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Digital supplementary material

Digital supplementary material to

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MicroCT Imaging of internal structures of *Colobopsis explodens*. For imaging of the internal structures of C. *explodens* minor workers, specimens were stored in 70% ethanol and prepared according to the following protocol: Specimens stored in 70% EtOH were transferred to ethanol:Bouins solution (1:1 v/v) for 3h – overnight; then to 70% EtOH (> 30 min); 96% EtOH (> 30 min); 100% EtOH (> 30 min); I2E stain (1% iodine, 96-100% EtOH; METSCHER 2009a, b) overnight; and finally stored in 100% EtOH until scanned. Fixed and stained specimens were then scanned in 100% EtOH. To mount specimens for scanning, they were inserted into pipette tips that were sealed with Parafilm and glued onto Lego blocks. X-ray microtomographic images were made with a high-resolution microCT system (Xradia MicroXCT-200, Zeiss X-Ray Microscopy, Pleasanton, CA) with a tungsten microfocus X-ray source and variable secondary optical magnification. Scans were made with an anode voltage setting of 60 kV and 66 µA with an exposure time of 30 sec for projection images every 0.20° over a half-rotation. Using 4X optical magnification, the specimen was scanned in two parts (anterior and posterior halves of body) and stitched after reconstruction. Images were reconstructed with the Xradia XMReconstructor software, and tomographic reconstructions were exported as image stacks with isotropic voxel sizes of 2.2 µm. The resulting images are shown in Figure S1 and depict the locations of the mandibular gland reservoirs (MGRs) in the body (from head to gaster) of an *C. explodens* minor worker (see also DAVIDSON & al. 2012 for histology). The microCT image data can be downloaded at https://figshare.com/articles/media/Colobopsis_explodens_microCT_image/12770810/1.

Ejection of MGRC without suicidal explosion. Figure S2 depicts the non-explosive COCY species "BBQ" ejecting its MGRC through the mouth openings. The gaster stays intact.

Fig. S2. A "BBQ" ant is ejecting its peach colored MGRC onto the head of an *Oecophylla smaragdina* individual.

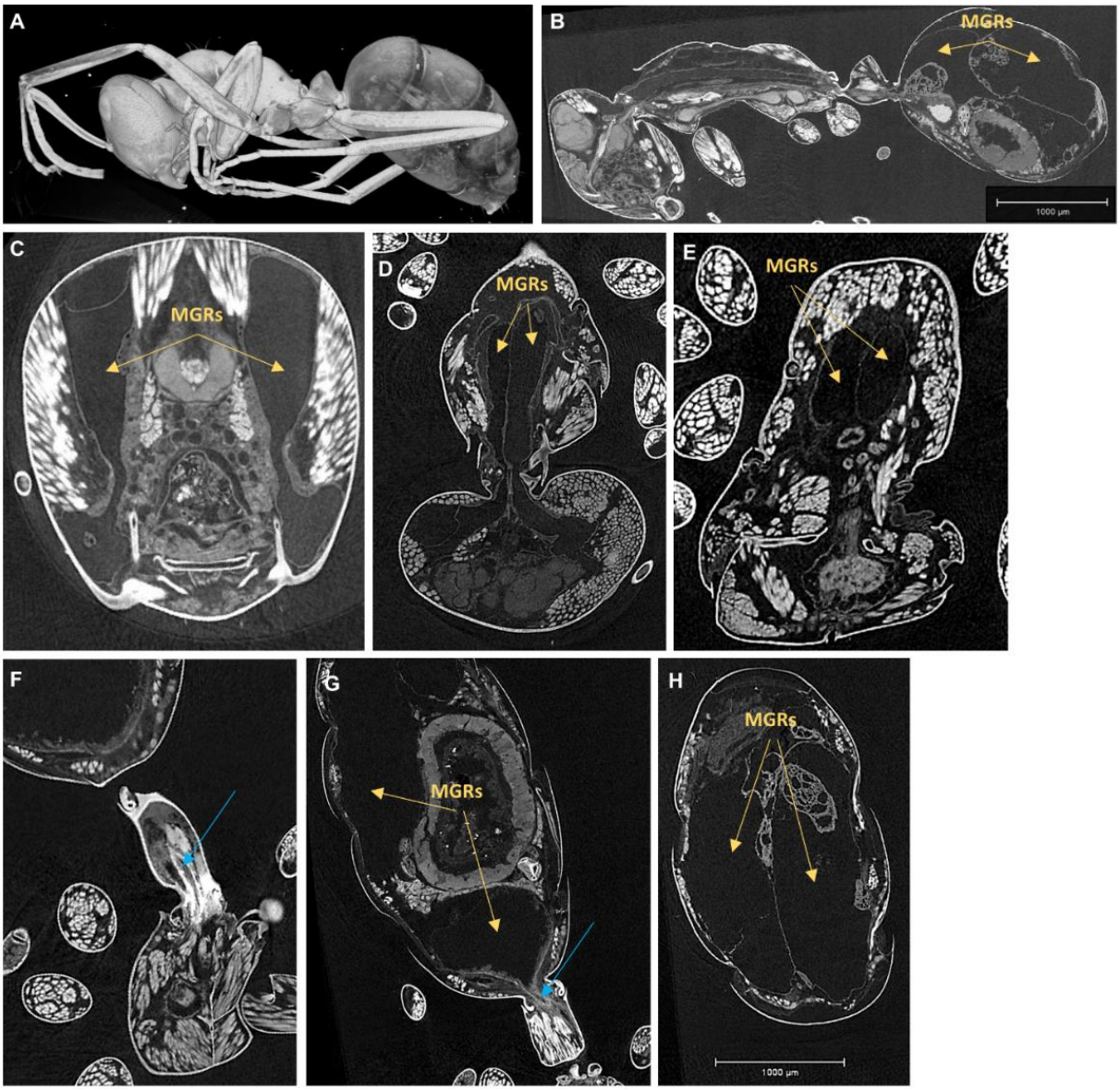

Fig. S1: Internal structures of a Colobopsis explodens minor worker visualized by microCT imaging. Arrows denote mandibular gland reservoirs (MGRs). A: Volume rendering, whole specimen, lateral view. Microtomographic sections: B: sagittal section, whole specimen, lateral view. C: head, frontal view. D: oblique dorsoventral section, posterior head to anterior mesosoma. E: mesosoma, oblique dorsoventral section. F: Anterior petiole to posterior mesosoma, dorsal view, note narrow passage (blue arrow, compare to Fig. 1C in Davidson & al. 2012). G: Posterior petiole to anterior gaster, dorsal view (narrow passage blue arrow). H: Gaster, dorsal view.

Defense behavior of *Colobopsis explodens*. To illustrate the defense behavior of *C. explodens*, confrontation assays were conducted and video recorded. To this end, a *C. explodens* minor worker was put into a Petri dish together with a putative opponent of approximately the same size, and placed under a light microscope (20x magnification) to which an 8 mega pixel eyepiece of an USB video microscope camera (O.W.L. Electronics Limited, Wilnecote, UK) was mounted. The single video sequences were recorded with the O.W.L. micro capture software (version 6.7). The assembled video "Defense behavior of Cexplodens" was annotated and cut using Corel VideoStudio X10 software (Corel Corporation, Ottawa, Canada). The exemplified autothysis behavior of *C. explodens* was similar throughout the tested confrontations. After actively approaching the putative enemy, the *C. explodens* minor worker turned over on its back and seized the opponent by biting into one of its

antennae or into a front leg. After having established this fixed bite-grip the *C. explodens* worker ejected its yellowish viscous MGRC by rupturing of the gastral wall. The secretion targeted large areas of the opponent's body (as mandibles, sensory organs, mesosoma and legs). Subsequently, the rival was restricted in its movements by the first liquid secretion, which dried out and irreversibly glued the opponent to the dead *C. explodens* worker.

In situ antimicrobial assays. The ejection of the MGRC was triggered by the soaking of an ant in 70% ethanol for 3 seconds (Fig. S2). Five µL of a suspension consisting of MGRCs obtained from 20 *Colobopsis explodens* worker ants in 200 µL of water was placed in the middle of a Potato Dextrose Agar (PDA, Sigma-Aldrich, Vienna, Austria) plate which had been inoculated with suspensions (OD_{600nm}: 0.1) of *Penicillium* sp. and *Fusarium* sp. by streaking the swab and incubated for 48 hours at ambient temperature (28-30 °C).

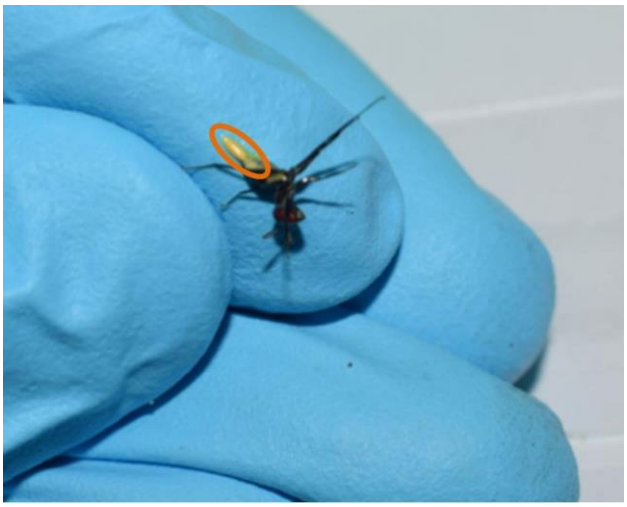

Fig. S3: In situ ejection of the MGRC by a *Colobopsis explodens* worker ant**.** The ant was soaked in 70% ethanol for 3 seconds to release the yellow MGRC (indicated by an orange circle).

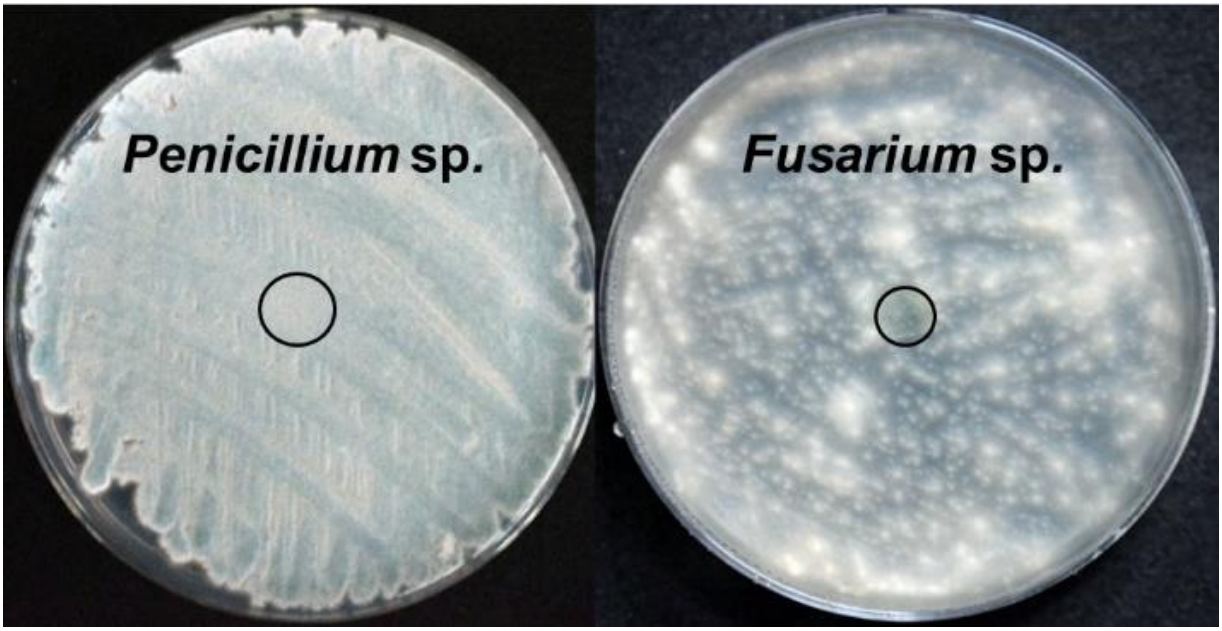

Fig. S4: In situ antimicrobial assays of the MGRC of *C. explodens* against filamentous fungi. Black circles indicate the area in which the MGRC suspension has spread (seen as visible edge of the suspension droplet).

Extraction efficiency. From the four replicate MGRC extractions, the extraction efficiency of the first extraction stage was estimated to be 93% and 94% for MAPG and noreugenin, respectively (Table S1).

Tab. S1: Extraction efficiency for MAPG and noreugenin after four successive extractions. Quantified averaged concentrations of respective compounds in µg mg^{-1} in each of the four successive extractions (n=5 samples extracted in parallel). Extraction efficiency $E\,[\%] = \left(\frac{c_1 - c_2}{c_1}\right) * 100$, whereby c_1 is the concentration of a compound calculated after first extraction and c_2 is the concentration calculated after second extraction.

Extraction	MAPG [µg mg^{-1}]	Noreugenin [µg mg^{-1}]
1	59 ± 5	16 ± 0.9
2	4 ± 0.9	1 ± 0.2
3	0.4 ± 0.1	0.4 ± 0.0
4	n.d.	n.d.
E[%]	93	94

In vitro antimicrobial assays with noreugenin. The antimicrobial activity of noreugenin was assessed by the broth microdilution method in 96-well polystyrene F-bottom plates at concentrations from 0 to 169 mg L^{-1}, as the biological relevant concentrations of (16 ± 0.9) µg of noreugenin per 1 mg MGRC corresponds to a concentration of 80 mg L^{-1} at an assay volume of 200 µL. A 13.3 µg $µL^{-1}$ stock concentration was prepared for noreugenin with DMSO as solvent (as noreugenin was not soluble in acetone). For the tests with the model organisms *Escherichia coli* TUCIM 4420 (Gram-negative, Enterobacterales, Proteobacteria; 16S rRNA, GenBank accession number: NC_000913.2), *Bacillus velezensis* TUCIM 5485 (Gram positive, Bacillales, Firmicutes; 16S rRNA, GenBank accession number: CP006890.1) and *Trichoderma guizhouense* NJAU 4742 (DRUZHININA & al. 2018) Mueller-Hinton Broth (MHB) and for *Debaryomyces* sp. TUCIM 5826 (Saccharomycetales, Ascomycota, Fungi; ITS1 and 2 rRNA, GenBank accession number: MG189903.1) yeast-nitrogen base medium containing noreugenin at concentrations ranging from 0.132 – 340 µg L^{-1} with a final volume of 100 µL were added to 100 µL microbial suspension with a concentration of $5x10^4$ CFU mL^{-1}. A sterility control with MHB only, YNB+1.5% glucose respectively, a growth control without noreugenin as well as a control with DMSO were done in the same microplate. The 96-well plates were then incubated on 28 °C for 24 hours for *E. coli* and *B. velezensis* or for 66 hours for *Debaryomyces sp*. and *T. guizhouense*, and the absorbance at 600 nm was determined after 12 h, 18 h and 24 h of incubation for *E. coli* and *B. velezensis* or after 18 h, 42 h, 66 hours of incubation for *Debaryomyces sp*. and *T. guizhouense*. Each experiment was performed in triplicates. The results showed that noreugenin, when applied in the tested concentration range between 0 and 169 mg L^{-1}, had no effect on the tested model organisms when compared to the control with DMSO only (Fig. S4).

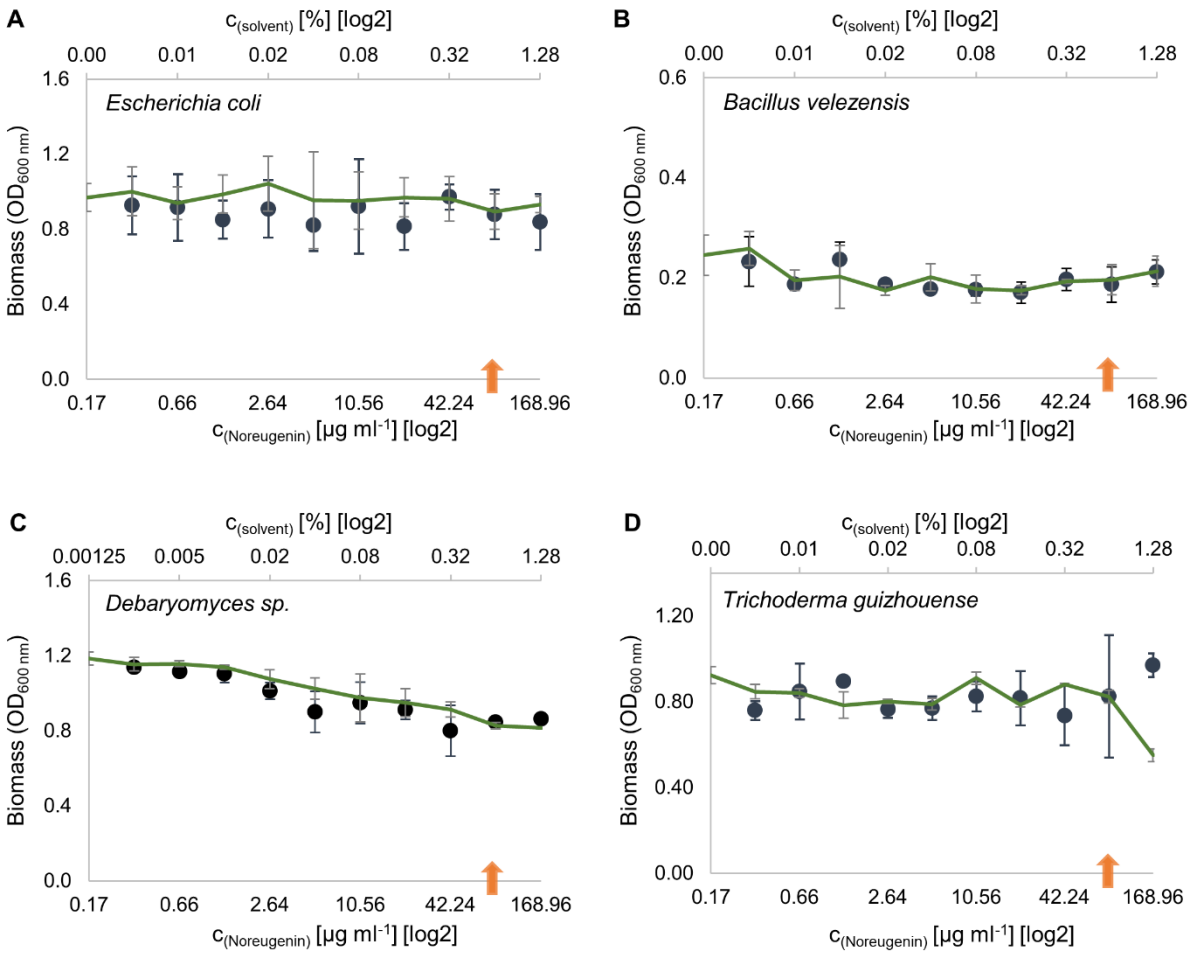

Fig. S5: Antimicrobial activity assay for noreugenin against four model microorganisms *E. coli* (A), *B. velezensis* (B) after 24 hours of incubation, *Debaryomyces sp.* (C) and *T. guizhouense* (D) after 66 hours of incubation. Blue dots indicate growth of the respective microorganism at different concentrations of noreugenin. Green line shows the control, that is the growth of the respective microorganism in presence of the solvent only. The orange arrow indicates the estimated mean concentration of noreugenin that would be present during the assay (80 µg mL^{-1}) based on the measures absolute concentration of this compound in the MGRC of *C. explodens.* Error bars are based on standard deviations calculated from three replicates.

Acidity of MGR secretions. The pH values of MGR secretions ejected by minor workers belonging to different species of the *Colobopsis cylindrica* group were determined in situ. For this, individual ants were stressed by the experimenter by gently touching a leg with forceps until the ants released clear, liquid droplets from the tips of their gasters. Since this liquid exhibited a pungent acid-like smell, we suggest that it originated from the poison gland and presumably contained formic acid (see also MASCHWITZ & MASCHWITZ 1974, JONES & al. 2004). After release of this droplets the ants were held tightly with forceps to trigger the ejection of their MGR secretions, which were subsequently smeared onto pH indicator stripes. The resulting color changes were assessed individually by two different experimenters (Fig. S5). The results showed acidity for all tested MGR secretions (Table S2). The pH value of *C. explodens'* MGR secretion was determined to be in the range of 3.0-3.5 (Table S2). Interestingly, the pH value of the MGRC calculated based on the concentrations of the dominant phenolic compounds MAPG and noreugenin also indicate an acidic milieu of the secretion (Table S3).

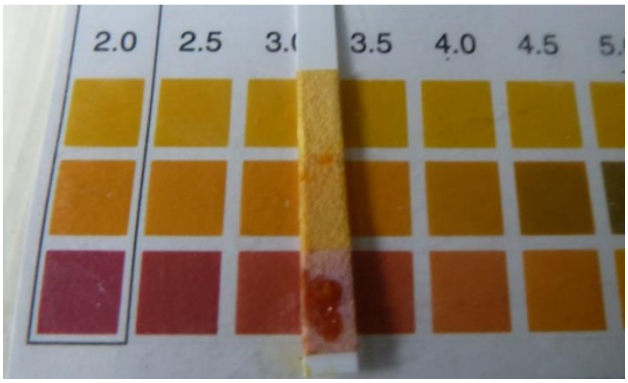

Fig. S6: PH indicator stripe with MGRC ejected by autothysis of one *C. explodens* individual.

Tab. S2: PH values determined for MGRCs of different COCY species. *MGR secretion ejected by autothysis; ΔMGRC ejected via mouth; x…no sample available. Acronyms of COCY species used as in COOK (2008), but taxonomic description is still required).

	pH value of MGR secretion						
Species	sample 1	sample 2	sample 3	sample 4	sample 5	sample 6	sample 7
C. explodens ("YG")	3.5*	3.5*	3.5*	3.5*	3.0*	3.5*	3.0*
"nrSA"	3.0*	2.5Δ	3.0*	3.0*	2.5 - 3.0*	2.5 - 3.0*	2.5 - 3.0*
"SA"	2.0 - 2.5Δ	2.5 - 3.0*	3.0*	2.5 - 3.0*	2.5 - 3.0*	3.0*	2.5 - 3.0*
"BBQ"	2.5 - 3.0Δ	2.0Δ	2.0Δ	2.0Δ	2.5 - 3.0Δ	x	x
"RHOG"	2.5Δ	2.5Δ	2.5 - 3.0Δ	2.5 - 3.0Δ	2.5Δ	2.5 - 3.0Δ	x
"AR"	3.5 - 4.0Δ	3.5*	3.5 - 4.0*	3.5Δ	3.5 - 4.0*	x	x

Tab. S3: PH values calculated for MGRCs of *C. explodens* based on the concentrations of MAPG and noreugenin determined via quantification. [1] pKa…values taken from literature; [2] concentrations of compounds determined via quantification in µg mg^{-1} (see main part of manuscript); [3] concentrations of compounds determined via quantification in g L^{-1} (with assumed density of MGRC of 1); [4] molecular weights of compounds; [5] concentrations of compounds in mol L^{-1}. The pH value was calculated with the formula $pH = 0.5 * (pKa - logc0)$

	pKa[1]	c [µg mg^{-1}][2]	c [g L^{-1}][3]	MW[4]	c [mol L^{-1}][5]	log_{c0}	**pH**
MAPG	8.03	59	59	168	0.35119	-0.45446	**4.24**
Noreugenin	6.65	16	16	192	0.083333	-1.07918	**3.86**

References not mentioned in main manuscript

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