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

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ABSTRACT

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

Introduction

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

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

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

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

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

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

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

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

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

Methods

Imaging morphology of staphyla and gongylidia

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

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

We used light and fluorescence microscopy to view the cultivar's internal anatomy (*e.g.* septa, vacuoles, nuclei, etc.). Staphyla samples were placed in a drop of mounting solution (dH_2O, PBS, 3% KOH) on a glass slide. Gongylidia were then separated under a stereo microscope (16x or 25x magnification) with 0.16-mm diameter acupuncture needles and stained using two methods. For visualization under white light, we placed samples in either 0.1% Congo-red (in 150 mM NaCl) for one minute followed by a wash with 150 mM NaCl, or 1.5% phloxine followed by a wash with 3% KOH. For nucleus visualization under UV light, we stained samples for 10 minutes using "Vectashield with DAPI" (Vector Laboratories, Burlingame, CA, USA). We then performed bright-field, dark-field, phase-contrast, and fluorescence microscopy under an Olympus BX63 microscope (Olympus, Tokyo, Japan). The microstructures were measured and photographed using a mounted QImaging Retiga 6000 monochrome camera and cellSens Dimension v1.18 (Olympus) image-processing software.

We used transmission electron microscopy (TEM) to visualize fungal cells with greater magnification and resolution (i.e. the 500 nm scale). Staphylae were collected from fragments of intact fungus gardens, fixed in 2% glutaraldehyde in 0.05 M PBS (pH 7.2) and then post-fixed in 1% w/v OsO_4 with 0.05M $K_3Fe(Cn)_6$ in 0.12 M sodium phosphate buffer (pH 7.2) for 2 hr at room temperature. Fixed samples were washed three times in ddH_2O for 10 minutes and dehydrated in a series of increasing ethanol concentrations series (70%, 96% and 99.9%). Each dehydration lasted 15 min and was performed twice per concentration. Samples were then repeatedly 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, Wienna,
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^2) 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 µm to 70 µm and filament lengths ranging from 40 µm to over 250 µm. 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***

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

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

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

Cellular reorganizations during gongylidium formation

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

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

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

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

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

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

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References

1. Solberg MF, Robertsen G, Sundt-Hansen LE, Hindar K, Glover KA. Domestication leads to increased predation susceptibility. Scientific Reports. 2020;10(1):1929.

2. Milla R, Osborne CP, Turcotte MM, Violle C. Plant domestication through an ecological lens. Trends Ecol Evol. 2015;30(8):463-9.

3. Gering E, Incorvaia D, Henriksen R, Conner J, Getty T, Wright D. Getting Back to Nature: Feralization in Animals and Plants. Trends Ecol Evol. 2019;34(12):1137-51.

4. Comai L. The advantages and disadvantages of being polyploid. Nature Reviews Genetics. 2005;6(11):836-46.

5. Stetter MG, Gates DJ, Mei W, Ross-Ibarra J. How to make a domesticate. Curr Biol. 2017;27(17):R896-R900.

6. Edger PP, Poorten TJ, VanBuren R, Hardigan MA, Colle M, McKain MR, et al. Origin and evolution of the octoploid strawberry genome. Nat Genet. 2019;51(3):541-7.

7. Piperno DR. Patterns, Process, and New Developments

The Origins of Plant Cultivation and Domestication in the New World Tropics. CurrAnthr. 2011;52(S4):S453-S70.

8. Renner SS, Wu S, Pérez-Escobar OA, Silber MV, Fei Z, Chomicki G. A chromosome-level genome of a Kordofan melon illuminates the origin of domesticated watermelons. Proc Natl Acad Sci. 2021;118(23):e2101486118.

9. Mueller UG, Gerardo NM, Aanen DK, Six DL, Schultz TR. The evolution of agriculture in insects. Annual Review of Ecology, Evolution, and Systematics. 2005:563-95.

10. Schultz TR, Brady SG. Major evolutionary transitions in ant agriculture. Proc Natl Acad Sci. 2008;105(14):5435-40.

11. Barrera CA, Sosa-Calvo J, Schultz TR, Rabeling C, Bacci Jr M. Phylogenomic reconstruction reveals new insights into the evolution and biogeography of Atta leaf-cutting ants (Hymenoptera: Formicidae). Syst Entomol. 2021;n/a(n/a).

12. Boomsma JJ. Lifetime Commitment between Social Insect Families and Their Fungal Cultivars Complicates Comparisons with Human Farming. In: Schultz TR, Gawne R, Peregrine PN, editors. The Convergent Evolution of Agriculture in Humans and Insects. Vienna Series in Theoretical Biology. Cambridge, Massachusetts: MIT Press; 2022. p. 73-86.

13. Frank SA. Host-symbiont conflict over the mixing of symbiotic lineages. Proceedings of the Royal Society of London Series B: Biological Sciences. 1996;263(1368):339-44.

14. Axelrod R, Hamilton WD. The Evolution of Cooperation. Science. 1981;211(4489):1390-6.

15. Hölldobler B, Wilson EO. The leafcutter ants: civilization by instinct. New York: W. W Norton & Co.; 2011.

16. Wirth R, Herz H, Ryel RJ, Beyschlag W, Hölldobler B. Herbivory of Leaf-Cutting Ants. Germany: Springer-Verlag Berlin Heidelberg; 2003. 233 p.

17. Crumiere AJJ, James A, Lannes P, Mallett S, Michelsen A, Rinnan R, et al. The multidimensional nutritional niche of fungus-cultivar provisioning in free-ranging colonies of a neotropical leafcutter ant. Ecol Lett. 2021;24(11):2439-51.

18. Mueller UG, Schultz TR, Currie CR, Adams RM, Malloch D. The origin of the attine ant-fungus mutualism. Q Rev Biol. 2001:169-97.

19. Quinlan RJ, Cherrett JM. The role of fungus in the diet of the leaf-cutting ant Atta cephalotes (L.). Ecol Entomol. 1979;4(2):151-60.

20. Weber NA. Dry season adaptations of fungus-growing ants and their fungi. The Anatomical Record. 1957;128(3):1.

21. Wheeler WM. The fungus-growing ants of North America. Bulletin of the American Museum of Natural History. 1907;23:139.

22. Möller A. Die Pilzgärten einiger südamerikanischer Ameisen. A.F.W. S, editor. Jena: Gustav Fischer Verlag; 1893.

23. Powell RJ. The influence of substrate quality on fungus cultivation by some Attine ants: University of Exeter; 1984.

24. Weber NA. Fungus-growing ants. Science. 1966;153(3736):587-604.

25. Swingle WT. Fungus Gardens in the Nest of an Ant (Atta tardigrada Buckl.) near Washington. Proc Am Assoc Adv Sci. 1896;44th Meet.:2.

26. Nygaard S, Hu H, Li C, Schiøtt M, Chen Z, Yang Z, et al. Reciprocal genomic evolution in the ant–fungus agricultural symbiosis. Nat Commun. 2016;7:12233.

27. Solomon SE, Rabeling C, Sosa-Calvo J, Lopes CT, Rodrigues A, Vasconcelos HL, et al. The molecular phylogenetics of Trachymyrmex Forel ants and their fungal cultivars provide insights into the origin and coevolutionary history of 'higher-attine' ant agriculture. Syst Entomol. 2019;44(4):939-56.

28. De Fine Licht HH, Schiøtt M, Rogowska-Wrzesinska A, Nygaard S, Roepstorff P, Boomsma JJ. Laccase detoxification mediates the nutritional alliance between leaf-cutting ants and fungus-garden symbionts. Proc Natl Acad Sci. 2013;110(2):583-7.

29. Kooij PW, Rogowska-Wrzesinska A, Hoffmann D, Roepstorff P, Boomsma JJ, Schiott M. *Leucoagaricus gongylophorus* uses leaf-cutting ants to vector proteolytic enzymes towards new plant substrate. ISME J. 2014;8(5):1032-40.

30. Schiøtt M, Rogowska-Wrzesinska A, Roepstorff P, Boomsma JJ. Leaf-cutting ant fungi produce cell wall degrading pectinase complexes reminiscent of phytopathogenic fungi. BMC Biol. 2010;8(1):156.

31. Aylward FO, Khadempour L, Tremmel DM, McDonald BR, Nicora CD, Wu S, et al. Enrichment and Broad Representation of Plant Biomass-Degrading Enzymes in the Specialized Hyphal Swellings of Leucoagaricus gongylophorus, the Fungal Symbiont of Leaf-Cutter Ants. PLOS ONE. 2015;10(8):e0134752.

32. Schiøtt M, Boomsma JJ. Proteomics reveals synergy between biomass degrading enzymes and inorganic Fenton chemistry in leaf-cutting ant colonies. eLife. 2021;10:e61816.

33. Martin MM. The Biochemical Basis of the Fungus-Attine Ant Symbiosis. Science. 1970;169(3940):16.

34. Martin MM, Martin JS. The biochemical basis for the symbiosis between the ant, Atta colombica tonsipes, and its food fungus. J Insect Physiol. 1970;16(1):109-19.

35. Khadempour L, Kyle JE, Webb-Robertson B-JM, Nicora CD, Smith FB, Smith RD, et al. From Plants to Ants: Fungal Modification of Leaf Lipids for Nutrition and Communication in the Leaf-Cutter Ant Fungal Garden Ecosystem. mSystems. 2021;6(2):e01307-20.

462 36. Shik JZ, Rytter W, Arnan X, Michelsen A. Disentangling nutritional pathways linking
463 leafcutter ants and their co-evolved fungal symbionts using stable isotopes. Ecology.
464 2018;99(9):1999-2009.

465 37. Martin MM, Carman RM, MacConnell JG. Nutrients Derived from the Fungus Cultured
466 by the Fungus-Growing Ant Atta colombica tonsipes1. Ann Entomol Soc Am. 1969;62(1):11-3.

467 38. De Fine Licht HH, Boomsma JJ, Tunlid A. Symbiotic adaptations in the fungal cultivar of
468 leaf-cutting ants. Nat Commun. 2014;5(1):5675.

469 39. Kikuma T, Ohneda M, Arioka M, Kitamoto K. Functional Analysis of the ATG8
470 Homologue Aoatg8 and Role of Autophagy in Differentiation and Germination in
471 Aspergillus oryzae. Eukaryot Cell. 2006;5(8):1328-36.

472 40. Pinan-Lucarré B, Paoletti M, Dementhon K, Coulary-Salin B, Clavé C. Autophagy is
473 induced during cell death by incompatibility and is essential for differentiation in the filamentous
474 fungus Podospora anserina. Mol Microbiol. 2003;47(2):321-33.

475 41. Liu X-H, Gao H-M, Xu F, Lu J-P, Devenish RJ, Lin F-C. Autophagy vitalizes the pathogenicity
476 of pathogenic fungi. Autophagy. 2012;8(10):1415-25.

477 42. Kües U, Navarro-González M. How do Agaricomycetes shape their fruiting bodies? 1.
478 Morphological aspects of development. Fungal Biology Reviews. 2015;29(2):63-97.

479 43. Elander PH, Minina EA, Bozhkov PV. Autophagy in turnover of lipid stores: trans-
480 kingdom comparison. J Exp Bot. 2017;69(6):1301-11.

481 44. Bartoszewska M, Kiel JA. The role of macroautophagy in development of filamentous
482 fungi. Antioxid Redox Signal. 2011;14(11):2271-87.

483 45. Levine B, Klionsky DJ. Development by Self-Digestion: Molecular Mechanisms and
484 Biological Functions of Autophagy. Dev Cell. 2004;6(4):463-77.

485 46. Klionsky DJ, Abdel-Aziz AK, Abdelfatah S, Abdellatif M, Abdoli A, Abel S, et al. Guidelines
486 for the use and interpretation of assays for monitoring autophagy (4th edition)1. Autophagy.
487 2021;17(1):1-382.

488 47. Angeli-Papa J, Eymé J. Le champignon cultivé par la" fourmi-manioc", Acromyrmex
489 octospinosus Reich en Guadeloupe; résultats préliminaires sur le mycélium en culturue pure et
490 sur l'infrastructure des hyphes. C r hebd Séanc Acad Sci, Paris D 1979;281:21-4.

491 48. Noda T, Ohsumi Y. Tor, a Phosphatidylinositol Kinase Homologue, Controls Autophagy
492 in Yeast*. J Biol Chem. 1998;273(7):3963-6.

493 49. Mauthe M, Orhon I, Rocchi C, Zhou X, Luhr M, Hijlkema K-J, et al. Chloroquine inhibits
494 autophagic flux by decreasing autophagosome-lysosome fusion. Autophagy. 2018;14(8):1435-
495 55.

496 50. Wu Y-T, Tan H-L, Shui G, Bauvy C, Huang Q, Wenk MR, et al. Dual Role of 3-
497 Methyladenine in Modulation of Autophagy via Different Temporal Patterns of Inhibition on
498 Class I and III Phosphoinositide 3-Kinase*. J Biol Chem. 2010;285(14):10850-61.

499 51. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis.
500 Nat Methods. 2012;9(7):671-5.

501 52. R Core Team. R: a language and environment for statistical computing. Foundation for
502 Statistical Computing. Vienna, Austria 2020.

503 53. Kassambara A. rstatix: Pipe-Friendly Framework for Basic Statistical Tests. R package
504 version 0.7.0. ed2021.

505 54. Kooij PW, Aanen DK, Schiøtt M, Boomsma JJ. Evolutionarily advanced ant farmers rear
506 polyploid fungal crops. J Evol Biol. 2015;28(11):1911-24.

507 55. Grell MN, Linde T, Nygaard S, Nielsen KL, Boomsma JJ, Lange L. The fungal symbiont of
508 *Acromyrmex* leaf-cutting ants expresses the full spectrum of genes to degrade cellulose and
509 other plant cell wall polysaccharides. BMC Genomics. 2013;14.

510 56. Mohali S. Ultrastructural and morphological study of the mutualistic fungus of the ant
511 Atta cephalotes. Rev Ecol Lat Am. 1998;5(3):1-6.

512 57. Plamann M, Minke PF, Tinsley JH, Bruno KS. Cytoplasmic dynein and actin-related
513 protein Arp1 are required for normal nuclear distribution in filamentous fungi. J Cell Biol.
514 1994;127(1):139-49.

515 58. Xiang X. Nuclear movement in fungi. Semin Cell Dev Biol. 2018;82:3-16.

516 59. Mundim FM, Costa AN, Vasconcelos HL. Leaf nutrient content and host plant selection
517 by leaf-cutter ants, Atta laevigata, in a Neotropical savanna. Entomol Exp Appl. 2009;130(1):47-
518 54.

519 60. Berish CW. Leaf-Cutting Ants (Atta cephalotes) Select Nitrogen-Rich Forage. The
520 American Midland Naturalist. 1986;115(2):268-76.

521 61. Howard JJ, Cazin J, Wiemer DF. Toxicity of terpenoid deterrents to the leafcutting
522 antAtta cephalotes and its mutualistic fungus. J Chem Ecol. 1988;14(1):59-69.

523 62. Howard JJ. Leafcutting Ant Diet Selection: The Role of Nutrients, Water, and Secondary
524 Chemistry. Ecology. 1987;68(3):503-15.

525 63. Howard JJ. Leafcutting and Diet Selection: Relative Influence of Leaf Chemistry and
526 Physical Features. Ecology. 1988;69(1):250-60.

527 64. Nichols-Orians CM, Schultz JC. Interactions among leaf toughness, chemistry, and
528 harvesting by attine ants. Ecol Entomol. 1990;15(3):311-20.

529 65. Crumière A, Mallett S, Michelsen A, Rinnan R, Shik J. Nutritional challenges of feeding a
530 mutualist: testing for a nutrient-toxin tradeoff in fungus-farming leafcutter ants. Ecology. in
531 press.

532 66. Mueller UG, Mikheyev AS, Hong E, Sen R, Warren DL, Solomon SE, et al. Evolution of
533 cold-tolerant fungal symbionts permits winter fungiculture by leafcutter ants at the northern
534 frontier of a tropical ant–fungus symbiosis. Proc Natl Acad Sci. 2011;108(10):4053-6.

535 67. Nichols-Orians CM. Environmentally Induced Differences in Plant Traits: Consequences
536 for Susceptibility to a Leaf-Cutter Ant. Ecology. 1991;72(5):1609-23.

537 68. Pinto-Tomás AA, Anderson MA, Suen G, Stevenson DM, Chu FST, Cleland WW, et al.
538 Symbiotic Nitrogen Fixation in the Fungus Gardens of Leaf-Cutter Ants. Science.
539 2009;326(5956):1120-3.

540 69. Khadempour L, Fan H, Keefover-Ring K, Carlos-Shanley C, Nagamoto NS, Dam MA, et al.
541 Metagenomics Reveals Diet-Specific Specialization of Bacterial Communities in Fungus Gardens
542 of Grass- and Dicot-Cutter Ants. Frontiers in Microbiology. 2020;11.

543 70. Sapountzis P, Zhukova M, Shik JZ, Schiott M, Boomsma JJ. Reconstructing the functions
544 of endosymbiotic Mollicutes in fungus-growing ants. eLife. 2018;7:e39209.

545 71. Nygaard S, Zhang G, Schiøtt M, Li C, Wurm Y, Hu H, et al. The genome of the leaf-cutting
546 ant Acromyrmex echinatior suggests key adaptations to advanced social life and fungus farming.
547 Genome Res. 2011;21(8):1339-48.

548 72. Bass M, Cherrett JM. Leaf-Cutting Ants (Formicidae, Attini) Prune Their Fungus to
549 Increase and Direct Its Productivity. Funct Ecol. 1996;10(1):55-61.

550 73. Schiøtt M, De Fine Licht HH, Lange L, Boomsma JJ. Towards a molecular understanding
551 of symbiont function: Identification of a fungal gene for the degradation of xylan in the fungus
552 gardens of leaf-cutting ants. BMC Microbiol. 2008;8(1):40.

553 74. Moller IE, De Fine Licht HH, Harholt J, Willats WGT, Boomsma JJ. The Dynamics of Plant
554 Cell-Wall Polysaccharide Decomposition in Leaf-Cutting Ant Fungus Gardens. PLOS ONE.
555 2011;6(3):e17506.

556 75. Shik JZ, Gomez EB, Kooij PW, Santos JC, Wcislo WT, Boomsma JJ. Nutrition mediates the
557 expression of cultivar–farmer conflict in a fungus-growing ant. Proc Natl Acad Sci.
558 2016;113(36):10121-6.

559 76. Shik JZ, Kooij PW, Donoso DA, Santos JC, Gomez EB, Franco M, et al. Nutritional niches
560 reveal fundamental domestication trade-offs in fungus-farming ants. Nat Ecol Evol.
561 2021;5(1):122-34.

562 77. Xiang X, Beckwith SM, Morris NR. Cytoplasmic dynein is involved in nuclear migration in
563 Aspergillus nidulans. Proc Natl Acad Sci. 1994;91(6):2100-4.

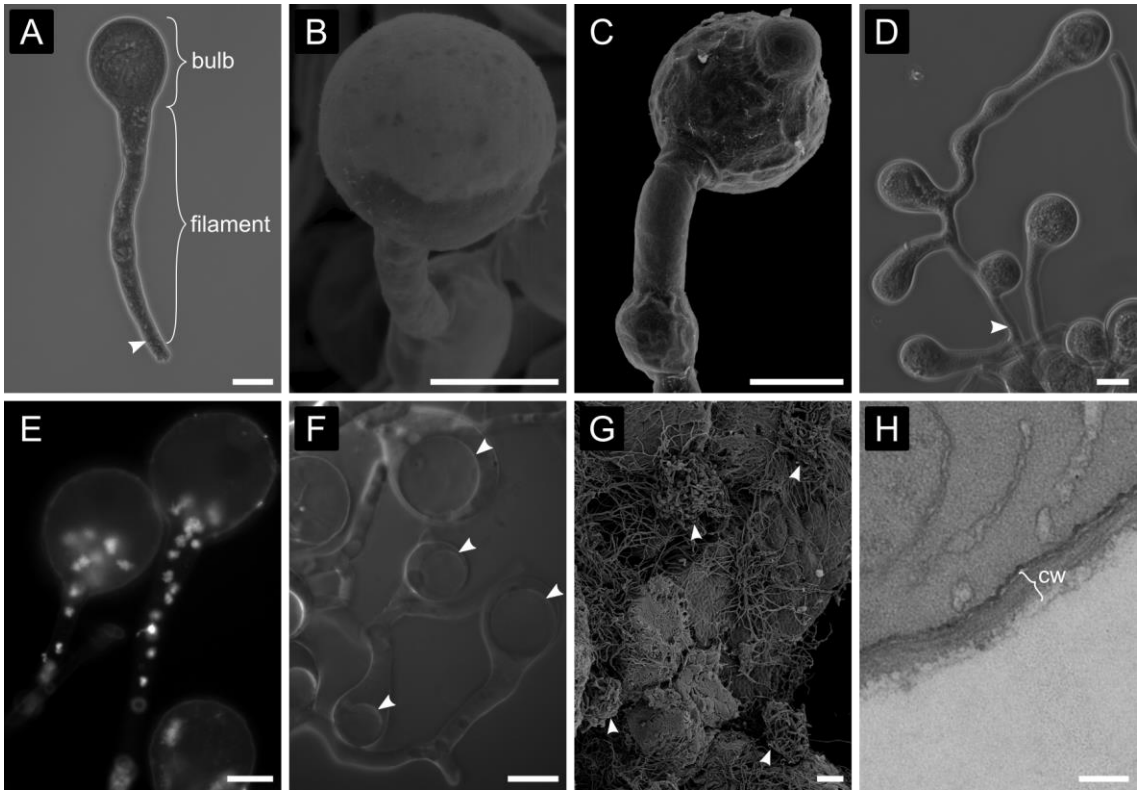
564 78. Yamamoto A, Hiraoka Y. Cytoplasmic dynein in fungi: insights from nuclear migration. J
565 Cell Sci. 2003;116(22):4501-12.

566 79. Gehrmann T, Pelkmans JF, Ohm RA, Vos AM, Sonnenberg ASM, Baars JJP, et al. Nucleus-
567 specific expression in the multinuclear mushroom-forming fungus Agaricus bisporus
568 reveals different nuclear regulatory programs. Proc Natl Acad Sci. 2018;115(17):4429-34.

569

570 **Figures**

571



572

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 µm, G = 100 µm, H = 200 nm.

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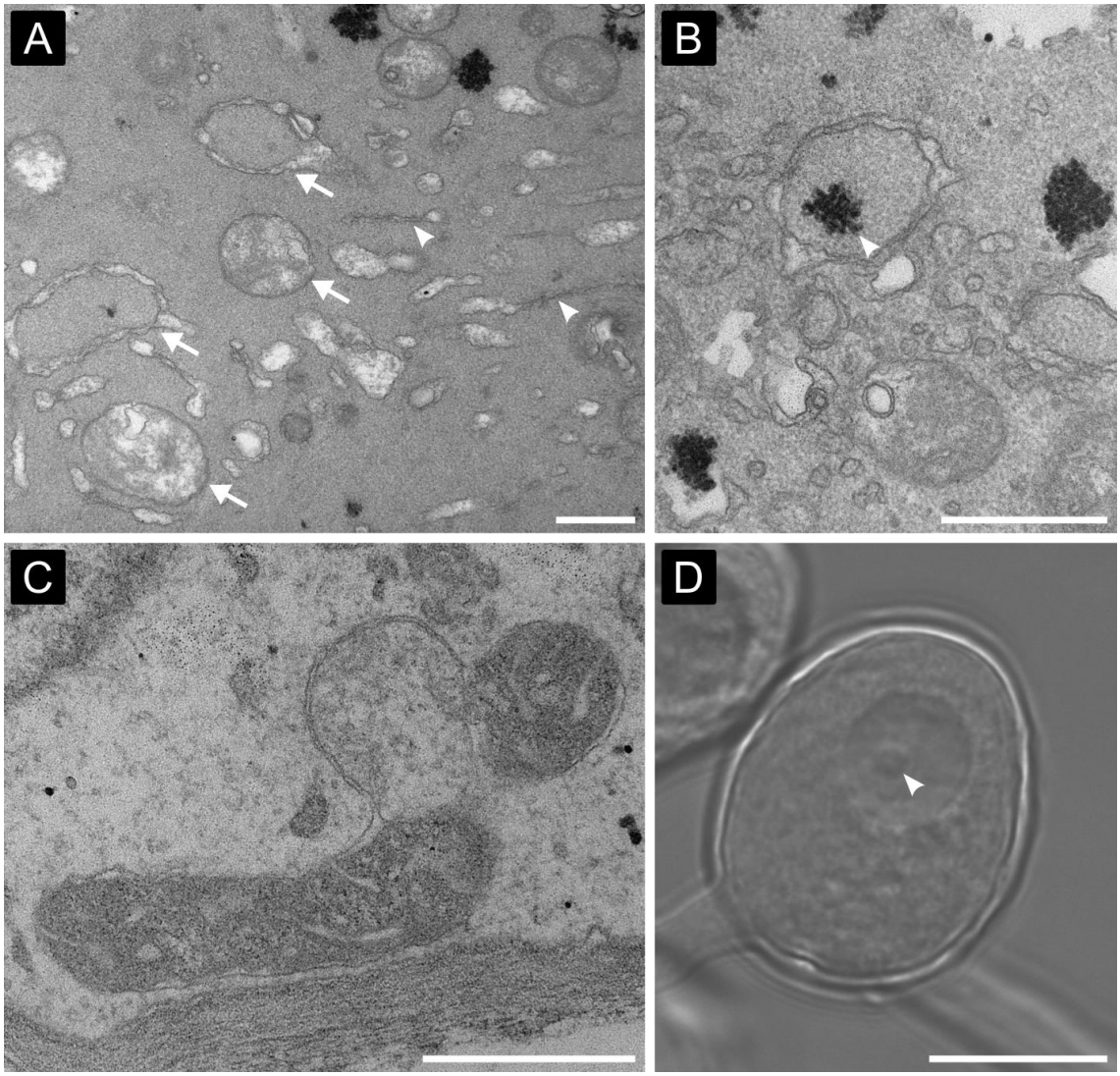
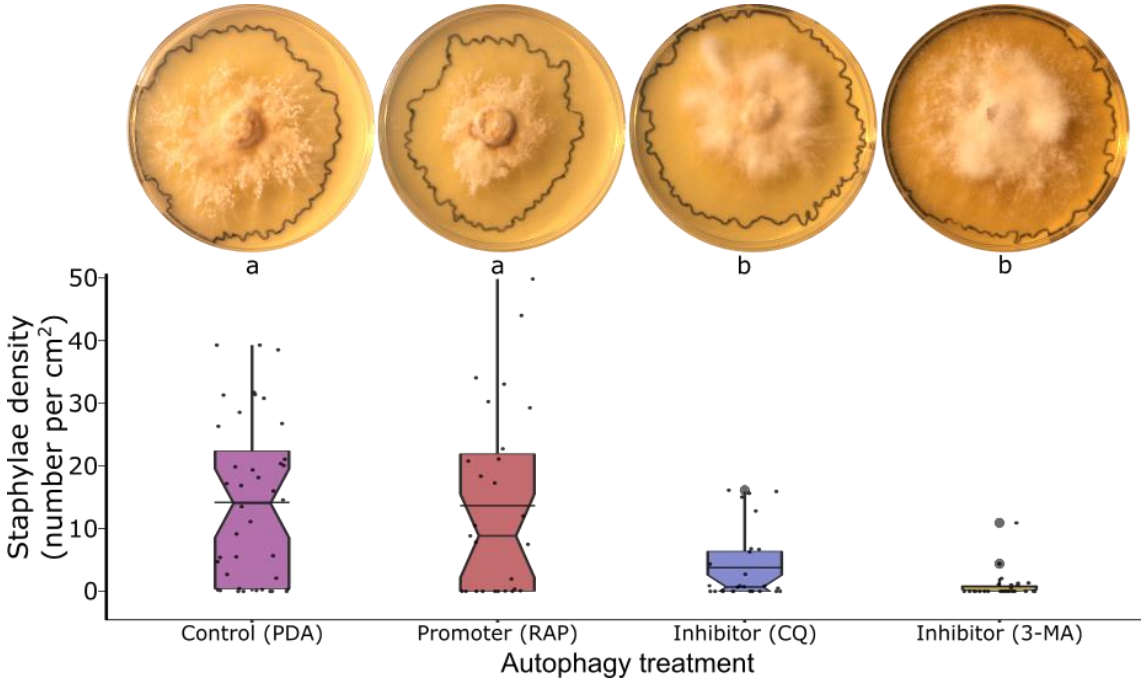


Fig. 2: Autophagic recycling of cellular material. A) The autophagosomes that are diagnostic of autophagy are vesicles with double-layered membranes (arrows) and are produced by stretches of endoplasmic reticulum membranes (arrowheads). **B)** Autophagosomes sequester a broad range of cytoplasmic components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



598

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, staphyla 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 staphylae (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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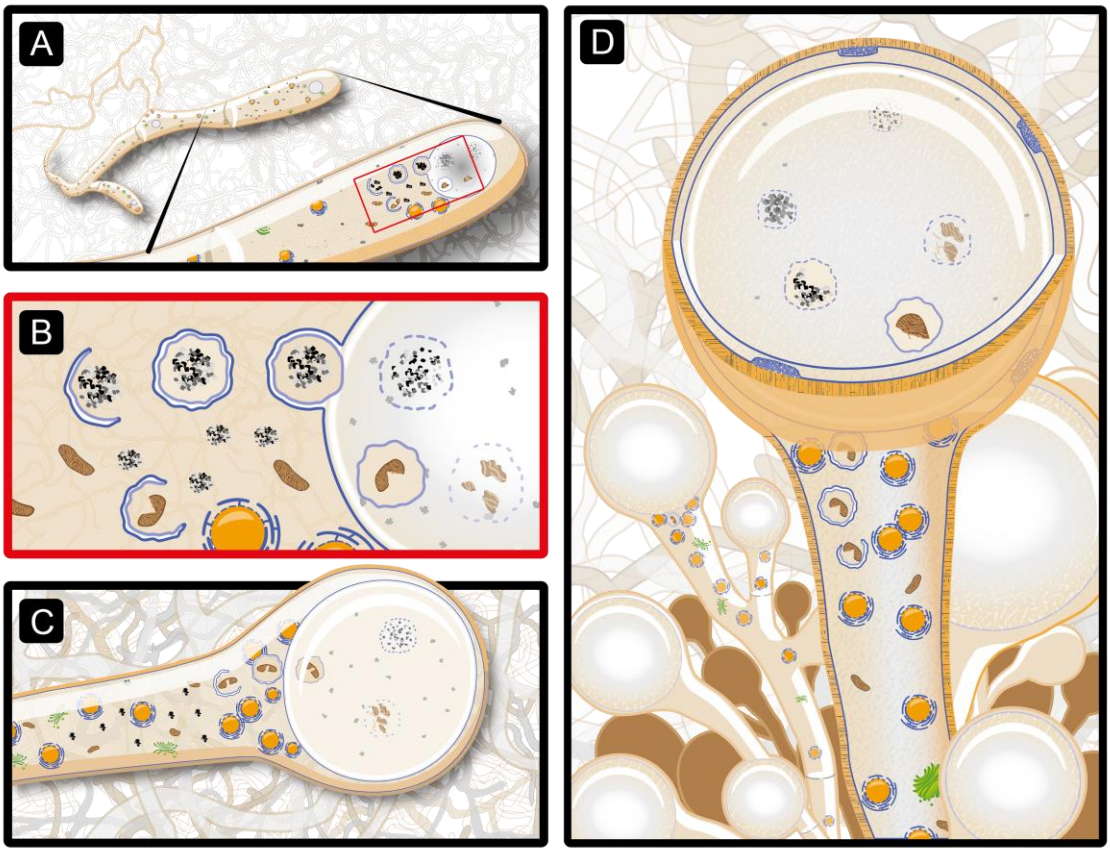


610

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

Supplementary figures

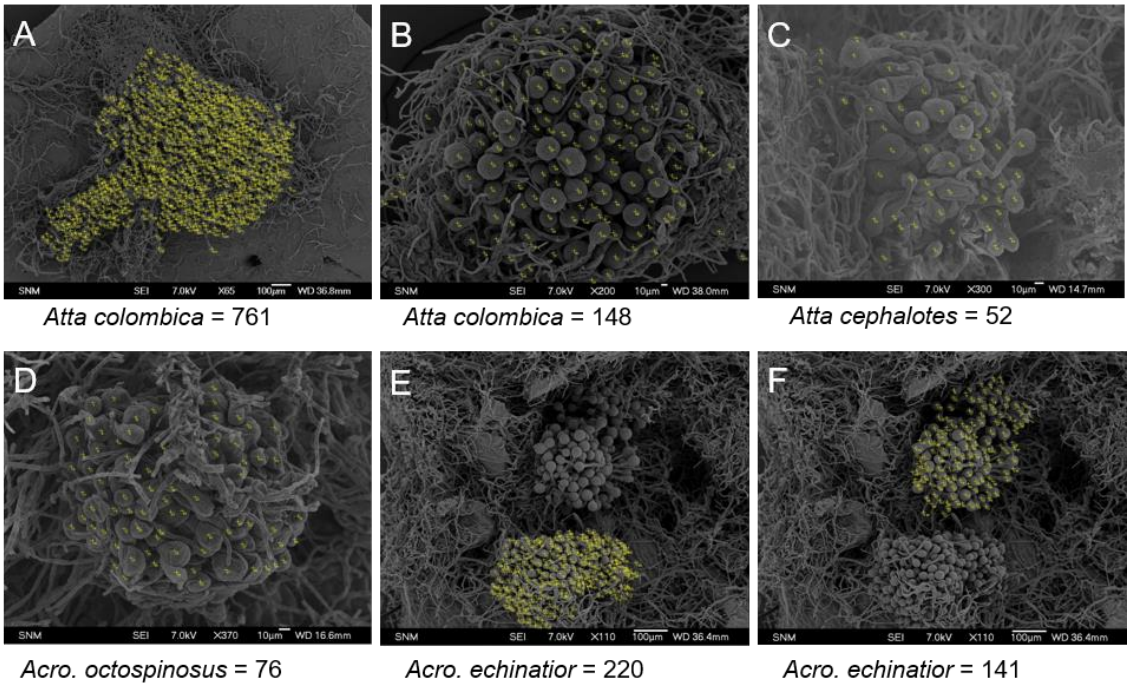
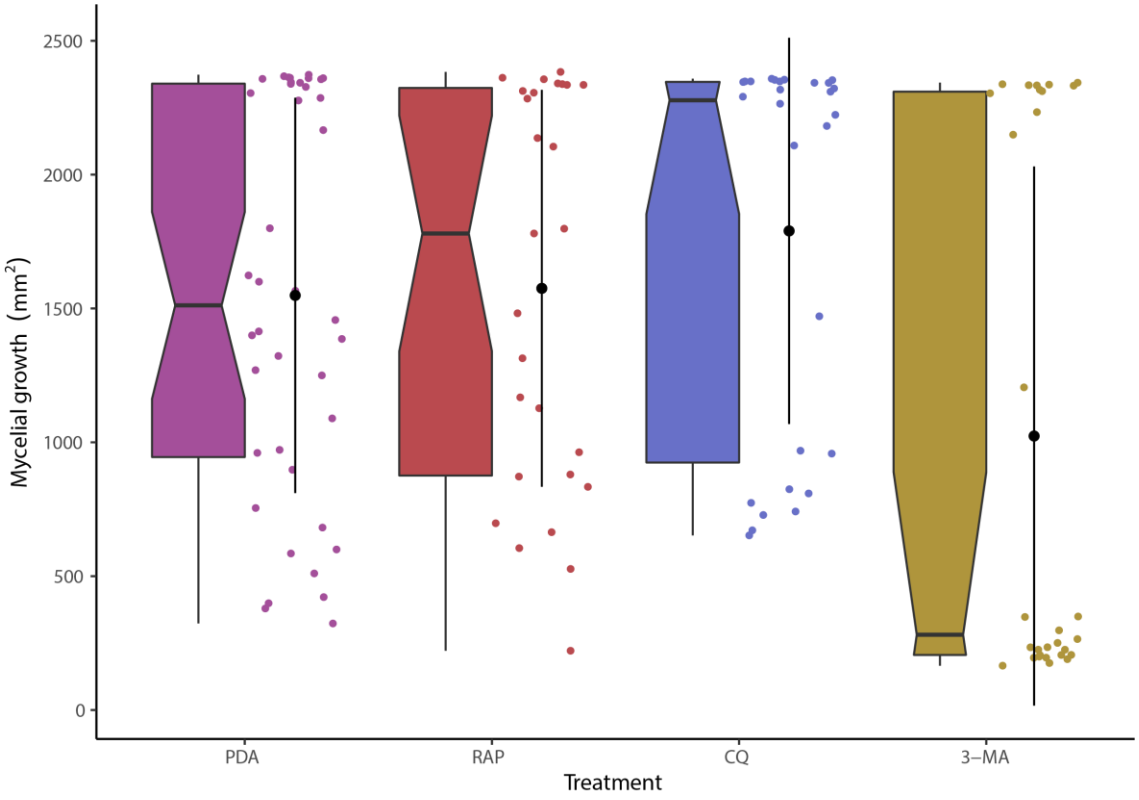


Fig. S1: Counting of gongylidia per staphylae in L. gongylophorus from different leafcutter ants' species. A) Staphylae from in vitro culture without ant manipulation. B-E) staphylae from colonies fungus garden. Scale bars sizes are indicated in each image.



630

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