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ORIGINAL PAPER

Nest sanitation through defecation: antifungal properties of wood cockroach feces

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Abstract The wood cockroach *Cryptocercus punctulatus* nests as family units inside decayed wood, a substrate known for its high microbial load. We tested the hypothesis that defecation within their nests, a common occurrence in this species, reduces the probability of fungal development. Conidia of the entomopathogenic fungus, *Metarhizium anisopliae*, were incubated with crushed feces and subsequently plated on potato dextrose agar. Relative to controls, the viability of fungal conidia was significantly reduced following incubation with feces and was negatively correlated with incubation time. Although the cockroach's hindgut contained abundant β-1,3-glucanase activity, its feces had no detectable enzymatic function. Hence, these enzymes are unlikely the source of the fungistasis. Instead, the antifungal compound(s) of the feces involved heat-sensitive factor(s) of potential microbial origin. When feces were boiled or when they were subjected to ultraviolet radiation and subsequently incubated with conidia, viability was "rescued" and germination rates were similar to those of controls. Filtration experiments indicate that the fungistatic activity of feces results from chemical interference. Because Cryptocercidae cockroaches have been considered appropriate models to make inferences about the factors fostering the evolution of termite sociality, we suggest that nesting in microbe-rich environments likely selected for the coupling of intranest defecation and feces fungistasis in the common ancestor of wood cockroaches and termites. This might in turn have served as a preadaptation that prevented mycosis as these phylogenetically related taxa diverged and evolved respectively into subsocial and eusocial organizations.

Keywords *Metarhizium anisopliae* · Termites · Preadaptation · *Cryptocercus punctulatus* · β-1,3-Glucanases · Sociality

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Introduction

The subsocial wood cockroach *Cryptocercus punctulatus* has been considered an ideal species for making inferences about the evolution of ancestral termites (Klass et al. 2008; Nalepa 2011). The phylogenetic relation between extant Cryptocercidae and basal termites together with similarities in the nesting and feeding habits, hindgut microbial communities, behavior, parental care, and altricial offspring suggests that termite eusociality evolved once from a *Cryptocercus*-like ancestor (Cleveland et al. 1934; Grassé 1986; Nalepa 1984, 1994, 2011; Thorne and Carpenter 1992; Grandcolas and Deleporte 1996; Grandcolas 1997; Lo et al. 2000; Nalepa et al. 2001; Bell et al. 2007; Lo and Eggleton 2011; Schauer et al. 2012).

Both *C. punctulatus* cockroach family units and the most basal "lower" termites feed on and nest in decayed wood (Bell et al. 2007). Although such environments buffer these insects from changes in temperature and humidity while providing protection against predators and fostering group living (Hamilton 1978; Kirkendall et al. 1997; Klass et al. 2008), they are also colonized by a dense and diverse microbial

community (Tunaz and Stanley 2009; Ohkuma 2006; Weiss 2006) which can pose significant risks to these xylophagous insects. *Cryptocercus* and lower termites not only possess similar cuticular microbial loads (Rosengaus et al. 2003), but also have similar nutritional requirements. Decayed wood is low in nitrogen and difficult to break down (Cleveland et al. 1934; Ohkuma et al. 2009). Not surprisingly, both *Cryptocercus* and lower termites share numerous prokaryotic and unicellular eukaryotic mutualists in their hindgut that help in the digestion of cellulose (Cleveland et al. 1934; Nalepa et al. 2001; Ohkuma et al. 2009; Schauer et al. 2012). Both *C. punctulatus* and lower termites engage in proctodeal feeding whereby excretion of hindgut fluid containing the necessary mutualistic gut symbionts are transferred via anus-to-mouth exchanges amongst altricial family members (Bell et al. 2007; Nalepa 2011). Finally, nest construction in both *C. punctulatus* and lower termites typically use solid feces, wood, and soil material in combination with saliva and fluid fecal matter, resulting in a cohesive nest structure (Bell et al. 2007). Their fecal material is also used to bury dead nestmates (Rosengaus et al. 1998; Bell et al. 2007). Because fungal overgrowth was never observed in galleries of *C. punctulatus* unless they were abandoned (Bell et al. 2007; Nalepa 2013, personal observation), it was suggested, but not proven, that their feces could have similar fungistatic properties as those reported in the primitive damp-wood termite *Zootermopsis angusticollis*, (Rosengaus et al. 1998; Bell et al. 2007). *C. punctulatus* could benefit from fecal fungistasis by controlling both fungal entomopathogens as well as overgrowth of wood-decaying fungi.

Given that the fossil evidence points to the existence of mycoparasites ~400 million years ago (mya) (Taylor et al. 2005), it is reasonable to assume that the *Cryptocercus*-like cockroach ancestor exploited microbe-rich environments during the late Jurassic–early Cretaceous (~145.0–152.1 mya) when the divergence of termites and the Cryptocercidae likely took place (Burnham 1978; Thorne and Carpenter 1992; Bell et al. 2007). These pathogens could have posed important pathogenic pressures on the common ancestor promoting multiple adaptations to resist bacterial and fungal infections. In turn, these adaptations could have been "carried over" the "sociality threshold" as *Cryptocercus* and termites evolved group living (Rosengaus et al. 2011). We propose that in the common ancestor of *Cryptocercus* and termites, intranidal defecation was likely the consequence of living in and exploiting enclosed rotten environments for extended periods of time. The accumulation of antifungal compounds through feces deposition may have in turn served as a preadaptation fostering not only the evolution of group living (in the form of subsocial or eusocial lifestyles; Nalepa et al. 2001) but also reduced risks of mycosis. Although we can only infer the selection pressures under which subsocial and eusocial behavior evolved in *Cryptocercus* and termites, respectively, there is no doubt that group living in termites increases resistance against pathogenic microorganisms (Rosengaus et al. 2011 and references therein). A similar scenario may have existed at the point of divergence between these two lineages.

The present study first tested the hypothesis that fecal material of the extant *C. punctulatus* has fungistatic activity. Also, through a series of in vitro experiments and the use of chromogenic gels, we investigated a few of the biological properties and origin of such fungistasis.

Methods

Insect collection and maintenance

To test if these feces have antifungal properties, four family units (~30 individuals each) were collected in Blacksburg, VA, USA in March 2009 by Dr. John Wenzel, director of the Center for Biodiversity and Ecosystems of the Carnegie Museum of Natural History. Wood containing cockroaches were placed in plastic boxes ($33\times19\times11$ cm^3) and maintained in the laboratory at 25 °C and lightly sprayed every 1–3 days to keep high humidity. Decayed red oak (*Quercus rubra* collected from Dublin, OH, USA) was added periodically as a supplementary food source.

Preparation of conidia suspensions

The entomopathogenic fungus *Metarhizium anisopliae* was used as a model pathogen. This is a cosmopolitan soil fungus that naturally co-occurs and infects a number of soil-dwelling organisms (Roberts and St Leger 2004). It has been also isolated from sites around *C. punctulatus* nests (Bulmer et al. 2012). Thus, this fungus is an ecologically relevant pathogen. A stock 0.1 % Tween 80 conidia suspension containing 6.4×10^8 conidia/mL was freshly prepared according to Rosengaus et al. (1998). The average conidia germination rate (±SD) was 97.4±6.0 % ($n=30$ fields of vision).

Preparation of conidia–feces suspensions

Five relatively moist fecal pellets (soft to the touch) weighing on average (±SD) 0.176 g (±0.0018; $n=20$ groups of five pellets each) were collected from each of the four different family units. These pellets were likely defecated by the adults and older offspring, given their size. The fecal pellets were placed into a sterile 1.5-mL microcentrifuge tubes and mashed with the use of a flamed but cooled glass rod. Ten microliters of a 6.4×10^7-conidia/mL suspension was added to each of the tubes and immediately mixed with a vortex for 2 min each. Subsequently, to avoid feces from sinking and to ensure a homogeneous conidia–feces suspension, the tubes were placed onto a shaking platform at 30 rpm and 25 °C and

incubated for 1, 3, 12, and 24 h. These conidia–feces suspensions were used to test whether *C. punctulatus* feces negatively affects conidia viability (see below). The possibility exists that the cockroach's proctodeal fluid may also have a significant fungistatic activity. Unfortunately, expressing proctodeal liquid proved unsuccessful; thus, we focused only on testing the fungistatic activity of fecal pellets.

Fungistatic activity of *C. punctulatus* feces

Following each incubation time, 5 μL of the conidia–feces suspension/family was collected and seeded onto a microscope slide containing 1 mL of solidified potato dextrose agar (PDA) layer as described in Rosengaus et al. (1998; $n=3$ replicates). In addition, control slides were also seeded with 5 μL of the 6.4×10^7-conidia/mL suspension lacking cockroach feces. The slides were then placed inside a covered plastic container lined with moist paper towels and incubated for 18 h at 25 °C. Percent germination was estimated by counting the number of conidia with visible germ tubes out of the total number of conidia for each of ten fields of vision per slide (a total of 30 fields of vision per family per incubation time; Rosengaus et al. 1998).

D-δ-Gluconolactone inhibition of β-1,3-glucanase activity

Active β-1,3-glucanase have been detected across several insect species including armyworms, mealworms, and bollworms (Pauchet et al. 2009; Genta et al. 2009; Bragatto et al. 2010) as well as several termite species (Bulmer et al. 2009; Schultheis 2009; Hamilton et al. 2011). Recently, these enzymes were also found in tissues of *C. punctulatus*, including its gut (Bulmer et al. 2012). These enzymes, if excreted along with the feces, could be responsible for the observed antifungal properties as they are known to break down β-1,3-glucans, the main component of fungal cell walls (Bowman and Free 2006). Because D-δ-gluconolactone (GDL) inhibits β-1,3-glucanase activity (Bulmer et al. 2009, 2012), we tested if the loss of enzymatic function rescued fungal growth. To this end, extracts of *C. punctulatus* feces were prepared by macerating 1.2 g of solid cockroach fecal pellets into 150 μL of 200 mM sodium acetate (NaAC). This sample extract was thoroughly mixed and filtered through biomashers (145-μm filter, Omni International) via centrifugation and then divided into two aliquots: one for running antifungal assays on *M. anisopliae* and the other for running chromogenic gels to visualize β-1,3-glucanase activity with and without the inhibitor GDL (see the "Chromogenic gels" section). To test the antifungal activity, 10 μL of feces extract was transferred into 10 μL of either 20 mM GDL or 200 mM NaAC (control). Subsequently, 10 μL of a 6×10^7-conidia/mL suspension was added to the feces/GDL or the feces/NaAC aliquots, which were incubated for 3 h at 25 °C. Five microliters of each sample was then seeded on three microscope slides (as described above). Additional slides seeded with conidia alone or a mixture of 10 μL of 6.4×10^7 conidia/mL and 10 μL of 20 mM GDL (no feces) were similarly prepared and served as controls. Percent germination rates were counted 18 h post-seeding across 30 fields of vision for each treatment.

Chromogenic gels

To test whether feces of *C. punctulatus* contain fungistatic β-1,3-glucanases, cockroaches were cold-immobilized and swabbed with 70 % ethanol, with their entire guts dissected. The latter was sectioned into the fore-, mid-, and hindgut regions. The hindgut region is where mutualistic cellulolytic protozoa are harbored (Bell et al. 2007), and work on *Zootermopsis angusticollis* provides strong evidence that the protozoa are the source of these β-1,3-glucanases (Schultheis 2009; Rosengaus et al. in preparation 2014, unpublished data). Both the hindgut contents and the feces ($n=5$ pellets) were homogenized separately inside a sterile biomasher (145-μm filter, Omni International) with 20 μL of 200 mM NaAC (pH 5.5). The biomashers were then centrifuged at $13.2\times g$ for 10 min at 25 °C. The fraction was collected and mixed with 10 μL of NBS running dye (Bio Rad) before loading 30 μL per lane of a curdlan–Ramazol Brilliant Blue acrylamide 14 % chromogenic gel (Bulmer et al. 2009). Gels were run in Tris–glycine buffer at 50 V until the samples reached the stacking portion of the gel. The voltage was then changed to 150 V for approximately one additional hour. Gels were washed in 100 mM NaAC (pH 5.5) overnight on a shaking platform. Evidence of β-1,3-glucanase activity of gut contents and feces samples can be detected as clearing zones on the gel. In a parallel experiment, cockroach gut contents and feces were treated with the inhibitor GDL (see the "D-δ-Gluconolactone inhibition of β-1,3-glucanase activity" section), and these samples were loaded onto a chromogenic gel as well.

Nature and origin of fungistatic compounds

A series of follow-up experiments were carried out in an attempt to narrow down the nature and origin of the fungistatic compound(s) of the cockroach feces; fecal pellets were boiled, irradiated, or filtered as described in the following subsections.

Heat sensitivity of antimicrobial compounds

To test if the fungistatic effect of the cockroach feces was heat sensitive, five fecal pellets were collected from each of the four *C. punctulatus* families and crushed as described above. Each tube received 20 μL of a 0.1 % Tween 80 solution, and the tubes were placed onto a shaking platform at 30 rpm and 25 °C for 2 min. The tightly closed microcentrifuge tubes

were then placed in boiling water for 30 min. After boiling, the tubes were allowed to cool down to room temperature. Subsequently, 10 μL of a 6×10^7-conidia/mL suspension was added to each of the tubes. The tubes were shaken with a vortex for 2 min followed by their placement onto the shaking platform and then incubated for 1, 3, 12, and 24 h as described above. Following each incubation time, 5 μL of the conidia–feces suspension per family was seeded to quantify germination rates (as described above). Control slides were similarly seeded with 5 μL of a 6×10^7-conidia/mL suspension that lacked cockroach feces.

UV irradiation

To test if fungistatic activity of cockroach feces was related to the microbial community colonizing the fecal pellets, five pellets per family were homogenized and placed in separate sterile disposable polystyrene Petri dishes (100×15 mm^2). Phosphate-buffered saline (1 mL) was added to each dish. The same suspension was then exposed to ultraviolet radiation at 250 nm for 0.5, 1, 2, 4, 6, 8, and 10 min. Following each irradiation time, the samples were centrifuged at $16\times g$, 4 °C for 5 min and then added to 10 μL of a 6.4×10-conidia/mL suspension, thoroughly mixed and immediately plated onto PDA as described earlier. Germination rates were counted 18 h post-seeding. Controls were established in a similar fashion, except that the feces/PBS buffer did not undergo irradiation. A second control treatment included plating untreated conidia (no UV and no feces) to calculate the baseline conidia viability of our original conidia suspension.

Because some natural antibiotic compounds such as quinolones, tetracyclines, sulfonamides, tylosin, and nitrofuran may be photodegradable while others are not (Thiele-Bruhn and Peters 2007; Kümmerer 2009), the UV protocols described above were repeated. In this instance, however, the UV-irradiated feces underwent DAPI staining (DAPI FluroPure Grade) to quantify the number of living microbial cells following UV treatment. Using sterile syringe filtration, the feces were removed from the buffer and mixed with 5 μL of a 0.1-mg/mL concentration of DAPI stain in the dark and left undisturbed for 15 min at 25 °C. Using UV microscopy, the luminescence of DNA from live cells colonizing the feces was detected. Live microbial cells (i.e., fluorescence) were enumerated as a function of UV treatment duration (0, 4, and 10 min). Live microbes were counted for five fields of vision for each of the three UV time exposures.

Filtration of feces extracts

To pinpoint if the presence of the microbial consortia colonizing the feces (the native gut microbes excreted along with the feces and/or the secondarily acquired microbes once feces were defecated) was responsible for the antifungal activity, feces extracts were filtered through a 0.22-μm filter (Spin-X , Costar), thus creating a microbe-free feces filtrate. *M. anisopliae* conidia (20 μL of a 6×10^7-conidia/mL suspension) was incubated in a shaking platform (200 rpm; 25 °C) along with 20 μL of either unfiltered or filtered fecal extracts for 3 and 24 h. If conidia viability improved when microorganisms larger than 0.22 μm were filtered out, then the microbial cells are likely responsible for the feces fungistatic effects. Such effects could be due to the synthesis of bioactive antifungal compounds by the microbes themselves (chemical interference) and/or competitive exclusion between such microbes and *M. anisopliae* (Chouvenc et al. 2012). On the other hand, if conidia viability remained reduced relative to controls (no feces) but the magnitude of the fungistasis was similar between the filtered (microbe-free) and unfiltered (with microbes) extracts, then chemical interference is likely the mechanism by which fungistasis occurred.

Results

Fungistatic activity of *C. punctulatus* feces

Conidia incubated with feces of each of the *C. punctulatus* families had significantly lower viability than that of controls (Fig. 1; $X^2=127.6$, 119.1, 118, 125; $df=2$, $p<0.0001$, Kruskal–Wallis test). The fungistatic effect of feces did not differ significantly across the four families (Fig. 1; $X^2=5.3$, $df=3$, $p\geq0.1$, Kruskal–Wallis test), but conidia germination

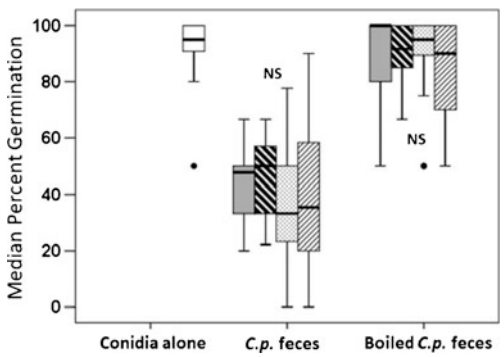

Fig. 1 Median percent germination of fungal conidia (±interquartile range) following mixing with fresh and boiled feces of four different family units and immediately plated onto potato dextrose agar (*gray*, *thick diagonal black and white*, *dotted*, *thin antidiagonal black and white*). For comparison, the percent germination of control untreated conidia is included (*white*). No significant (*NS*) differences in the germination rates across the four families were observed when conidia were incubated with *C. punctulatus* (*C.p.*) feces ($X^2=5.3$, $df=3$, $p>0.05$, Kruskal–Wallis test) or when conidia were incubated with boiled feces ($X^2=6.6$, $df=3$, $p>0.05$, Kruskal–Wallis test). However, relative to our control (conidia alone, *open bar*), germination was significantly lower when conidia were incubated with *C.p.* feces ($X^2=127$, $df=3$, $p\leq0.0001$). Outliers, identified by *small filled circles*, include cases with values 3 box lengths from the edge of the box

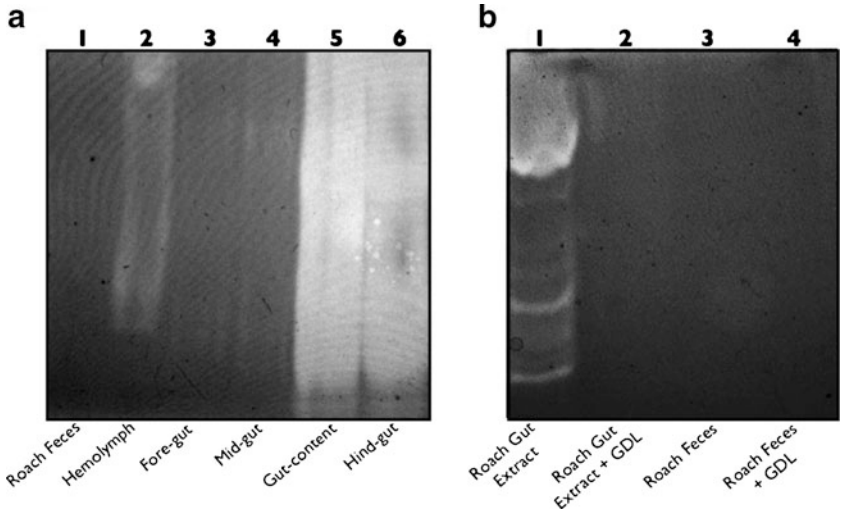

Fig. 2 **a** Curdlan–Ramazol Brilliant Blue chromogenic gel showing a lack of β-1,3-glucanase activity in the cockroach fecal pellets (*lane 1*), but an abundant presence of these enzymes in the hindgut of *C. punctulatus*. **b** *Lane 1* loaded with cockroach gut extract, *lane 2* cockroach gut extract and the inhibitor GDL, *lane 3* cockroach feces extract, *lane 4* loaded with cockroach feces extract and GDL. The addition of GDL eliminates the activity of the gut extract (*lane 2*) but does not affect the profile of the cockroach's feces extract (*lane 3* vs. *lane 4*). Hence, β-1,3-glucanases are not responsible for the fungistatic properties of the roach feces as activity in normal feces was lacking and conidia viability was not rescued when extracts of *C. punctulatus* feces and GDL were incubated with fungal conidia (Table 1)

was significantly reduced as incubation time increased (X^2= 81.7, df=3, $p \leq 0.0001$, Kruskal–Wallis test, Fig. 1 Supplemental Material). Because conidia incubated with boiled *C. punctulatus* fecal material had a viability that approached that of controls (Fig. 1, mean rank=270 and 227, respectively; Mann–Whitney U test=10,637, z=−2.1, p=0.03), we concluded that the fungistatic compound(s) was (were) heat sensitive. Boiling, however, not only denatured any putative antifungal enzymes excreted by the cockroach, but also eliminated the microbial community naturally associated with the feces. To narrow down between these two possibilities, we ran chromogenic gels together with the GDL experiments as well as the UV exposure and filtration experiments below.

Chromogenic gels

Our results, similar to those of Bulmer et al. (2012), indicate that *C. punctulatus* contains β-1,3-glucanase activity in its hindgut region (Fig. 2a). To our surprise, however, cockroach feces contained no detectable β-1,3-glucanase activity (Fig. 2a). When the inhibitor GDL was added to the gut extracts, the enzymatic activity within the gel was clearly eliminated (Fig 2b). Yet, GDL-treated feces continued exhibiting antifungal activity (Table 1; Fig. 2b). Taken together, our results indicate that the antifungal properties of cockroach feces resulted from the excretion of unknown heat-sensitive compounds produced by the cockroach itself, the hindgut symbiont consortia, or the acquired secondary microbial community following defecation. Contrary to expectation, the antifungal activity of fecal pellets is not due to the excretion of β-1,3-glucanases.

UV exposure, DAPI staining, and filtration experiments

Since microbes are known to be the source of the many antibiotic compounds (Thomashow et al. 2008), we treated feces with UV radiation to destroy the microbial assemblage. However, because some antimicrobials may be also photodegradable (Thiele-Bruhn and Peters 2007), the results of the

Table 1 Median percent germination±MAD after fungal conidia were incubated with and without feces treated with GDL

	Treatment				
	Conidia alone	Conidia and GDL	Conidia and feces extract	Conidia, feces extract, and GDL	p value
Median percent germination±MAD	100±0 a	100±0 a	35.4±14 b	42.8±7 b	Overall X^2=98.3, df=3, $p \leq 0.0001$

The overall comparison was significant (Kruskal–Wallis test). Treatments followed by different letters represent significant differences in pairwise comparisons following a Bonferroni correction

MAD median absolute deviation

UV experiment are inconclussive unless interpreted alongside the DAPI staining and filtration experiments. Such results suggest that the observed fungistatic activity was likely of microbial origin since conidia viability was reestablsihed with increased UV duration (Fig. 3) while negatively correlated with number of live microbes in the feces (Fig. 4; Pearson's correlation=−0.8, df=13, $p \leq 0.0001$). Moreover, our filtration experiments indicate that relative to the control conidia [mean rank=60.7 (3 h) and 56.6 (24 h)], both unfiltered [mean rank=33.1 (3 h) and 37.9 (24 h)] and filtered [mean rank=42.6 (3 h) and 42.1 (24 h)] feces had reduced conidia viabilities (overall X^2=17.8, df=2, $p \leq 0.0001$ at 3 h and X^2=9.1, df=2, $p \leq 0.01$ at 24 h). The fact that the mean rank conidia viabilities between unfiltered and filtered feces extracts were not significantly different after a 3-h incubation [mean rank=26.9 vs. 34.1, respectively; (Mann–Whitney U test=341, z=−1.6, p=0.10)] or 24-h incubation [mean rank=28.5 vs. 32.5, respectively; (Mann–Whitney U test=390, z=−0.9, p=0.3)] suggests that the fungistatic activity of the feces is due to chemical interference rather than competitive exclusion, as microbial cells were absent in the filtered extract. The bioactive compound(s) produced by the microbial community likely passes through the filter resulting in the recorded lower conidia viability. Interestingly, such compound(s), in the albescence of its secreting microbial community, do not appear to have differential fungistasis with longer incubation time (mean ranks=973.5 and 856.5 for 3 and 24 h, respectively; Mann–Whitney U test=391.5, z=−0.8, p=0.4). This suggests that the presence of a continuous biosynthetically active microbial community colonizing the feces is required for maximum antifungal activity: while higher fungistasis of unfiltered feces was recorded as incubation time increased (Fig. 1, Supplemental Material), no differences in antifungal effects were recorded for filtered feces (without its microbial consortia). Whether the microbes synthesizing the antifungal compound(s) are native to the cockroach's gut microbiome, secondarily acquired from the nest environment once pellets are defecated, or a combination of both remains to be determined.

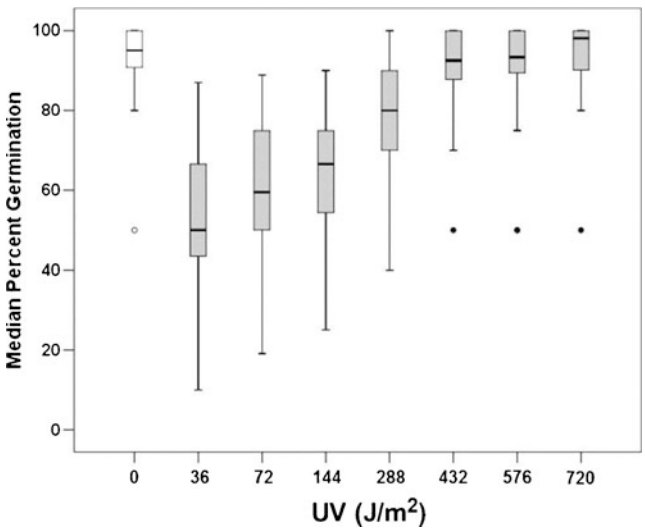

Fig. 3 Median percent germination (±interquartile range) of conidia incubated with fecal material previously exposed to UV radiation for various time intervals. Germination rates were significantly different as a function of UV treatment (X^2=696.5, df=7, $p \leq 0.0001$, Kruskal–Wallis test). The increased irradiation times (0, 0.5, 1, 2, 4, 6, 8, and 10 min) correspond to the joule per square meter (J/m^2) units on the x-axis. Outliers are identified by *small circles* which include cases with values 3 box lengths from the edge of the box. For ease of comparison, the white box indicates the median percent germination of conidia with no feces added

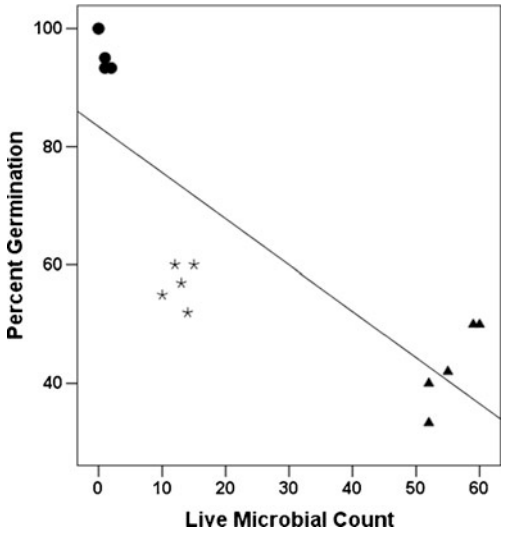

Fig. 4 Scatter plot of conidia germination as a function of live microbial loads colonizing feces following different UV irradiation times (*black-filled triangle*=0 min, *asterisk*=4 min, is *black-filled circle*=10 min). *Line* represents best fit relation between the two variables (overall R^2=0.643). Irradiation time was negatively correlated with live microbial loads (Pearson's correlation=−0.90, df=13, $p \leq 0.0001$). Irradiation time and conidia germination were positively correlated (Pearson's correlation=0.96, df=13, $p \leq 0.0001$) as the microbial load of feces decreased with irradiation time. Note that two of the five data points for the 10-min UV irradiation treatment are overlapping at coordinates (0, 100)

Discussion

Given the phylogenetic relationship and the notably shared nesting and feeding habits, nutritional needs, microbial mutualism, and behavioral characteristics between *C. punctulatus* and the lower Isoptera, termite eusociality likely evolved once from a subsocial, *Cryptocercus*-like ancestor (Bell et al. 2007). Although only four families were tested here, the lack of variability in the fungistatic activity of feces among them (Fig. 1) suggests that this phenomenon is widespread and not necessarily influenced by genetic variability and/or differences in wood diet and/or the accompanying ingestion of different plant secondary compounds.

Our results indicate that, similar to the basal damp-wood termite *Z. angusticollis* (Rosengaus et al. 1998), *C. punctulatus* fecal pellets have fungistatic properties. The exact source(s) of

these compounds, however, remains elusive. Recently, the presence of several β-1,3-glucanases was visualized in the gut of *C. punctulatus* (Bulmer et al. 2012). Because of the abundant β-1,3-glucanase content in their hindguts (Fig 2a; Bulmer et al. 2012) and the fact that these enzymes have proven a potent antifungal activity (Bulmer et al. 2009, 2012; Schultheis 2009; Hamilton et al. 2011), it was reasonable to assume that these enzymes were responsible for the antifungal properties of the feces. Yet, our data indicate that β-1,3-glucanases are not incorporated into and excreted along with the cockroach's solid waste material in spite the high levels of enzymatic activity in their hindguts (Fig. 2a). This observation strongly suggests that these enzymes are reabsorbed at the distal portion of their digestive system (Fig. 2, Supplemental Material). The insect's rectum is known for its resorptive power (Ramsay 1971). The questions of whether *C. punctulatus* sequesters these antifungal enzymes at the region of the rectum before defecation and the mechanisms by which this is achieved remain to be addressed. Comparative analyses of the presence of active β-1,3-glucanases in the fecal pellets of the damp-wood *Z. angusticollis* and subterranean *Reticulitermes flavipes* termites suggest that the reabsorption of β-1,3-glucanases is not unique to *C. punctulatus* (Fig. 2, Supplemental Material).

The lack of β-1,3-glucanases in the solid feces points to other alternative sources of antifungal activity including the cockroaches' endogenous glandular secretions, the insects' symbiotic gut microbiota (the native gut flora such as bacteria, protozoa, and fungi), or the secondarily acquired microbial community naturally associated with soil and/or decayed wood that may also exploit the cockroach's feces. Either of these alternative sources on its own or in combination may explain the feces' antifungal activity.

The fungistatic properties of cockroach feces may have resulted from the inclusion of glandular compounds into their fecal material. Cockroaches in general have numerous glands (Batra and Batra 1966; 1973; Olagbemiro et al. 1988). Up to 93 compounds have been isolated, and of these, several appear to have fungistatic activity in other insects (Brossut and Sreng 1985; Dillon and Charnley 1986, 1995; Bell et al. 2007). The fact that such glandular secretions are spread throughout their body via intense self-grooming is reminiscent of the protective metapleural gland secretions of many ants (Schluns et al. 2009). The possibility exists that some of these glandular secretions become incorporated into the feces before and/or during defecation. A second and nonexclusive alternative source of fungistatic compounds includes the secretion and excretion of bioactive molecules by the mutualistic bacterial and protozoan symbionts inhabiting the digestive tract of these cockroaches. While the bacterial community throughout the entire cockroach's gut includes members of the phyla Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes, Synergistetes, Verrucomicrobia, and candidate phylum Termite Group 1, the eukaryotic protozoa, mostly located in the cockroach's hindgut, are members of the orders Trichomonadida, Cristamonadida, Hypermastigida, and Oxymonadida (Berlanga et al. 2009). These microorganisms, many of which are shared with the lower termites, may synthesize and excrete antimicrobial compounds into the gut's lumen and ultimately become incorporated onto the feces. Their presence and metabolic by-products in the guts of wood cockroach and several lower termite species may help explain why fungal conidia become unviable during their transit through the alimentary canals (Chouvenc et al. 2009, 2010). Such symbiont-mediated protection has been recognized as an important interaction that renders the insect hosts less susceptible to infection (Oliver et al. 2003; Scarborough et al. 2005; Moran 2006; Haine 2007; Schultheis 2009).

Finally, following defecation, the cockroach's feces may acquire microorganisms from the surrounding environment, with up to a 100-fold increase in the microbial load from the time of their deposition (Bell et al. 2007). Such microbial colonizers may use feces as a suitable substrate as these possess relatively high moisture content, high surface area, relatively high pH, and nutritional resources that support microbial growth (Bell et al. 2007). The results of the boiling, UV and filtration experiments, as well as those from DAPI staining indicate that feces are imbued with heat-sensitive fungistatic compounds likely of microbial origin. Unfortunately, while there has been intensive effort to identify the prokaryotic and eukaryotic gut microbiota of these cockroaches (Bell et al. 2007; Berlanga et al. 2009; Ottesen and Leadbetter 2010), little is known about the microbial communities colonizing cockroach feces which have been described as containing protozoan cysts, bacterial cells, and spores (Bell et al. 2007). It should not be surprising that microbes colonizing feces may be the source of antifungal compounds. Potent antibiotics have been isolated from a variety of microbes. A case in point is the widespread mutualistic association of Actinobacteria (i.e. *Streptomyces*) with both solitary and social insects (Currie et al. 2003; Kaltenpoth et al. 2006; 2009; Hulcr et al. 2011; Mathew et al. 2012; Chouvenc et al. 2013).

Studies on the ecological significance of defecation and coprophagy as well as the variety of adaptations evolved by insects for dealing with waste disposal and/or management have indicated important costs and benefits associated with this most basic consequence of feeding (Weiss 2006; Jackson and Hart 2009). Costs of feces accumulation include prey attraction and, consequently, high predation rates (Weiss 2006). However, some insects capitalize on the accumulation of their own excrement to use as physical or chemical (toxic or noxious) defenses to deter natural enemies, camouflage, building material, mate attractants, adornments, and/or fertilizers (Weiss 2006). Additionally, defecation within the nest provides a substrate to a diverse microbial community. The accumulation of antimicrobial by-products from these communities and/or competitive exclusion among members of the

microbial consortia can reduce fungal growth and ultimately render these insects less susceptible to infection (Chouvenc et al. 2013). Our filtration results indicate that the microbial community negatively affects *M anisopliae* development by chemical interference.

Currently, it is impossible to conclude that intranidal defecation evolved as a specific mechanism to cope with pathogens or if restricted fungal growth within the nest was just a beneficial consequence of the microbial community colonizing wood cockroach feces. Regardless of the original selection pressures favoring within-nest defecation, the coupling of both behavioral responses (coprophagy, proctodeal feeding, burial of dead nestmates) and biochemical protection by the feces (either through glandular secretions, its associated acquired microbiota, and/or hindgut symbionts from the host) likely reduced mycosis risks during the evolution and further diversification of the ancestral cockroach that gave rise to *C. punctulatus* and the Isoptera. The fact that the extant *C. punctulatus* cockroach shares many life history attributes with lower termites, including exploiting microbe-rich environments, points to the possibility that the subsocial cockroach-like prototermite may have benefitted from reduced risks of mycosis through intranidal defecation (Rosengaus et al. 2011). Nest sanitation through defecation may have been (and still be) an adaptive and common trait in other soil-dwelling and xylophagous organisms, particularly if large amounts of feces accumulate within a nest due to the insect's social lifestyle.

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Author contributions RBR conceived and designed the experiment, analyzed the data, and wrote the manuscript. KM performed all the germination enumeration and DAPI staining. RBR together with WD, RB, and VG ran the glucanase gels and GDL experiments, while RB was involved in running all filtration experiments.

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