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1	Burrowing and casting activities of three endogeic earthworm species
2	affected by organic matter location
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### 8 Abstract

9 Earthworms are crucial for production and maintenance of soil structure and their activities 10 can strongly impact soil functioning (e.q. water regulation, nutrient dynamics). This 11 laboratory study investigated the bioturbation activity of three endogeic species, A. 12 chlorotica, A. icterica and A. caliginosa, as affected by different locations of organic matter 13 (OM) in the soil profile: OM scattered on the soil surface (surface-OM) or homogeneously 14 mixed into the soil (mixed-OM). Microcosms, each containing a combination of one species 15 (three individuals) and one OM location, were subjected to controlled environmental 16 conditions (temperature, humidity and day/night cycle) for 60 days. At the end of the 17 experiment, microcosms were cut into multiple horizontal cross-sections every centimetre 18 and bioturbation activities were analysed based on the number of burrows, the burrowed 19 area and the percentage of burrowed area totally refilled with casts.

Results showed that regardless of species, there was significantly fewer burrows and a greater percentage of burrowed area refilled with casts under mixed-OM than under surface-OM. *A. chlorotica* and *A. caliginosa* had a significantly greater burrowed area under mixed-OM than under surface-OM. Regardless of OM location, as depth increased, burrow number and area decreased for *A. chlorotica* and generally increased for *A. icterica*. In contrast, burrowing activity of *A. caliginosa* was affected by OM location as depth increased: under

Page 1 of 22

26 mixed-OM, burrow number decreased but burrowed area remained constant, whereas under

27 surface-OM, burrow number remained constant and burrowed area increased.

These results improve understanding of effects of endogeic species on soil structure and highlight effects of OM location on earthworm bioturbation. Especially this study gives information about the burrowing activity of *A. icterica* which has so far been little documented, and also informs about refilled burrows which is a major parameter for soil functioning.

33

34 Keywords: soil structure, behaviour, Allolobophora chlorotica, Allolobophora icterica,
35 Aporrectodea caliginosa

36

### 37 1. Introduction

38 Earthworms have been described as soil engineers (Jones et al., 1994; Lavelle et al., 1997) 39 because of their ability to modify their own environment and notably the soil structure via 40 their bioturbation activity which consists of burrowing and producing casts. This ingestion-41 egestion of soil strongly affects soil structure (Dexter, 1988; Lee and Foster, 1991) depending 42 on the context, earthworms increase porosity (Pérès et al., 2010; Lamandé et al., 2011; van 43 Schaik et al., 2014) or increase bulk density (Blanchart et al., 1997). Consequently, 44 earthworms affect several soil functional properties and ecosystem services, such as soil 45 moisture, water infiltration and water regulation, soil organic matter (OM) availability, 46 nutrient cycling and primary production (Jouquet et al., 2006; Capowiez et al., 2009; Blouin 47 et al., 2013; Crittenden et al., 2014). To understand the functional links between earthworms 48 and soil structure, scientists have focused on one aspect of bioturbation (*i.e.* burrows) (Pérès

Page 2 of 22

49 et al., 2010; Lamandé et al., 2011; van Schaik et al., 2014) but without integrating other
50 aspects of its complexity, especially casts.

Earthworm bioturbation results from complex interactions and can be affected by various
parameters, such as the location of OM, which is a food resource for earthworms and affects
earthworm foraging activity (Jeanson, 1968; Martin, 1982; Pérès et al., 2010). However, this
aspect needs further study, especially in relation to cast production.

Bioturbation properties of anecic earthworms are well described: these dwelling earthworms
build a relatively permanent burrow system, vertically oriented, bring soil from the depth to
the soil surface and cover their burrow walls with their casts (Kretzschmar, 1990;
Kretzschmar and Aries, 1990; Daniel et al., 1997; Jégou et al., 1999, 2001; Shipitalo and Butt,
1999; Bastardie et al., 2003; Nuutinen and Butt, 2003).

60 Bioturbation properties of endogeic earthworms, however, are less well known, despite 61 existing studies (Bolton and Phillipson, 1976; Capowiez et al., 2001; Jégou et al., 2001; Felten 62 and Emmerling, 2009), these earthworms are reported to burrow through the soil, creating 63 horizontal and randomly oriented burrows considered to be temporary structures (Bouché, 64 1972). Only few data exist about their casting activity in soil and especially the proportion of 65 burrows refilled with casts which is an important parameter for soil functioning (Schrader, 66 1993; Francis et al., 2001; Perreault and Whalen, 2006; Capowiez et al., 2014). Additionally, 67 some endogeic species have received attention, e.g. A. chlorotica (Capowiez et al., 2001, 68 2014) and A. caliginosa (Schrader, 1993; Francis et al., 2001; Jégou et al., 2001; Perreault and 69 Whalen, 2006; Capowiez et al., 2014), but other species are not well documented such as A. 70 icterica which has been only assessed once, through its burrow network (Bastardie et al., 71 2005a).

A study of earthworm bioturbation encounters several challenges, of which difficulty in
accessing burrows and casts due to soil opaqueness is one. Several authors have used Page 3 of 22

3/3

74 transparent 2D-terrariums (Schrader, 1993; Whalen et al., 2004; Perreault and Whalen, 2006; 75 Felten and Emmerling, 2009). Their relative thinness, however, may influence earthworm 76 behaviour and therefore this 2D approach appears less relevant than 3D microcosms 77 (Capowiez et al., 2001, 2014). Several authors have used 3D X-ray tomography (Joschko et al., 78 1989; Jégou et al., 1997; Capowiez et al., 1998), which is relevant for burrow network 79 assessment but this does not allow direct assessment of cast production (Joschko et al., 80 1993). As an alternative, the study by Hirth et al., 1996 is particularly interesting because it used cross sections of cylindrical microcosms to analyse both burrowing and casting by 81 82 endogeics. Another challenge to studying earthworm bioturbation is correctly identifying 83 which species produced observed burrows and casts, especially in natural conditions, in 84 which several species bioturbate the soil (Capowiez et al., 1998; Pérès, 2003; Bastardie et al., 85 2005b). Thus, despite their artificiality, microcosms remain necessary to describe 86 bioturbation activity of a species (Bastardie et al., 2005b).

The aim of this study was to assess under controlled conditions, burrowing and casting activities of three endogeic earthworms (i) as a function of OM location in the soil profile, and (ii) as a function of soil depth. The destructive method used is based on soil cross sections of microcosms and was used to observe, classify and quantify bioturbation, *i.e.* number of burrows, burrowed area and percentage of total burrowed area totally refilled with casts.

### 93 2. Materials and Methods

94 Our experimental system takes benefits from previous studies such as Jégou et al. (2001) for
95 the design of microcosms and from Hirth et al. (1996) for the assessment of burrowing
96 activity.

#### 97 2.1. Experimental system

98

### 2.1.1. Microcosm characteristics

99 Twenty four microcosms were built using PVC cylinders (20 cm in length and 15 cm in 100 internal diameter). They were cut lengthwise into two equal halves to facilitate their final 101 opening. A 500 μm nylon mesh was placed at the upper and lower openings to retain 102 earthworms.

103

### 3 **2.1.2.** Soil and organic matter features

The soil was collected from an arable field in Le Rheu, Brittany, France (N 48°09, W 1°81) and was a silt loam soil (FAO, 1988) with 16% sand, 69% silt, 15% clay. Soil organic matter content (2%) and  $pH_{H2O}$  (6.1) were in accordance with the values observed in cultivated soils in Brittany ("BDAT," 2002). Soil was air-dried before being passed through a 2 mm sieve to remove biostructures already present.

We used ryegrass (*Lolium perenne* L.) from an unmown and untreated grassland as the OM resource for earthworms. This OM was oven-dried for 48h at 60°C before being ground to a maximum width of 1 mm. OM was supplied at 20.7 g dry weight (dw) per microcosm, *i.e.* 100 g of soil with 0.6 g dw of OM, corresponding to a non-limiting food resource for earthworms (Curry and Schmidt, 2007). Two OM treatments were defined: OM mixed with all the soil (mixed-OM treatment, 12 microcosms) and OM evenly scattered on the soil surface (surface-OM treatment, 12 microcosms).

### 116 **2.1.3.** Microcosm filling

117 Microcosms were filled with five layers of soil. Each layer had a bulk density of 1.3 g.cm<sup>-3</sup>: it 118 was made of 690 g dw of soil that was packed down to obtain a 3-cm-high layer. Thus, soil in 119 the columns was 15 cm deep. The bulk density of each layer has been recorded in some 120 cultivated fields (Peigné et al., 2009; Bottinelli et al., 2013) and in other microcosm 121 experiments (Jégou et al., 1999). Additionally, the use of dry soil and thin layers prevented 122 inter-layer smoothing when soil was packed down. Once constructed, microcosms were re-123 moistened by capillary absorption and freely-drained for 48 h to reach field capacity which 124 was kept by re-wetting the surface every two weeks.

125

#### 2.1.4. Earthworm introduction

126 Three endogeic species, according to Bouché (1972), were collected from an arable field: 127 *Allolobophora chlorotica* (Savigny, 1826), *Allolobophora icterica* (Savigny, 1826) and 128 *Aporrectodea caliginosa* (Savigny, 1826). These species are commonly found in cultivated soil 129 in France (Cluzeau et al., 2012). All earthworms were sub-adults or adults and were 130 acclimated to the soil for one week before being introduced into microcosms (Fründ et al., 131 2010).

132 Experiments involved use of earthworm monocultures: one endogeic species employing three individuals per microcosm and corresponding to 170 earthworms.m<sup>-2</sup> and a mean fresh 133 134 biomass (± standard deviation) of 0.8±0.1 g for A. chlorotica, 2.1±0.1 g for A. icterica and 135 1.3±0.1 g for A. caliginosa per microcosm. Earthworm biomass and density values were 136 consistent with those found in cultivated fields in Brittany (Pélosi et al., 2014). The 137 experimental design had two OM locations with three earthworms species replicated four 138 times (2x3x4=24 microcosms). Earthworms were placed on the soil surface and allowed to 139 burrow down. Then, microcosms were placed on a raised grid in a climatic chamber at 10°C 140 with a day/night cycle corresponding to the external one (18/6h in June and July).

Page 6 of 22

Microcosms were maintained for 60 days after earthworm inoculation, at which time they
were microwaved to stop earthworm activity by killing them *in situ* (5 minutes, 400 Watts).
They were then slowly oven dried at 45°C for one week to help cutting cross-sections and to
prevent their smoothing.

145

#### 2.2. Bioturbation assessment

Each microcosm was cut from top into eleven cross sections every centimetre corresponding to sections  $z_0$  to  $z_{10}$ . The surface of each section was lightly brushed using a paintbrush and blown using a compressor at its lowest pressure to remove dust and studied with the following procedure:

- Outlines of burrows were identified with the naked eye and traced with pen on a
   transparent sheet of plastic placed on the surface. Casts that completely obstructed
   the burrows were similarly recorded.
- After digitizing the drawings (resolution: 600 ppi), they were analyzed with Fiji
  software (Schindelin et al., 2012) and a homemade script in the Jython programming
  language (http://www.jython.org/). Each burrow was identified and described by its
  total area and the area occupied by casts. The percentage of burrow refilled with
  casts (area occupied by casts divided by the burrowed area) was calculated.
- 158

#### 2.3. Statistical analysis

Data analysis was performed using R software (R. Core Team, 2013). If the normality of residues (Shapiro test) and the heteroscedasticity (Bartlett test) were verified, we used multiway ANOVA and post-hoc LSD Tukey's tests with species, depth and OM location as factors. Otherwise, the Kruskall-Wallis test checked for factor effect, and pairwise Wilcoxon tests with Bonferroni correction were used as post-hoc tests. Linear regressions were calculated to test the relation between bioturbation and depth. If linear regression was not significant, the *nls* 

Page 7 of 22

165 function of R was used to estimate parameters of a non-linear model. The goodness of fit of 166 the non-linear model was assessed with a Pearson test of correlation between estimated and 167 observed values. Significance threshold was set at  $\alpha = 5\%$ .

168 3. Results

169 At the end of the experiment, some OM remained on the surface of the surface-OM 170 treatment, which suggested that excess OM had been applied. We observed that surface 171 casts were still being produced at the end of the experiment which suggests that earthworms 172 were still active.

- 173 **3.1.** Bioturbation of earthworms as affected by organic matter location
- 174 3.1.1. Number of burrows:

The number of burrows per section was significantly (p < 0.001) affected by earthworm</li>
species, OM location, and by 2-way interactions: species × OM location, species × depth, and
OM location × depth.

178 The number of burrows per section was significantly higher under surface-OM vs. mixed-OM

179 regardless of species (Fig. 1a). It was approximately 2.3, 2.1 and 1.5 times as large under

180 surface-OM vs. mixed-OM for *A. icterica, A. caliginosa* and *A. chlorotica,* respectively.

181 Under surface-OM, the number of burrows per section decreased from A. icterica to A.

182 caliginosa to A. chlorotica, with a significant difference between each pair. Under mixed-OM,

- 183 A. icterica had a significantly higher number of burrows per section than A. chlorotica.
- 184 3.1.2. Burrowed area per section

185 Burrowed area was significantly (p < 0.001) affected by species, OM location, and the 2- and

186 3-way interactions (species × OM location × depth).

Page 8 of 22

187 *A. chlorotica* and *A. caliginosa* had significantly higher burrowed area under mixed-OM than
188 under surface-OM (Fig. 1b). Their burrowed area was approximately 3.7 and 2.2 times as
189 large, respectively, under mixed-OM vs. surface-OM. Conversely, *A. icterica* burrowed area
190 was not significantly affected by OM location.

191 Under surface-OM, the area burrowed per section significantly decreased from A. icterica to

192 A. caliginosa to A. chlorotica.

193 3.1.3. Percentage of burrowed area refilled with casts

194 The percentage of refilled area was significantly (p < 0.05 for species × depth and p < 0.001

195 for other factors) affected by species, OM location, and all 2- and 3-way interactions.

196 Earthworms refilled their burrows more under mixed-OM than under surface-OM (Fig. 1c).

197 This percentage was approximately 3.1, 1.7 and 2.5 times as high under mixed-OM vs.

198 surface-OM for *A. chlorotica*, *A. icterica* and *A. caliginosa*, respectively.

Under mixed-OM, *A. caliginosa* had a higher percentage of burrowed area refilled with casts
than the two other species. This percentage was not significantly different for the three
species under surface-OM.

202 **3.2.** Effect of soil depth on bioturbation

Earthworms bioturbated all sections of all microcosms, except one microcosm with *A*. *chlorotica* under surface-OM, in which no burrow was found in the two deepest sections. The top section (*i.e.*  $z_0$ ) of several microcosms could not be analyzed, placing the first analyzed section at a depth of 1 cm (*i.e.*  $z_1$ ).

207 3.2.1. Number of burrows as depth increased

208 Under both OM-location treatments, the number of burrows produced by *A. chlorotica*209 decreased as depth increased (Figs. 2a and 2d) whereas it increased as depth increased for *A.*210 *icterica* (Figs. 2b and 2e). *A. caliginosa* had a varied response: the number of burrows

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Page 9 of 22

decreased as depth increased under mixed-OM, but remained constant as depth increased
under surface-OM (Fig. 2f). For all combinations of species and OM location, except *A*. *caliginosa* under surface-OM, regressions predicting the number of burrows as depth
increased were statistically significant.

215

#### 3.2.2. Burrowed area as depth increased

216 A linear relation exists between burrowed area and depth in most treatments (Fig. 3). The 217 burrowing activity of A. chlorotica was concentrated within the first 3 cm under mixed-OM 218 (Fig. 3a). Unlike the mixed-OM treatment, the upper sections under surface-OM did not 219 noticeably differ from others, even though food resources were located at the surface. 220 However, A. icterica and A. caliginosa under surface-OM had burrowed areas at the surface 221 (*i.e.*  $z_1$ ) that were higher than those at 1 cm (Figs. 3e and 3f). In both cases, this surface point 222 was excluded from linear regression since its standard error was extremely high, and the 223 surface burrowing could reflect a specific behaviour due to OM location on the soil surface. 224 For both OM locations, burrowing of A. icterica linearly increased as depth increased (Figs. 3b 225 and 3e). There was no significant linear correlation between the area refilled with casts and 226 depth (data not shown).

### 227 4. Discussion

### 228 4.1. Earthworm sensitivity to OM location

Our results showed major differences in the bioturbation of the three species studied, even though all belong to the endogeic group. According to this study, species is a key factor that must be considered when linking earthworms to bioturbation. Additionally, our results highlighted the need to account for effects of OM location on earthworm bioturbation, since it might influence the number of burrows produced, the burrowed area, and the burrowed area refilled with casts. Total burrowed area of *A. icterica* was noteworthy unaffected by OM

Page 10 of 22

235 location, even though its behaviour was changed, notably by increasing the number of 236 burrows. However, A. chlorotica and A. caliginosa were greatly influenced by OM mixed with 237 soil, which led them to increase the area burrowed. These observations are consistent with 238 their food consumption habits: under spruce forest conditions, Bernier (1998) observed that 239 A. icterica's digestive tract contained a higher mineral content than that of other species, 240 including A. caliginosa. Conversely, the diets of A. chlorotica and A. caliginosa are known to 241 be similar (Piearce, 1978). Since OM is a known food resource for the three endogeic species, this study showed that less of it in the soil could lead earthworms to build more burrows 242 243 supporting previous study under field conditions (Pérès et al., 2010). It should be noted that 244 interspecific competition can affect the burrowing activity of earthworm species. In 245 particular, the burrow system of A. chlorotica was unaffected by Aporrectodea nocturna 246 (Capowiez et al., 2001) and A. caliginosa had a significantly lower burrowing activity in the 247 presence of several other species (Felten and Emmerling, 2009). Thus both OM location and 248 interspecific competition should be included in further studies of earthworm bioturbation 249 activity.

250 It is worthwhile to note that the measured percentage of burrowed area refilled with casts is 251 far from equal among documented studies. For example, the percentage for A. caliginosa in 252 our study (10-35%) was much lower than that of Francis et al. (2001) and Capowiez et al. 253 (2014) (40-85%) but higher than that observed by Perreault and Whalen (2006) (<10%). 254 Differences among studies may stem from the methods used, but also from the influence of 255 food quality, food quantity, temperature and bulk density, which differed among studies. For 256 example, Perreault and Whalen (2006) observed that surface casting of A. caliginosa was greater with wetter soil. Our results showed that OM location is another factor that affects 257 258 the percentage of burrowed area refilled. We observed a lower percentage of burrowed area 259 refilled with casts under surface-OM vs. mixed-OM. There could be several explanations for

Page 11 of 22

260 this: (i) casts were less stable under surface-OM and were undetectable at the end of the 261 experiment; (ii) casts under the surface-OM treatment had a greater bulk density; (iii) 262 burrows were built by pushing particles; (iv) casts were egested at the soil surface and (v) 263 more casts were crushed against burrow walls under surface-OM vs. mixed-OM. Further 264 studies are needed to explain this difference between the two OM locations and to study the 265 mechanisms of cast production. The measured percentage of burrowed area refilled with 266 casts is an indicator of burrow continuity, notably because reported casts are those that 267 completely obstructed the burrows. Our study suggests that burrows built under mixed-OM 268 are more discontinuous, which can impact on water movements (Allaire-Leung et al., 2000) 269 and burrow lifespan (Capowiez et al., 2014).

#### 270 4.2. Bioturbation activity as depth increased

271 We assessed differences in burrow number and burrowed area as depth increased. A. 272 chlorotica burrowing activity was concentrated in the top few centimeters which was not 273 observed by Capowiez et al. (2001). This result agrees with an intermediate position of A. 274 chlorotica between the ecological categories "endogeic" and "epigeic" (Bouché, 1977; Pérès, 275 2003). Nevertheless, we observed that this epi-endogeic behaviour was more marked with 276 OM mixed into the soil than with OM on the soil surface. This seems counterintuitive, 277 because OM on the soil surface would induce earthworms to burrow at the surface to feed. 278 But our study does not reveal whether earthworms expressed true epigeic behaviour when 279 OM was on the surface by feeding in the thin organic layer at the top of microcosms. 280 Nevertheless, the effect of A. chlorotica on the soil volume was smaller when OM was on the 281 surface. The burrowed area of A. caliginosa slightly increased as depth increased when OM 282 was located on the soil surface, which contradicts other studies (McKenzie and Dexter, 1993; 283 Jégou et al., 1997), but no trend was found when OM was mixed into the soil. A. icterica is 284 reported to be a typical endogeic earthworm (Bastardie et al., 2005a). This corresponds with

Page 12 of 22

the observation that OM location significantly influenced *A. icterica*, but less than the two other species in our study. Like Francis et al. (2001), we observed no significant decrease in the area refilled with casts as depth increased, which is contrary to results of Capowiez et al. (2014).

289

### 290 Conclusion

291 Data on the effect of endogeic earthworms on soil structure are rare in the literature, 292 especially concerning *A. icterica*. Our study provides insights into the burrowing activity of 293 three endogeic species according to organic matter location. Bioturbation is crucial in 294 agriculture particularly in no-till fields which are not mechanically treated to create 295 favourable soil structure and in which structure and soil functioning are strongly affected by 296 biological activity (Capowiez et al., 2009; Peigné et al., 2009; Crittenden et al., 2014).

297 Our results obtained under controlled conditions have now to be confirmed under field 298 conditions, in which soil heterogeneity can be integrated and interspecific competition 299 between earthworms occur. Among other parameters such as bulk density, OM location may 300 change according to tillage practices: for example, no-till systems keep OM on the soil 301 surface and ploughed systems mix OM into the soil. Our results suggest that the three 302 species do not bioturbate the soil in the same way under these tillage practices because of 303 these differences in OM location. However the combined effect of OM location and other 304 parameters, e.g. bulk density, must be studied to confirm this statement. Moreover, even 305 though these species are endogeic, they do not preferentially burrow the soil at the same 306 depth, but are complementary and thus our results suggest that these species do not occupy 307 the same ecological niche. This is one reason why species diversity within the same 308 ecological category must be maintained or increased. Results of this study will be integrated

- 309 into a computer model that simulates impacts of earthworms on soil structure and accounts
- 310 for tillage practices.
- 311 Earthworm ecological categories are still disputed. This study shows that the bioturbation
- 312 activity of A. chlorotica and A. icterica agrees with their classification as epi-endogeic and
- 313 true endogeic, respectively. Recent work has begun to focus on addressing the ecological
- 314 traits of earthworm species (Lowe and Butt, 2007; Fernández et al., 2010; Pey et al., 2014)
- and this needs to be continued.

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Figure 1. Measured bioturbation parameters of three endogeic earthworm species. Error
bars represent one standard error. Bars sharing a letter are not significantly (p > 0.05)
different. (a) Mean number of burrows per section; (b) Mean burrowed area per section; (c)
Mean percentage of burrowed area refilled with casts.

324

325 Figure 2. Mean number of burrows per section by depth of three endogeic earthworm 326 species. Error bars represent one standard error. Linear regressions are given if significant. a) 327  $1.58 \times \text{depth} + 21.04$ , r = 0.63, p < 0.001; b)  $-1.52 \times \text{depth} + 7.90$ , r = 0.74, p < 0.001; c)  $0.87 \times$ 328 depth + 18.09, r = 0.57, p < 0.001; d)  $1.27 \times \text{depth} + 24.69$ , r = 0.65, p < 0.001; e)  $-2.68 \times$ 329 depth + 24.74, r = 0.69, p < 0.001; f) Regression not significant.

330

Figure 3. Mean burrowed area per section by depth of three endogeic earthworm species. Error bars represent one standard error. Linear or non-linear regressions are given if significant a) area =  $3458.67 \times e^{0.36 \times depth} - 0.1$ , Pearson correlation coefficient = 0.79, p < 0.001; b) area =  $-85.35 \times depth + 331.76$ , r = 0.60, p < 0.001; c) Regression not significant; d) area =  $25.84 \times depth + 397.17$ , r = 0.54, p < 0.001; e) area =  $-78.85 \times depth + 405.35$  (first layer excluded), r = 0.69, p < 0.001; f) area =  $-25.67 \times depth + 364.61$  (first layer excluded), r = 0.34, p < 0.05.

338

Page 18 of 22

- 338 Bioturbation activity of endogeic species was affected by organic matter location
- 339 Burrowed area refilled with casts were higher under *mixed-OM* vs. *surface-OM*
- 340 A. icterica was less affected by OM location than A. chlorotica and A. caliginosa
- 341 A. chlorotica expressed an epi-endogeic behavior
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