Rearing and storage in mung beans reduce medically significant molds by the seed beetle, *Callosobruchus maculatus* (Coleoptera: Chrysomelidae), utilized in science classrooms

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To examine whether colonies of seed beetles, *Callosobruchus maculatus*, may be a health concern as a source of mold allergens to students and teachers, we compared the amount and type of molds present on adult beetles reared with mung beans and cowpeas, *Vigna radiata* and *Vigna unguiculata*, respectively (Family Fabaceae). Specimens were analyzed from two elementary schools, a commercial supplier and a university insectary. A total of nine fungal genera were isolated from live and dead adult beetles, consisting primarily of *Aspergillus* (a large proportion of which was *Aspergillus niger*) and *Penicillium*, with lower, variable numbers of *Alternaria*, *Cladosporium*, *Fusarium*, *Mucor*, *Rhizopus*, *Scopulariopsis*, and *Trichoderma*. *Absidia*, *Geotrichum*, and *Paecilomyces* were additional genera isolated from dead beetles. These molds are medically significant as potential allergens, and others (*Aspergillus*, *Rhizopus*, *Mucor*) can cause secondary infections in people. The mold profile of different beetle colonies are similar in quality and quantity, thus suggesting that beetles are acquiring molds that are commonly found or can develop on seeds in their rearing/storage containers. Rearing on mung beans suppresses total mold growth by nearly 63% compared to the amount observed when raised on cowpeas. In fact, most cultures of beetles reared in mung beans were entirely fungus-free. Our conclusion is that the amount and diversity of allergenic molds from seed beetles for use in classrooms can be minimized considerably by using mung beans for beetle rearing, along with routine colony maintenance and proper hygiene.

**Keywords:** student projects; handling precautions; insects; childhood asthma

**Introduction**

Novel approaches designed to stimulate hands-on investigative learning in the classroom have frequently involved insects (Wagler & Wagler 2011). The seed beetle, *Callosobruchus maculatus* (F) (Coleoptera: Chrysomelidae), is one of the more recent additions to the list of insects that are being promoted as an educational tool, aimed mainly at undergraduate biology course laboratories (Beck & Blumer 2011; Schlueter & D’Costa 2013). Furthermore, enthusiasm for seed beetle colonies and their biology have spread to elementary and secondary schools. User-friendly, detailed instructional websites about this beetle’s care, ease of handling (they do not bite), small rearing space requirement, hardness, manageable size, and inability to fly under prescribed colony conditions make this beetle ideal for scientific investigation in the classroom (Beck & Blumer 2011). The typical colony setup for seed beetles is that adults are stored in containers of mung beans, *Vigna radiata*, or cowpeas, *Vigna unguiculata*. After mating, females lay eggs on the surfaces of the seeds, larvae hatch from the eggs and then burrow into the seeds to consume the seed’s internal contents (Mitchell 1975; Messina & Mitchell 1989). The seed fills with white frass (fecal waste) from larval development. Pupation occurs within the seed and new adults emerge by chewing through the seed coat. Adults live for ca. 2 weeks (Mitchell 1975), defecating amongst the seeds as they mate and oviposit. These beetles are dry-adapted (Appel et al. 1999; Yoder et al. 2010), require no water (Mitchell 1975; Messina & Mitchell 1989), and thrive in both dry seeds and under arid conditions within their storage containers (Schade & Vamosi 2012). A typical mature beetle colony contains numerous adult beetles crawling over and through layers of dry, hollowed out seeds, dead adults, and large amounts of beetle frass.

Mitosporic fungi are frequently associated with insects, their colonies, and debris (Arlian 2002), producing copious quantities of spores (conidia) that circulate throughout insect colonies. Certain rapid spore producing molds have the potential to be medically significant if they are routinely handled with long exposure to the insects and their debris (e.g., feces, old food, dead individuals) during colony rearing. The medical issues are due to inhalation of spores or placement around the face or in the broken skin.
which leads to allergic reactions in sensitized people such as contact dermatitis, swollen, itchy watery eyes, sneezing, labored breathing, asthmatic attacks, and in severe cases anaphylaxis (Arlian 2002). Children and the elderly are more vulnerable, as well as people that are prone to infection, mold-sensitivity, or have compromised immune systems. This is of concern when children are handling insects directly and are involved with colonies during the course of experimental projects. This is likely to be an ongoing concern as the popularity of seed beetles and their incorporation into science curricula increases. We have recently outlined methods for suppressing mold allergens associated with the Madagascar hissing cockroach, Gromphadorhina portentosa (Yoder et al. 2008, 2012) often utilized in educational settings (Wagler & Wagler 2011). The purpose of this paper is to provide public health information for educators that are presently, or are considering, using seed beetles in the classroom. Our hypothesis is that seed beetle colony may be a source of mold allergens.

Materials and methods

Beetles

Containers of seeds bearing Callosobruchus maculatus (Brazil strain; also known as cowpea strain) were obtained from: a commercial supplier, Carolina Biological Supply Co. (Burlington, NC, USA); a large-scale rearing operation, Biological Sciences Greenhouse Insectary (The Ohio State University, Columbus, OH, USA); and two elementary schools where these beetles are currently being utilized in projects by students: Miami Co., OH, USA (science classroom-1) and Erie Co., PA, USA (science classroom-2). Names of elementary schools are kept confidential at the request of school officials. All of these beetles were maintained at 25°C (± 2°C), 40–60% RH, variable photoperiod, and were fed mung beans (V. radiata) or cowpeas (V. unguiculata) purchased from area grocers. Specimens were sent by overnight mail and set up in culture upon arrival. Adults were sedentary, flightless morphs; i.e., the form that predominates under storage conditions. Sex determination was based on black color and stripes on the pygidium for females, and brown color and lack of stripes and smaller pygidium for males (Bandara & Saxena 1995). Colonies were about 1-month old, except for OSU colonies that were 4 to 5 months old. Beetles that remained unresponsive to stimuli (e.g., prodding), did not crawl, and/or failed to self-right when overturned were classified as dead as observed by 40× microscopy. Colony ranged from 75–100 beetles/250–500 cm³ rearing container. Mass of beetles and seeds was determined with a Cahn electrobalance (±0.2 µg precision, ±6 µg accuracy at 1 mg; Ventron Co., Cerritos, CA, USA).

External fungus isolation

A beetle was placed in a 1.5 cc microfuge tube with 1 ml autoclave-sterilized (121°C, 15 psi), deionized double-distilled (DI) water and mixed with a vortex (Scientific Industries, Bohemia, NY, USA). This extract was diluted serially with sterile DI water to 0.1, 0.01, and 0.001 dilutions for enumeration (Brown 2007). A calibrated glass micropipette (accuracy ± 0.25%, precision CV < 0.6%; Fisher Scientific, Pittsburgh, PA, USA) was utilized to measure out 0.1 ml of the extract and each dilution that was plated by distributing over the agar surface on different media: potato dextrose agar (PDA), Sabouraud agar, and corn meal agar (Difco, Fisher). Petri plates were sealed with Parafilm (Pechiney Plastic Packaging, Menasha, WI) prior to incubation so that treatments were not subjected to contaminants within the incubator. Petri plates were incubated at 25 ± 1°C, darkness, in a programmable incubator (Fisher) and examined daily for hyphae. Fungal colonies were counted with an automatic colony counter (Bantex Co., Burlingame, CA, USA). An embedding technique (one beetle embedded per plate in cooled molten agar) and subculturing hyphal tips that could be traced back to having originated from the beetle’s body surface by 100× microscopy was also employed to recover molds (Yoder et al. 2003; Benoit et al. 2004). Molds were isolated from live beetles, dead beetles, unused seeds from an unopened bag of seeds, and used seeds from beetle rearing. Water-treated plates and plates treated by the same manipulations except without using a beetle, or seed, served as controls.

Fungal identification

A 1 cm³ block of agar was removed with a scalpel from the edge of a fungal mycelium and placed on to a plate of fresh agar, incubated (25°C, darkness), and examined by microscopy for conidia after 1 to 2 weeks. Identification was based on obverse and reverse pigmentation of fungal colonies, and morphological structure of conidia and phialides by 1000× light microscopy under oil. Molds were identified using keys (Barnett & Hunter 2003) and by pure culture comparison. Cultures that failed to initiate reproductive structures (e.g., conidia) in pure culture after prolonged (>2 weeks) incubation were assignable to Mycelia sterilis. Bacteriological methods and tentative identification using the Enterotube (Becton Dickinson, Sparks, MD, USA) and API (bioMérieux Vitek, Hazelwood, MO, USA) systems were carried out on bacterial colonies of interest.

Preparation of images

Images were acquired by means of a SPOT digital camera (Diagnostic Systems Laboratories, Webster, TX, USA) fitted on an Olympus stereoscope (Olympus America, Center
Figure 1. Mold colonies on potato dextrose agar plates that were extracted from a single mung bean (*Vigna radiata*), cowpea (*Vigna unguiculata*), and female beetle, *Callosobruchus maculatus*. Whitish materials on infested seeds are beetle eggs (oval shaped) and the speckles are frass from beetle larvae. Unused seed, seed from an unopened package from grocery store; used seed, seed that had been employed to rear beetles in a 4-week old colony of about 100 beetles; live beetle, adult female taken from a colony. Petri plates were incubated at 25°C, in darkness for 4 days; 100 µl of a 1.0 ml DI water rinse. Petri plates were randomly selected from over 100 plates, not selecting the ones that had the most or the least amount of molds. Photo credit: K.M. Gribbins, Wittenberg University, Springfield, OH.

**Sample sizes and statistics**

The experiment was replicated three times per sampling site, with each replicate consisting of a different subcolony of 75–100 beetles selected at random at each location. Ten live beetles were selected from each subcolony for external fungus culturing. Of the ten beetles, four were plated on PDA, three on corn meal agar, and three on Sabouraud agar. The same was done for dead beetles, unused seeds, and used seeds, using enumeration and embedding techniques. Data were compared using an analysis of variance (ANOVA; \( p < 0.05 \); SPSS 14.0 for Windows, IBM, Armonk, NY, USA; Excel, Microsoft, Redmond, WA; Minitab, Chicago, IL, USA; Sokal & Rohlf 1995). Mass measurements were compared with an analysis of covariance (ANCOVA; Sokal & Rohlf 1995).

**Results**

**Culture plate observations**

Figure 1 depicts mold colonies that were cultured on PDA from the surface of a seed, before and after beetle rearing, and a beetle. Results were similar for Sabouraud agar and corn meal agar (data not presented). Plates where only bacteria were observed and no fungal colonies were present were typical for cultures of beetles that had been reared and stored on mung beans (bottom left, Figure 1). From 100 different plates, twenty colonies of these bacteria were randomly chosen from cultures of used seed (\( n = 10 \) from mung beans and 10 from cowpeas) and also for cultures of beetles (\( n = 10 \) from mung beans and 10 from cowpeas); thus, total \( n = 40 \) bacterial colonies. All 40 of these bacterial colonies showed similar characteristics (short, Gram negative, pleomorphic rods, oxidase-negative, catalase-positive) and all were identified as *Enterobacter* sp., likely related to *Enterobacter cloacae* based on the Enterotube and API identification system.
Impact of beetle rearing on mold levels in seeds

Before beetle rearing (Table 1), the composition of the external mycoflora of unused mung beans was dominated by Aspergillus (mean 40% across sampling sites), primarily by unidentified species (mean 24%) and secondarily by Aspergillus niger (mean 15%) and Penicillium (mean 44%). Alternaria, Cladosporium, Fusarium, Paecilomyces, Rhizopus, and Trichoderma were present as minor components (mean 9% across these components) compared to percentages of Aspergillus and Penicillium ($p < 0.05$; Table 1), and occurrence of these minor components varied among the different sampling sites. In contrast, cowpeas yielded higher number of fungal isolates than mung beans ($p < 0.05$; Table 1). Molds present on cowpeas were Aspergillus (mean 21% across sampling sites), A. niger (mean 15%) (total Aspergillus mean 39%), and Penicillium (mean 27%) as major components, and Alternaria, Fusarium, Rhizopus, and Trichoderma were present as minor components (mean 7%) compared to large percentages of Aspergillus and Penicillium ($p < 0.05$). Also, present on cowpeas were Absidia, Cladosporium, Mucor, and Paecilomyces, but these molds were present in low percentages (mean 3% across these components) and their presence varied among sampling sites.

After beetle rearing (Table 2), consistent components in the external mycoflora of mung beans were Aspergillus (secondarily A. niger, mean 17% of total mean 44% Aspergillus), Penicillium (mean 27%), and Trichoderma (mean 10%), and these components appeared in large percentages than the irregular components, Absidia, Alternaria, Cladosporium, Fusarium, Geotrichum, Mucor, Paecilomyces, Rhizopus, and Scopulariopsis (mean 6% across components) ($p < 0.05$; Table 2). Composition of molds on cowpeas consisted of Aspergillus (mean 40%, with 15% A. niger) and Penicillium (mean 34%) dominating, and Trichoderma, Alternaria, and Rhizopus present as minor (mean 6%), regular components ($p < 0.05$; Table 2). On cowpeas, Absidia, Cladosporium, Fusarium, Geotrichum, Mucor, and Paecilomyces were in low percentages (mean 4%) compared to more frequent molds ($p < 0.05$) and were irregular in frequency across different sampling sites (Table 2). When the percentage of total isolates before and after seeds had been employed for rearing (Tables 1 and 2), the mung beans showed an increase in fungal levels at the OSU site and a decrease at SC2 ($p < 0.05$), and no significant difference at SC1 ($p > 0.05$), whereas cowpeas showed an increase at OSU ($p < 0.05$), but no significant difference for SC1 and SC2 sites ($p < 0.05$).

External mycoflora of beetles

Amounts of molds recovered from beetles that were reared and stored on cowpeas were two to three times greater than the number of molds recovered when on mung beans (Table 3; $p < 0.05$). Evidence of this quantitative effect is apparent in Figure 1. Beetles reared on mung beans yielded Aspergillus (38% mean across sampling sites), A. niger (variable proportion within total percentage of Aspergillus), Penicillium (mean 27%), as the major isolates, and Trichoderma in comparatively lower amounts (mean 14%) ($p < 0.05$); components appearing with irregular frequency were Alternaria, Cladosporium, Fusarium, Mucor, and Rhizopus (mean 13%; Table 3). For beetles reared on cowpeas, the mycoflora was dominated by

Table 1. Molds present on unused mung beans (Vigna radiata) and cowpeas (Vigna unguiculata). Number of isolates is combined from potato dextrose agar, Sabouraud agar, and corn meal agar growth media.

| Molds            | Mung beans | Cowpeas | $n = 10$ Untreated controls |
|------------------|------------|---------|-----------------------------|
|                  | CB | OSU | SC1 | SC2 |              | OSU | SC1 | SC2 |              |
| Absidia          | -  | 0   | 0   | 0   | 0             | 0   | 1   | 2   | 0             |
| Alternaria       | -  | 1 (6)| 0   | 0   | 0             | 2 (7)| 1 (2)| 3 (9)| 0             |
| Aspergillus      | -  | 4 (25)| 2 (22)| 5 (24)| 5 (17)| 8 (20)| 9 (26)| 0             |
| Aspergillus flavus| - | 0     | 0   | 1 (5) | 1 (3) | 0   | 2 (6)| 0             |
| Aspergillus niger| - | 3 (19)| 1 (11)| 3 (14)| 5 (17)| 8 (20)| 4 (11)| 0             |
| Cladosporium     | -  | 0   | 0   | 1 (5) | 0   | 2 (5)| 1 (3) | 0             |
| Fusarium         | -  | 0   | 0   | 2 (10)| 3 (10)| 2 (5)| 1 (3) | 0             |
| Mucor            | -  | 0   | 0   | 0   | 1 (3) | 0   | 0   | 0             |
| Mycelia sterilia | -  | 0   | 0   | 0   | 0   | 0   | 1 (3) | 0             |
| Paecilomyces     | -  | 0   | 1 (11)| 0   | 0   | 0   | 1 (3) | 0             |
| Penicillium      | -  | 6 (38)| 5 (55)| 8 (38)| 6 (21)| 13 (32)| 10 (29)| 0             |
| Rhizopus         | -  | 0   | 0   | 0   | 2 (7) | 3 (7) | 2 (6) | 1 (100)       |
| Trichoderma      | -  | 2 (13)| 1 (11)| 1 (5) | 4 (14)| 3 (7) | 1 (3) | 0             |
| Total            | 16 | 10  | 21  | 7   | 29            | 41  | 35  | 1             |

Source: CB, Carolina Biological Supply Co.; OSU, The Ohio State University; SC1, science classroom-1; SC2, science classroom-2. Note: — = not available for analysis.
Table 2. Molds recovered from used mung beans (Vigna radiata) and cowpeas (Vigna unguiculata) after beetle rearing from beetle colonies. Number of isolates combined from potato dextrose, Sabouraud, and corn meal agar media.

| Molds       | CB       | OSU      | SC1       | SC2       | OSU      | SC1       | SC2       | n = 10 Untreated controls |
|-------------|----------|----------|-----------|-----------|----------|-----------|-----------|----------------------------|
| Absidia     | 1 (5)    | 0        | 0         | 0         | 2 (5)    | 0         | 0         | 0                          |
| Alternaria  | 1 (5)    | 0        | 0         | 1 (7)     | 3 (7)    | 1 (3)     | 2 (5)     | 0                          |
| Aspergillus | 4 (21)   | 6 (26)   | 3 (38)    | 2 (15)    | 9 (20)   | 9 (25)    | 6 (16)    | 0                          |
| Aspergillus flavus | 1 (5) | 0        | 0         | 0         | 0        | 2 (6)     | 3 (8)     | 0                          |
| Aspergillus niger | 3 (16) | 3 (13)   | 2 (25)    | 2 (15)    | 6 (14)   | 5 (14)    | 6 (16)    | 0                          |
| Cladosporium| 1 (5)    | 0        | 0         | 0         | 1 (2)    | 0         | 2 (5)     | 0                          |
| Fusarium    | 0        | 1 (4)    | 0         | 0         | 2 (5)    | 0         | 0         | 0                          |
| Geotrichum  | 0        | 2 (9)    | 0         | 0         | 0        | 1 (3)     | 0         | 0                          |
| Mucor       | 1 (5)    | 0        | 0         | 1 (7)     | 0        | 2 (6)     | 0         | 0                          |
| Paecilomyces| 1 (5)    | 0        | 0         | 0         | 0        | 1 (3)     | 0         | 0                          |
| Penicillium | 3 (16)   | 8 (35)   | 2 (25)    | 4 (31)    | 14 (32)  | 10 (28)   | 16 (42)   | 0                          |
| Rhizopus    | 0        | 0        | 0         | 2 (15)    | 3 (7)    | 1 (3)     | 1 (3)     | 0                          |
| Scopulariopsis | 0    | 2 (9)    | 0         | 0         | 0        | 0         | 0         | 0                          |
| Trichoderma | 3 (16)   | 1 (4)    | 1 (13)    | 1 (7)     | 4 (9)    | 4 (11)    | 3 (8)     | 0                          |
| Total       | 19 (10)  | 23 (10)  | 8 (8)     | 13 (10)   | 26 (10)  | 33 (10)   | 38 (10)   | 1 (100)                   |

Source: CB, Carolina Biological Supply Co.; OSU, The Ohio State University; SC1, science classroom-1; SC2, science classroom-2.

Table 3. List of molds found on live female beetles Callosobruchus maculatus from colonies at Carolina Biological Supply Co. (CB), The Ohio State University (OSU), and two science classrooms (SC1, SC2). Number of isolates combined from potato dextrose, Sabouraud, and corn meal agar media.

| Molds       | Beetles raised on mung beans | Beetles raised on cowpeas | n = 10 Untreated controls |
|-------------|------------------------------|---------------------------|----------------------------|
|              | CB   | OSU | SC1 | SC2   | OSU | SC1 | SC2 |                           |
| Alternaria   | 0    | 1 (13) | 2 (12) | 0 | 0 | 4 (12) | 3 (8) | 0                          |
| Aspergillus  | 1 (8) | 2 (25) | 2 (12) | 3 (30) | 7 (27) | 6 (18) | 4 (11) | 0                          |
| Aspergillus flavus | 0   | 1 (13) | 0 | 0 | 0 | 2 (6) | 3 (8) | 0                          |
| Aspergillus niger | 2 (17) | 1 (13) | 2 (12) | 2 (20) | 6 (23) | 4 (12) | 3 (8) | 0                          |
| Cladosporium | 0    | 0     | 1 (10) | 0 | 0 | 1 (3) | 0 | 0                          |
| Fusarium    | 0    | 0     | 2 (12) | 0 | 0 | 0 | 2 (5) | 0                          |
| Mucor       | 0    | 0     | 0 | 1 (10) | 0 | 0 | 2 (6) | 0                          |
| Penicillium | 4 (33) | 2 (25) | 5 (29) | 2 (20) | 8 (32) | 7 (21) | 11 (30) | 0                          |
| Rhizopus    | 2 (17) | 0 | 3 (18) | 0 | 1 (4) | 2 (6) | 2 (5) | 0                          |
| Scopulariopsis | 0   | 0     | 0 | 0 | 0 | 0 | 0 | 0                          |
| Trichoderma | 3 (25) | 1 (13) | 1 (6) | 1 (10) | 3 (12) | 5 (15) | 9 (24) | 1 (100)                   |
| Total       | 12   | 8     | 17 | 10 | 26 | 33 | 37 | 1                          |

Aspergillus (mean 38%, with A. niger comprising mean 14%). Penicillium (mean 28%), Trichoderma (mean 17%), and Rhizopus (mean 5%) were the minor components (p < 0.05). The variable components were Alternaria, Cladosporium, Fusarium, Mucor, and Scopulariopsis (mean 6%) that were present at some sampling sites and not others (Table 3). Nearly identical results were obtained for male beetles (data not presented). No significant differences were detected across the sampling sites in the mean percentages of Aspergillus, A. niger, Penicillium, and Trichoderma between used mung bean seeds and corresponding beetles (p > 0.05; Tables 2 and 3). Similarly, the mean percentage of Aspergillus, A. niger, Penicillium, and Rhizopus did not differ significantly between used cowpeas and adult beetles reared on cowpeas (p > 0.05; Tables 2 and 3). For beetles raised on cowpeas, however, the mean percentage of Trichoderma was ca. two times higher on the beetles compared to the amount present on used cowpeas seeds (p < 0.05; Tables 2 and 3).

Dead beetles harbored a mycoflora that was composed predominately of Aspergillus (mean 36% across sampling sites, mean 13% was A. niger) and Penicillium
Table 4. List of molds isolated from dead female beetles, *Callosobruchus maculatus*, from sampling sites: CB, Carolina Biological Supply Co.; OSU, The Ohio State University; and SC1 and SC2, two science classrooms. Results from potato dextrose, Sabouraud, and corn meal agar media were combined to give total number of isolates.

| Molds               | Beetles raised on mung beans | Beetles raised on cowpeas | n = 10 Untreated controls |
|---------------------|------------------------------|---------------------------|---------------------------|
|                     | CB  | OSU | SC1 | SC2 | OSU | SC1 | SC2 |                  |
| Absidia             | 0   | 2   | 1   | 1   | 2   | 0   | 1   | 3                 |
| Alternaria          | 0   | 1   | 5   | 0   | 0   | 2   | 5   | 1                 |
| Aspergillus         | 3   | 33  | 4   | 20  | 6   | 15  | 4   | 15               |
| Aspergillus flavus  | 0   | 1   | 5   | 7   | 0   | 2   | 5   | 8                |
| Aspergillus niger   | 2   | 22  | 2   | 10  | 6   | 15  | 5   | 19               |
| Cladosporium        | 0   | 0   | 0   | 0   | 4   | 10  | 2   | 8                |
| Geotrichum          | 0   | 0   | 0   | 0   | 2   | 5   | 0   | 0                |
| Fusarium            | 0   | 0   | 1   | 7   | 0   | 2   | 8   | 1                |
| Mucor               | 0   | 1   | 5   | 0   | 0   | 2   | 8   | 0                |
| Paecilomyces        | 0   | 0   | 1   | 7   | 0   | 0   | 0   | 0                |
| Penicillium         | 2   | 22  | 4   | 20  | 8   | 20  | 3   | 11               |
| Rhizopus            | 0   | 1   | 5   | 7   | 3   | 7   | 2   | 8                |
| Scopulariopsis      | 0   | 1   | 5   | 0   | 0   | 1   | 4   | 0                |
| Trichoderma         | 2   | 22  | 3   | 15  | 6   | 15  | 3   | 11               |
| Total               | 9   | 20  | 14  | 15  | 41  | 27  | 31  | 0                |

(mean 25%), with *Trichoderma* (mean 18%) being a lesser component (*p* < 0.05) for beetles on mung beans. *Absidia*, *Alternaria*, *Cladosporium*, *Fusarium*, *Mucor*, *Paecilomyces*, *Rhizopus*, and *Scopulariopsis* (mean 8% for these isolates combined) were irregular components that were not recovered from all sampling sites (Table 4). Mean percentage across all sampling sites of *Aspergillus*, *A. niger*, *Penicillium*, and *Trichoderma* was similar between live beetles and dead beetles when reared and stored in mung beans (*p* > 0.05; Tables 3 and 4). For dead beetles that had been reared and stored on cowpeas, the composition of the mycoflora (Table 4) was similar to that for dead beetles that had been in mung beans (*p* > 0.05), except there was the addition of *Cladosporium* (mean 7%) and *Rhizopus* (mean 8%) as regular components in the cowpeas setting. More molds were recovered from dead beetles that had been raised on cowpeas than on mung beans (*p* < 0.05; Table 4). When comparing live versus dead beetles that had been reared on mung beans, there was an increase in the number of molds at the OSU site (*p* < 0.05), and no significant differences at CB, SC1, and SC2 (*p* > 0.05) (Tables 3 and 4). The same trend was apparent between live and dead beetles on cowpeas: there was an increase in the number of fungal isolates in dead beetles from OSU (*p* < 0.05), and no significant differences were detected at the other sites (*p* > 0.05) (Tables 3 and 4).

**Size information of specimens**

Except for the SC1 site, fresh mass was larger for female beetles than males (Table 5; *p* < 0.05). Masses were lower for dead beetles than live beetles (except for SC2 specimens; *p* < 0.05), which we attributed to loss of water weight because they were brittle and dried out. Mass of cowpeas was greater than mung beans (Table 5; *p* < 0.05) and this was also evident from Figure 1. Comparing used and unused seeds, the mung beans experienced a percentage decrease in mass of 25.3% (CB), 34.7% (OSU), 10.6% (SC1), and 32.7% (SC2), and these were significantly different than the percentage decrease in mass of 53.9% (CB), 49.9% (OSU), 63.5% (SC1), and 68.2% (SC2) for cowpeas (*p* < 0.05), thus suggesting that there is more feeding activity on cowpeas or that cowpeas dry more readily after they have been fed upon by beetles.

**Discussion**

Evidence indicates that seed beetle colonies are notorious for harboring low levels of fungal inoculum when these insects are reared in captivity in well-ventilated containers containing dry seed food sources. These low fungal levels are attributed to the relatively low 40–60% RH within the beetle colony coupled with the beetle’s ability to resist desiccation (Appel et al. 1999; Jennings & Lysek 1999; Yoder et al. 2010). Moreover, food reserves contained within the seed are known to contain antifungal compounds that are released upon mechanical damage to the seed coat (Baily 1973), and this is thought to discourage fungal entry and proliferation (Bailey 1973; Ye et al. 2000; Wang et al. 2005). Salivary or fecal components from the beetles may also have antifungal capabilities (Lorito et al. 1993). Beetle activity does not seem to increase mold levels, as the levels between unused and used seeds compare favorably to each other. Beetles being dry-adapted (Appel et al. 1999; Yoder et al. 1999; Yoder et al. 2010) were dry-adapted (Appel et al. 1999; Yoder et al. 2010).
et al. 2010) have the ability to thrive in a relatively mold-free habitat of dry seeds. The mycoflora of beetles and seeds were remarkably similar despite differences in sampling sites, how beetles were handled, and handling by different people. This similarity probably derives from the beetles being raised on the same kinds of seeds at these different sites. Beetles on mung beans show fewer molds than on cowpeas implying that the amount of molds (i.e., conidia) on mung beans is lower.

Molds are the competitors to beetles and can quickly overtake them for resources (Jennings & Lysek 1999), faster than beetles can complete a generation if ambient conditions are right, and consequently wipe out the local beetle population in the colony. The Brazil strain of C. maculatus for this study and in most educational settings tolerates multiple larvae per seed (Guedes et al. 2003); i.e., cowpeas show a larger decrease in mass after being infested with beetles than mung beans, as an indicator that there are more larvae feeding on cowpeas than on mung beans. In contrast to mung beans, the cowpeas can support a much higher number of beetle larvae per seed, and this likely plays a role in fungal growth through increased respiratory activity from numerous feeding beetle larvae that elevate moisture levels. Increased heat is also associated with large numbers of feeding larvae (Rivers et al. 2010; G. D. Keeney, The Ohio State University, Columbus, OH, USA; unpublished observations, 2013), essentially making the cowpeas a ‘small incubator’ that contributes to enhancing mold growth. We attribute cowpeas having high mold levels to increased temperature and moisture generated from more beetle larvae feeding on individual seeds at any one time. In this scenario, the feeding activity on mung beans, which typically supports only one larva per seed, apparently is not sufficient to generate the increased moisture and temperature that perpetuates mold growth like what is seen in cowpeas.

There was a bacterium that consistently appeared in high abundance that was identified as Enterobacter sp. similar to cloacae. Enterobacter cloacae is a common insect resident bacterium found mainly in the insect gut and feces (Tanada & Kaya 1993), and it has been previously identified in beetles from agricultural settings (Sami et al. 2008). Thus, recovery of E. cloacae from colonies of these seed beetles is not surprising. Enterobacter cloacae was found on seed surfaces after seeds had been employed for beetle rearing and not before on unused seeds. We also found E. cloacae on the beetles. The most likely scenario is that E. cloacae originates from frass that is abundant within the beetle colony. Larvae exit the egg via the side attached to the seed. Adults deposit frass amongst the seeds as they mate and oviposit, already carriers of the bacterium in their gut. The larval frass is not typically abundant until after the new adults emerge from their host seed, releasing an amount of the frass into the colony and carrying the bacterium on their bodies and in their gut as well. Identifying E. cloacae from the surface of beetles indicates that they are incidentally coating themselves with frass while crawling through layers of used seeds in rearing/storage containers. Enterobacter cloacae is listed as an opportunistic pathogen and is largely regarded as being nosocomial (Barnes et al. 2003). Enterobacter cloacae is an antifungal bacterium (Lorito et al. 1993), thus implying that part of the reduced mold levels in beetle colonies is due to E. cloacae that comes from beetle frass.

Our results also have implications related to fungal ecology. Essentially, the seed is a closed system thanks to

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### Table 5. Mass measurements of beetles Callosobruchus maculatus, mung beans Vigna radiata, and cowpeas Vigna unguiculata, (each n = 30) from suppliers: CB, Carolina Biological Supply Co.; OSU, The Ohio State University; SC1, science classroom-1; SC2, science classroom-2.

| Specimen     | CB         | OSU         | SC1         | SC2         |
|--------------|------------|-------------|-------------|-------------|
| Adult female |            |             |             |             |
| Live         | 3.34 ± 0.36a | 2.14 ± 0.21a | 2.61 ± 0.25a | 3.05 ± 0.27a |
| Dead         | 1.86 ± 0.19b | 1.22 ± 0.30b | 1.98 ± 0.31b | 2.19 ± 0.28b |
| Adult male   |            |             |             |             |
| Live         | 2.26 ± 0.16c | 1.71 ± 0.18c | 2.37 ± 0.26c | 1.97 ± 0.11c |
| Dead         | 1.07 ± 0.22b | 0.98 ± 0.15b | 1.16 ± 0.13c | 1.44 ± 0.16c |
| Mung beans   |            |             |             |             |
| Unused       | –          | 59.17 ± 3.16d | 74.87 ± 3.55d | 63.38 ± 4.37d |
| Used         | 50.23 ± 2.08c | 38.61 ± 2.57c | 66.93 ± 1.94c | 37.67 ± 3.23c |
| Cowpeas      |            |             |             |             |
| Unused       | –          | 252.90 ± 16.04f | 190.20 ± 14.67f | 234.01 ± 8.52f |
| Used         | –          | 126.49 ± 4.81f | 69.34 ± 6.27f | 74.49 ± 5.31f |

Note: – = Carolina Biological Supply ships only colonies of seed beetles on mung beans, and also no unused mung beans were available from Carolina Biological for analysis. Data followed by the same letter within a column are not significantly different (p > 0.05).
the durable testa (seed coat). Once breached by mechanical injury (mandible action), the protein-rich cotyledons are vulnerable to water imbibition and subsequent fungal exploitation, with the beetle serving as a source of inoculum via conidia that adhere to the exoskeleton. From the perspective of the beetle, encouraging fungal entry and proliferation would be detrimental given that the invading fungus would likely compete for the same seed nutrient reserves. By targeting bean species known to harbor antifungal compounds, these beetles have gained the upper hand with the opportunistic molds. Moreover, the beetle’s ability to resist desiccation and thrive in a dry environment further reduces the competition with molds. Considering that the beetle’s life cycle requires considerable time to complete, it is imperative that mold suppression be achieved.

From a medical standpoint, the molds we cultured are common saprobes that are known for producing copious quantities of spores (Jennings & Lysek 1999). Composition of the mycoflora of beetles was dominated by mitosporic fungi (primarily Aspergillus, Penicillium, Trichoderma) and few zygomycetes (Rhizopus, Mucor, Absidia). Zygomycetes typically require previously broken down simple sugars for growth and development, whereas mitosporic fungi utilize more complex sugars (Moore-Landecker 1996). Seed endosperm is mainly a complex carbohydrate (Evert & Eichhorn 2012). This would cause a shift from the zygomycetes to favor proliferation of mitosporic fungi in the beetle colony, because seeds are the sole food source for the beetles and seeds alike. The heavy mitosporic fungal load associated with the beetles and their colonies is of concern because many of these molds function as potential allergens (namely Rhizopus, Mucor, Alternaria, Penicillium, Aspergillus) and fungal opportunists (Aspergillus, Mucor, Rhizopus) that can cause secondary infections in people. Of special concern is Aspergillus that is known to infect people by broken skin or inhalation of conidia leading to aspergillosis. Colonies of Rhizopus and Mucor, although fewer in number revealed by this study, are more virulent and are capable of invading the central nervous system, primarily in immunocompromised individuals (e.g., leukemia and AIDS patients; Moore-Landecker 1996). There is also evidence that these beetle colonies occasionally harbor Aspergillus flavus – a well-known, seriously problematic mold known to release neurotoxic aflatoxins into foodstuffs (Keller et al. 2012). Although it is unlikely that aflatoxin-laced seeds harboring A. flavus will be directly ingested by those tending the beetle colony, conidia from this fungus have the potential to escape into wind currents, spreading inoculum into areas where human food is prepared and consumed. Transmission of these molds to people is largely through spores. The mycoflora of the beetle reflects the fungal composition of their surroundings, most similar to the mycoflora of the host seed. The fact that a beetle colony is suppressive toward mold growth has the effect of restricting transmission of these medically significant mold strains by reducing spore production.

Practical application of our results to healthcare (disease prevention) and science education was the original intent of this investigation. Following colony set up guidelines proposed by Beck and Blumer (2011), the results indicate that the beetle colony generates few molds. There is also an age effect: OSU colonies were older by a few months and harbored more molds than more newly established colonies at other sites. Proper colony maintenance by disposing of old, used seeds to prevent mold buildup, avoiding small containers for rearing/storage (a large, 150 mm Petri dish is recommended; Beck & Blumer 2011), and promoting proper hygiene (hand washing, keeping hands away from eyes and face, wearing dust masks) could reduce further risk of mold growth and transmission that may lead to possible infection and allergic reactions. We advocate for maximum mold control by rearing seed beetles on mung beans.

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