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ACQUISITION AND RETENTION OF BACTERIAL SPORES
(*BACILLUS ATROPHAEUS*) BY EIGHT INSECT SPECIES

A Thesis submitted in partial fulfillment of the requirements for the degree of Masters of
Science at Virginia Commonwealth University.

by

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July 2006

Acknowledgements

This project was funded by a contract from Johns Hopkins University Applied Physics Laboratory to KM Kester & BL Brown. I thank Sumona Kabir for her assistance with the majority of the data collection. I thank Drs. Karen M. Kester, Bonnie L. Brown, Joseph P. Chinnici, and W. Scott Street of VCU for their support. I thank Dr. Coby Schal of North Carolina State University (Raleigh, North Carolina) for the German cockroaches, Wes Watson of North Carolina State University (Raleigh, North Carolina) for the common house flies, and Robert Matthews of University of Georgia (Athens, Georgia) for the wasp cages. I express my appreciation to Michelle Peace of VCU, for the use of her pig cadavers and for both her and her students' help in collecting blue bottle flies and hairy rove beetles.

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Abstract

ACQUISITION AND RETENTION OF BACTERIAL SPORES (*BACILLUS ATROPHAEUS*) BY EIGHT INSECT SPECIES

By Kieron Marie Torres, M.S.

A Thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2006

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Acquisition and retention of spores of an anthrax surrogate, *Bacillus atrophaeus* Nakamura ("BG") were evaluated in eight insect species. Species included: house cricket (*Acheta domesticus* L.), German cockroach (*Blatella germanica* L.), common house fly (*Musca domestica* L.), blue bottle fly (*Calliphora vomitoria* L.), hairy rove beetle (*Creophilus maxillosus* L.), yellow mealworm (*Tenebrio molitor* L.), common paper wasps (*Polistes exclamans exclamans* Viereck), red paper wasps (*Polistes carolina* L.), red harvester ant (*Pogonomyrmex barbatus* Smith). Individual insects were offered BG-

treated food and sacrificed at specified time intervals following one, two or three meals. Resulting samples were surface-washed five consecutive times then homogenized to release gut contents, and the homogenate and first and fifth washes were cultured on Trypticase Soy Agar to determine recovery of BG spores. All species delivered spores but BG retention among species varied over time. Results demonstrate the potential of insects to serve as biosentinels for detecting the presence of spore-forming bacteria in the environment.

Introduction

Insects have adapted to utilize many food sources and occupy many habitats, including those inhabited by humans and domesticated animals. Although many insect species are considered pests, others have been exploited for human benefit. For example, honey bees are cultivated throughout the world for their honey, wax and pollen and used for pollinating agricultural crops, and predatory and parasitic insects are introduced to reduce population levels of pest species. Aquatic insect communities are often used as indicators of water and wetland habitat quality (e.g., Patrick and Palavage 1994, Gore *et al.* 2001). More directly, mosquitoes are assayed routinely for the presence of West Nile Virus and other arboviruses (McIntosh *et al.* 1976, Diallo *et al.* 2005, Higgs *et al.* 2005). Insects and other arthropods can also be used for surveillance of bioterrorism agents.

The concept of employing insects for the surveillance of bioterrorism agents and emerging diseases is well grounded by reports that naturally occurring microorganisms can be cultured from insects (Gilliam and Valentine 1976, Gilliam and Morton 1978, Gilliam *et al.* 1990, Jang and Nishijima 1990, Goerzen 1991). This concept is supported further by the demonstrated capacity of several insect species to serve as mechanical vectors of human pathogens. For example, the common house fly (*Musca domestica*), which feeds and breeds on fecal matter and comes into contact with humans and human food, has been implicated as a mechanical vector of many human pathogens (Graczyk *et*

al. 2001), including *Helicobacter pylori* (Grübel *et al.* 1997), *Escherichia coli* (Kobayashi *et al.* 1999, Alam and Zurek 2004), *Salmonella* spp. (Sulaiman *et al.* 1988), *Vibrio cholerae* (Fotedar, 2001) and *Yersinia pseudotuberculosis* (Zurek *et al.* 2001). Similarly, calliphorid flies have been implicated as mechanical vectors of *E. coli* (Paraluppi *et al.* 1996). The German cockroach (*Blattella germanica*) is an implicated mechanical vector of *E. coli* (Zurek and Schal 2004), *Pseudomonas aeruginosa* (Fotedar *et al.* 1993), and *Salmonella enteritidis* (Ash and Greenberg 1980), and American, German, and Oriental cockroaches are possible vectors of *Salmonella typhimurium* (Kopanic *et al.* 1994). The lesser mealworm, *Alphitobius diaperinus*, is a demonstrated mechanical vector of turkey coronavirus (Watson *et al.* 2000), as well as a potential vector for Newcastle disease, fowl pox (De las Casas *et al.* 1976), and infectious bursal disease (McAllister *et al.* 1995).

Although several insect species have been implicated or even demonstrated to serve as mechanical vectors of human and animal diseases, very few studies have considered how long insect-borne pathogens can be retained and detected. Ash and Greenberg (1980) detected *Salmonella enteritidis* serotype typhimurium in German cockroach feces for 3-20 days and in the gut for 10 days longer. *Pseudomonas aeruginosa* was detected in German cockroach feces for up to 114 days (Fotedar *et al.* 1993). Kopanic *et al.* (1994) detected *Salmonella typhimurium* in American, German, and Oriental cockroaches for up to 96 hr. Grübel *et al.* (1997) found *Helicobacter pylori*

in house fly gut and excreta for up to 30 hr. House flies excreted *Escherichia coli* O157:H7 for at least three days (Kobayashi *et al.* 1999). Watson *et al.* (2000) detected active turkey coronavirus in lesser mealworm gut 1 hr after feeding but not after 12 hr. Infectious bursal disease virus was isolated from the lesser mealworm foregut for up to 14 days, but isolation was erratic (McAllister *et al.* 1995).

Recent work by Kester *et al.* (2004) has demonstrated that many arthropod species can passively collect and deliver detectable quantities of spore-forming bacteria from the environment. In a study conducted at Dugway Proving Ground, Utah, in 2003, arthropods were collected before and after a point-source release of *Bacillus atrophaeus* Nakamura (a commonly used surrogate for *Bacillus anthracis* known as "BG" in reference to its previous classification as *B. globigi*). Whereas only 8% of all arthropods collected from the target area prior to the BG release produced BG colonies on culture (there was residual contamination due to releases made several months earlier), 67% of all arthropods collected from the same area 2 weeks after the release were positive (Kester *et al.*, unpublish.). Delivery rates varied by species, e.g., scorpions and darkling beetles showed high rates of delivery relative to ants and flies. Results of this study indicate the need for more controlled studies to evaluate variation in acquisition and retention of spore-forming bacteria among arthropod species. This information is essential for predicting when a sentinel most likely encountered a bioterrorism agent and

in combination with knowledge of the dispersal capacity of this sentinel can be used to localize the area of contamination.

An important parameter of sentinel capacity is the ability of a species to acquire and retain a sufficient quantity of an agent long enough to permit detection. Since arthropods are highly diverse and have adapted to a variety of diets and habitats, they will most likely vary in the ability to acquire and retain an ingested pathogen. For example, generalist predatory species that are adapted for feeding irregularly may retain and bioaccumulate prey-borne bacterial spores for days to weeks. In contrast, actively foraging scavengers that feed frequently may retain spores for hours or days. One way to evaluate the potential of an arthropod to serve as a sentinel is to determine how soon and for how long an agent of interest can be delivered in detectable quantity under controlled laboratory conditions.

Acquisition and retention of BG among insect species was compared by feeding individuals a single BG-treated meal and then sacrificing them at specified intervals of time after feeding; prepared samples were cultured on Trypticase Soy Agar (TSA). Bioaccumulation of BG was tested in a few species. A secondary objective was to evaluate the effectiveness of two sample preparation methods—surface washing and homogenization—by determining the association of positive results obtained by each insect species.

Materials and Methods

I. Experimental Design

This study employed a series of feeding trials with selected insect species designed to evaluate acquisition of spores of *Bacillus atrophaeus* (“BG”) and to determine the window of spore retention. Prior to each trial, experimental insects were starved for 24 hr to control for variable physiological states and to increase the likelihood of feeding. The experimental designs, including insect species, sample sizes, food types, and time intervals sampled in each trial are shown in Table 1. With the exception of house flies and blue bottle flies, individual insects were placed in separate containers to allow for ease of observation and data recording. To prevent secondary exposure to BG through contact with excreta or vomitus (flies) some experimental confinements included paper liners that were removed with the food and replaced daily. To evaluate BG retention, insects were offered a single meal of BG-treated food that was removed after the observed feeding bout; insects were then assayed at selected subsequent time intervals; control individuals were offered similar types and amounts of food untreated with BG. To assess the possibility of bioaccumulation, individuals were offered BG-treated food every 24 hr for one, two, or three feedings. Insects were starved during the 24 hr period between feedings, i.e., food was removed after eating was observed unless otherwise noted. Those insects that received only one or two BG-treated meals were

offered water and untreated food. Randomly selected individuals from the group fed one BG-treated meal were sacrificed at 24, 48, and 72 hr. Individuals from the group fed two BG meals were sacrificed at 48 and 72 hr. Individuals from the group fed three BG meals were sacrificed at 72 hr. To control for variation due to health or age that could result in poor feeding, only insects that were observed feeding were used in these studies.

II. Experimental Organisms

Spores of BG were obtained from Dugway Proving Ground, Utah as a dry powder estimated to contain 2×10^{11} BG spores/g. These spores were suspended at 10^8 BG spores/mL in an aqueous solution of 1% phenol to be used as a stock solution. To standardize the effect of dosage, a solution of 10^6 BG spores/mL in 25% glycerol: 5% sucrose was applied to relevant food types at a constant ratio of 100 μ L spores per 0.1 g of food.

Insect species used in this project were selected for study because they are common, abundant, and can be easily reared or obtained. All are either cosmopolitan or have congeners around the world and several are synanthropic. Only adult insects were used. For each experiment, every effort was made to achieve sample sizes of 10 individuals per treatment. However, sample size ultimately varied across species-specific experiments due to insect availability, morbidity, and time constraints. Insects that died before being sacrificed were not used. As a result, the number of individual insects

treated in some studies differed from the number of individuals ultimately sacrificed and assayed for BG.

House cricket. The house cricket (*Acheta domesticus* L.) (Orthoptera: Gryllidae) is a synantrophic species found in warm areas within domestic and industrial structures. Crickets were a gift from Ghann's crickets (Atlanta, Georgia). They were maintained in the lab in plastic terrariums and fed apple and ground dog kibble (Ol' Roy or Alpo Adult) until experimental exposure.

German cockroach. The German cockroach (*Blattella germanica* L.) (Blattodea: Blatellidae) is a synantrophic scavenger that is considered a pest. Cockroaches were a gift from Dr. Coby Schal of North Carolina State University (Raleigh, North Carolina, U.S.A.). Young adults of mixed sexes were maintained in the lab in sealed glass aquaria and fed ground rodent food until experimental exposure.

Common house fly. The common house fly, *Musca domestica* L. (Diptera: Muscidae), is a synantrophic scavenger that is considered a pest and disease vector especially where associated with domesticated animals. Flies used in this study were a gift from W. Watson, North Carolina State University (Raleigh, North Carolina, U.S.A.). Until experimental exposure 1-2 day old flies were maintained in the lab in 30 cm x 30 cm x 30cm collapsible stainless steel cages (BioQuip, 1405B) and fed powdered sugar and water.

Blue bottle fly. The blue bottle fly (*Calliphora vomitoria* L.) (Diptera: Calliphoridae) is a detritivore associated with human refuse and cadavers. Flies were captured at the VCU Farm in the proximity of decomposing pig carcasses. They were maintained in the lab in 30 cm x 30 cm x 30cm collapsible stainless steel cages and fed powdered sugar and water prior experimental exposure.

Hairy rove beetle. The hairy rove beetle (*Creophilus maxillosus* L.) (Coleoptera: Staphylinidae) is a predator found mainly in habitats that support fly larvae and other detritivores; e.g., carrion, rotted refuse, and other decaying organic matter. Hairy rove beetles were captured at the VCU Farm from the carcasses of decomposing pigs. Prior to experimental exposure, beetles were held in plastic shoe boxes (Sterlite™) with sufficient detritus from the farm to cover the bottom and they were fed ground dog kibble.

Yellow mealworm. The yellow mealworm (*Tenebrio molitor* L.) (Coleoptera: Tenebrionidae) is a scavenger found in grain and food storage facilities and therefore is considered a pest. Mealworm pupae were a gift from Ghann's crickets (Atlanta, Georgia, U.S.A.). Pupae were reared in plastic shoe boxes filled with wheat bran. Emerging adults were fed apple and the wheat bran was lightly sprayed with water regularly. Adults were allowed to breed to create a colony, and the wheat bran was replaced as it was consumed.

Paper wasps. Paper wasp (Hymenoptera: Vespidae) larvae are predators, but their prey must be captured and delivered by nectar and pollen feeding adults. Entire

nests with queens and adults of common paper wasps (*Polistes exclamans exclamans* Viereck) and red paper wasps (*Polistes carolina* L.) were collected locally and maintained in the lab for 2-8 weeks in separate 20 cm x 20 cm x 20 cm Plexiglas cages. Plexiglas cages were a gift from Robert Matthews of the University of Georgia (Athens, Georgia, U.S.A.). Cages were provisioned with filter paper for nest expansion, and with water and 5% sucrose that was changed daily. Prior to use, wasps were chill-anaesthetized by holding at 4°C for 1 hr.

Red harvester ant. The red harvester ant, *Pogonomyrmex barbatus* Smith (Formicidae), feeds on seeds and other insects. Although most *Pogonomyrmex* spp. occur in the US west of the Mississippi River, “harvester ants” are distributed worldwide in dry arid regions. Harvester ants used in this study were obtained from the wild in Arizona. Upon arrival, ants were held in a 10-gal glass aquarium and fed on dog kibble and apple.

III. Sample Preparation

Acquisition of spores is most likely to occur through ingestion or grooming (internal BG) or by adherence to the exoskeleton or other body parts (surface BG). To separate internal BG from surface BG, each insect was washed in sterile DNA-grade water five consecutive times, in 2 mL for the first wash and 1 mL each for the remaining four washes. One mL each of the first and fifth washes was retained for culturing, and all other washes were discarded. The remaining 1 mL of the first wash was retained for future analyses. Wash 1 was expected to contained most of the surface BG of each

insect, and Wash 5 was cultured to determine whether the surface had been cleansed thoroughly before homogenization. Wash 1, split into two 1 mL aliquots, and Wash 5 were frozen in separate 2.0 mL screwcap microfuge tubes with O-rings (Fisher 05-669-4) and held at -20°C . To release gut contents, each washed insect was homogenized with a grinding pestle (PGC 81-6791-03) in 3 mL of sterile DNA grade water. Each homogenate was filtered through a $10\ \mu$ filter created from a 1.7 mL microfuge tube (Fisher 02-681-320) and a piece of Nitex nylon $10\ \mu$ mesh (SEFAR America, Depew, New York, U.S.A.) that formed a funnel in the tube. The filtered homogenate was frozen at -20°C in three 1 mL portions each in 2.0 mL screwcap microfuge tubes with O-rings. One portion was used for culturing and the remaining two aliquots were retained for future analyses.

For each individual specimen, one 1 mL portion of each type of sample (homogenate, Wash 1, and Wash 5) was processed to allow BG culture. The samples were centrifuged at $12,500\times g$ for 15 min, the liquid discarded, and solid material was resuspended in $50\ \mu\text{L}$ Trypticase Soy Broth (TSB). These reconstituted samples were transferred to and incubated in a 96-well PCR plate at 80°C for 30 min, and then held at ambient temperature (23°C) for 90 min. Following incubations, the processed homogenate, first wash, and fifth wash for each specimen were placed in separate sections of a single Petri dish. Culture media consisted of TSA (TSB plus 1.2% starch agar) and were held at $30\text{--}35^{\circ}\text{C}$ for 48 hr. Positive and negative (blank) controls were run

with each batch of samples cultured. The positive control enabled tests for methods and media quality, whereas the negative control enabled tests for contamination. Following the culture period, Petri dishes were refrigerated at 4°C and digitally photographed for archiving.

IV. Data Collection and Analyses

The BG colonies resulting from each cultured sample were counted, and samples were considered positive if they exhibited at least one BG colony. For each species tested, retention and bioaccumulation were visualized by graphing the percentage of individuals that delivered BG positive samples (SigmaPlot V9.0, SPSS V13.0). Acquisition and retention were compared among insect species, across common time intervals (2, 4, 12, 24, 48, and 72 hr) and among species across time intervals using a univariate general linear model (GLM, SPSS V13.0) procedure. Separate analyses were performed to compare the proportion of positive surface washes, homogenates and individuals (wash or homogenate). Resulting parameter estimates were visualized by graphing and used to predict BG delivery rates by species. Finally, the association of positive surface washes and positive homogenates was tested for each species using separate Chi-Square Tests of Independence, and contingency coefficients were calculated to determine the strength of the association (SPSS V13.0).

Results

I. BG Acquisition and retention by insect species

House cricket. The house cricket acquired BG as early as 0.5 hr and delivered at every time interval tested up to 72 hr. Between 1 and 12 hr the proportions of positive samples ranged from 40-100% positive samples; thereafter rates were highly variable (Fig. 1). Rather than showing a discrete peak, crickets exhibited consistently high rates of spore acquisition and retention. Based on results, there was no evidence of bioaccumulation in this species (Fig. 2) since there was no observed increase in the proportion of positive samples over time or with repeated feeding events.

German cockroach. The German cockroach acquired BG as early as 0.5 hr and delivered up to 48 hr, except at 36 hr. Delivery was most reliable between 0.5 and 10 hr, with a possible peak at 2 hr (60% positive samples) (Fig. 3). Only one round of testing was included in analyses because all resulting samples in the second study were negative. Likewise, BG was not detected in cockroaches receiving multiple feedings so bioaccumulation could not be tested (Fig. 4).

Common house fly. The common house fly acquired BG between 2 and 6 hr (10-30%). Except for a pulse at 24 hr, samples collected between 0.25 and 1 hr or from 8 to 72 hr post-feeding were negative for BG, (Fig. 5). Because there were no positive

samples in the multiple feeding study beyond 24 hr, bioaccumulation could not be tested (Fig. 6).

Blue bottle fly. Acquisition of BG by the blue bottle fly was somewhat sporadic. In Study 1, BG was detected (10-40% positive samples) at all time intervals tested (2-72 hr) except at 6, 10, 12 and 36 hr, whereas in a replicate study, BG was detected only at 2 and 24 hr (10% positives samples each interval) (Fig. 7). Due to the low number of positive samples, bioaccumulation could not be tested (Fig. 8).

Hairy rove beetle. The hairy rove beetle acquired BG as early as 0.25 hr and delivered up to 2 hr, with peak delivery at 2 hr (100% positive samples). Although BG was detected at 48 hr (40% positive samples), it was not detected at other time intervals between 4 and 72 hr (Fig. 9). The proportion of positive samples increased over time and with repeated feeding events, demonstrating that bioaccumulation occurs in this species (Fig. 10).

Yellow mealworm. BG was detected in yellow mealworm samples only at 0.5, 4, 8, and 24 hr (10-20% positive samples) (Fig. 11). Because no BG was detected in mealworms that received multiple feedings, bioaccumulation could not be tested (Fig. 12)

Paper wasps. Paper wasps acquired BG as early as 0.25 hr and delivered up to 72 hr post-feeding, but not detected at 0.5 and 1 hr. The proportions of positive samples ranged from 40-80% positive samples between 2 and 24 hr; thereafter, rates were highly variable (Fig. 13). Although the first trial was compromised by the presence of

contaminated control samples, results of the second trial were similar. There was no evidence of bioaccumulation; the proportion of positive samples was sporadic after 24 hr and with multiple feedings (Fig. 14).

Red harvester ant. In the first trial, none of the red harvester ant samples tested positive for BG ($n=28$) and in a second trial, only one individual (both wash and homogenate) tested positive at 0.083 hr (5 min) (Fig. 15).

II. BG acquisition and retention among insect species

Acquisition and retention of BG among species, across time intervals common to all insect species, and among species over time were compared using a general linear model procedure (GLM; SPSS V13.0). Separate analyses were performed to compare the percent positive surface washes, percent positive homogenates, and percent positive individuals (a positive wash or homogenate from a single specimen). Negative controls were excluded from analyses.

Overall, BG acquisition and retention varied among insect species over time. With one exception, results of the GLM analyses were similar for washes, homogenates and combined samples (Table 2). The proportion of BG-positive surface washes differed significantly among species over time ($P=0.044$) and among species ($P<0.001$) but not across time intervals ($P=0.227$; $n=674$). For homogenates, the proportion of positive samples varied significantly among species, across time intervals and for species over time ($P<0.001$ for each, $n=674$). Likewise, the proportion of BG-positive individuals

(wash or homogenate) differed significantly among species, across time intervals and among species over time ($P<0.001$, $P<0.001$, and $P=0.007$, respectively, $n=674$).

Predicted rates and patterns of BG delivery by insect species based on parameter estimates are shown in Figure 16.

III. BG delivery with respect to sample preparation method

The association of positive surface washes and positive homogenates was tested for six species using separate Chi-Square Tests of Independence; two species (red harvester ant and yellow mealworm) were excluded from analyses due to insufficient sample size (Table 3). For three species (hairy rove beetle, blue bottle fly and house cricket), positive washes and homogenates were associated, and this association was especially strong for the hairy rove beetle. For the remaining three species (common house fly, German cockroach and paper wasps), positive results from two sample preparation methods were not associated and for each, more homogenates than surface washes yielded positive results.

Discussion

All insect species acquired spores of *Bacillus atropheaus* (BG) through a single meal of BG-treated food and as anticipated, BG acquisition and retention varied among insect species. For some species, BG retention was relatively constant (e.g., house cricket), whereas in others BG retention appeared to reach a maximum and thereafter declined (e.g., hairy rove beetle). BG retention among species varied over time (Fig. 16). For three species (German cockroach, house fly, and paper wasps), positive results obtained from surface washes and homogenates were not associated (Table 3), indicating that for these species homogenization yields more valid results than surface washing alone. Results are discussed in consideration of the biosentinel potential of each species for detection of spore-forming bacteria in the environment.

House cricket. All (100%) of the house crickets sacrificed up to 12 hr post-feeding tested positive for BG and 20-30% were BG-positive up to 72 hr (Fig. 1). Due to variability, results of the bioaccumulation studies are difficult to interpret but overall, suggest that the house cricket bioaccumulates ingested spores (Fig. 2). Predicted BG delivery suggests that delivery of BG decreases over time (Fig. 16); however, since crickets are likely to remain in the same microhabitat where they initially acquired BG, repeat exposure is likely to occur under natural conditions. This may be the first study to investigate acquisition or retention of microbes by the house cricket.

German cockroach. Although 10-60% individuals tested positive for BG up to 24 hrs and 10% tested positive at 48 hr (Fig. 3), no evidence of bioaccumulation was found in this species (Fig. 4). The predicted BG delivery pattern suggests a fairly rapid decline in the proportion of positive individuals over time (Fig. 16). Although no comparable studies on retention of spore-forming bacteria are available, Fischer *et al.* (2003) reported recovery of *Mycobacterium avium* at 3 and 10 days post-exposure in the Oriental cockroach, *Blatta orientalis*, and Fotedar *et al.* (1993) reported recovery of *Pseudomonas aeruginosa* in the German cockroach for up to 114 days. Thus, longer retention times should be tested. Further, roaches are likely to remain in the same microhabitat where they initially acquired BG so repeat exposure is likely to occur under natural conditions. In addition, BG may be transferred among roaches aggregating during daylight hours. Future studies should consider these factors.

Because roaches are nocturnal, they offer tremendous stealth potential and should make excellent sentinels for surveillance of spore-forming bacteria in human-inhabited structures and animal facilities. Studies investigating exposure dosage levels and secondary transfer of BG spores through aggregation are now underway.

Common house fly. Of house flies sacrificed up to 4-6 hr and at 24 hr post-feeding, 10-30% of all individuals tested positive for BG (Fig. 5). The pulse at 24 hr is most likely due to secondary exposure through contact with vomitus or excreta, which is supported by the absence of the 24-hr pulse in the second round of trials when paper

liners were removed daily. These data indicated that the house fly does not bioaccumulate BG (Fig. 6), a finding that is consistent with those of the acquisition and retention studies. The predicted BG delivery pattern suggests that the most reliable delivery of evidence of BG occurs within a few hours of exposure (Fig. 16), indicating that the house fly may retain bacterial spores for less time than vegetative bacteria. Grübel *et al.* (1997) reports recovery of *Helicobacter pylori* in the house fly for up to 30 hr and Kobayashi *et al.* (1999) and Zurek *et al.* (2001) both report recovery of *Escherichia coli* O157:H7 and *Yersinia pseudotuberculosis*, respectively, for 3 days. Possibly, the larger BG spores are retained in the foregut and released in the vomitus; this should be examined in future studies. In any case, the short retention of BG ensures that positive individuals have acquired BG in proximity to the site at which they are collected.

Blue bottle fly. Results for acquisition and retention of BG in the blue bottle fly were similar to those obtained for the house fly; 20-30% of individuals retained BG for up to 8 hrs and delivered BG again at 24, 48 and 72 hrs (Fig. 7). When paper liners were removed daily to prevent secondary exposure to BG through vomitus or excreta, BG was recovered from individuals at 2 and 24 hrs only. Slightly more individuals fed two BG-treated meals tested positive for BG than those fed only one BG-treated meal only (Fig. 8) suggesting that BG is bioaccumulated in the blue bottle fly. Predicted BG delivery patterns for the house fly and blue bottle fly are similar except that the blue bottle fly may retain BG for a longer period time (Fig. 16). The utility of the blue bottle fly as a sentinel

for detecting the presence of spore-forming bacteria in cadavers deserves further investigation. Although no comparable studies could be found in the literature, Paraluppi *et al.* (1996) reported the recovery of several bacteria from field-collected calliphorids and Fischer *et al.* (2004) have demonstrated the capacity of calliphorids of *Mycobacteria*. Such reports in combination with the results reported herein suggest that calliphorids are potentially valuable biosentinels.

Hairy rove beetle. Results indicate that BG is acquired quickly and retained for as long as 48 hr; 30-100% of individuals retained BG up to 2 hr and 38% tested positive at 48 hr (Fig. 9). The positive results for beetles at 48 hr may be due to secondary exposure through contact with BG delivered from excreta. Alternatively, beetles may retain BG much longer than 2 hr, and in consideration of the bioaccumulation study, this is more likely. Of all species studied, the hairy rove beetle presented the most definitive evidence of BG bioaccumulation (Fig. 10). The hairy rove beetle has excellent potential as a sentinel for detection spore-forming bacteria in and in proximity to cadavers, and this deserves further attention.

Yellow mealworm. Compared to the other species in this study, the yellow mealworm showed low BG acquisition rates and inconsistent retention times; only 0-20% of individuals at any time interval tested positive for BG sporadically up to 24 hr (Fig. 11). The low acquisition and retention of BG is similar to the results of Watson *et al.* (2000), who did not detect active turkey coronavirus in exposed lesser mealworms after 1

hr. In addition, the yellow mealworm does not appear to bioaccumulate BG (Fig. 12). The predicted BG delivery pattern suggests that the yellow mealworm would not be an effective sentinel for detecting spore-forming bacteria, largely due to poor acquisition rates (Fig. 16). However, poor acquisition rates could be compensated by using pooled samples, and if so, this species could prove useful as a “early-alert” sentinel for detection of spore-forming bacteria in stored grain.

Paper wasps. Cumulative results of the two BG acquisition and retention studies suggest that paper wasps acquire BG easily and retain it for a relatively long period of time (Fig. 13). Results of BG bioaccumulation studies are difficult to interpret and could reflect contamination observed in the first trial or large variations in retention time observed in other species investigated in this study (Fig. 14). The predicted BG delivery pattern suggests that paper wasps would be excellent sentinels for detecting the presence of spore-forming bacteria in the environment (Fig. 16). However, due to the possibility of sample contamination, acquisition rates and delivery rates may be overestimated. Because results were compromised, experiments with paper wasps should be repeated and based on reports of bacteria recovered from larval provisions in other hymenopterans (e.g., Gilliam *et al.* 1984, 1985), paper wasp larvae should be considered in future work. Since paper wasps tend to remain in proximity to their nests, they would most likely make reliable sentinels for detecting spore-forming bacteria near structures. In addition

to contact with plants during foraging, wasps may also encounter spores while flying, as demonstrated experimentally for honey bees (Lighthart *et al.* 2000).

Red harvester ant. The red harvester ants did not acquire BG easily or they retained it only briefly. Only one of the 47 ants fed BG-treated food tested positive for BG at any time interval (Fig. 15), suggesting that this species would not be a good sentinel for detecting spore-forming bacteria in the environment. BG spores are fairly large, ca. 1078 nm in diameter (Plomp *et al.* 2005). Possibly, spores are not ingested by red harvester ants or more likely, most spores are filtered out by the proventriculus and then ejected via the buccal cavity rather than entering the hindgut. For comparison, Cannon (1998) reported that 300-10000 nm microspheres were contained by the proventricular valve in the much larger carpenter ant (*Camponotus pennsylvanicus* De Geer).

Summary. This study illustrates the potential utility of insects as reporters of environmentally-acquired *Bacillus* spores and the exceptionally wide range of variation expected for retention time across species. Variation in rates of acquisition and retention among species could be due to factors other than species-specific rates of acquisition and retention. Physiological factors affecting intake and retention included nutritional status, life stage, and age. As several species were caught in the wild or obtained as gifts, these factors could not be controlled. Another source of potential variation relates to sample handling. Only one portion of each homogenate and Wash 1 was cultured for each

sample. Although the portions had been placed in separate tubes immediately after washing or homogenization, there may have been some disparity between portions. However, the data are not likely to be underestimates because a subset of archived samples was later tested using a different and possibly more sensitive method, polymerase chain reaction (PCR), and all samples that had been negative by culturing were also negative by PCR (data not shown). Another possible factor contributing to variation in rates over time is that germination rate for the stock BG could have diminished over time. Size may have affected delivery, in that the larger insects were capable of acquiring and delivering greater numbers of spores purely based on the surface area available for spore adhesion. For example, the house cricket and paper wasps are larger than the yellow mealworm and the red harvester ant, and the former types of insects had higher percentages of delivery. In application, pooling individuals may increase the probability of detection especially for small species and those with low rates of acquisition.

Future research tasks include continuing research on the species used in this study, such as increasing the time increments before and after 72 hr and studying a broader range of species. More time increments between 0 and 72 hr would help determine where BG delivery ends in species with short ranges of delivery, such as the house fly, blue bottle fly, and yellow mealworm. Some species had ranges of deliver up to 72 hr, such as the German cockroach. This indicates that more time increments after 72

hr would help determine where BG delivery ends. Congener species should be studied to determine whether there is similarity in retention among species from the same genus or family. Spiders, other predators, and herbivores such as moths, true bugs, and beetles would be logical groups to study with respect to acquisition and retention of spores. Although paper wasp larvae are predators, only paper wasp adults were used in this study. Wasp larvae depend on the adults to bring prey from the nearby environment and might be good for studying bioaccumulation. Larval studies should be conducted, particularly where the larvae are associated with humans and the adults are not.

The acquisition and retention of BG varied with time and insect species, and some insects showed obvious peaks between time of acquisition and elimination. Delivery of BG differed with intake, digestion, and elimination. For most species, surface washing was a sufficient method of sample preparation for detection of acquired BG. All species except the red harvester ant reliably acquired and delivered BG spores. Overall, results demonstrate that insects can serve as passive biosentinels for detecting spore forming bacteria in the environment.

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Table 1: Experimental plan for BG retention and bioaccumulation studies. Information includes time intervals, food type, and number of individuals for retention and bioaccumulation studies. Bioaccumulation studies were not performed on the red harvester ant. Unless otherwise noted, each insect in the feeding trials was given 0.1 g of an appropriate food mixed with 100 μL 10^6 spores/mL BG in 25% glycerol: 5% sucrose.

Insects	Study	N	Time Intervals (hr)	Food
House cricket (<i>Acheta domesticus</i> L.) (Orthoptera: Gryllidae)	1 ^a	22	1, 2, 4, 8, 12, 24, 48, and 72	Ground adult dog kibble
	2 ^b	78	0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, and 72	Crushed apple
German cockroach (<i>Blatella germanica</i> L.) (Blattodea: Blattellidae)	1 ^a	140	0.5, 1, 2, 4, 6, 8, 10, 12, 24, 48, and 72	Ground rodent food
Common house fly (<i>Musca domestica</i> L.) (Diptera: Muscidae)	1 ^c	150	2, 4, 6, 8, 10, 12, 24, 36, 48, and 72	4 g ground adult dog kibble (treated with 800 μL 10^6 BG)
	2 ^d	159	0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, and 72	10 g 9:1 graham crackers: confectioners' sugar (treated with 10 mL 10^6 BG)
Blue bottle fly (<i>Calliphora vomitoria</i> L.) (Diptera: Calliphoridae)	1 ^c	150	2, 4, 6, 8, 10, 12, 24, 36, 48, and 72	2 g Pork (coated with 400 μL 10^6 BG)
	2 ^d	150	0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, and 72	10 g 9:1 graham crackers: confectioners' sugar (treated with 10 mL 10^6 of BG)
Hairy rove beetle (<i>Creophilus maxillosus</i> L.) (Coleoptera: Staphylinidae)	1 ^e	35	0.25, 0.5, 1, 2, 4, 12, 24, 48, and 72	Ground adult dog kibble

Insects	Study	N	Time Intervals (hr)	Food
Yellow mealworm (<i>Tenebrio molitor</i> L.) (Coleoptera: Tenebrionidae)	1 ^f	140	0.25, 0.5, 1, 2, 4, 12, 24, 48, and 72	Crushed apple
	2 ^f	140	0.25, 0.5, 1, 2, 4, 12, 24, 48, and 72	Crushed apple
Common paper wasps (<i>Polistes exclamans exclamans</i> Viereck) (Vespidae) and red paper wasps (<i>Polistes carolina</i> L.) (Vespidae)	1 ^g	51	2, 4, 12, 24, 48, and 72	5% sucrose 25% glycerin water
	2 ^h	62	0.25, 0.5, 1, 2, 4, 12, 24, 48, and 72	5% sucrose 25% glycerin water
Red harvester ant (<i>Pogonomyrmex barbatus</i> Smith) (Formicidae)	1 ^a	28	0.5, 1, 2, 4, 8, 12, and 24	Ground adult dog kibble
	2 ⁱ	19	0.083, 0.167, 0.25, 0.5, and 2	Ground adult dog kibble

^a Container was an inverted 175 mL plastic cup where the domed lid was lined with filter paper and served as the container bottom (Anchor-Hocking; SB0-6, LD-6). Filter paper was removed with the food.

^b Container was an inverted 175 mL plastic cup where the domed lid was lined with filter paper and served as the container bottom (Anchor-Hocking; SB0-6, LD-6). Filter paper was replaced daily.

^c Thirty flies were confined in a 4 L plastic candy jar with a 200 mm pantyhose tube secured over the opening.

^d Fifty flies were confined to a 30 cm x 30 cm x 30cm collapsible stainless steel cage (BioQuip, 1405B). Freezer paper was used to line the cage bottom and liners were replaced daily.

^e Container was a 30 mL Solo™ soufflé cup (P100) with a lid (PL1). The entire container was replaced daily.

^f Container was a 60 mL Solo™ soufflé cup (B200) with a lid (BL2). The entire container was replaced daily.

^g Container was a 0.5L Solo™ drinking cup (TP16) inverted over a plastic lid (624TS).

^h Container was a 400mL Glad™ food container with holes punched into the sides and the lid for ventilation. The entire container was replaced daily.

ⁱ Container was an inverted 175 mL plastic cup where the domed lid. The harvester ants created holes in the filter paper in the first study, so filter paper was not used in the second study.

Table 2. BG retention among eight insect species: Results of GLM analyses. Separate analyses (GLM, SPSS V13.0) were performed to compare the proportion of positive surface washes, homogenates, and combined samples (positive surface wash or homogenate) among insect species (see Table 1), across common time intervals (2, 4, 12, 24, 48, and 72 hr), and among species over time.

Dependent Variable: Surface wash

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	10.831(a)	14	0.774	12.712	0.000
Species	5.582	7	0.797	13.103	0.000
Time_Interval	0.089	1	0.089	1.464	0.227
Species * Time_Interval	0.793	6	0.132	2.171	0.044
Error	40.169	660	0.061		
Total	51.000	674			

$R^2 = 0.212$ (Adjusted $R^2 = 0.196$)

Dependent Variable: Homogenate

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	17.160	14	1.226	16.564	0.000
Species	14.069	7	2.010	27.161	0.000
Time_Interval	2.834	1	2.834	38.294	0.000
Species * Time_Interval	2.771	6	0.462	6.241	0.000
Error	48.840	660	0.074		
Total	66.000	674			

$R^2 = 0.260$ (Adjusted $R^2 = 0.244$)

Dependent Variable: Surface wash and homogenate together

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	29.401(a)	14	2.100	21.130	0.000
Species	19.770	7	2.824	28.416	0.000
Time_Interval	2.204	1	2.204	22.174	0.000
Species * Time_Interval	1.777	6	0.296	2.979	0.007
Error	65.599	660	0.099		
Total	95.000	674			

$R^2 = 0.309$ (Adjusted $R^2 = 0.295$)

Table 3. Association of sample preparation methods by insect species. A Chi-Square Test of Independence (SPSS V13.0) was used to evaluate the association of positive surface washes and positive homogenates by insect species; values close to zero indicate a weak association. Contingency coefficients measure the strength of the association; values close to zero indicate a strong association. Insect species are listed in Table 1; two species (red harvester ant and yellow mealworm) were excluded from analyses due to insufficient sample sizes. Numbers of BG-positive individuals are separated by “Positive W” and “Positive H” where W = surface washing, and H = homogenization.

Species	N	Chi-Square Value	Contingency Coefficient	Positive W	Positive H
Hairy rove beetle	11	0.665	0.092	6	7
Blue bottle fly	15	0.269	0.198	7	10
House cricket	43	0.174	0.145	31	25
Common house fly	11	0.087	0.343	4	8
German cockroach	25	0.001	0.435	8	20
Paper wasps	29	0.000	0.423	12	25

Figure 1. BG acquisition and retention: House cricket (*Acheta domesticus*). Study 1 ($n=22$) and Study 2 ($n=78$). Individuals were sacrificed at 0.5 hr in Study 2 only; lines in place of bars indicate negative samples.

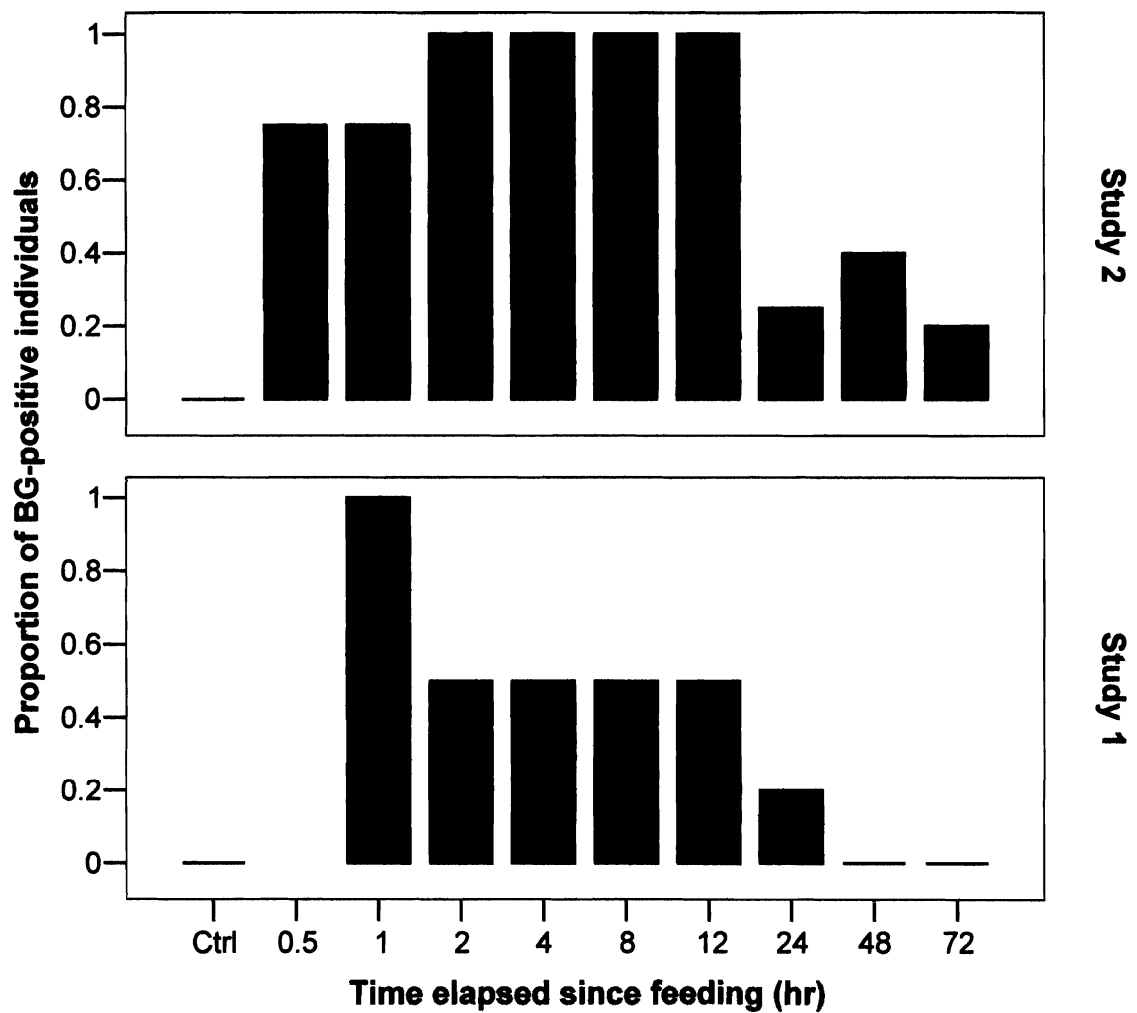


Figure 2. BG bioaccumulation: House cricket (*Acheta domesticus*). Individuals were fed a single meal at $t=0$ ($n=18$), two meals at $t=0$ and $t=24$ hr ($n=12$) or three meals at $t=0$, $t=24$ and $t=48$ hr ($n=11$).

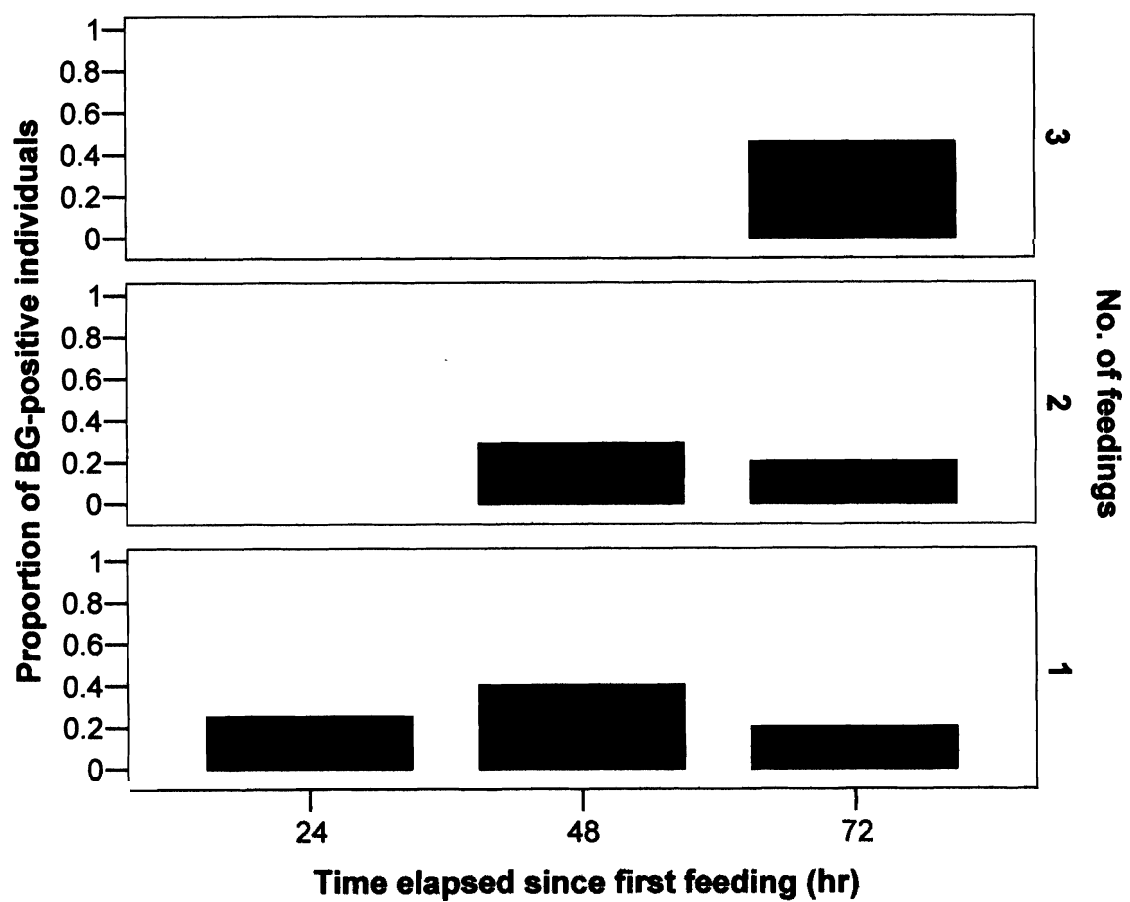


Figure 3. BG acquisition and retention: German cockroach (*Blatella germanica*). Individuals ($n=113$) were sacrificed at all intervals shown from 30 min to 72 hr; lines in place of bars indicate negative samples.

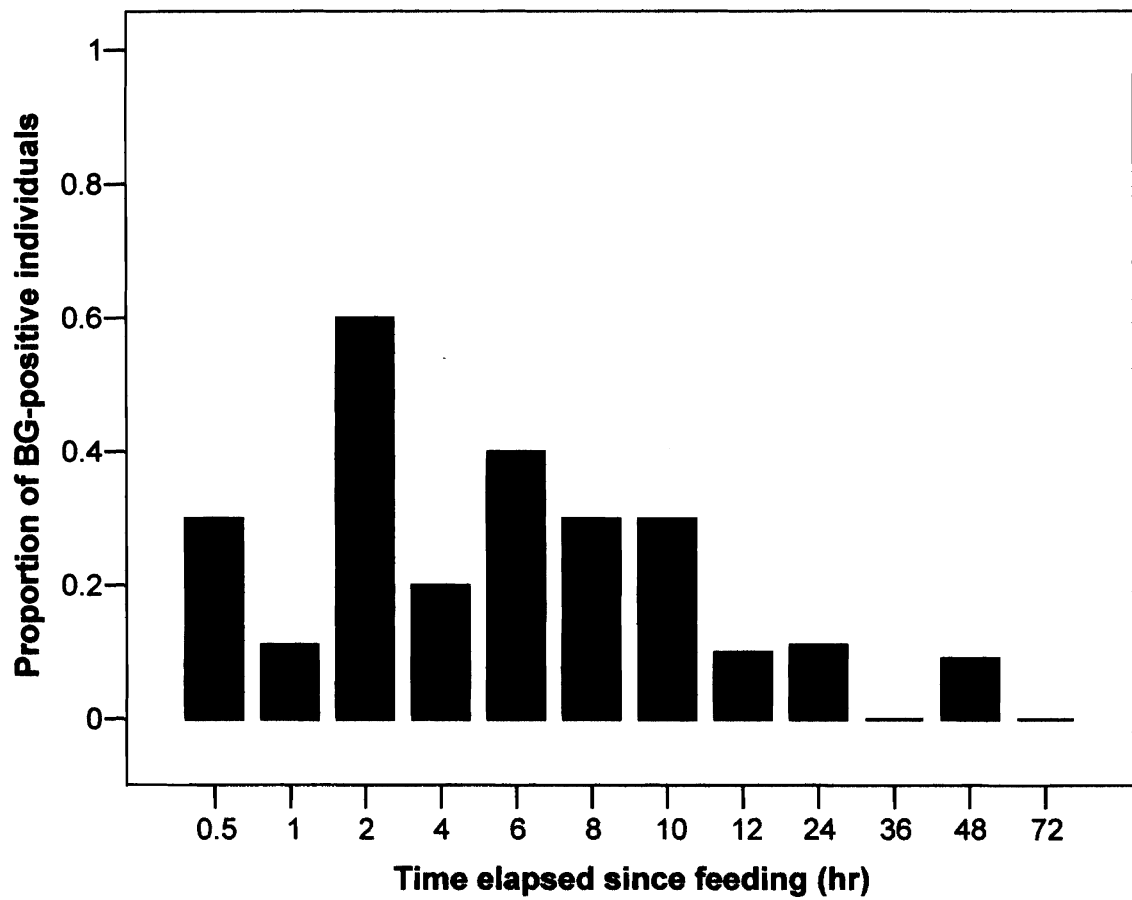


Figure 4. BG bioaccumulation: German cockroach (*Blatella germanica*). Individuals were fed a single meal at $t=0$ ($n=23$), two meals at $t=0$ and $t=24$ hr ($n=6$) or three meals at $t=0$, $t=24$ and $t=48$ hr ($n=6$); lines in place of bars indicate negative samples.

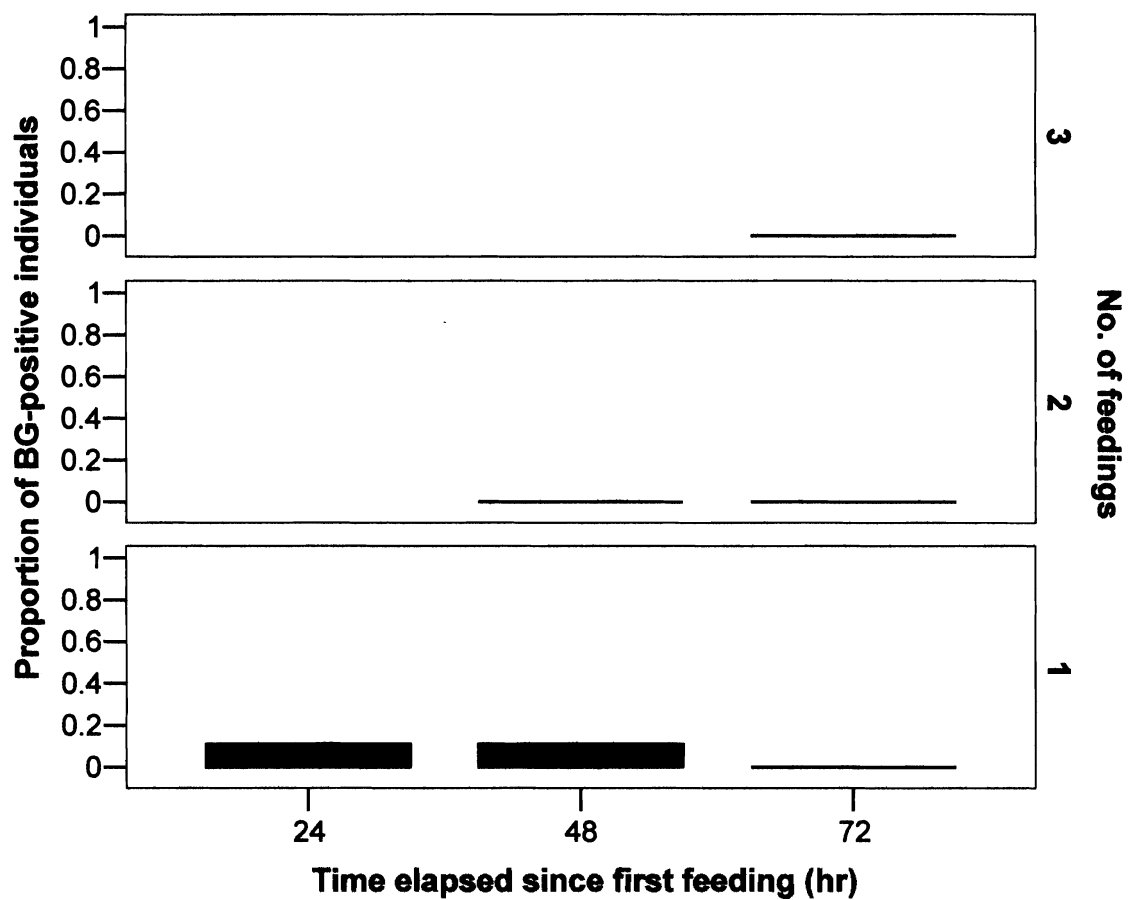


Figure 5. BG acquisition and retention: Common house fly (*Musca domestica*). Individuals were sacrificed at 6, 10, and 36 hr in Study 1 ($n=88$) and at 0.5 and 1 hr in Study 2 ($n=67$); lines in place of bars indicate negative samples. Study 1 controls were misplaced.

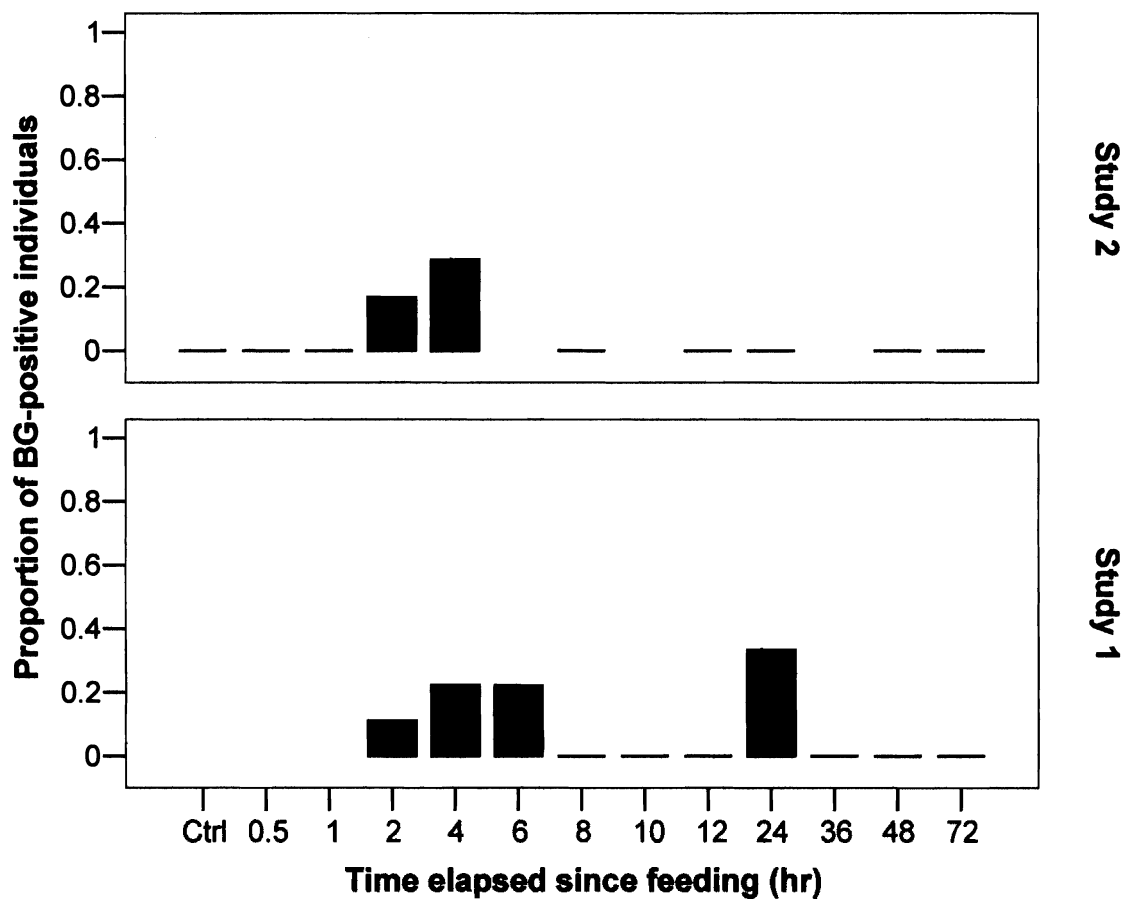


Figure 6. BG bioaccumulation: Common house fly (*Musca domestica*). Individuals were fed a single meal at $t=0$ ($n=20$), two meals at $t=0$ and $t=24$ hr ($n=16$) or three meals at $t=0$, $t=24$ and $t=48$ hr ($n=10$); lines in place of bars indicate negative samples.

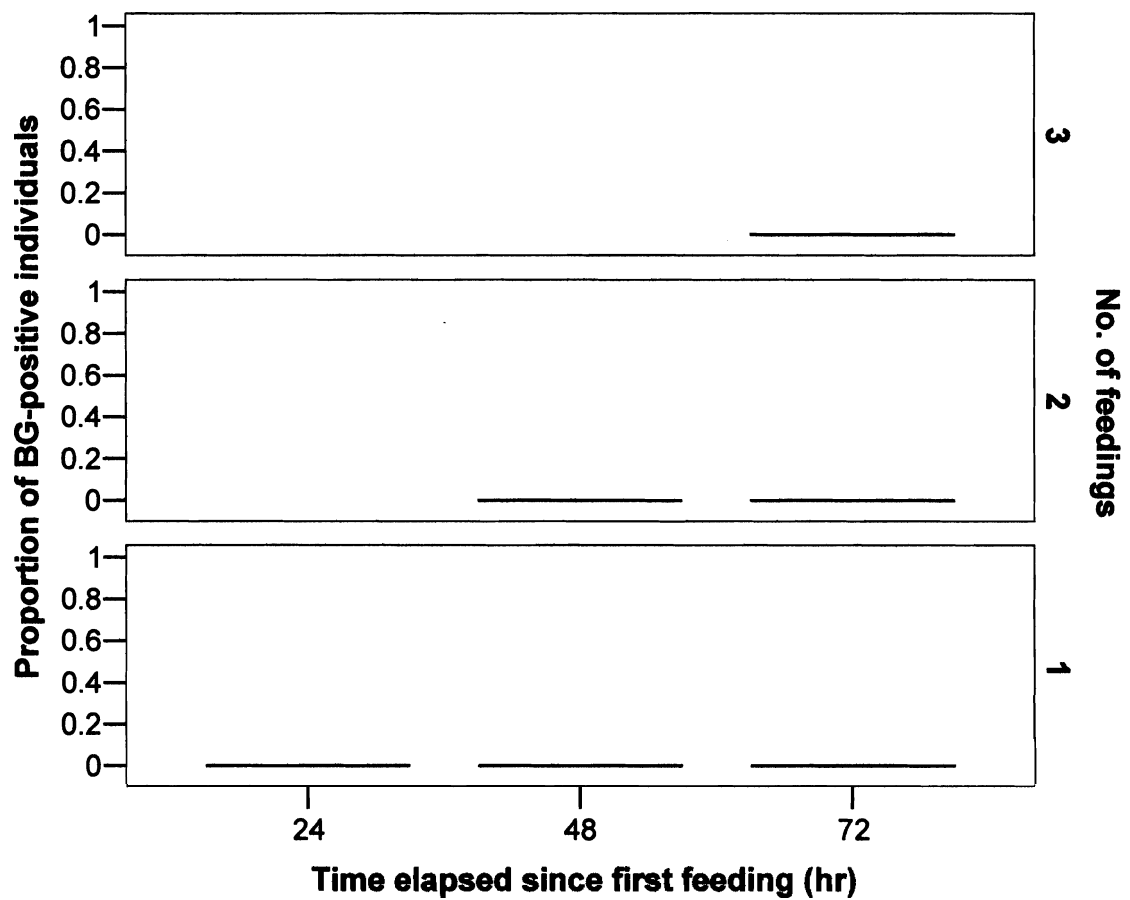


Figure 7. BG acquisition and retention: Blue bottle fly (*Calliphora vomitoria*).
 Individuals were sacrificed at 6, 10, and 36 hr in Study 1 ($n=90$) and at 0.5 and 1 hr in Study 2 ($n=107$) only; lines in place of bars indicate negative samples.
 Study 1 controls were misplaced.

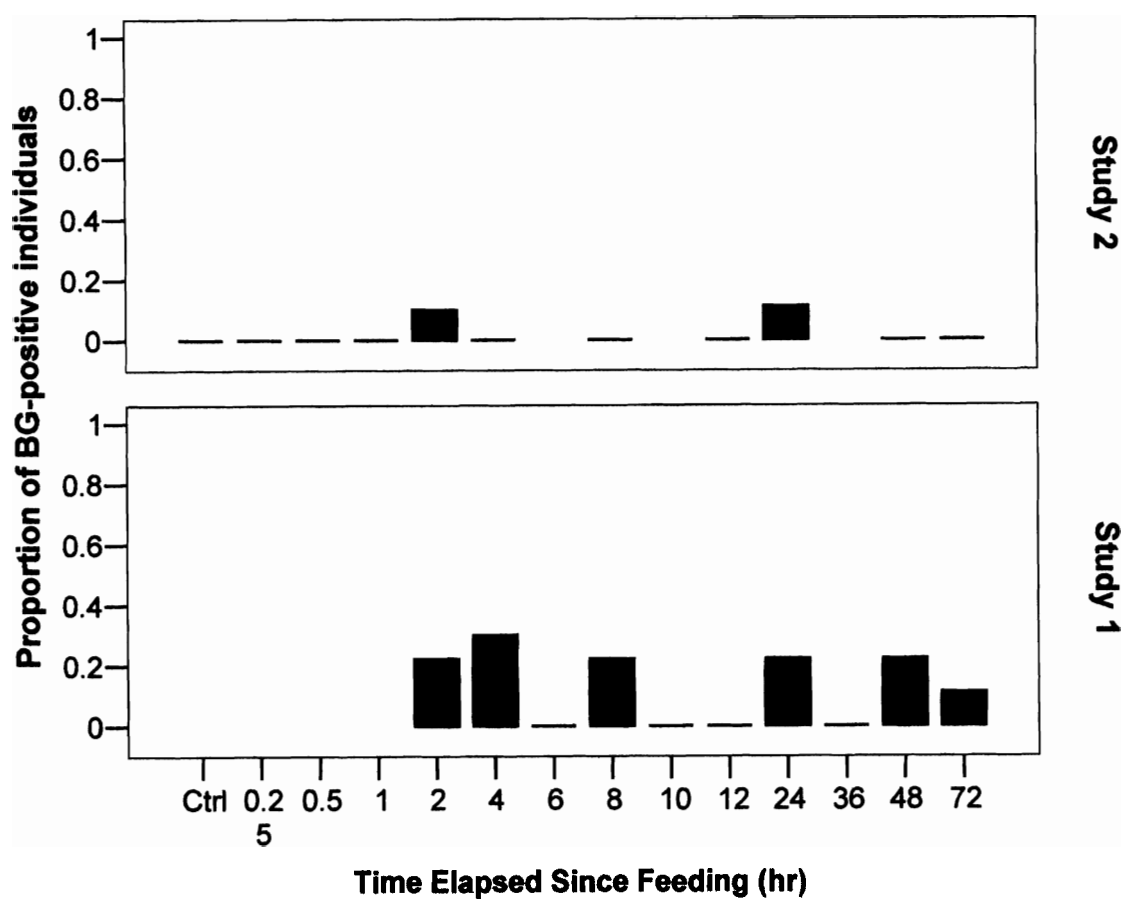


Figure 8. BG bioaccumulation: Blue bottle fly (*Calliphora vomitoria*). Individuals were fed a single meal at $t=0$ ($n=28$), two meals at $t=0$ and $t=24$ hr ($n=17$) or three meals at $t=0$, $t=24$ and $t=48$ hr ($n=10$); lines in place of bars indicate negative samples.

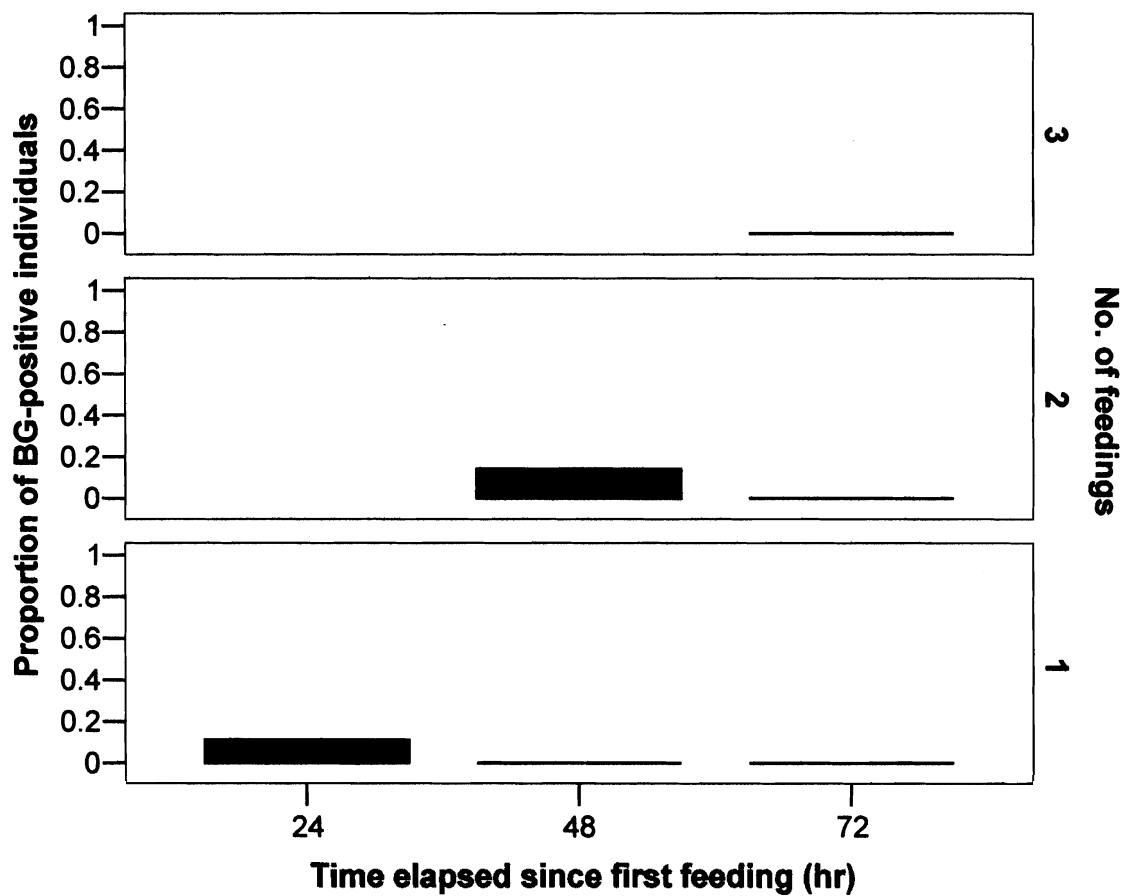


Figure 9. BG acquisition and retention: Hairy rove beetle (*Creophilus maxillosus*). A single study was conducted ($n=28$); lines in place of bars indicate negative samples.

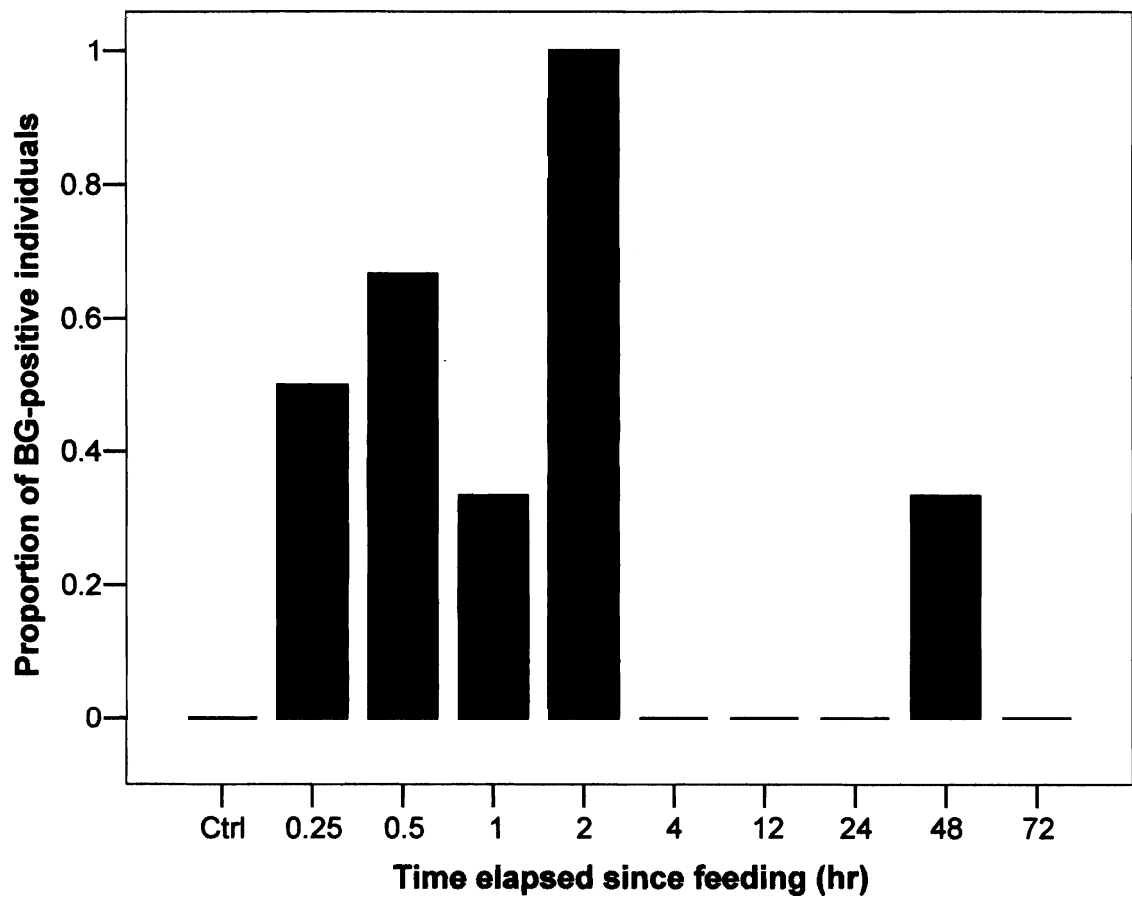


Figure 10. BG bioaccumulation: Hairy rove beetle (*Creophilus maxillosus*). Individuals were fed a single meal at $t=0$ ($n=8$), two meals at $t=0$ and $t=24$ hr ($n=6$) or three meals at $t=0$, $t=24$ and $t=48$ hr ($n=1$); lines in place of bars indicate negative samples.

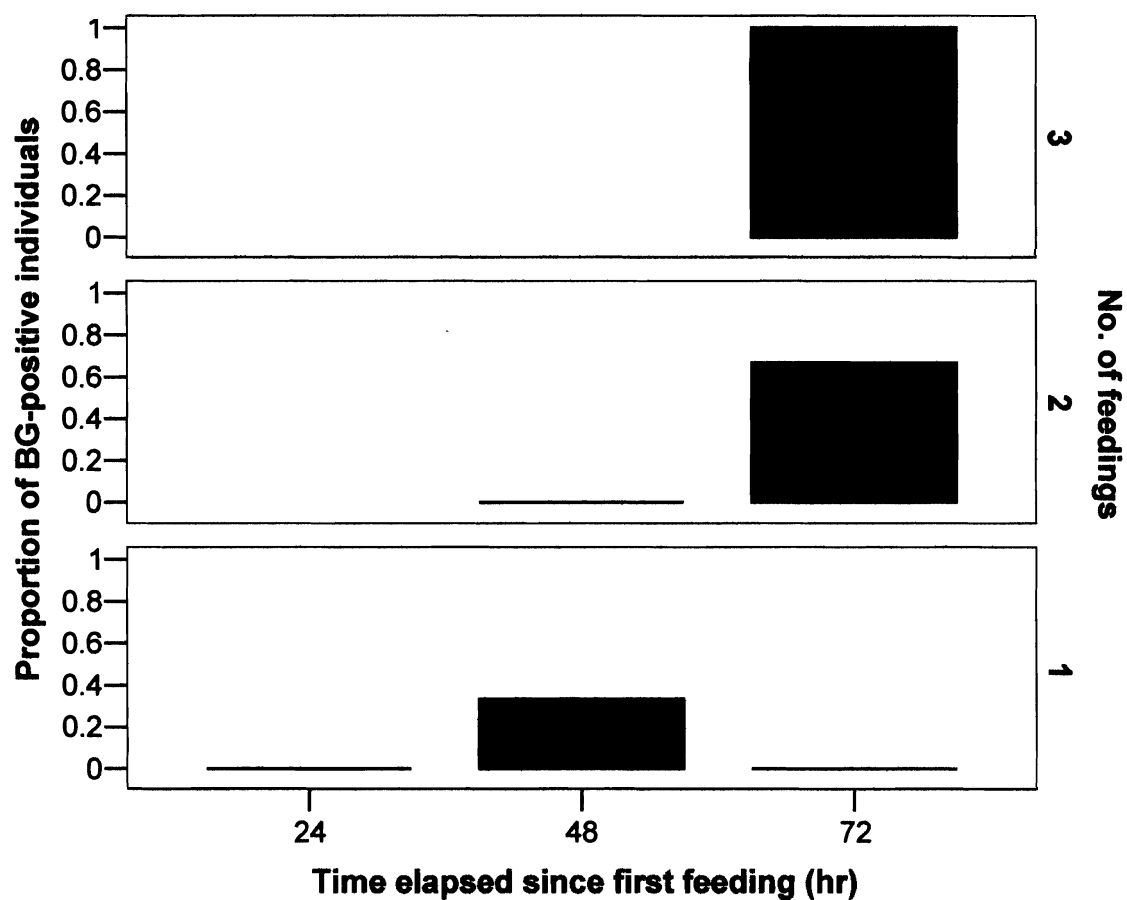


Figure 11. BG acquisition and retention: Yellow mealworm (*Tenebrio molitor*). Study 1 ($n=89$) and Study 2 ($n=110$); lines in place of bars indicate negative samples.

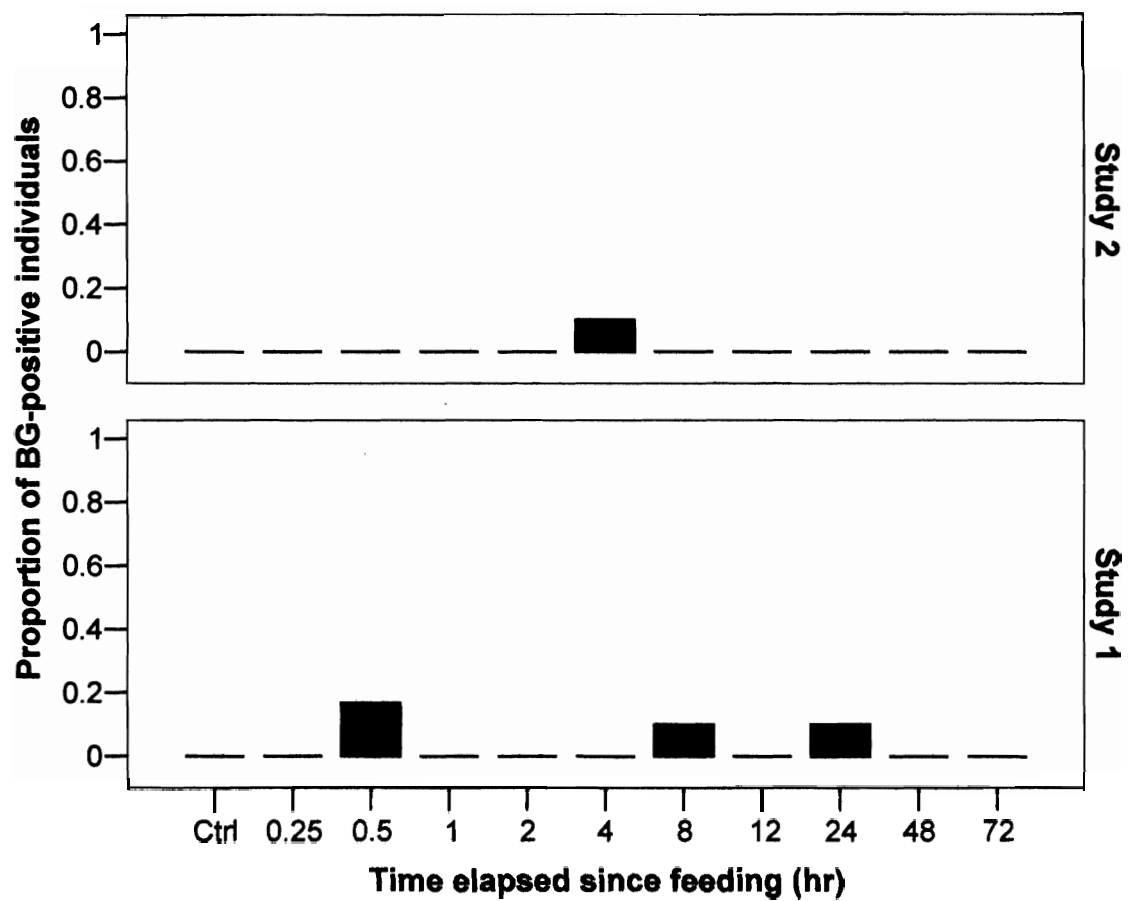


Figure 12. BG bioaccumulation: Yellow mealworm (*Tenebrio molitor*). In Study 1 individuals were fed a single meal at $t=0$ ($n=30$), two meals at $t=0$ and $t=24$ hr ($n=20$) or three meals at $t=0$, $t=24$ and $t=48$ hr ($n=10$). In Study 2 individuals were fed a single meal at $t=0$ ($n=30$), two meals at $t=0$ and $t=24$ hr ($n=20$) or three meals at $t=0$, $t=24$ and $t=48$ hr ($n=10$). Lines in place of bars indicate negative samples.

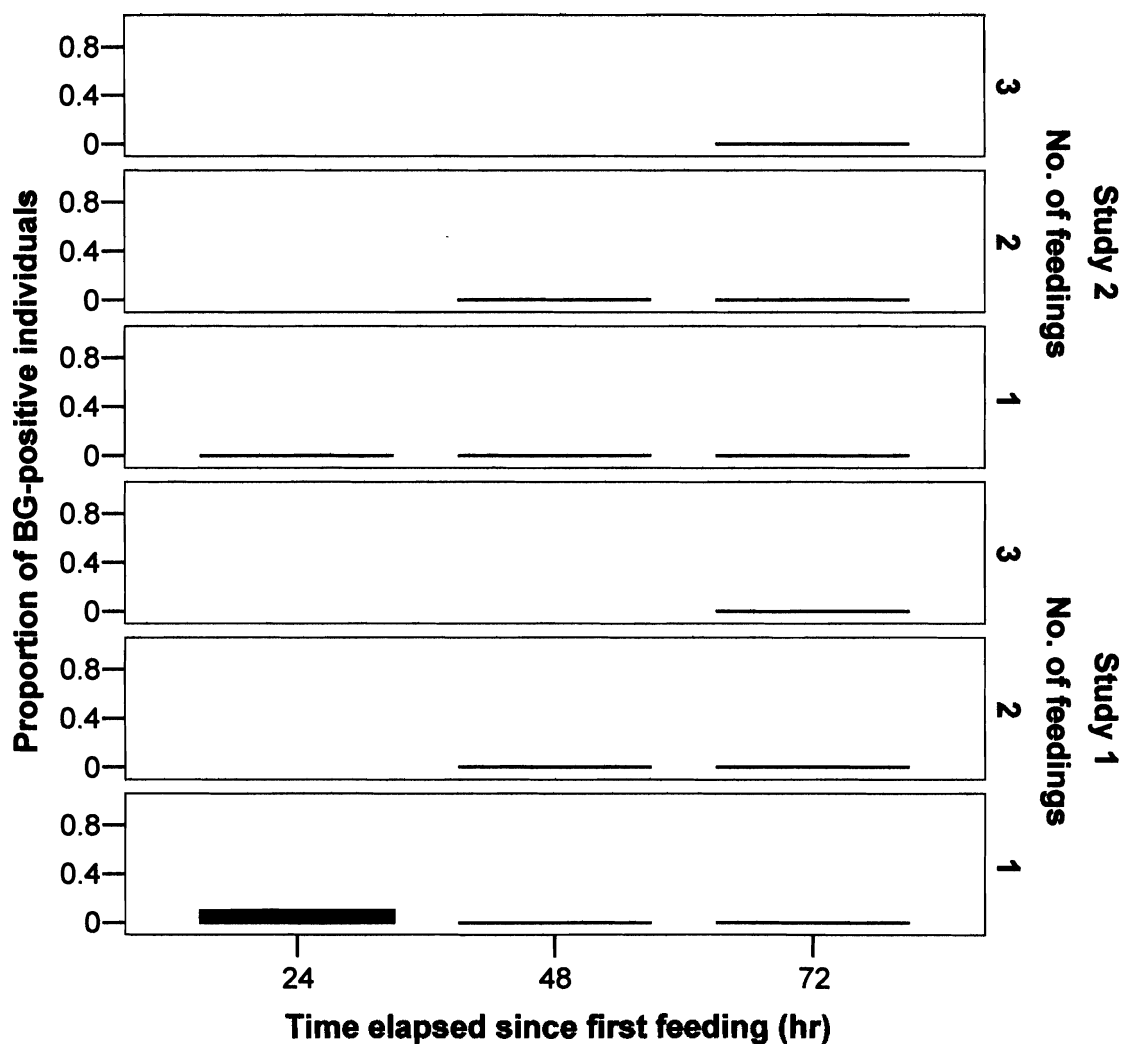


Figure 13. BG acquisition and retention: Paper wasps (*Polistes exclamans exclamans* and *Polistes carolina*). Study 1 ($n=37$) and Study 2 ($n=43$); lines in place of bars indicate negative samples. Individuals were sacrificed at 0.25, 0.5, 1, and 8 hr in Study 2 only. Study 1 controls were contaminated.

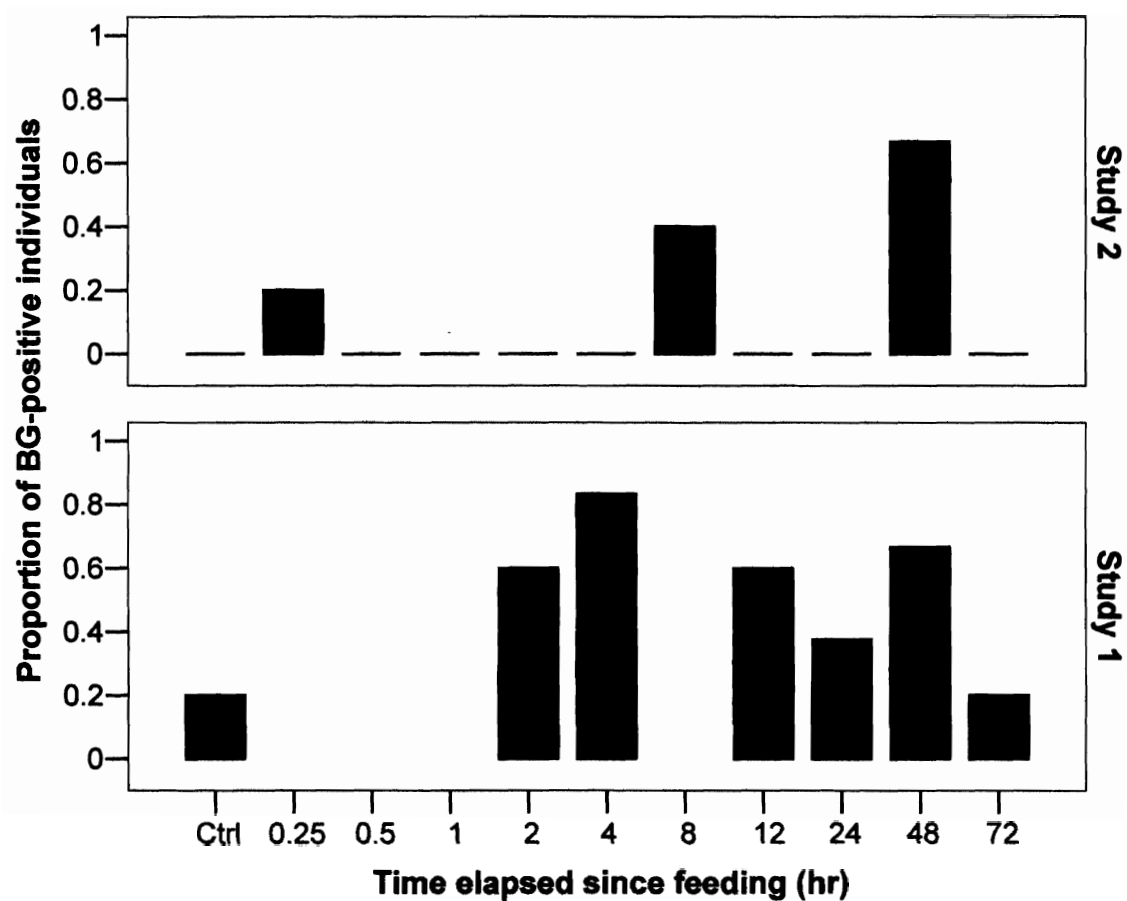


Figure 14. BG bioaccumulation: Paper wasps (*Polistes exclamans exclamans* and *Polistes carolina*). In Study 1 individuals were fed a single meal at $t=0$ ($n=21$), two meals at $t=0$ and $t=24$ hr ($n=7$) or three meals at $t=0$, $t=24$ and $t=48$ hr ($n=2$). In Study 2, individuals were fed a single meal at $t=0$ ($n=14$), two meals at $t=0$ and $t=24$ hr ($n=8$) or three meals at $t=0$, $t=24$ and $t=48$ hr ($n=3$). Lines in place of bars indicate negative samples.

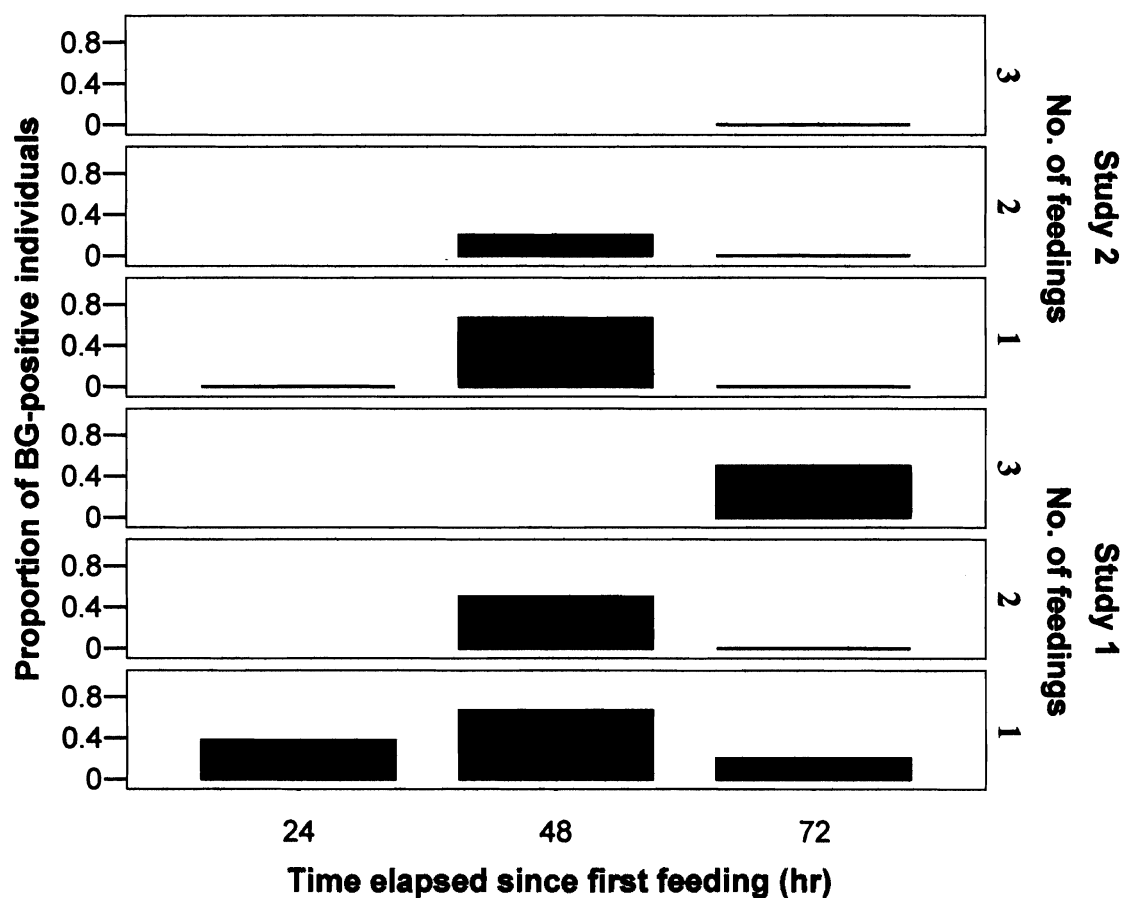


Figure 15. BG acquisition and retention: Red harvester ant (*Pogonomyrmex barbatus*). In Study 1 ($n = 28$), individuals were sacrificed at 30 min and 1, 2, 8, 12, and 24 hr. In Study 2 ($n = 19$), individuals were sacrificed at 5, 10 and 30 min and 2 hr. Lines in place of bars indicate negative samples.

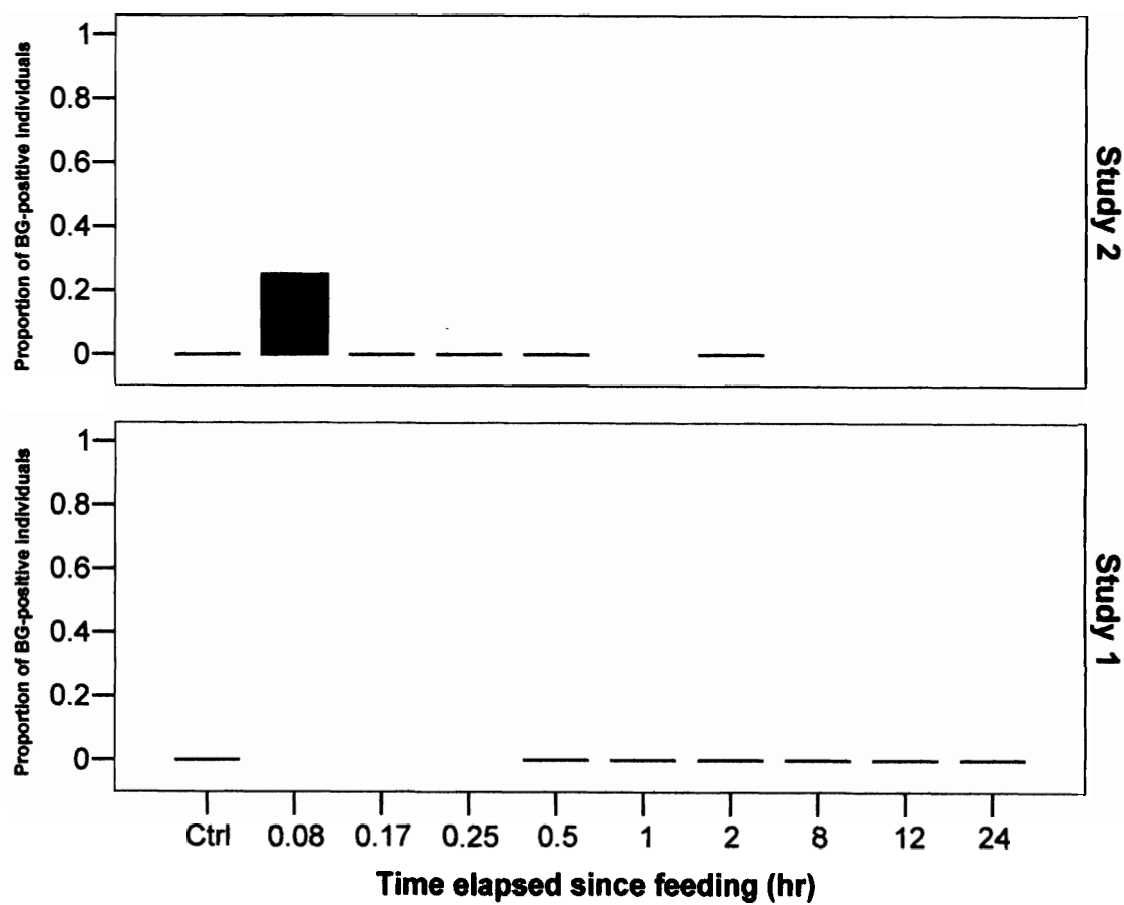
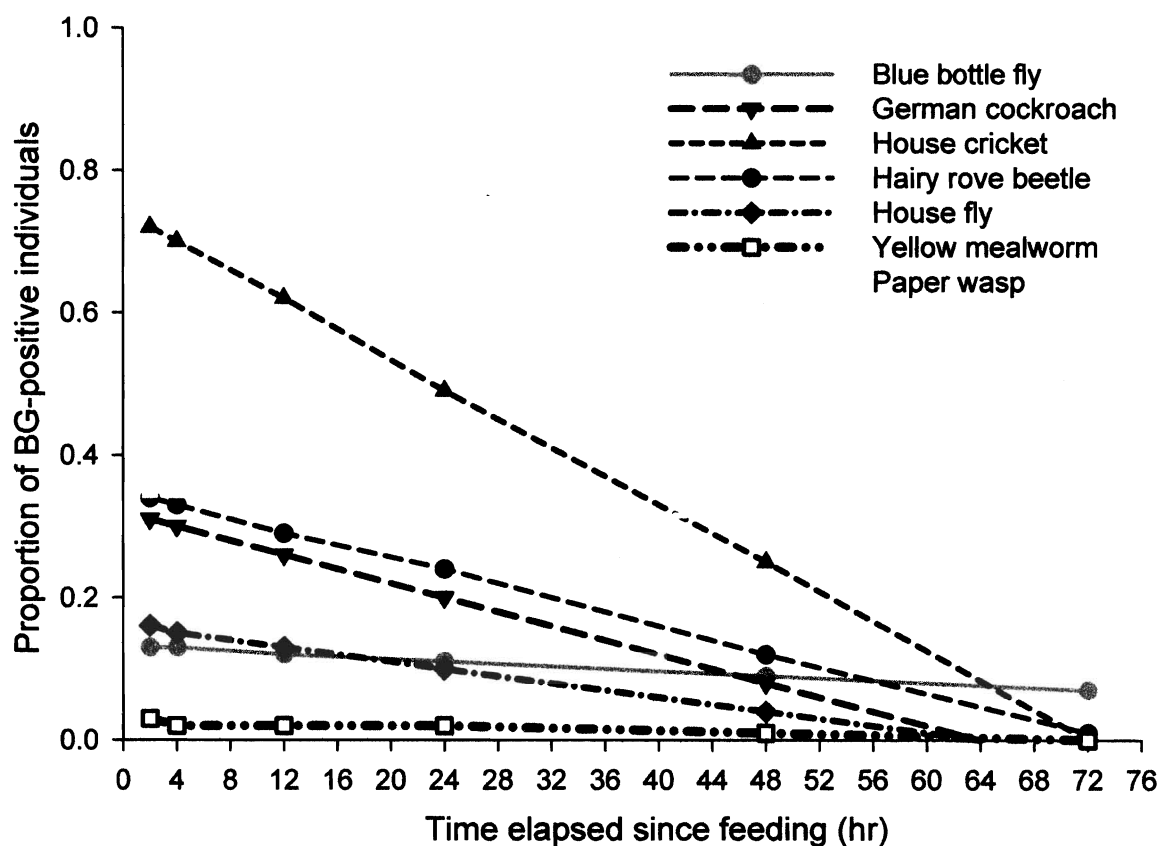


Figure 16. Predicted BG delivery by insect species. Species include blue bottle fly (*Calliphora vomitoria* L.), German cockroach (*Blatella germanica* L.), house cricket (*Acheta domesticus* L.), hairy rove beetle (*Creophilus maxillosus* L.), house fly (*Musca domestica* L.), yellow mealworm (*Tenebrio molitor* L.) and paper wasp (= *Polistes exclamans exclamans* Viereck and *Polistes carolina* L.). Values are based on GLM parameter estimates for insect species x common time intervals (see Appendix A).



APPENDIX A

GLM parameter estimates used to predict BG delivery patterns. Results of GLM analyses are shown in Table 2 and BG delivery patterns are visualized in Fig. 16.

Dependent Variable: Combined sample methods (surface wash or homogenate positive)
95% Confidence Interval

Parameter	B	Std. Error	t	Sig.	Lower Bound	Upper Bound
German cockroach	0.320	0.059	5.404	0.000	0.204	0.436
House cricket	0.668	0.070	9.497	0.000	0.530	0.806
Blue bottle fly	0.140	0.043	3.284	0.001	0.056	0.224
House fly	0.160	0.047	3.449	0.001	0.069	0.252
Yellow mealworm	0.025	0.041	0.624	0.533	-0.054	0.105
Hairy rove beetle	0.273	0.108	2.533	0.012	0.061	0.485
Paper wasps	0.412	0.058	7.073	0.000	0.297	0.526
Time_Interval	-0.003	0.001	-2.360	0.019	-0.006	-0.001
German cockroach *	-0.002	0.002	-1.001	0.317	-0.006	0.002
Time_Interval						
House cricket *	-0.003	0.002	-1.566	0.118	-0.007	0.001
Time_Interval						
Blue bottle fly *	0.002	0.002	1.055	0.292	-0.001	0.005
Time_Interval						
House fly *	0.001	0.002	0.341	0.733	-0.003	0.004
Time_Interval						
Yellow mealworm *	0.003	0.002	1.730	0.084	0.000	0.006
Time_Interval						
Hairy rove beetle *	0.004	0.003	1.325	0.186	-0.002	0.009
Time_Interval						
Paper wasps *	0	.	.	.		
Time_Interval						

VITA

Kieron Marie Torres was born in Fort Huachuca, Arizona, on October 6, 1979. She graduated from New Kent High School, New Kent, Virginia, with honors in 1997 and received her Bachelors of Science in Biology at Virginia Commonwealth University in Richmond, Virginia, in December 2002. Her M.S. in Biology from Virginia Commonwealth University is expected in August 2006. Ms. Torres was an Adjunct Professor at V.C.U. from August 2002 to December 2005, a Graduate Assistant at V.C.U. from August 2002 to May 2005, and a Project Assistant for the V.C.U. Department of Education and Life Science Department from August 2004 to May 2005. She is a coauthor of *Students as "Human Chromosomes" in Role-Playing Mitosis & Meiosis* with Dr. Joseph P. Chinnici and Joyce W. Yue in *The American Biology Teacher*.