Hindawi Publishing Corporation
Psyche
Volume 2013, Article ID 162964, 7 pages
http://dx.doi.org/10.1155/2013/162964

Research Article

Carcass Fungistasis of the Burying Beetle *Nicrophorus nepalensis* Hope (Coleoptera: Silphidae)

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Received 14 January 2013; Accepted 8 April 2013

Academic Editor: Vladimir Kostal

Our study investigated the fungistatic effects of the anal secretions of *Nicrophorus nepalensis* Hope on mouse carcasses. The diversity of fungi on carcasses was investigated in five different experimental conditions that corresponded to stages of the burial process. The inhibition of fungal growth on carcasses that were treated by mature beetles before burial was lost when identically treated carcasses were washed with distilled water. Compared with control carcasses, carcasses that were prepared, buried, and subsequently guarded by mature breeding pairs of beetles exhibited the greatest inhibition of fungal growth. No significant difference in fungistasis was observed between the 3.5 g and the 18 to 22 g guarded carcasses. We used the growth of the predominant species of fungi on the control carcasses, *Trichoderma* sp., as a biological indicator to examine differences in the fungistatic efficiency of anal secretions between sexually mature and immature adults and between genders. The anal secretions of sexually mature beetles inhibited the growth of *Trichoderma* sp., whereas the secretions of immature beetles did not. The secretions of sexually mature females displayed significantly greater inhibition of the growth of *Trichoderma* sp. than those of sexually mature males, possibly reflecting a division of labor in burying beetle reproduction.

1. Introduction

Burying beetles (*Nicrophorus* spp.) use small vertebrate carcasses as food for their larval broods by depositing their eggs around a buried carcass [1, 2]. Carcasses are nutritious yet rare resources [3, 4]. During the lifetime of a beetle, it may find only one carcass that is suitable for reproduction [5]. Competition for carcasses is intense [6–8], and burying beetles of the same or different species may fight to maintain occupancy of the carcass [1, 9–11].

Bacterial and fungal decomposers destroy carcasses, and scavenging animals have evolved behavioral and physiological counterstrategies to maintain food sources [12]. Before burying a carcass, the burying beetles remove the fur or feathers from the carcass, compact the carcass by rolling it repeatedly, and smear its surface with their anal secretions [1]. Carcasses used by beetles typically vary in size from 1 to 75 g [9, 10, 13] and are encountered in variable states of decay. Burying beetles exhibit adaptive strategies that enable them to manage the carrion resources in such diverse conditions, such as adjusting the number of eggs laid [13, 14] and practicing infanticide [15, 16], with the number of surviving larvae positively correlated with carcass size [9, 10, 17].

Although the loss of biomass resulting from microbial growth on a carcass is not large, microorganisms often produce toxins that can affect beetle-larvae survival [18–20]. The oral and anal secretions of various burying beetle species have bacteriostatic effects [21]. The oral secretions contain phospholipase A_2 that may disrupt the cell membranes of bacteria [22]. Fungal growth may also be inhibited following the preburial treatments by burying beetles [23]. The temperature and the composition of food materials can influence the antimicrobial activity of the oral secretions [24], and the antibacterial activity of the anal secretions has been shown to be upregulated following the discovery of carrion [25].

Burying beetles' preference for appropriately sized carcasses for reproduction may be related to their capacity to secrete antimicrobial substances [26]. Although burying

beetles can feed more offspring on a larger carcass, the energy expenditure for the preburial preparation of larger carcasses is also higher. Scott [27] proposed that microorganisms are more serious competitors on larger carcasses because of the difficulties associated with preburial preparations. Scott [27] also reported that mold often renders substantial amounts of large carcasses unusable, and Hwang and Shiao [26] reported that large carcasses decay more rapidly than small carcasses, resulting in lower trophic efficiency for large carcasses. Therefore, communal breeding observed in some species of burying beetles may prevent the decay of a large carcass, contributing to better breeding efficiency [28–31].

Both uniparental and biparental breedings are commonly observed in burying beetles [8, 32]. Females reproducing without the assistance of a male do not display reduced reproductive success [33]. However, with the help of a male, the carcass can be better preserved [6, 34], and dipteran larvae and conspecific competitors can be more efficiently excluded [32, 35–38]. In addition, the male can also substitute for the female in brood care [39]. However, because the primary role of the male in brood care is providing protection against competitors, we propose that the female likely makes a greater antimicrobial contribution to the carcass.

In our current study, we assessed fungistasis in carcasses in the laboratory that were colonized by *Nicrophorus nepalensis*, a common burying beetle in southern Taiwan. We investigated whether the fungistatic efficiency of beetles correlated with the sexual maturity or the sex of the parent, and we examined whether fungistatic capacity of beetles was sex or age dependent.

2. Materials and Methods

2.1. Field Collection and Laboratory Rearing of Beetles. The *N. nepalensis* Hope beetles were collected using 15 hanging pitfall traps that were baited with 40 g of chicken meat each and placed at 100 m intervals along the Fengkang Forest Road (22°00′N, 120°41′E) in Kaohsiung City in southern Taiwan at altitudes of 1100 to 1600 m above sea level from January to July, 2009. All field-collected beetles were anesthetized with carbon dioxide, and any mites were removed under a microscope using forceps. To avoid the influence of parasites, only the laboratory-reared F1 and F2 offspring of field-collected beetles were used in our experiments. All beetle cages used in our study were 10.4 × 10.4 × 6 cm transparent plastic containers. All the beetles used in our study were reared at 20°C with 12 h light-dark cycling.

A breeding pair of field-collected beetles and a 20 g mouse carcass were added to a cage with 4 cm thick moist peat. Following oviposition, the eggs were removed and placed on wet toilet paper in an 8.5 cm Petri dish for hatching. The larvae and the parents were transferred to a new cage with 1 cm thick moist peat. When the larvae emerged from the burrow to pupate, up to 8 were placed in a new cage with 4 cm thick moist peat. Groups of up to 6 newly eclosed adults of the same sex were transferred to new cages with 3 cm thick moist peat. Prior to the fungistasis experiments, the laboratory-reared adult beetles were fed twice a week with freshly decapitated *Tenebrio molitor* or cut sections of *Zophobas morio*. No beetles were exposed to carcasses before being used in the fungistasis experiments.

2.2. Experimental Design. To investigate whether preburial preparations affect fungal growth on carcasses, fungal growth on mouse (ICR strain) carcasses was assessed in five different conditions. All fresh frozen mice were purchased in CMLAC in National Taiwan University and thawed before the experiments. Untreated (control) carcasses, treated carcass balls, washed carcass balls, protected carcass balls, and large protected carcass balls were examined for visible fungal growth over the course of 14 days under the standard rearing conditions. The control carcasses (approximately 3.5 g) were not exposed to burying beetles and were placed on the surface of the moist peat in an otherwise empty cage. The treated carcass balls were obtained at 3 days following presentation of a carcass (approximately 3.5 g) to a mating pair of sexually mature adults by removing the carcass immediately after burial. During this stage, the fur removal, the carcass compaction, and the deposition of anal excretions had occurred prior to the removal of the carcass, but larval hatch had not yet occurred. The treated carcasses were each transferred to a new cage with moist peat and no beetles. The washed carcass balls were obtained using the same procedure as the treated carcass balls, with an additional step in which the carcass balls were rinsed with distilled water before being transferred to a new cage. The protected and large protected carcass balls were obtained using the same procedure as the treated carcass balls, except that the same mating pair of beetles was added after the carcass was transferred to a new cage, and 18 to 22 g mouse carcasses were used for the large protected carcass balls. To remove newly oviposited eggs, the carcass ball and the adult beetles were transferred to a new cage daily, with the transfers performed under red light to avoid disrupting the light-dark cycle.

2.3. Cultivation and Identification of Carcass Fungi. Any fungus that grew on a carcass within 14 days in any of the 5 experimental conditions was cultivated for identification. Solid malt extract agar (MEA) medium was prepared from 26 g of malt extract agar (Fluka) in 500 mL distilled water and sterilized at 121°C for 15 min. To prepare the solid cornmeal agar (CMA) medium, 10 g of cornmeal was boiled in distilled water. Following filtration, the volume of the cornmeal filtrate was adjusted to 500 mL, and 10 g of agar (Sigma-Aldrich, St. Louis, MO, USA) was added before sterilization at 121°C for 15 min. After cooling the media to 50°C, 40 ppm of Streptomycin sulfate and 40 ppm of Penicillin G were added to both the MEA and CMA media, and the media were poured into Petri dishes before solidification. A hypodermic needle was used to remove a specimen of the carcass skin containing the fungi, and the specimen was used to inoculate MEA plates, and the fungi were cultured for 7 days at 25°C. The fungi cultured on MEA plates were used to inoculate CMA plates that were subsequently cultured at 25°C. The cultured fungi were dyed with cotton blue in lactoglycerol, and the various taxa were identified to genus or species.

2.4. Fungistasis Quantification Assays. To examine differences in the fungistatic capacity of the anal secretions from males versus females and sexually mature adults versus sexually immature adults, the anal secretions were collected from each and used in fungistasis quantification assays. We observed that *Trichoderma* sp. was the predominant species on control carcasses and that *Trichoderma* sp. growth was inhibited on carcasses treated by sexually mature beetles. Therefore, we used *Trichoderma* sp. growth as a biological indicator of the fungistatic efficiency of burying beetle secretions. Green colonies of *Trichoderma* sp. formed on CMA plates after culturing for 7 days at 25°C. One colony was suspended in 300 μL of ultrapure water. Inoculums were prepared by mixing 30 μL of the fungal suspension with 30 μL of ultrapure water (control group) or 30 μL of anal secretions from male or female beetles that were taken at 6 days (sexually immature) or 35 days (sexually mature) after eclosion, and 2 μL of each inoculum was separately used to inoculate CMA plates that were subsequently cultured at 25°C. The secretory volume of each individual was different; 30 μL of anal secretions were collected from different individuals. The number of *Trichoderma* sp. colonies present on the CMA plates at 7 days after inoculation was recorded. The number of days at which the fungal growth reached confluency on the CMA plates was also recorded.

2.5. Statistical Analysis. We used the Fisher exact test to compare the fungal growth in the various experimental conditions, and we used a χ^2 test to compare the differences in the fungal species that were isolated in each set of experimental conditions. An independent sample Student's t test was used to examine the differences in fungistatic efficiency between sexually mature males and females. All statistical analyses were conducted using the SPSS version 17.0 computer software, with an alpha value of 0.05 as the accepted level of statistical significance.

3. Results

3.1. Inhibition of Fungal Growth on Carcasses. Compared with the control carcasses ($n = 19$), fungal growth was significantly inhibited on the treated and protected carcass balls (Fisher exact test: treated carcass balls: $P < 0.01$, $n = 18$; protected carcass balls: $P < 0.001$, $n = 16$). However, the fungistatic effect was significantly greater on protected carcass balls than on treated carcass balls (Fisher exact test: $P = 0.01$). The fungistasis on the washed carcass balls ($n = 18$) was not significantly different than that of the control carcasses (Fisher exact test: $P = 0.08$), and the fungistasis on the protected carcass balls ($n = 16$) was not significantly different than that of the large protected carcass balls ($n = 17$; Fisher exact test: $P = 0.68$; Figure 1).

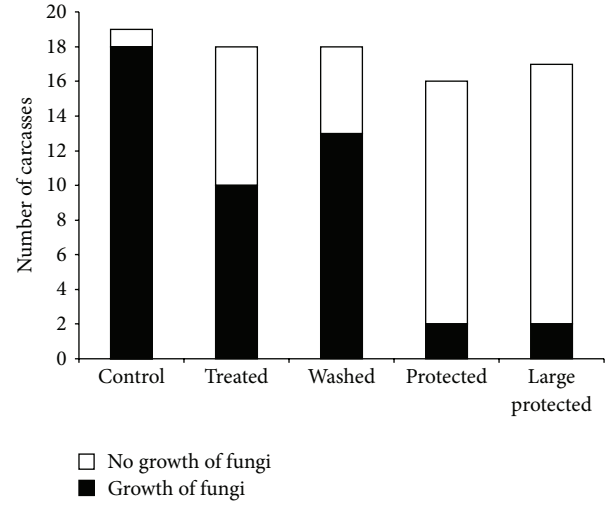

Figure 1: Number of control carcasses (untreated, $n = 19$), treated carcass balls ($n = 18$), washed carcass balls ($n = 18$), protected carcass balls (3.5 g, $n = 16$), and large protected carcass balls (18 to 22 g, $n = 17$) on which fungi grew within 14 d. The beetles had opportunity to come into contact with carcass on a protected carcass ball, while beetles were removed at 3 days after burial on a treated carcass ball.

3.2. Fungus Diversity on Carcasses. The following 12 fungal species were isolated from the mouse carcasses in the various experimental conditions: *Alternaria* sp., *Aspergillus fumigates*, *Cladosporium herbarum*, *Cladosporium* sp. 1, *Cladosporium* sp. 2, *Conidiobolus* sp., *Dactylaria* sp., *Graphium* sp., *Mucor* sp., *Phoma* sp., *Trichoderma* sp., and *Verticillium* sp. On control carcasses, 23 fungal samples were acquired, which consisted of the following 7 species: *Aspergillus fumigates*, *Alternaria* sp., *Cladosporium* sp. 1, *Graphium* sp., *Mucor* sp., *Trichoderma* sp., and *Verticillium* sp. The *Trichoderma* sp. Had the highest frequency of occurrence on control carcasses, accounting for 72% of the acquired fungal samples. On the treated carcass balls, 10 fungal samples were acquired, which consisted of the following 4 species: *Aspergillus fumigates*, *Cladosporium herbarum*, *Mucor* sp., and *Phoma* sp. The *Mucor* sp. was the most predominant fungus on treated carcass balls, accounting for 70% of the acquired fungal samples. On the washed carcass balls, 14 fungal samples were acquired, which consisted of the following 8 species: *Aspergillus fumigates*, *Alternaria* sp., *Cladosporium* sp. 2, *Conidiobolus* sp., *Dactylaria* sp., *Graphium* sp., *Mucor* sp., and *Trichoderma* sp. The *Mucor* sp. was the predominant fungus on washed carcass balls, accounting for 62.5% of the acquired fungal samples (Figure 2). Only *Aspergillus fumigates* and *Cladosporium* sp. 1 were identified on the protected carcass balls, and only *Aspergillus fumigates* and *Mucor* sp. were identified on the large protected carcass balls.

The incidences of the various fungal species were not significantly different between the control carcasses and the washed carcass balls (χ^2 test: $P = 0.07$, control carcasses: $n = 23$, washed carcass balls: $n = 14$), or between the treated carcass balls and the washed carcass balls (χ^2 test: $P = 0.41$, treated carcass balls: $n = 10$). However, there were significant differences in the incidences of the various fungal species between the control carcasses and the treated carcass balls (χ^2 test: $P = 0.001$, treated carcass balls: $n = 10$).

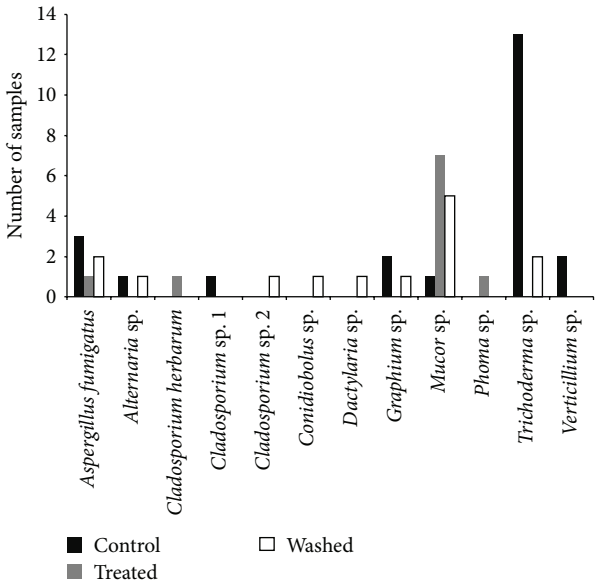

Figure 2: The sample number of the fungi species that were collected from control carcasses (n = 23), treated carcass balls (n = 10), and washed carcass balls (n = 14).

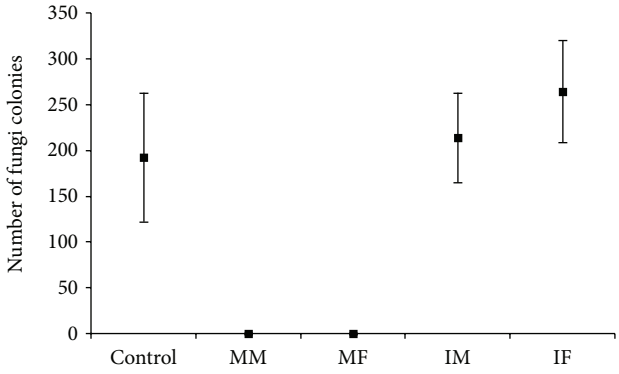

Figure 3: The number of *Trichoderma* sp. colonies (mean ± standard error) on CMA plates at 7 d after inoculation that were produced from the various inoculums. The inoculums were prepared from *Trichoderma* sp. suspended in ultrapure water (control group: n = 16) and the anal secretions of sexually mature males (MM: n = 16), sexually mature females (MF: n = 15), immature males (IM: n = 16), and immature females (IF: n = 16).

3.3. Effects of Beetle Sexual Maturity on Fungistasis. After cultivation for 7 days, 192.56 ± 70.41 colonies of *Trichoderma* sp. were present on the CMA plates that had been inoculated with the control inoculums (n = 16). The inoculums that contained the anal secretions of sexually mature beetles produced no colonies on the CMA plates. Compared to the control inoculums, the anal secretions from both sexually mature males and females could significantly inhibit fungal growth (independent sample Student's t test: $P < 0.001$; sexually mature male: n = 16, sexually mature female: n = 15). Inoculums containing the anal secretions of immature beetles produced fungal growth on the CMA plates, regardless of sex (immature male: 214.06 ± 48.86 colonies, n = 16; immature female: 264.38 ± 55.95 colonies, n = 16). The numbers of *Trichoderma* sp. colonies produced from the control inoculums were not significantly different than that produced from the immature male inoculums (independent sample Student's t test: P = 0.32). The inoculums that contained the anal secretions of sexually immature females produced a significantly greater number of colonies compared with the control inoculums (independent sample Student's t test: P = 0.003) and the inoculums that contained the anal secretions of sexually immature males (independent sample Student's t test: P = 0.011) (Figure 3).

The fungal growth reached confluency on the CMA plates in 3.0 ± 0.0 days using the control inoculums, 6.0 ± 0.0 days using the sexually mature male inoculums, and 9.0 ± 0.0 days using the sexually mature female inoculums. Thus, although the anal secretions from sexually mature beetles inhibited the growth of *Trichoderma* sp., the number of days required for fungal growth to reach confluency was significantly longer for inoculums containing mature female anal secretions than those produced from mature males (independent sample Student's t test: $P < 0.001$, sexually mature male: n = 16, sexually mature female: n = 15), which is considered a better fungistasis from mature females.

4. Discussion

Vertebrate carcasses are a high-quality source of nutrition for many species, with insects, scavengers, and microbes competing for the food resources. Insects typically begin to consume carcasses before the arrival of the larger scavenging species, and microbes release toxins that may drive away competitors [40]. Burying beetles use a small vertebrate carcass as a source of nutrition of their larval broods [1, 14], putting them in direct competition with intraspecific or interspecific insects, bacteria, fungi, microorganisms, and so on [27].

The decomposition rate of a buried carcass is slower than that of an exposed carcass because subsequent access to the carcass may be hindered for many insects and other scavengers [40]. Thus, the burial behavior of burying beetles is an adaptation that reduces competition for food resources. Observed in our study, the adults of *N. nepalensis* often feed on the intestines of carcasses before removing the fur, which may reduce the rate of decay of the carcass by eliminating the bacteria that are normally present in intestines. However, soil is also rich in microorganisms, such as fungi, that may subsequently diminish the quality of a carcass after burial.

The efficiency of carcass preservation may thus directly affect the successful production of burying beetle offspring. The deposition of oral and anal secretions on a carcass is one burying beetle behavior that reduces the rate of decay [23, 27, 33, 35]. Before burying the whole carcass, *N. nepalensis* removes hair or feathers prior to coating the carcass with secretions first. Unlike other species in North America, burying beetles bury the whole carcass first [41]. In our study, fungistasis was most efficient when a breeding pair of beetles remained present with the carcass. Thus, it is likely that the anal secretions of beetles are continuously deposited on

the carcass. Our findings support the claim that the activity of the antimicrobial chemicals in the secretions is maintained over time [23, 24] because the fungistasis was also observed on the treated carcass balls without attending adult beetles. But when a prepared carcass was given a rinse in water, the protection of fungistatic effect was absent in the washed carcass balls. However, the diversity of fungal species isolated from washed carcass balls was nonetheless influenced by the preexisting anal secretions because the dominant fungal species on both treated and protected carcass balls was *Mucor* sp., whereas *Trichoderma* sp. was the dominant species on control carcasses.

The preparation of large carcasses for burial requires more time and energy and often leads to reduced quality of maintenance by beetles [27]. A previous study showed that *N. nepalensis* was unable to efficiently use the resources of a 130 g carcass, resulting in lower offspring weight to carcass weight ratios, compared with that of smaller carcasses, because the larger carcasses decayed rapidly [26]. Compared with the 3.5 g protected carcass balls, the 18 to 22 g protected carcass balls exhibited no significant difference in fungistasis. The 2 sizes of carcasses that were used in our study are within the size range of carcasses typically used by *N. nepalensis*. Nonetheless, the fungistatic capacity of burying beetle behavior in the field may be limited by the size of a carcass because the maintenance of carcasses in the field may involve greater competition with microorganisms and other competitors than was replicated in our laboratory experiments. Therefore, the effects of carcass size on reproduction success should be further investigated in the field.

In our study, the 12 fungal species that were collected from the carcasses are common in the natural environment of the burying beetle, especially in the soil and the decaying organic matter of leaf litter [42]. However, whether the source of the fungi in our experiments was the mouse carcasses, the beetles, or the moist peat used in the cages was not determined. *Conidiobolus* sp. and *Mucor* sp. belong to the Zygomycotina, and the other 10 species that were identified in our study are members of Ascomycotina. Fungi of the Zygomycotina are commonly found in leaf litter and soil, and some species may parasitize insects [43, 44]. Members of Ascomycotina may cause disease in certain plants, and other members, such as *Cordyceps sinensis*, may parasitize insects [45]. The dominant species on the control carcasses in our study was *Trichoderma* sp., which is widespread in soil [46, 47].

The oral and anal secretions of burying beetles contain antimicrobial chemicals [48]. The antimicrobial and lytic activities of the anal secretions in *N. vespilloides* are upregulated following the discovery of a carcass [25]. Cotter and Kilner [25] suggested that the antimicrobial activity may be influenced by juvenile hormone. Our findings support the role of juvenile hormone in the fungistatic properties of anal secretions because the secretions from sexually immature adults did not inhibit fungal growth. Thus, it is doubtful that the fungistatic properties of secretions from sexually immature beetles are upregulated following contact with a carcass, despite the burying of carcasses by sexually immature beetles [26]. We suggest that the burying behavior of sexually immature beetles may serve to protect the carcass for subsequent feeding or reproduction.

Burying beetles rear their offspring by biparentally caring for the brood [1]. The participation of the male in biparental care can significantly improve resistance to alien invaders, compared with uniparental care by a female [32, 37, 49]. However, no significant increase in larval weight or brood weight occurs with biparental care, compared with uniparental care by a female [33, 35, 36]. In biparental care, males spend more time protecting the larvae and carcass from invaders than females, whereas females spend more time feeding larvae than males [50–53]. The division of labor in reproduction among male and female burying beetles may extend to the deposition of oral and anal secretions on the carcass. Our results indicate that both sexually mature male and female burying beetles produce anal secretions that inhibit the growth of fungi. However, the degree of fungistasis conferred by the secretions was significantly different between the sexes.

Conflict of Interests

The authors have no conflict of interests with the SPSS used inside the paper.

Acknowledgments

The authors wish to thank Professor P.-J. Lee for her help with identifying the fungi. This research was supported by Grants from the National University of Tainan (99AB2-14 and AB100-319) and the National Science Council, Taiwan (NSC 97WFA0F00038).

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