

1 **A domesticated fungal cultivar recycles its cytoplasmic contents as nutritional**  
2 **rewards for its leafcutter ant farmers**

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23 **Competing Interests**

24 The authors declare no competing interest.

25

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27 attines

28

29 **ABSTRACT**

30 Leafcutter ants farm a fungal cultivar (*Leucoagaricus gongylophorus*) that converts inedible  
31 vegetation into food that sustains colonies with millions of workers. Like fruits of crops  
32 domesticated by humans, *L. gongylophorus* has evolved specialized nutritional rewards—tiny  
33 swollen hyphal cells called gongylidia that package metabolites eaten by ant farmers. Yet, little is  
34 known about how gongylidia form, and whether ants regulate this formation through plant-  
35 fragment provisioning. We used microscopy and *in vitro* manipulations to explain the cellular  
36 mechanisms governing gongylidium formation. First, *L. gongylophorus* is polykaryotic (up to 17  
37 haploid nuclei/cell) and our results suggest intracellular nucleus distributions govern gongylidium  
38 morphology with their absence in expanding edges arresting apical growth and their presence  
39 mediating complex branching patterns. Second, nanoscale TEM imaging shows that the cultivar  
40 recycles its own cellular material (e.g. cytosol, mitochondria) through a process called ‘autophagy’  
41 and stores the resulting metabolites in gongylidia. This autophagic pathway is further supported  
42 by gongylidium inhibition when isolated fungal cultures are grown on media with autophagy  
43 inhibitors (chloroquine, 3-methyladenine). We hypothesize that autophagic nutritional reward  
44 production is *the* ultimate cultivar service and reflects a higher-level organismality adaptation  
45 enabled by strict symmetric lifetime commitment between ant farmers and their fungal crop.

46

## 47 Introduction

48 The advent of domesticated agriculture some 10 000 years ago was a turning point for  
49 humans and for the domesticated crops whose derived traits would likely have been maladaptive  
50 in their free-living ancestors (1-3). Key crop adaptations include whole genome duplication events  
51 (resulting in polyploidy) that can increase functional heterozygosity (4) and selection for specific  
52 regulatory genes that can reduce seed shattering or enhance fruit size, color, and sweetness (5-  
53 8). Fascinatingly, humans are not the only farmers. Several insect lineages independently evolved  
54 obligate farming systems of fungal cultivars that produce specialized edible reward structures (9).  
55 However, while human farmers modify growth environments in well-known ways to maximize crop  
56 yield (e.g. adding fertilizers, controlling watering conditions, etc.), the analogous mechanisms by  
57 which insect farmers promote expression of edible reward structures in fungal cultivars remain  
58 poorly understood.

59 The largest-scale insect farmers are the *Atta* leafcutter ants, the crown group of the  
60 fungus-farming 'attine' lineage (10, 11). Despite obvious analogies with farming systems of  
61 humans (9), farming by leafcutter ants is fundamentally different because it is 'organismal' in the  
62 sense that it represents a strictly symmetrical obligate mutualistic dependence(12). Such an  
63 arrangement never allows alternative crops, but does sustain selection for co-evolutionary  
64 integration and higher-level adaptation that cannot evolve when farming practices are  
65 asymmetrically promiscuous (13, 14). These differences make it interesting to explore how insect  
66 farmers regulate crop productivity, which is relevant for understanding the broad eco-evolutionary  
67 success of these naturally selected farming systems.

68 A mature rainforest colony of the leafcutter ant *Atta colombica* can have millions of  
69 specialized ants that divide the work of foraging fresh plant fragments and caring for the fungal  
70 cultivar (15). In this way, colonies convert foraged fragments from hundreds of plant species (16)  
71 into a mulch used to provision their fungal cultivar *Leucoagaricus gongylophorus*. In return, the  
72 cultivar converts inedible plant biomass into edible reward structures called gongylidia, tiny (*ca.*  
73 30  $\mu\text{m}$  diameter) swollen hyphae that grow in bundles called staphylae (17-25). Gongylidia are a  
74 defining trait of irreversible crop domestication and are unique to the fungal lineage farmed by  
75 leafcutter ants and other higher-neoattine genera including *Trachymyrmex*, *Sericomyrmex*,  
76 *Mycetomoellerius*, and *Paratrachymyrmex* (18, 26, 27).

77           Gongylidia mediate functional integration with their ant symbionts in two main ways. First,  
78 they contain enzymes (e.g., laccases, pectinases, proteases) that gardening ants ingest and then  
79 vector to patches of newly deposited vegetation to catalyze fungus-mediated digestion and  
80 detoxification (28-32). Second, they contain nutrients (e.g., a suite of amino acids, lipids and  
81 glycogen) that are the ants' primary food source (33, 34). The ability to regulate the quantity and  
82 quality of gongylidia would thus provide clear benefits for the ant farmers. However, the  
83 mechanisms linking substrate provisioning by farming ants and the production of the cultivar's  
84 edible yield have remained poorly known. To better understand these mechanisms, we: 1)  
85 visualized the morphology of gongylidia and staphylae using scanning electron microscopy  
86 (SEM), and 2) described the cellular reorganizations that mediate gongylidium formation by  
87 combining light, fluorescence, and transmission electron microscopy (TEM).

88           We next examined the cellular origins of the edible resources contained in the large  
89 vacuole that fills each gongylidium cell. Previous evidence suggests that *L. gongylophorus* directly  
90 metabolizes provisioned plant fragments to produce these edible resources. First, the cultivar can  
91 metabolize lipids rich in alpha-linoleic acid (18:3) from foraged plant fragments into linolenic acid  
92 (18:2) that is enriched in gongylidia (35). This synthesized metabolite is thought to mediate  
93 interkingdom communication by eliciting attractive behaviors in ant workers, in contrast to the  
94 precursor 18:3 lipid that elicits antagonistic behaviors (35). Second, isotopic enrichment studies  
95 have shown that the cultivar quickly (within days) shunts C and N from provisioned substrates  
96 (glucose and ammonium nitrate, respectively) into edible gongylidia (36). Third, different substrate  
97 types are associated with increased expression of a variety of genes regulating specific pathways  
98 for nutritional metabolism (19, 34, 37, 38). However, it is also reasonable to predict that the  
99 diversity of compounds found within gongylidia have varied biochemical origins.

100           Autophagy is a plausible alternative pathway underlying gongylidia formation that  
101 involves the recycling of metabolic source material and potentially the fine-tuning of its  
102 composition. The metabolic pathways for autophagy are conserved across eukaryotes and are  
103 known to mediate development, cellular differentiation (39-42), and housekeeping (43, 44) in  
104 fungal cells. During autophagy, cytoplasmic components (i.e., glycogen, proteins, organelles) are  
105 incorporated into a vacuole for enzymatic degradation and the resulting catabolites are then  
106 recycled as nutrients to sustain other cellular processes and produce new cellular components

107 (45, 46). Initial evidence for autophagy in *L. gongylophorus* was first obtained in 1979 by Angeli-  
108 Papa and Eymé (47) who used TEM imaging to observe endoplasmic reticulum membranes  
109 engulfing mitochondria during gongylidia formation. However, to our knowledge, this preliminary  
110 evidence for autophagic recycling of the cultivar's own intracellular content during gongylidia  
111 formation has not been subsequently explored.

112 We propose that confirmation of an autophagic pathway(s) would have important  
113 implications for understanding the leafcutter symbiosis since it implies that natural selection has  
114 targeted the farming symbiosis in ways that made provisioning more robust and less dependent  
115 on the variable quality and quantity of foraged vegetation. Specifically, we predict that autophagic  
116 nutrient recycling of cellular contents would: 1) reduce variability in the quality of the cultivar's  
117 nutritional rewards by optimizing the composition of metabolic source material, 2) constrain the  
118 ability of ants to directly regulate cultivar productivity through their provisioning decisions, and 3)  
119 provide metabolic precursor substrates during periods of environmental vegetation shortage.

120 We tested for autophagic gongylidium formation in two ways. First, autophagy  
121 encompasses two main types of cellular recycling mechanisms: 1) macroautophagy in which  
122 cytoplasmic content (i.e. cytosolic metabolites and organelles) are sequestered into double-  
123 membrane vesicles that fuse with vacuoles, and 2) microautophagy in which the vacuolar  
124 membrane invaginates and directly engulfs cytoplasmic cargo at smaller scales than  
125 macroautophagy (45, 46). We analyzed TEM images to determine whether and how these  
126 specific autophagic processes influence gongylidia formation. Second, we tested whether  
127 autophagy is necessary for gongylidia formation by performing an *in vitro* experiment where the  
128 density of staphyla was measured in cultivars grown with known inhibitors and promoters of  
129 autophagy in fungal cells.

130

## 131 **Methods**

### 132 ***Imaging morphology of staphyla and gongylidia***

133 We isolated fungal cultivar (*L. gongylophorus*) from two colonies of *Atta colombica*  
134 (Ac2012-1 and Ac2019-1) that were collected in Soberanía National Park, Panama and are  
135 maintained at the University of Copenhagen in a climate-controlled room (25°C, 70% RH, minimal

136 daylight). Axenic fungal isolates were grown in the dark in Petri dishes on potato dextrose agar  
137 (PDA) at 25°C. We first used scanning electron microscopy (SEM) to visualize the external  
138 morphology of gongylidia and staphylae. We sampled fungal tissues from both Petri dish cultures  
139 and directly from colonies and fixed them in PBS with 0.1% Tween 20 and fixatives (4%  
140 glutaraldehyde, 4% formaldehyde). Samples were: 1) dehydrated in an ethanol series (35%, 55%,  
141 75%, 85%, 95%, and 2x in 100%) for 30 minutes per concentration, 2) critical-point dried, 3)  
142 coated with platinum and 4) imaged on a JSM-840 scanning electron microscope (JEOL, Tokyo,  
143 Japan) at 7.0 kV at the Zoological Museum of the University of Copenhagen. A slight wrinkled  
144 appearance of the surface of gongylidium cells in the resulting SEM images was due to  
145 unavoidable plasmolysis caused by the preparation process.

146 We used light and fluorescence microscopy to view the cultivar's internal anatomy (e.g.  
147 septa, vacuoles, nuclei, etc.). Staphyla samples were placed in a drop of mounting solution (dH<sub>2</sub>O,  
148 PBS, 3% KOH) on a glass slide. Gongylidia were then separated under a stereo microscope (16x  
149 or 25x magnification) with 0.16-mm diameter acupuncture needles and stained using two  
150 methods. For visualization under white light, we placed samples in either 0.1% Congo-red (in 150  
151 mM NaCl) for one minute followed by a wash with 150 mM NaCl, or 1.5% phloxine followed by a  
152 wash with 3% KOH. For nucleus visualization under UV light, we stained samples for 10 minutes  
153 using "Vectashield with DAPI" (Vector Laboratories, Burlingame, CA, USA). We then performed  
154 bright-field, dark-field, phase-contrast, and fluorescence microscopy under an Olympus BX63  
155 microscope (Olympus, Tokyo, Japan). The microstructures were measured and photographed  
156 using a mounted QImaging Retiga 6000 monochrome camera and cellSens Dimension v1.18  
157 (Olympus) image-processing software.

158 We used transmission electron microscopy (TEM) to visualize fungal cells with greater  
159 magnification and resolution (i.e. the 500 nm scale). Staphylae were collected from fragments of  
160 intact fungus gardens, fixed in 2% glutaraldehyde in 0.05 M PBS (pH 7.2) and then post-fixed in  
161 1% w/v OsO<sub>4</sub> with 0.05M K<sub>3</sub>Fe(CN)<sub>6</sub> in 0.12 M sodium phosphate buffer (pH 7.2) for 2 hr at room  
162 temperature. Fixed samples were washed three times in ddH<sub>2</sub>O for 10 minutes and dehydrated  
163 in a series of increasing ethanol concentrations series (70%, 96% and 99.9%). Each dehydration  
164 lasted 15 min and was performed twice per concentration. Samples were then repeatedly  
165 infiltrated with increasing Resin Epon:Propylene oxide ratios (1:3, 1:1, 3:1) with each step lasting

166 from 20 to 40 min. Samples were then embedded in 100% Epon and polymerized overnight at  
167 60°C. Sections, ca. 60-nm thick, were cut with an Ultra cut 7 ultramicrotome (Leica, Vienna,  
168 Austria), collected on copper grids with Formvar supporting membranes, and stained with uranyl  
169 acetate and lead citrate. Samples were TEM imaged on a CM100 BioTWIN (Philips, Eindhoven,  
170 The Netherlands) at an accelerating voltage of 80 kV. Digital images were recorded with a side-  
171 mounted OSIS Veleta digital slow scan 2 × 2 k CCD camera and the ITEM software package  
172 (Olympus Soft Imaging Corp, Münster, Germany). TEM sample preparation and imaging were  
173 performed at the Core Facility for Integrated Microscopy at the University of Copenhagen.

174

### 175 ***Autophagy inhibition assay***

176 We tested the role of autophagy in gongylidia production with an *in vitro* growth assay  
177 containing four treatment groups. First, autophagy is often initiated in cells when the target of  
178 rapamycin kinase (TOR) is inhibited. Autophagy can thus be induced *in vitro* by adding rapamycin  
179 (RAP) (48) an allosteric TOR inhibitor (46). In contrast, autophagy is often inhibited *in vitro* using  
180 Chloroquine (CQ) or 3-methyladenine (3-MA), as these compounds are known to respectively  
181 block autophagosome-vacuole fusion (49) and suppress a class III PtdIns3K enzyme required to  
182 initiate autophagosome formation (50). We used these chemicals to experimentally induce (RAP)  
183 or inhibit (CQ, 3-MA) autophagy in *L. gongylophorus* Petri dish cultures and we then measured  
184 the effects on staphyla density relative to control. Briefly, 5-mm diameter fungus plugs from  
185 previously isolated and reinoculated PDA culture were inoculated in 60-mm Petri dishes  
186 containing 10 ml of PDA (control), PDA + 300 ng/ml rapamycin (Medchem Express, Monmouth  
187 Junction, NJ, USA), PDA + 1.5 mM chloroquine diphosphate (Sigma-Aldrich, St. Louis, Missouri,  
188 USA), or PDA + 10 mM 3-MA (Medchem Express). We grew cultures for 46 days at 25°C in the  
189 dark, after which we photographed plates to measure growth area (mm<sup>2</sup>) using ImageJ (51) and  
190 then directly counted the staphylae on these Petri dishes under a stereo microscope (40x  
191 magnification) to quantify staphyla density (number of staphylae/growth area). The measurement  
192 of growth area provided a means of assessing general non-target effects of the chemical  
193 treatments on cultivar performance. We tested for treatment effects (PDA-Control, RAP, CQ, 3-  
194 MA) on mycelial growth and staphyla density in R version 4.0.2 (52) using a Kruskal-Wallis test

195 in *rstatix* version 0.7.0 (53) with pairwise post-hoc tests performed using Dunn's Test in *rstatix*  
196 with adjusted p-values calculated using the false-discovery rate method.

197

## 198 **RESULTS**

### 199 ***Nutritional reward structures***

200 Each gongylidium cell consists of two sections that we term the bulb (swollen section)  
201 and the filament (elongated section) ([Fig. 1 A](#)). Contrary to their typical depiction, gongylidia often  
202 have intercalary bulbs (between filaments) and intercalary filaments (between bulbs) ([Fig. 1 B-C](#)).  
203 Branching gongylidia are also common, with a single hyphal cell bearing two or more terminal  
204 bulbs ([Fig. 1 D](#)). Individual gongylidia also exhibit variable sizes, with bulb diameters ranging from  
205 12  $\mu\text{m}$  to 70  $\mu\text{m}$  and filament lengths ranging from 40  $\mu\text{m}$  to over 250  $\mu\text{m}$ . We hypothesize this  
206 size variation reflects indeterminately expanding gongylidia growth trajectories. Each gongylidium  
207 cell contains at least eight nuclei usually concentrated at the intersection of the bulb and the  
208 filament (54) ([Fig. 1 E](#)) and one large vacuole ([Fig. 1 F](#)) whose volume tends to comprise half of  
209 bulb volume.

210 Individual staphylae range widely in size and can contain from tens to hundreds of  
211 gongylidia ([Fig. 1 G and Fig. S1](#)), but always form on the surface mycelium of the fungus garden  
212 matrix where they are easily detachable from the surrounding hyphae. Staphylae also form in the  
213 absence of ants under *in vitro* (Petri dish) growth conditions, but they have key morphological  
214 differences compared to staphylae that grow in ant-tended fungus gardens. Staphyla growing  
215 isolated in Petri dishes tend to be: 1) less detachable because they are usually covered by  
216 filamentous hyphae, 2) larger in area and with more individual gongylidia, and 3) comprised of  
217 gongylidia that grow continuously until they burst. We thus propose that under farming conditions,  
218 ants likely harvest staphylae earlier in their development before vacuoles can produce turgor  
219 pressure exceeding the retaining capacity of their exceptionally thin (ca. 120 to 220 nm) cell walls  
220 ([Fig. 1 H](#)).

221

### 222 ***An autophagic mechanism of gongylidia formation***



223 TEM and light microscopy images revealed a variety of cellular features related to the  
224 influx of metabolites that are typically diagnostic of macroautophagic processes. First, gongylidia  
225 were enriched with long stretches of endoplasmic reticulum that produce double-membraned  
226 vesicles called autophagosomes ([Fig. 2](#)). It is within these autophagosomes that recycling of  
227 cellular materials is initiated. We observed that autophagosomes contained cytosol ([Fig. 2 A](#)),  
228 glycogen ([Fig. 2 B](#)) and mitochondria ([Fig. 2 C](#)). Other likely abundant metabolites (e.g., lipids,  
229 amino acids, enzymes (26, 28, 35, 38, 55)) contained in vesicles are too small to be detected with  
230 TEM imaging. Second, vacuoles within bulbs often contained single-membraned autophagic  
231 bodies ([Fig. 2 D](#)) that are remnants of autophagosomes that lost their outer membrane after  
232 vacuolar fusion. This indicates that autophagosomes deliver metabolites into gongylidia vacuoles.  
233 Third, large numbers of damaged mitochondria occurred in gongylidia and were often associated  
234 with endoplasmic reticulum membranes. This suggests they were destined to be sequestered into  
235 autophagosomes, digested, and recycled into edible metabolites. Given these hallmarks of  
236 macroautophagy (and the lack of evidence for microautophagy), we henceforth use ‘autophagy’  
237 to refer to macroautophagy.

238 An autophagic mechanism for gongylidia formation was further supported by significant  
239 treatment effects on staphyla density in the *in vitro* experiment represented in [Fig. 3](#) (Kruskal-  
240 Wallis:  $H_3 = 34.7$ ,  $p < 0.001$ ). Pairwise post-hoc comparisons indicated that both autophagy  
241 inhibitors (3-MA and CQ) were associated with significantly reduced staphyla density compared  
242 to the control (PDA) ( $p_{adj} < 0.001$  and  $p_{adj} = 0.001$ , respectively) and to the autophagy-induction  
243 (RAP) treatment ( $p_{adj} < 0.001$  and  $p_{adj} = 0.026$ , respectively). We also detected a significant  
244 treatment effect on mycelial growth area ( $H_3 = 16.3$ ,  $p = 0.001$ ). However, this was due to  
245 differences between 3-MA and all other treatments (PDA:3-MA,  $p_{adj} = 0.004$ ; RAP:3-MA,  $p_{adj} =$   
246  $0.015$ ; CQ:3-MA,  $p_{adj} = 0.001$ ), with no other significant pairwise tests ([Fig. S2](#)). Thus, while both  
247 autophagy inhibition treatments resulted in staphyla reduction, it is possible that 3-MA negatively  
248 influenced staphyla density through unknown indirect effects on cultivar performance. In contrast  
249 to the deleterious effects of autophagy inhibitors, the autophagy promotor (RAP) did not  
250 significantly increase staphyla density relative to control (PDA) or either of the autophagy  
251 inhibition treatments.

252 Combined these *in vitro* results reveal two key additional ways that autophagic recycling  
253 pathway may function in the production of nutritional rewards. First, neither of the autophagy  
254 inhibition treatments completely eliminated gongylidium formation—highlighting that the  
255 autophagic recycling of cultivar tissue pathway likely coincides with metabolic degradation of  
256 plant-derived substrates during gongylidium production. Second, the RAP autophagy promoter  
257 treatment did not enhance gongylidia density relative to control, suggesting that the autophagy  
258 pathway cannot be accelerated, and possibly that the process is constrained by an upstream  
259 process (e.g. pre-gongylidia differentiation) is already maximized given how gongylidia have  
260 evolved.

261

### 262 ***Cellular reorganizations during gongylidium formation***

263 With this evidence in hand, we propose the following developmental stages of gongylidia  
264 formation (Fig. 4). First, gongylidia formation begins when a hyphal cell growing at surface  
265 interstices within the fungus garden matrix becomes wider (Fig. 4 A). Second, the vacuole starts  
266 to expand as a response to autophagy and an accelerating rate of vesicle fusion delivers  
267 cytoplasmic cargo (Fig. 4 B). Third, the position of the expanding vacuole can determine the fate  
268 of the gongylidia. This is because *L. gongylophorus* has features analogous to polyploidy in crops  
269 domesticated by humans, being polykaryotic (7-17 haploid nuclei per cell) and heterogeneously  
270 haploid (5-7 distinct haplotypes per cell) (54, 56). While the functional implications of this  
271 ‘polyploidy’ are poorly understood, our results suggest the positions of nuclei in expanding  
272 gongylidia govern the morphology of these nutritional reward structures. The nuclei in most  
273 filamentous fungi are distributed evenly throughout the hyphal compartment and promote  
274 elongation in the growing tip while migrating apically as growth proceeds (57, 58). In contrast, the  
275 hyphal cells destined to become gongylidia bulbs are filled with an expanding vacuole that  
276 appears to exclude nuclei from the apical tip (Fig. 4 C) and then arrests the apical growth of the  
277 cell. Moreover, when nuclei happen to be distributed in different regions of the filament and bulb,  
278 they are associated with alternative gongylidia branching patterns and intercalary bulb formation.  
279 This suggests that the stereotypical single-bulb gongylidium cell shape depicted in Fig. 1 A is  
280 mediated by the absence of nuclei anterior to the vacuole. Finally, the staphyla forms as

281 developing gongylidia arise from the initial cell and from these ramifications, entangling within  
282 each other ([Fig. 4 D](#)).

283

## 284 **Discussion**

285 Leafcutter ants are ecologically dominant herbivores across neotropical ecosystems (15),  
286 but much remains unknown about how their fungal cultivar converts provisioned plant-fragment  
287 phytochemicals into edible nutritional rewards. Our results reveal that: 1) autophagy is linked to  
288 the vacuolar growth that governs expansion of gongylidia bulbs, and 2) autophagic recycling  
289 provides an active nutritional pathway where the fungal cultivar converts its own cellular material  
290 (e.g. cytosol, glycogen, mitochondria) into edible metabolites packaged into gongylidia vacuoles.  
291 Microscopic imaging shows the cellular hallmarks of autophagy (e.g. autophagosomes,  
292 autophagic bodies, abundant endoplasmic reticula), and a controlled *in vitro* experiment shows  
293 that gongylidium density is reduced when autophagy is suppressed. We hypothesize that this  
294 autophagic recycling pathway represents a final domestication step where the cultivar came to  
295 unambiguously prioritize nutritional services to its hosts even at the expense (up to a point) of its  
296 own mycelial health. In this sense, the autophagic recycling pathway is the expression of an  
297 obligately symmetric commitment between symbionts achieved after the fungal cultivar lost the  
298 capacity for a free-living existence.

299 The benefits to ant farmers of being able to regulate the production of edible yield by their  
300 crop through their phytochemical provisioning decisions are clear. Yet, these farmers also forage  
301 across hundreds of biochemically diverse plant species (59-61) and the plant fragments they  
302 collect contain key nutrients—but these nutrients can occur in suboptimal ratios and  
303 concentrations (17). Moreover, plant fragments contain a wealth of recalcitrant compounds (e.g.  
304 cellulose and lignin) and toxic metabolites that can reduce cultivar performance (62-65).  
305 Furthermore, the seasonal and spatial availability of preferred plant fragments may fluctuate in  
306 suboptimal ways (66, 67). Autophagic recycling may thus provide important benefits since the  
307 cultivar's organelles would yield reliably available and chemically predictable metabolic precursor  
308 compounds during periods of plant-fragment shortage. In this way, autophagy may stabilize the  
309 nutritional quality of the cultivar's nutritional rewards.

310 Published transcriptomic data also support the cultivar's use of autophagic recycling as  
311 gongylidium cells exhibit elevated expression levels of genes related to intracellular trafficking,  
312 secretion and vesicular transport (KOG group U) relative to undifferentiated hyphae (38). Yet,  
313 while gongylidia-linked autophagic recycling appears to be common, we propose that its primacy  
314 in nutritional reward production coexists with other known ant-mediated farming mechanisms.  
315 First, leafcutter ants frequently ingest gongylidia and vector the cultivar's enzymes (29, 30, 32,  
316 33) and nutrients (33, 34) as fecal droplets to catalyze degradation and detoxification of newly  
317 deposited plant fragments (28). Metabolites within these fecal droplets are assimilated by the  
318 cultivar and some subset likely enters biosynthetic pathways linked to gongylidium formation.  
319 Second, nutrients also appear to derive from bacterial symbionts (rather than plant fragments or  
320 autophagic recycling) (68-70). For instance, attine ants have lost the ability to synthesize arginine  
321 (71)—and depend on the cultivar's metabolism to produce this nitrogen-rich amino acid (26). In  
322 turn, the ants have evolved an association with a specialized bacterial symbiont (EntAcro1) that  
323 converts excess arginine in the ants' gut into N-rich ammonia fertilizer that the ants vector back  
324 to their fungal symbiont (70). As further evidence that the autophagic-recycling is one of several  
325 mechanisms by which gongylidia fill with metabolites, we note that staphyla production was still  
326 possible (even though significantly reduced) when autophagy was inhibited in the *in vitro*  
327 experiment. Resolving whether and how these nutritional pathways fluctuate relative to the  
328 specific resource needs of the colony thus represents an important next step.

329 Autophagic recycling provides a new lens to interpret well-known gardening behaviors in  
330 leafcutter ants. For instance, gardening ants constantly prune the cultivar's fungal mycelia which  
331 has been hypothesized to cause mechanical disruptions that stimulate gongylidium formation  
332 (72). However, the mechanism has remained unknown. Results of the present study suggest that  
333 pruning may induce gongylidium formation by causing an autophagic response to starvation—  
334 which is a common driver of autophagy in cells (48). Specifically, we reason that ant pruning  
335 severs connections with the main hyphal network and blocks the flow of nutrients to newly isolated  
336 fungal cells. Additionally, previous *in vitro* studies have observed highest staphylae densities at  
337 the lowest nutrient concentrations and after a period of days or weeks suggesting that staphyla  
338 formation was preceded by a period of nutrient depletion (17, 38, 71, 73, 74). Thus, while the  
339 focus on cultivar production typically hinges on optimized nutritional provisioning (17, 75, 76), the

340 behaviors linked to nutritional suppression are perhaps also important for optimized production of  
341 nutritional rewards.

342 We next explore the mechanisms by which nuclei may shape the trajectory of gongylidia  
343 development through their spatial distributions in hyphal cells. As the apical portion a gongylidium  
344 bulb develops, it is filled with a single large vacuole, which then obstructs nuclear migration and  
345 causes the clustering of nuclei at the bulb-filament interface. At the transcript level, gongylidia  
346 formation coincides with a structurally modified and upregulated transcript carrying a domain  
347 associated with microtubule related proteins (38) which could mediate such nuclear migration in  
348 association with motor protein complexes (58, 77, 78). We hypothesize that this obstruction of  
349 nuclear migration arrests apical bulb growth by blocking communication between the nuclei and  
350 the Spitzenkörper—the centralized machinery for hyphal growth located in the hyphae tip. This  
351 would then cause the bulb's balloon-like expansion.

352 These findings are just a starting point in our understanding of the functional  
353 consequences of the *L. gongylophorus* cultivar's status as: 1) a polykaryon (having 7-17 haploid  
354 nuclei per cell) and 2) being heterogeneously haploid (having 5-7 distinct haplotypes per cell)  
355 (54). The leafcutter cultivar likely inherited such polykaryotic condition from a *Leucoagaricus*  
356 ancestor that also gave rise to the polykaryotic gongylidium-bearing *Leucoagaricus* cultivars  
357 farmed by the other ant genera comprising the Higher-Neoattines. Yet the leafcutter cultivar is the  
358 only attine-farmed fungus that gained high haploid diversity (54). Thus, next steps involve moving  
359 beyond distributions of nuclei to testing whether factors like nucleus-specific expression and  
360 nuclear dominance are linked to gongylidium formation. Such a mechanism has been observed  
361 in the production of edible reward structures produced by the heterokaryon human-domesticated  
362 fungus *Agaricus bisporus*, where two distinct nuclear types exhibit differential expression in  
363 distinct tissues during mushroom formation (79). More generally, it will be important to link our  
364 proposal for gongylidium formation stages (Fig. 3: initiation, expansion, and bundling) to the  
365 specific regulatory mechanisms in the ant-fungus-bacterium provisioning symbiosis regulating the  
366 quality and quantity of these specialized nutritional rewards.

367

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377

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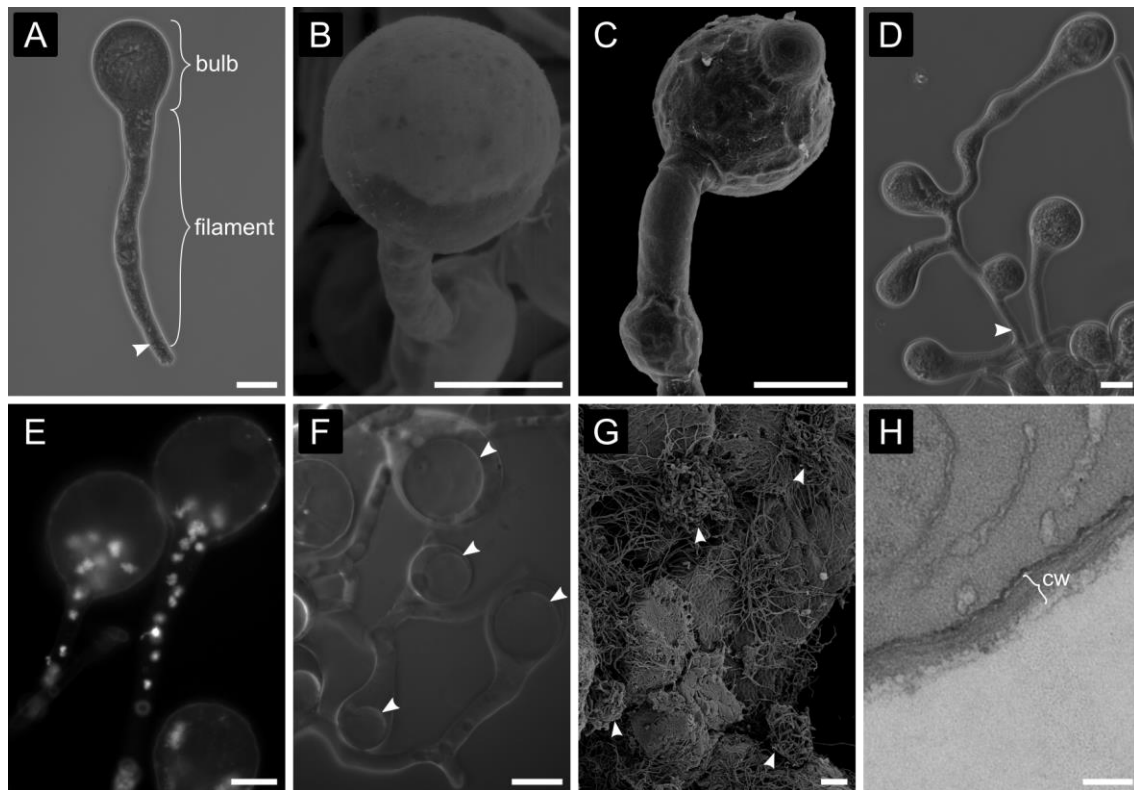
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570 **Figures**

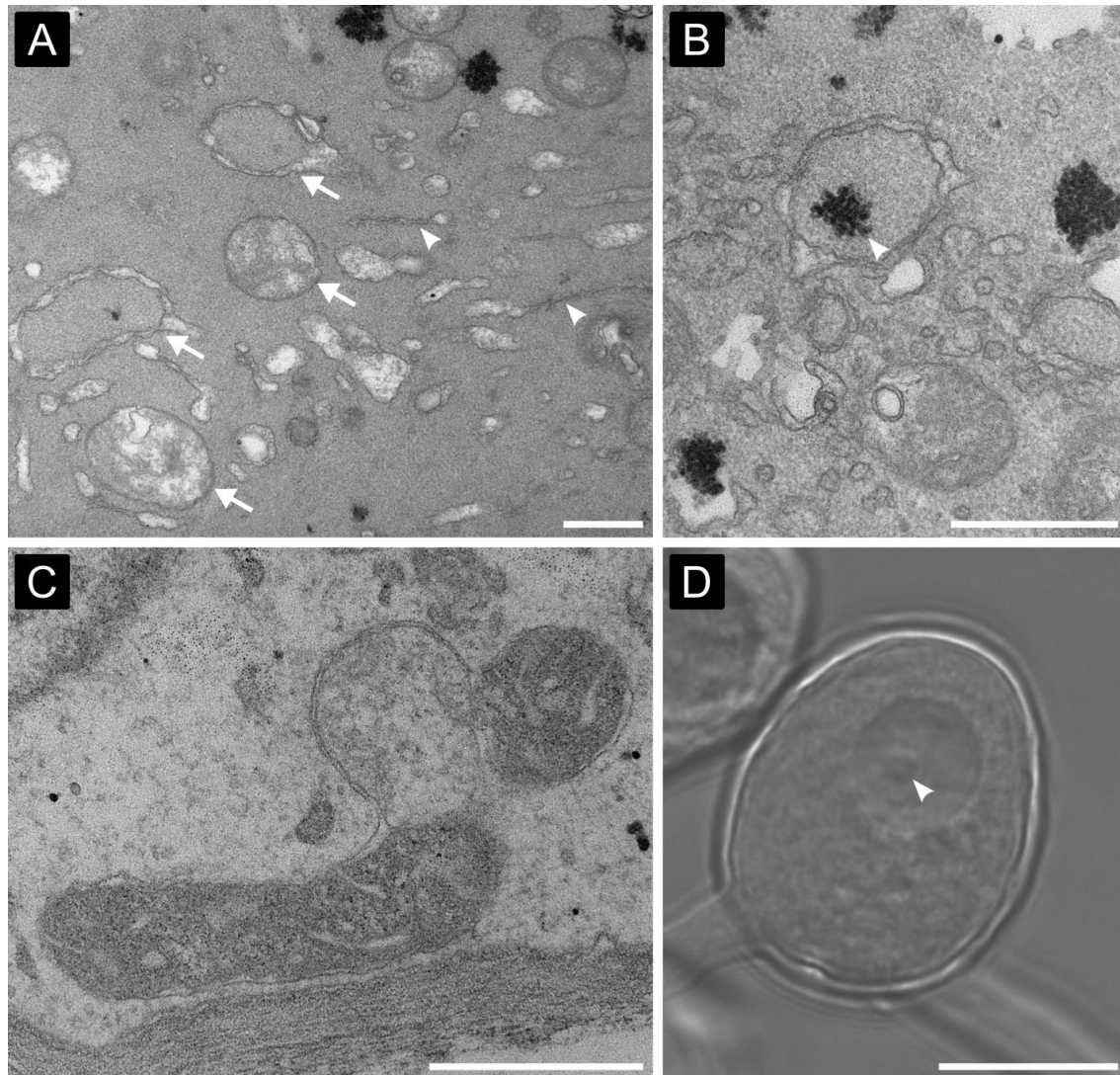
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573 **Fig. 1: The *Leucoagaricus gongylophorus* fungal cultivar produces gongylidia as**  
574 **specialized nutritional reward structures for leafcutter ants. A-C)** Gongylidia cells are  
575 typically depicted as a bulb at the end of a filament in the apical hyphal compartment separated  
576 by a septum (arrow). **D)** Gongylidia frequently exhibit more complex branching, with bulbs  
577 between filaments or in lateral branches of single hyphal cells delimited by septa. **E)** Individual  
578 gongylidia are polykaryotic, meaning that they have many haploid nuclei (white spots). In mature  
579 non-branching gongylidia cells, these nuclei occur at the base of the bulb (below a single large  
580 vacuole) and in the filament. Nuclei were visualized using DAPI staining. **F)** Each gongylidium  
581 contains a large vacuole (arrowheads). **G)** Staphyla grow in discrete patches at the surface of the  
582 fungus garden matrix in the middle garden stratum (arrowheads). **H)** Gongylidia cell presents very  
583 thin cell wall with ca. 120 to 220 nm (cw). Images produced by light microscopy (panels A, D, F),  
584 fluorescence microscopy (panel E), SEM (panels B, C, G) and TEM (panel H). Scale bars: A-F =  
585 20  $\mu$ m, G = 100  $\mu$ m, H = 200 nm.

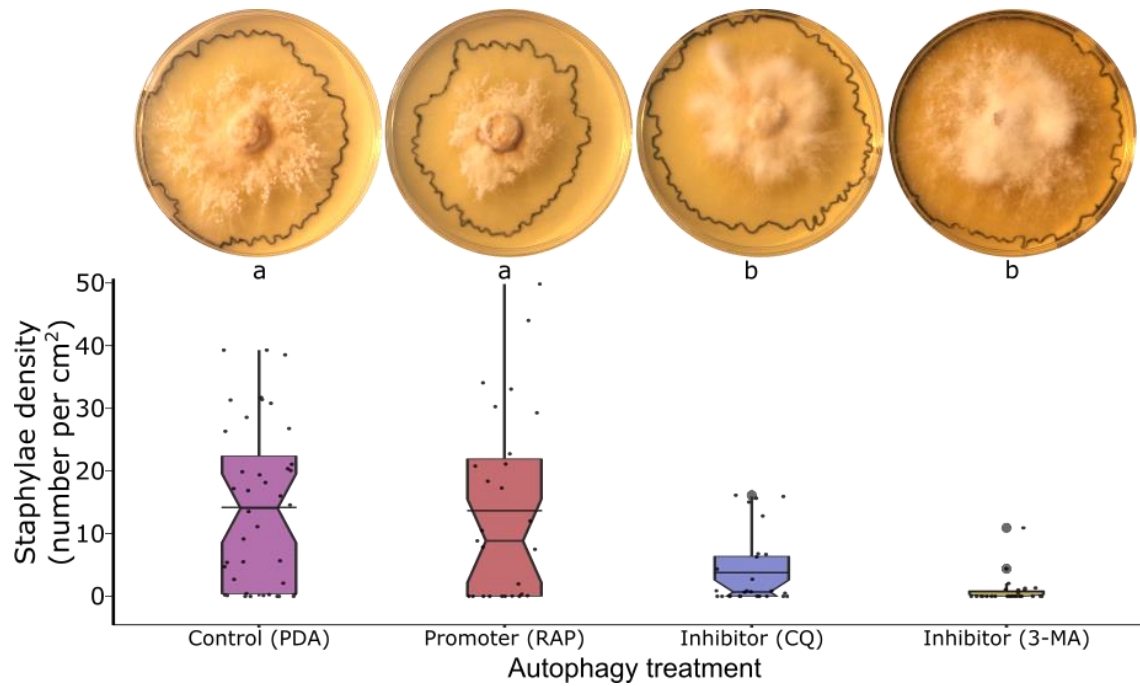
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588 **Fig. 2: Autophagic recycling of cellular material. A)** The autophagosomes that are diagnostic  
589 of autophagy are vesicles with double-layered membranes (arrows) and are produced by  
590 stretches of endoplasmic reticulum membranes (arrowheads). **B)** Autophagosomes sequester a  
591 broad range of cytoplasmic components including glycogen (arrowhead) and **C)** mitochondria  
592 which are then delivered to vacuoles in gongylidia bulbs for further degradation. **D)** Vacuolar  
593 expansion is mediated by fusion of autophagosomes that lose their outer membrane after fusing  
594 with the vacuole. These newly formed autophagic bodies are single-membrane vesicles that can  
595 be seen inside the vacuole prior to their degradation (arrows) using phase contrast microscopy.  
596 Images A-C acquired by TEM. Scale bars A-C = 500 nm, D = 20 μm.

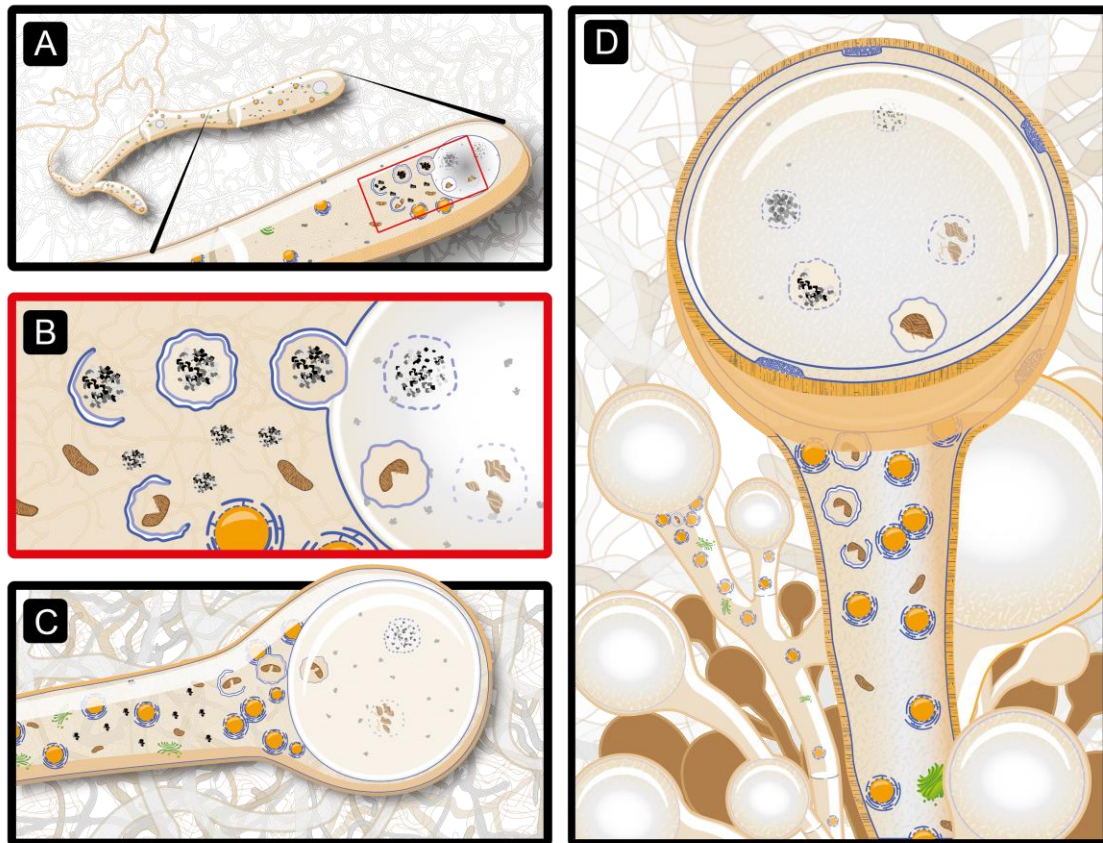
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599 **Fig. 3: Experimental evidence that autophagic recycling of the fungal cultivar's own**  
600 **cellular material mediates gongylidia formation.** Gongylidia density was significantly inhibited  
601 when *L. gongylophorus* was grown on potato dextrose agar supplemented with one autophagy  
602 inhibitor (PDA + chloroquine (CQ) or 3-methyladenine (3-MA)) relative to control (PDA) and an  
603 autophagy promoter (PDA + rapamycin (RAP)). Additionally, staphylococci density was not increased  
604 relative to control on Petri dishes containing the autophagy promoter (RAP). Black outlines on  
605 representative Petri-dish images for each treatment group indicate the radial growth area of  
606 cultivars and white clusters are the staphylococci (clearly visible in PDA and RAP). Different letters  
607 above bars indicate significant differences determined by a post-hoc Dunn's pairwise test ( $p <$   
608 0.05).

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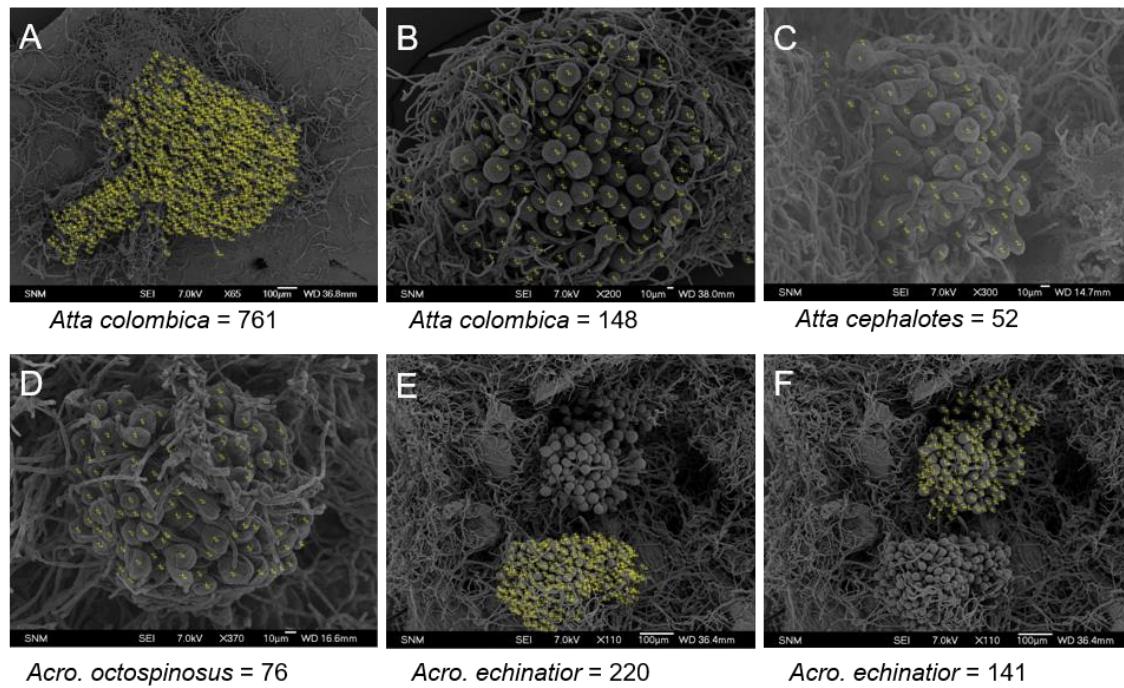


610

611 **Fig. 4: The hypothesized stages of autophagy-mediated gongylidia development.** **A)** An  
612 unknown mechanism (potentially starvation mediated by ant pruning (72)) triggers the widening  
613 of ordinary hyphae. Nuclei (orange circles) then begin to migrate terminally. **B)** Mediated primarily  
614 by an autophagic process, a large vacuole expands with the fusion of newly formed double  
615 membrane vesicles called autophagosomes (blue membraned vesicles) that sequester material  
616 present in the cytosol (e.g., glycogen (black and gray aggregates) and damaged mitochondria  
617 (shown here as brown indented ovals)). A key signature of this process is the proliferation of  
618 endoplasmic reticulum membranes (blue membranes around nuclei) that produce  
619 autophagosomes. **C)** The fusion of autophagosomes into vacuoles drives their expansion and  
620 forces the cellular swelling (bulb formation) while also halting further apical growth by excluding  
621 nuclei from the hyphal tip. **D)** This process repeats in up to hundreds of adjacent hyphae that  
622 become tangled and form the staphyla.

623

624 **Supplementary figures**



625

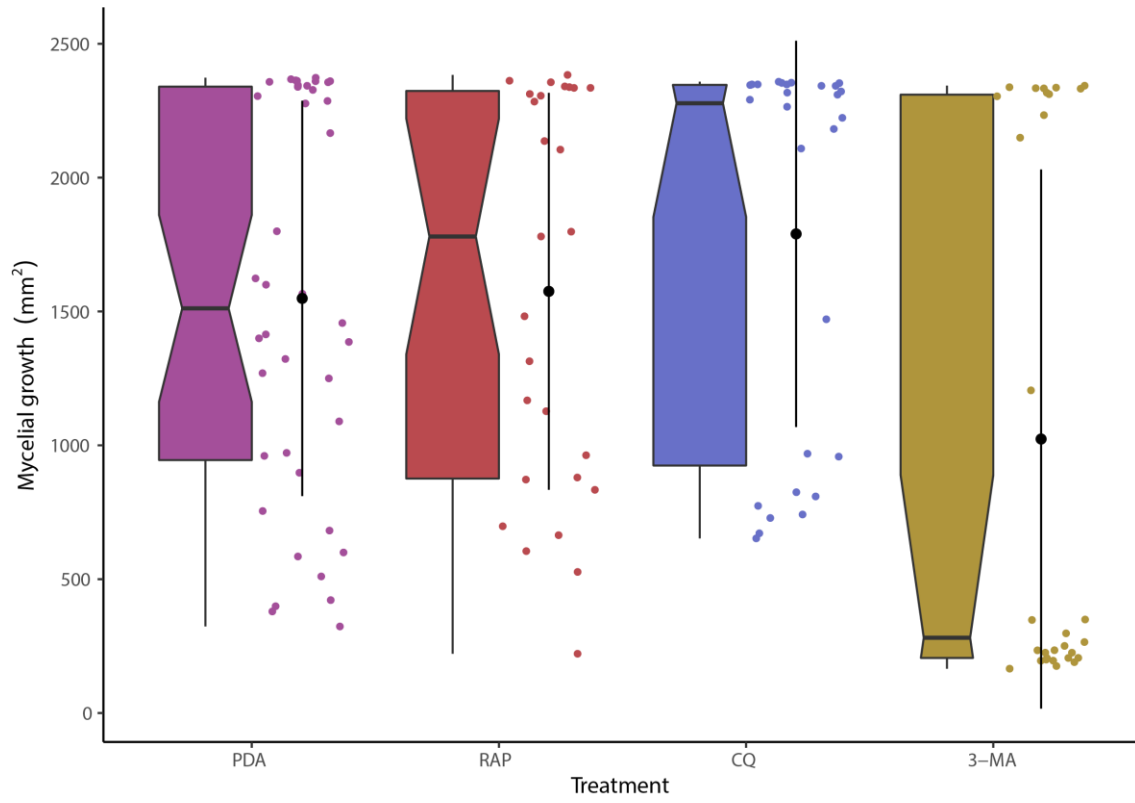
626 **Fig. S1: Counting of gongylidia per staphylae in *L. gongylophorus* from different leafcutter**

627 **ants' species.** A) Staphylae from in vitro culture without ant manipulation. B-E) staphylae from

628 colonies fungus garden. Scale bars sizes are indicated in each image.

629





630

631 **Fig. S2: Mycelial growth distribution (area per treatment).**