing species. We hypothesize that larger and anterior pieces show
83 increased survival and growth than smaller and posterior pieces, especially mouthless ones,
84 since posterior regions seem to have a limited regenerative capacity in many species. In
85 addition, the absence of a mouth in small pieces will force them to regenerate this organ before
86 being able to ingest food. If the piece is too small, it may not be able to regenerate a mouth
87 before depleting its resources by rearranging and consuming its own tissues.

88
89 **Methods**

90
91 We captured specimens of *Luteostriata abundans* in gardens, parks, and forest borders in the
92 cities of Ivoti, Montenegro, Novo Hamburgo, and São Leopoldo, state of Rio Grande do Sul,
93 Brazil. In the laboratory, they were kept in the dark in small plastic containers with moistened
94 earth and log fragments under a temperature ranging from 18 to 20 °C.

95
96 We used 35 animals in the study, of which 9 were left intact (group I) and 26 were cut
97 transversely into two pieces, an anterior and a posterior piece, using a razor blade. Three
98 different cuts were performed in different animals, resulting in the following arrangement (Fig.
99 1):
100

- 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).
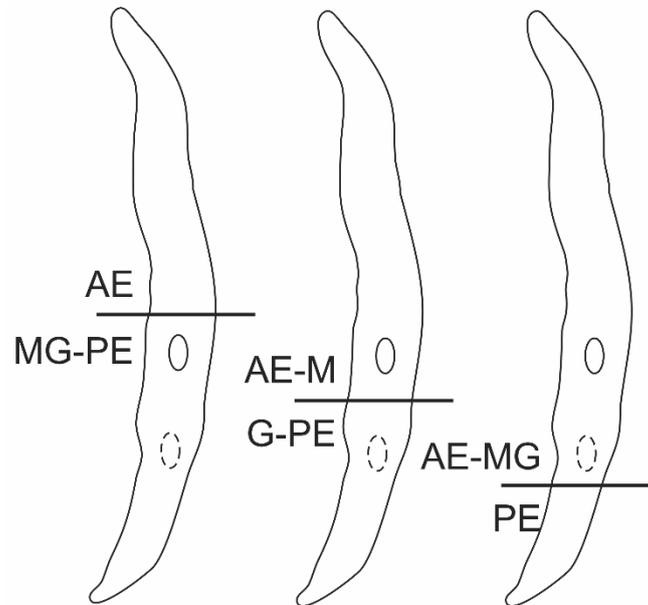
Figure 1. Representation of transversal cuts on specimens of *Luteostriata abundans* at different body regions. Horizontal lines represent the point at which a transversal cut was performed. Ellipsis with a continuous outline represents the location of the mouth and ellipsis with a dashed line represents 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 monitored the 9 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 (family Philosciidae) as food after every measurement. We calculated the size of the planarian as the elliptic area that it occupied while creeping using the 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 millimeters while creeping and Wc is the planarian's maximum width in millimeters while creeping. Although this formula does not provide the exact area occupied by the animal, it is a reasonable approximation and keeps the relation 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.

134
135   We compared the survival of planarians in each treatment from Kaplan-Meier survival curves
136   with a two-sided log-rank test. Additionally, we performed a Cox proportional hazards
137   regression analysis using the initial size of the specimens as an additional variable with the
138   treatment.
139
140   For pieces of each treatment having a mouth at the start of the experiment, we compared the
141   time they took to eat for the first time after amputation from Kaplan-Meier survival curves with
142   a two-sided log-rank test. Intact animals were used as a control. Since we only monitored the
143   pieces twice a week, we considered the day that a woodlouse was eaten as the day immediately
144   before the day in which we found the empty exoskeleton of the woodlouse.
145
146   For specimens that survived the 50 days of monitoring, we generated a scatter plot relating time
147   in days and size and calculated the linear equation for the growth during the whole period. The
148   slope of the line was used as the specimen's growth rate. We also calculated the size increase
149   of each specimen 26 and 50 days after amputation by dividing their size on those days by their
150   original size on day 1.
151
152   For pieces that lacked the pharynx and the mouth after amputation (AE, G-PE, PE), we counted
153   the days until the pharynx and the mouth regenerated and the animal restarted to eat. For all
154   pieces with a mouth, we measured twice a week the distance from the end where the animal
155   was cut to the mouth.
156
157   To compare the growth and regeneration ability of each piece, we performed a series of
158   Kruskal-Wallis and Mann-Whitney tests comparing the groups by (1) the growth rate across
159   the 50 days of monitoring, (2) the size increase of the animals after 26 and 50 days, (3) the
160   number of days for the mouthless pieces to regenerate the mouth, (4) the relative distance from
161   the mouth to the posterior end for anterior fragments (AE, AE-M, AE-MG) after 50 days, (5)
162   the relative distance from the mouth to the anterior end for posterior fragments (MG-EP, G-EP,
163   EP) after 50 days. We conducted all analyses in the program IBM SPSS Statistics 20. Detailed
164   data about each specimen and its measurements are presented in Suppl. material 1.
165
166   **Results**
167
168   Table 1 shows the size of all specimens at the start of the experiment and the mean size per
169   group. Groups AE-M, AE-MG, and I were formed by significantly larger pieces than groups
170   G-PE and PE, while AE and MG-PE were not significantly different from any group (Kruskal-
171   Wallis, $\chi^2(6) = 45.060$, $p < 0.001$) (Fig. 2).
172
173

Table 1. Initial size (in mm²) 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	57.48	75.49	81.73	45.55	33.64	18.15	95.91
±SD	±20.18	±16.33	±11.89	±12.39	±10.47	±5.43	±42.08
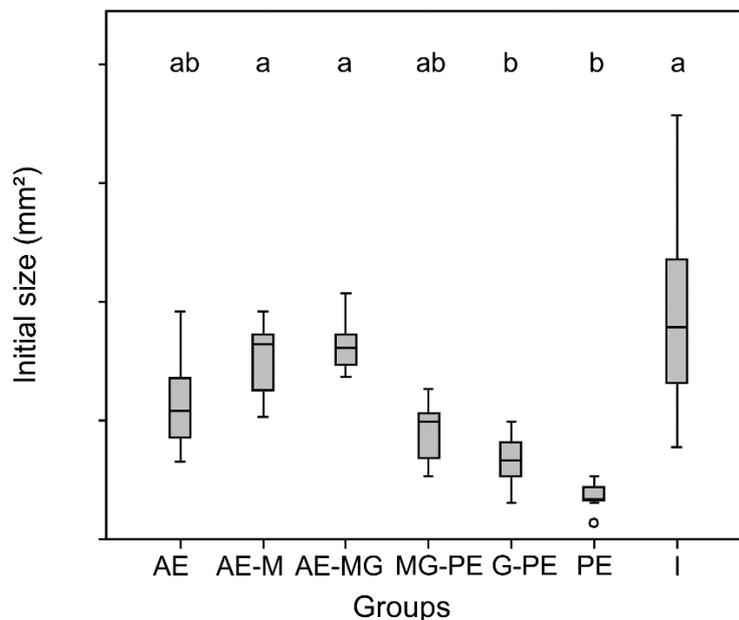
Figure 2. Boxplots showing the initial size of intact and regenerating specimens of *Luteostriata abundans*. AE: anterior end; AE-M: anterior end plus mouth; AE-MG: anterior end plus mouth and gonopore; MG-PE: posterior end plus mouth and gonopore; G-PE: posterior end plus gonopore; PE: posterior end; I: intact animals. Groups that do not share the same lowercase letters are significantly different.

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

195   Pieces of the MG-PE group took significantly more time to eat for the first time after amputation
196   than intact animals and pieces in the AE-M and AE-MG groups, whereas the latter three did
197   not differ significantly from each other (log-rank test, p < 0.001) (Fig. 4).
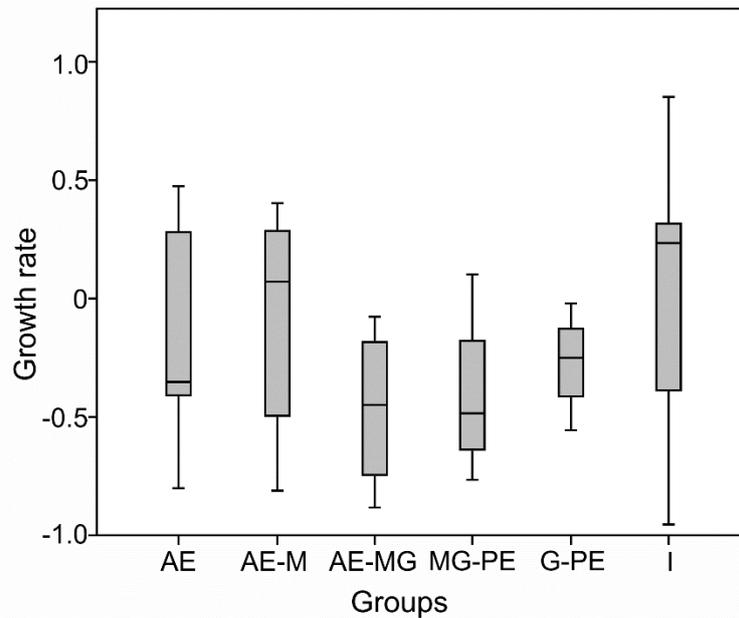198
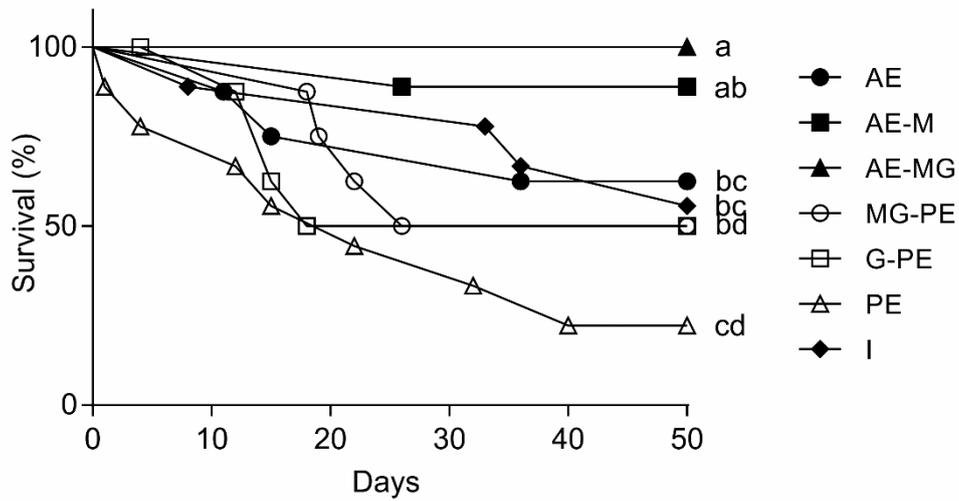
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200   **Figure 3.** Meier-Kaplan survival curves for intact and regenerating specimens of *Luteostriata*
201   *abundans*. AE: anterior end; AE-M: anterior end plus mouth; AE-MG: anterior end plus mouth
202   and gonopore; MG-PE: posterior end plus mouth and gonopore; G-PE: posterior end plus
203   gonopore; PE: posterior end; I: intact animals. Groups that do not share the same lowercase
204   letters are significantly different.
205
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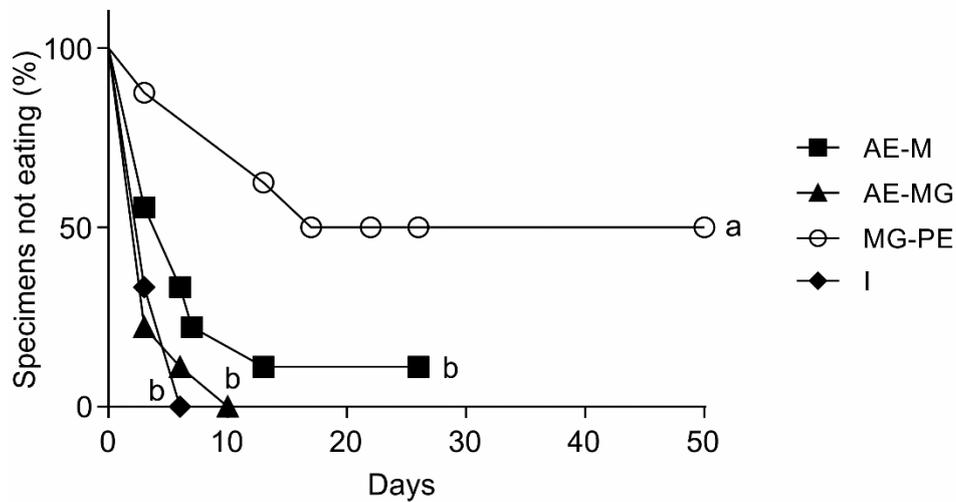
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208   **Figure 4.** Meier-Kaplan survival curves comparing the time for intact and mouth-bearing
209   regenerating specimens of *Luteostriata abundans* to eat for the first time since the start of the
210   experiment. AE-M: anterior end plus mouth; AE-MG: anterior end plus mouth and gonopore;
211   MG-PE: posterior end plus mouth and gonopore; I: intact animals. Groups that do not share the
212   same lowercase letters are significantly different.
213
214   The growth rate across 50 days of monitoring was not significantly different between the groups
215   (Kruskal-Wallis, $\chi^2(5) = 3.807$, p = 0.578) (Fig. 5).
216

217
218 **Figure 5.** Boxplots showing the growth rate of intact and regenerating specimens of
219 *Luteostriata abundans* over 50 days. AE: anterior end; AE-M: anterior end plus mouth; AE-
220 MG: anterior end plus mouth and gonopore; MG-PE: posterior end plus mouth and gonopore;
221 G-PE: posterior end plus gonopore; PE: posterior end; I: intact animals.
222
223
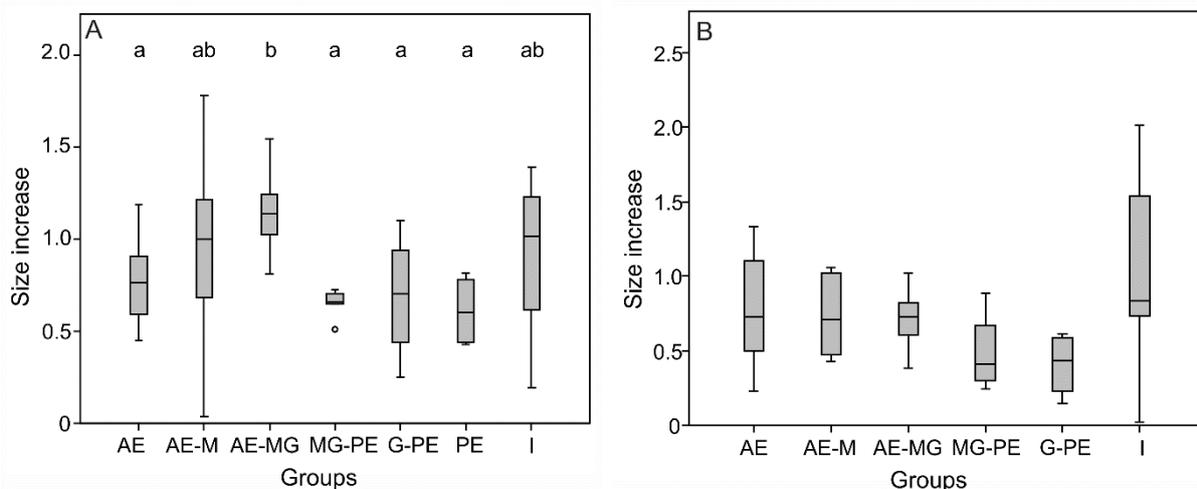
224
225 **Figure 6.** Boxplots showing the increase in the size of intact and regenerating specimens of
226 *Luteostriata abundans* after 26 (A) and 50 days (B). AE: anterior end; AE-M: anterior end plus
227 mouth; AE-MG: anterior end plus mouth and gonopore; MG-PE: posterior end plus mouth and
228 gonopore; G-PE: posterior end plus gonopore; PE: posterior end; I: intact animals. Groups that
229 do not share the same lowercase letters are significantly different from each other.
230
231 The increase in size after 26 days was significantly different between the groups (Kruskal-
232 Wallis, $\chi^2(6) = 12.748$, p = 0.047). Group AE-MG increased significantly more than groups AE,
233 MG-PE, G-PE, and PE. Groups AE-M and I did not differ significantly from any group (Fig.
234 6A). After 50 days, however, the increase in size was not significantly different between groups
235 anymore (Kruskal-Wallis, $\chi^2(5) = 6.941$, p = 0.225) (Fig. 6B).
236
237

238   The time for regenerating the mouth and restarting eating was significantly different between
239   the originally mouthless groups (Kruskal-Wallis, $\chi^2(2) = 10.964$, p = 0.004). It took longer for
240   group G-PE to regenerate a mouth than for group AE (p = 0.005), while group PE was not
241   significantly different from both (Fig. 7). Anterior fragments did not differ regarding the
242   relative distance of the mouth to the posterior end after 50 days (Kruskal-Wallys, $\chi^2(2) = 0.347$,
243   p = 0.841) (Fig. 8A) and neither did posterior fragments regarding the relative distance of the
244   mouth to the anterior end after 50 days (Mann-Whitney, U = 10.000, p = 0.686) (Fig. 8B).
245
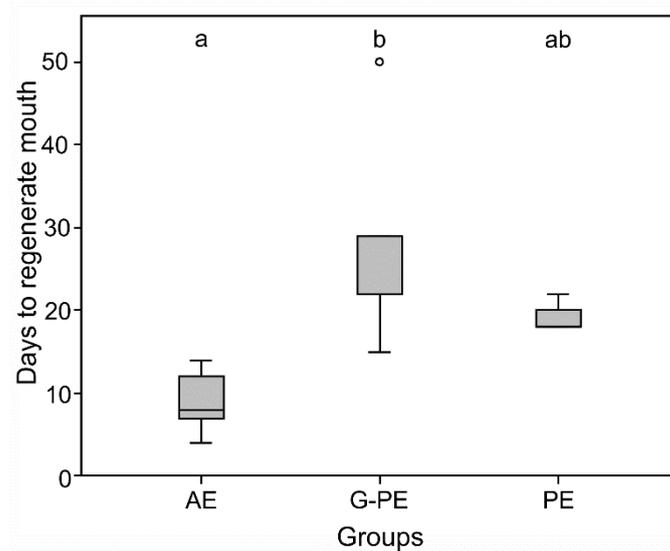
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247   **Figure 7.** Boxplots showing the time in days that mouthless regenerating specimens of
248   *Luteostriata abundans* took to regenerate the mouth. AE: anterior end; G-PE: posterior end
249   plus gonopore; PE: posterior end. Groups that do not share the same lowercase letters are
250   significantly different.
251
252
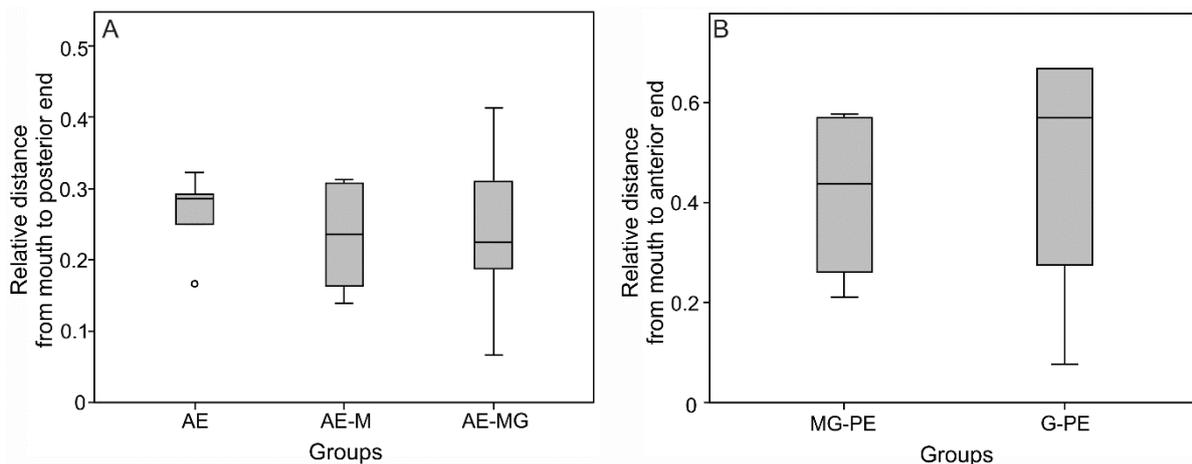
253
254   **Figure 8.** Boxplots showing the relative distance from the mouth to the regenerating end of
255   anterior (A) and posterior (B) pieces of *Luteostriata abundans* after 50 days. AE: anterior
256   end; AE-M: anterior end plus mouth; AE-MG: anterior end plus mouth and gonopore; MG-
257   PE: posterior end plus mouth and gonopore; G-PE: posterior end plus gonopore.
258

259 **Discussion**
260
261 Regeneration in freshwater planarians has been studied for the past two centuries and
262 approached from different perspectives, especially in more recent decades (Rink 2018). Among
263 land planarians, only the genus *Bipalium* received increased attention on its regenerative
264 capacity, and only by two Japanese researchers in the 1970s and 1980s (Shirasawa and Makino
265 1979, 1985, 1988). Our study is the second to investigate regeneration in a Neotropical land
266 planarian. A single previous study was conducted almost a century ago (Goetsch 1933) with a
267 Chilean species, *Pseudogeoplana pulla*, in which specimens were cut into two, four, or nine
268 pieces and monitored for a few months. Herein, for a preliminary observation, we limited the
269 experiments to only a single cut resulting in two pieces and monitored them for only 50 days.
270 However, as we performed the cuts at different points along the body, we can compare some of
271 our results to those of *P. pulla*.
272
273 Because Neotropical land planarians do not reproduce asexually and, therefore, are expected to
274 have a smaller number of neoblasts (Baguñà and Romero, 1981), we expected smaller pieces
275 to have a lower regenerative capacity than larger ones, which was supported by our results. The
276 group formed by the largest pieces (AE-MG) had the highest survival rate, while that with the
277 smallest pieces (PE) had the lowest survival rate. Goetsch (1933) observed the same with *P.
278 pulla*, where smaller pieces had a lower survival rate than larger ones. However, the statistical
279 analyses suggest that size alone was not responsible for the different survival rates, which could
280 result from the relatively low survival of intact animals, an unexpected and unexplainable
281 outcome. One possible explanation could be the now century-old hypothesis that regeneration
282 triggers rejuvenation in flatworms, but more recent investigations did not support this (Mouton
283 et al. 2018).
284
285 Besides size alone, we also expected posterior pieces to show a decreased capacity for
286 regeneration based on this general trend in many planarian groups (Reddien and Sánchez
287 Alvarado 2004). Specimens formed by only the posteriormost piece, cut behind the copulatory
288 apparatus, showed indeed the lowest survival rate, with only two surviving the 50 days of
289 monitoring. However, as these were also the smallest pieces, we cannot confirm whether their
290 low survival is due to their size, posterior position, or both. Goetsch (1933) suggested that the
291 low survival rate of posterior pieces of *P. pulla* resulted from the small number of neoblasts in
292 the posterior half since this region has much of its space occupied by complex organs such as
293 the pharynx and the copulatory apparatus. Therefore, there would be little room left for the
294 parenchyma, which contains the neoblasts. However, more recent studies on neoblast
295 distribution indicate that they are somehow uniformly distributed along the body, although they
296 are concentrated on the dorsal part of the parenchyma, especially in three longitudinal rows
297 running along the body (Orii et al. 2005).
298
299 Among the three pieces that kept the mouth and pharynx after amputation, only the two anterior
300 fragments continued to ingest food normally soon after, feeding like normal intact planarians.
301 Posterior pieces with a pharynx took a significantly longer time to eat, with half of spending
302 the 50 days of monitoring without ingesting food. Goetsch (1933) observed that posterior pieces
303 of *P. pulla* that kept the pharynx lost this organ soon after amputation and built a new one,
304 differently from anterior fragments with a pharynx, which kept the original one. However, in
305 *L. abundans*, according to our observations, which were only external in live animals, the
306 pharynx was retained in posterior pieces, although its function was lost and took more time to
307 be restored than in other pharynx-bearing fragments. This agrees with observations on
308 freshwater planarians of the families Dugesiidae and Planariidae, in which posterior pieces do

309 not shed the original pharynx, but its function takes a longer time to be recovered than in
310 anterior fragments (Sheiman et al. 2010). This temporary loss of function in the pharynx of
311 posterior fragments is likely the result of the loss of part of the nervous system, especially of
312 the head ganglia, which seem responsible for controlling the pharynx function (Sheiman et al.
313 2010). In other flatworms, such as polyclads, the pharynx may retain its function after the
314 amputation of the brain, but the animal's capacity to detect food is impaired (Schadt et al. 2021).
315
316 Although we did not find a significant difference in growth between the groups across the whole
317 period, the group with the largest pieces (AE-MG), which had the highest survival rate, also
318 showed the highest increase in size in the first 26 days. Since these pieces were the largest and
319 needed to regenerate only a small posterior fragment lacking important organs (Sluys and
320 Riutort 2018), it seems reasonable that they would have completed their regeneration in a few
321 days and, afterward, slow down their increase in size.
322
323 Anterior mouthless fragments (AE) regenerated a pharynx and mouth significantly faster than
324 posterior fragments (with gonopore, G-PE). The pharynx is known as one of the few parts of a
325 planarian's body that lacks neoblasts (Reddien and Sánchez Alvarado 2004, Orii et al. 2005).
326 However, as neoblasts are the only cells capable of reproduction, they must participate in the
327 regeneration of the pharynx as well. Studies on pharynx regeneration with freshwater planarians
328 indicate that it is rebuilt, like other organs, with neoblasts, which migrate from other body
329 regions following an injury (Kreshchenko 2009). According to the observations of Shirasawa
330 and Makino (1991) with *Bipalium kewense*, cells of the intestinal wall also participate in the
331 formation of the pharynx by undergoing dedifferentiation, but the possibility of
332 dedifferentiation in planarians needs further investigation (Reddien and Sánchez Alvarado
333 2004). Observations with other species indicate that, although the pharynx seems to start
334 regenerating sooner in posterior fragments, it becomes functional in a shorter time in anterior
335 fragments (Kreshchenko 2009), which supports our observations. This is likely caused by the
336 pharynx depending on the head ganglia to function properly (Sheiman et al. 2010).
337
338 The difference in the distance from the mouth to the posterior end between anterior pieces and
339 to the anterior end between posterior pieces was not significantly different after 50 days. This
340 suggests that they all approached the same level of regeneration by the end of the monitoring.
341 In fact, the position of the mouth approached the expected position of intact animals in all
342 fragments. This indicates that all fragments seem to have the same capacity to regenerate a
343 whole organism, similarly to what occurs in many freshwater species in the family Dugesiidae
344 (Reddien and Sánchez Alvarado 2004), the sister group of land planarians (Sluys et al. 2009).
345 Species in the more distantly related families Planariidae and Dendrocoelidae (superfamily
346 Planarioidea), on the other hand, usually have a more limited regenerative capacity (Ball et al.
347 1969, Brøndsted 1969), although some species are also as good as or even better at regenerating
348 than dugesiid planarians (Sheiman et al. 2010). Therefore, the high regenerative capacity
349 observed in *L. abundans* may be a shared trait of the superfamily Geoplanoidea (consisting of
350 Dugesiidae plus land planarians) or a shared trait of the suborder Continenticola that decreased
351 in some lineages.
352
353 **Conclusion**
354
355 *Luteostrata abundans* is a Neotropical land planarian with exclusive sexual reproduction, but
356 our results indicate that it can regenerate a whole organism from both anterior and posterior
357 fragments of different sizes, although small posterior fragments have a lower survival rate.
358 Albeit preliminary, our findings suggest that Neotropical land planarians may have a

359 regenerative capacity similar to asexually reproducing land planarians and dugesiid freshwater
360 planarians. Further studies can be extended to other Neotropical species and explore the
361 regenerative capacity of small pieces across an anteroposterior gradient, as well as examine the
362 process through histological and molecular techniques.
363
364 **Acknowledgments**
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369
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