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Using high-throughput amplicon sequencing to determine diet of generalist lady beetles in agricultural landscapes

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HIGHLIGHTS

- High-throughput amplicon sequencing (HTS) was used to characterize lady beetle diets.
- Prey detection was observed in 33–55% of field and lab specimens, respectively.
- Omnivory was prevalent in corn where prey richness and breadth were low.
- Omnivory was lower in prairie where prey richness and breadth were high.
- HTS is a useful tool for assessing biocontrol potential of predators in the field.

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ABSTRACT

Determining feeding relationships is central to understanding biological control potential in the field. However, methods to differentiate actual (or realized) feeding relationships from potential feeding relationships is lacking especially for small, generalist predators such as lady beetles. In this study, we used DNA metabarcoding approaches to characterize actual feeding relationships of lady beetles (Coccinellidae) in the field and validated our methods with a lab study. We first asked whether high-throughput amplicon sequencing (HTS) can characterize diets of lady beetles ranging from monotypic diets to diverse diet mixtures in the lab. We then examined whether diet composition and breadth of lady beetles collected from different habitat types in southern WI varied between monocultures of soybean and corn, diverse tallgrass prairie, and urban habitats. Lastly, we asked whether different body or tissue types (partial-body versus whole-body specimens) would change the likelihood of prey detection for both studies. In our controlled lab study, we found that HTS can accurately assess diet composition and diet breadth for lady beetle populations, but at the individual level, HTS has limitation for individuals feeding on more than three species of prey at any given time. In our field study, we documented lower prey richness and diet breadth in corn compared to soybean and grassland, and greater prey DNA in regurgitants than in whole-body specimens. Finally, we found that reduced diet diversity was associated with an increased prevalence of intraguild predation, but that habitat and prey diversity are not necessarily correlated. The prey detection rates (proportion of specimens with prey DNA) in our study were comparable to other studies (55% in the lab study, 33% in field study) and varied with diet composition and habitat type. The relatively low detection rates suggest that many consumer individuals would need to be assayed to fully assess diet diversity, especially in diverse systems.

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1. Introduction

Determining feeding relationships between predators and their prey is one of the greatest challenges in food-web ecology (Traugott et al., 2013; Eitzinger et al., 2013). Logistical limitations can make determining trophic linkages problematic, especially for organisms such as small insects that are difficult to observe and manipulate. Traditional approaches to quantifying feeding relationships include extensive direct and video-based observations (Paine, 1980; Grieshop et al., 2012; Zou et al., 2017), feeding trials (Muller et al., 1999; Schonrogge and Crawley, 2000), and gut content or fecal matter analyses (Pierce and Boyle, 1991; Petty, 1999). While these traditional approaches have been successful for large mammals or avian predators that are easier to track and observe (Kelly, 2000; Jordan, 2005; Garnick et al., 2018), they have been difficult for small generalist predators such as those with diverse diets, cryptic feeding behaviors, and a propensity to feed on soft-bodied prey or fluid-feeders. Moreover, these traditional approaches are labor-intensive, time-consuming, and/or conducted in artificial settings such as laboratories where the full spectrum of prey resources encountered by consumers is generally not identified or evaluated. One promising approach for determining feeding relationships is the use of metabarcoding techniques for identifying the DNA of consumed prey within the guts of predators (Symondson, 2002; Traugott et al., 2013; Sow et al., 2020).

High-throughput amplicon sequencing (HTS) is increasingly used as these approaches are sensitive to relatively small amounts of prey DNA (including both soft-bodied and hard-bodied prey), can be done with field collected specimens, and many samples can be processed in parallel (Pompanon et al., 2012; Greenstone et al., 2014; Paula et al., 2015; Sow et al., 2020). Metabarcoding has been widely used to estimate biodiversity patterns of insects, especially in species-rich locations (Yu et al., 2012; Dopheide et al., 2019; Elbrecht et al., 2019) because it can help alleviate specimen identification bottlenecks that can be time consuming and result in misidentification if proper taxonomic expertise is not available. Other, similar approaches can be used to estimate prey use and feeding behavior of insects. For example, PCR-based gut content analyses have been used to quantify levels of intraguild predation (IGP) in lady beetles, a phenomenon that can lead to reduced biocontrol potential with different lady beetle species and systems (Gagnon et al., 2011; Thomas et al., 2013; Rondoni et al., 2018). Gut-content analyses involving molecular approaches can be used to determine how different habitat types and land management practices affect feeding relationships in predators (Penn et al., 2017; Tiede et al., 2017). For example, Penn et al. (2017) found evidence of IGP and reduced prey use in ants with increasing habitat fragmentation suggesting that the efficacy of pest suppression decreases with habitat fragmentation regardless of changes in ant abundances and species richness.

In this study, we use high-throughput amplicon sequencing (HTS) to identify predator–prey associations and diet breadth of lady beetles, a common and important group of predatory insects in many agricultural landscapes. Previous work in lady beetles have only used species-specific primers to characterize potential use of prey (Gagnon et al., 2011; Thomas et al., 2013; Paula et al., 2015; Rondoni et al., 2018), however, these species-specific primers cannot capture the full spectrum of prey available to lady beetles, especially in diverse systems such as tallgrass prairie. HTS has been used to characterize feeding relationships in other arthropods (e.g., tiger beetles (Pons, 2006), ground beetles (Tiede et al., 2016), spiders (Piñol et al., 2014) and vertebrates (e.g., bats (Jusino et al., 2019), birds (Sow et al., 2020)) suggesting their potential use in lady beetles.

We used two methodological approaches to assess feeding relationships in lady beetles. First, we conducted a feeding trial as a proof of concept where lady beetles were fed known prey in either monotypic or diverse diets, and gut contents were analyzed using HTS ("Study 1"). In this study, we were primarily interested in whether HTS methods could accurately reconstruct prey use when lady beetles were fed under controlled conditions, and whether prey detection was limited to simple, monotypic diets. Second, we collected lady beetles in different habitats (soybean, corn, grasslands, and urban environments) to examine prey consumed under natural conditions ("Study 2"). Results from our previous research show that perennial systems such as tallgrass prairie and mixed grasslands supported a greater abundance and diversity of prey for lady beetles such as soft-bodied and immature herbivores (e.g., aphids, thrips, grasshoppers and hopper nymphs) than annual systems (Liere et al., 2015; Fox et al., 2016; Kim et al., 2017). We therefore predicted that lady beetles captured agricultural habitats would have lower diet breadth than those captured in grasslands. In addition, because the large presence of predator DNA can obscure the detectability of the prey DNA in the alimentary canal, we examined whether using different types of body tissue (whole specimens vs. partial-body specimens such as regurgitant, dissected alimentary canals) can alter the likelihood of detecting prey DNA. Because the preparations of the partial body specimens can be logistically challenging and time consuming, we wanted to determine whether the benefits of increased prey detection rates could offset the logistical challenges of partial-body insect preparation. To our knowledge, this study is the first to use HTS to characterize the diet of lady beetles, an important group of biocontrol agents.

2. Methods

2.1. Laboratory feeding trial (Study 1)

In 2014, we established a colony of lady beetles (*Hippodamia convergens)* purchased from Arbico Organics (Oro Valley, AZ). Upon arrival, adult lady beetles were placed in 473 ml glass jars (approximately 100 individuals per jar) filled with straw for cover, a cotton wick with water, and strips of mesh fabric with frozen diamond back moth eggs (*Plutella xylostella* purchased from Benzon Research, Carlisle, PA). Water and eggs were replaced as needed and the colony was held in a growth chamber set to 22 ± 1 °C, 50% RH, and a 12:12 (L:D) photoperiod. After 1 week of feeding and acclimation, lady beetles were starved for 48 h to remove any food from their digestive system. Individuals were placed within 118 ml soufflé cups (Dart Conex Complements, Mason, MI). They were then fed one of seven different diets *ad libitum* for 24 h before being frozen (N = 20 replicates per diet). This amount of time is within the detectability period of lady beetle prey and prey symbiont DNA as they reside in the guts (Paula et al., 2015). There were four monotypic diets fed to beetles: corn earworm eggs (*Helicoverpa zea* from Benzon Research, Carlisle, PA); beet armyworm larvae (*Spodoptera exigua*, 1st-2nd instars, from Benzon Research, Carlisle, PA); cabbage looper larvae (*Trichoplusia ni* 1st-2nd instars, from Benzon Research, Carlisle, PA), and pea aphid (*Acyrthosiphon pisum,* mixed instars from field collections in southern Wisconsin). The diverse diets consisted a four-species diet mixture (all four prey types) and two two-prey species diet mixes. Because we did not have enough beetles to do all pairwise combinations of prey types for the two-prey species diet mixes, we selected two combinations that would account for variability in prey detection between different life stages (corn earworm eggs + cabbage looper larvae) and variability due to differences in taxon type (pea aphid + beet armyworm larvae). We also had a control treatment where lady beetles were not fed anything for 24 h (N = 20). Feeding arenas consisted of the souffle cups, 1 lady beetle, and an excess of prey given lady beetle consumptions rates of ~100 prey per day (Krengel et al., 2013; Delgado-Ramírez et al., 2019). Because we wanted all food items to be eaten during the 24 h period, we placed approximately 50 prey items within each container (Delgado-Ramírez et al., 2019).

After 24 h of feeding, all lady beetles (including control groups) were surface sterilized with ethanol, bleach, and distilled water following protocols from Cooper et al. (2016) and frozen at −20 °C until DNA extraction. Because we were also interested in whether partial or whole-body specimens would yield different results in prey detection, we dissected alimentary canals from half of the lady beetle specimens just prior to DNA extraction and placed contents into 1.5 ml vials. For all specimens, we extracted and purified total DNA from homogenized samples using the DNeasy Blood and Tissue Kits (Qiagen Inc., Valencia CA USA) following the animal tissue protocol.

2.2. Field-captured specimens (Study 2)

In 2015–2016, we collected different species of lady beetles from soybean, corn, and mixed tallgrass prairie fields across southern WI using sweep nets and aspirators. Fifteen fields (sampled within a 50 m × 100 m area each, five fields per field type) within Dane County near Arlington, WI were searched for 30 min in a zig-zag pattern and any lady beetle observed was collected. Lady beetles were stored on ice upon return to the lab. We collected beetles monthly from June-August each year until 50 specimens were collected from each field type. We also collected lady beetles from within the city of Madison, WI in and around urban structures such as buildings and homes to determine whether urban environments offered food resources for lady beetles.

Upon return to the lab, lady beetles were surface sterilized and then individually placed into clean 1.5 ml tubes. Half the lady beetles were then immediately frozen at −20 °C. For the other half of the field captured specimens, we were interested in whether we could detect prey DNA in regurgitant of lady beetles, therefore we induced regurgitation by submerging tubes into a bath of warm water for 2–3 min and then centrifuging tubes for 1 min at low speed (300 RPM). If any regurgitant was observed (>90% of tested individuals regurgitated), we placed the lady beetles into separate tubes and processed both the regurgitant and whole-body specimen separately. Again, we extracted and purified total DNA from homogenized samples using the DNeasy Blood and Tissue Kits (Qiagen Inc., Valencia CA USA) following the animal tissue protocol.

2.3. PCR and Illumina workflow

Purified genomic DNA was submitted to the University of Wisconsin-Madison Biotechnology Center (Madison, WI). DNA concentration was verified fluorometrically using either the Qubit® dsDNA HS Assay Kit or Quant-iT™ PicoGreen® dsDNA Assay Kit (ThermoFisher Scientific, Waltham, MA, USA). Samples were prepared in a similar process as described in Illumina's 16 s Metagenomic Sequencing Library Preparation Protocol, Part # 15044223 Rev. B (Illumina 2013) with the following modifications. Purified DNA was PCR amplified using primers specific to the mitochondrial cytochrome *c* oxidase subunit I region (COI) that also contained Illumina adapters. The LCO1490/CO1-CFMRa primer set was used because it has been successful in detecting invertebrates in other species (Folmer et al., 1994; Jusino et al., 2019) and the sequences of these primers that were complementary to the COI locus were LCO1490: 5′- GGTCAACAAATCATAAAGATATTGG-3′ (Folmer et al., 1994) and CO1-CFMRa: 5′-GGWACTAATCAATTTC-CAAATCC-3′ (Jusino et al., 2019). PCR amplifications were as follows: 94 °C for 60 s followed by 35 cycles of 94 °C for 60 s, 50 °C for 90 s, 72 °C for 60 s, and a final extension of 72 °C for 7 min. Following initial amplification, reactions were cleaned using a 1x volume of AxyPrep Mag PCR clean-up beads (Axygen Biosciences, Union City, CA). Barcodes were added using the following thermal cycling conditions: 94 °C for 60 s followed by five cycles of pre-amplification at 94 °C for 60 s, 45 °C for 90 s, and 72 °C for 90 s, and a final extension of 72 °C for 7 min. The reactions were then cleaned using a 0.8x volume of AxyPrep Mag PCR clean-up beads (Axygen Biosciences) and the quality and quantity of the finished libraries were assessed using an Agilent DNA 1000 kit (Agilent Technologies, Santa Clara, CA) and Qubit® dsDNA HS Assay Kit (ThermoFisher Scientific), respectively. Libraries were pooled in an equimolar fashion and appropriately diluted prior to sequencing. Paired end, 150 bp sequencing was performed using the Illumina MiSeq Sequencer and a MiSeq Reagent Kit v2 (300-cycles) kit. Images were analyzed using the standard Illumina Pipeline, version 1.8.2. Three pools of 90, 192, and 104 samples were sequenced to a depth of approximately 10 million reads per pool, resulting in an approximate depth of 115,000, 54,000, and 103,000 reads per sample for the three library pools, respectively. Raw MiSeq reads have been deposited in NCBI's Sequence Read Archive (SRA) under Bioproject PRJNA735709.

2.4. Sequence analysis

The amplicon data were processed and analyzed using the Amplicon Toolkit Pipeline (AMPtk) (Palmer et al., 2018). In brief, overlapping paired end reads were merged using the USEARCH algorithm with default parameters (Edgar, 2010) as implemented in AMPtk's pre-processing tool. Reads that did not contain identifiable forward or reverse primer sequences or that contained more than one mismatched base with its assigned barcode were removed along with amplicons whose lengths were <170 nt after merging. All merged reads were subsequently trimmed to a standardized length of 180 nt. Reads from the three sequencing pools were quality filtered separately and then concatenated together for the remainder of the pipeline. Next, the Divisive Amplicon Denoising Algorithm (DADA2) was used to model sequencing errors in the amplicon dataset and subsequently infer the true sequence composition of each amplicon (Callahan et al., 2016). This denoising step is essential for amplicon experiments as errors can be introduced into the amplicons through the sequencing process or by merging overlapping paired end reads. Once DADA2 has modeled the error profiles present in the data, it generates a corrected set of amplicon sequencing variants (ASVs) that represent the true nucleotide sequences of all unique reads in a particular sample. Chimeras were also detected and removed by DADA2. Once ASVs were inferred, they were assigned to operational taxonomic units (OTUs) at 97% nucleotide sequence similarity. Samples containing <500 total reads after this stage were removed from the analysis. Additionally, OTUs were filtered using the AMPtk filter command, which is designed to identify and remove OTUs that are likely erroneously assigned to samples due to index bleed. Index bleed occurs when a small percentage of reads from one sample are misassigned to an incorrect sample (Schnell et al., 2015) and is estimated to impact approximately 0.5% of the total reads on the MiSeq instrument. To remove these reads, all amplicons were first mapped back to each OTU identified in the previous step and the number of reads that were predicted to bleed into each sample was determined. OTUs that were represented by read counts that fell below this threshold in each sample were identified as artefacts and removed from the analysis. Additionally, singleton OTUs were also identified and removed with this command. Finally, the taxonomy command was used to predict taxonomic assignment using a hybrid method that uses a combination of USEARCH (Edgar, 2010), SINTAX (Edgar, 2016) and UTAX (Edgar, 2013) for taxonomic predictions. The results for the three methods were compared and the method that produced the finest taxonomic resolution within the confidence thresholds were used to assign taxonomy. COI was used as the search database available at https://amptk.readthedocs.io/en/latest/taxonomy.html, 0.8 was used as the confidence threshold for taxonomic assignment by SINTAX and UTAX, and 0.7 was used as the threshold for USERACH percent identity. All non-arthropod reads were removed from the analyses.

2.5. Statistical analyses

2.5.1. Sequence analyses

Of the approximately 31.7 million paired-end reads that were sequenced for the three library pools, 27.8 million reads were successfully merged, contained both forward and reverse primers, and were of sufficient length for downstream processing and analysis. Actual read yields per sample ranged from 319 reads to 217,400 with an average number of reads per sample of approximately 72,000 and a median of 61,000. After denoising and removal of chimeras, 402 ASVs remained, which were ultimately clustered into 142 OTUs. Two samples did not

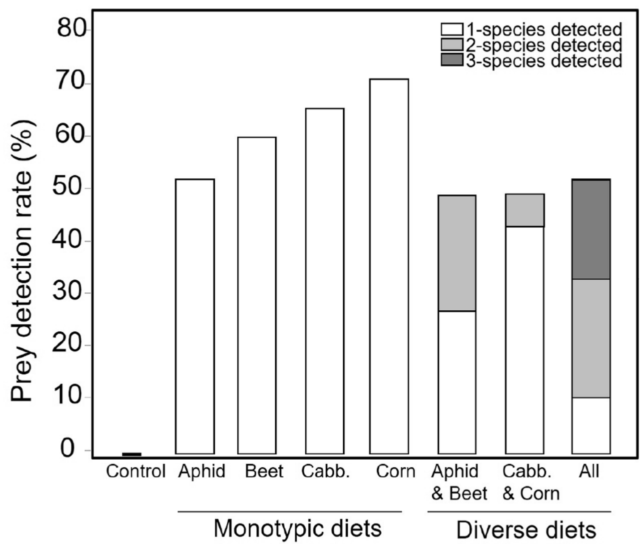

Fig. 1. Diet treatment effects on prey detection rates (i.e., the number of lady beetle individuals with prey items detected). Monotypic diets include only 1 species of prey offered to the lady beetles (aphids, beet armyworm, cabbage looper larvae, or corn earworm larvae). Diverse diets include two-species mixtures (aphids and beet armyworm mix or cabbage lopper and corn earworm larvae mix) or "all" four-species mixture (aphids, beet armyworm, cabbage looper, and corn earworm larvae offered simultaneously). Legend corresponds to the number of prey species detected in the diverse diets; 1 species only (white), 2 species only (light grey), and 3 species only (dark grey). No individual lady beetles had all four prey species detected in the diversity 4-species diet.

contain at least 500 reads after quality filtering (372 and 448) and were removed from the analysis.

2.5.2. *Diet analyses*

For each study, we first constructed a prey OTU presence-absence (1/0) matrix that contained each lady beetle individual assayed (rows) and all the insect prey OTUs that were detected (columns). We removed host ladybeetle DNA reads from the matrix. To determine prey OTU richness, we summed the prey presence values across the columns for each lady beetle individual. To determine whether individual lady beetles had any prey in their guts, we assigned richness values of 1 for individuals that had prey and 0 when no prey was detected.

We used a logistic regression to determine how the presence/absence of prey (i.e., prey detection rates) varied as a function of diet treatments (lab feeding assay in Study 1) or habitat type (prairie, soybean, corn, and urban habitats in Study 2). We were also interested in the interaction of diet treatment or habitat type with tissue type (whole or partial body). In a follow up analysis, we also used a GLM (gaussian error structure) to determine how the number of host lady beetle reads (log transformed) or prey reads (sum of all prey reads, log-transformed) varied as a function of diet treatment (Study 1) or habitat type (study 2) and their interactions with tissue type.

To determine how prey richness in the diet of beetles (total number of OTUs detected in each lady beetle) was affected by diet treatment (for Study 1) or habitat (for Study 2) and tissue type (Studies 1 and 2), we used a GLM with a Poisson distribution. Finally, we used a permutational MANOVA (PERMANOVA, Bray–Curtis dissimilarity) to examine how diet treatment (Study 1) or habitat type (Study 2) influenced prey OTU community composition. To determine how diet breadth varied with diet treatment (Study 1) and habitat type (Study 2), we performed multivariate homogeneity of variance tests (Bray-Curtis dissimilarity, betadisper function) which measures the object distance from the group centroid. All analyses were performed in R v4.0.2 (R Development Core Team, 2014).

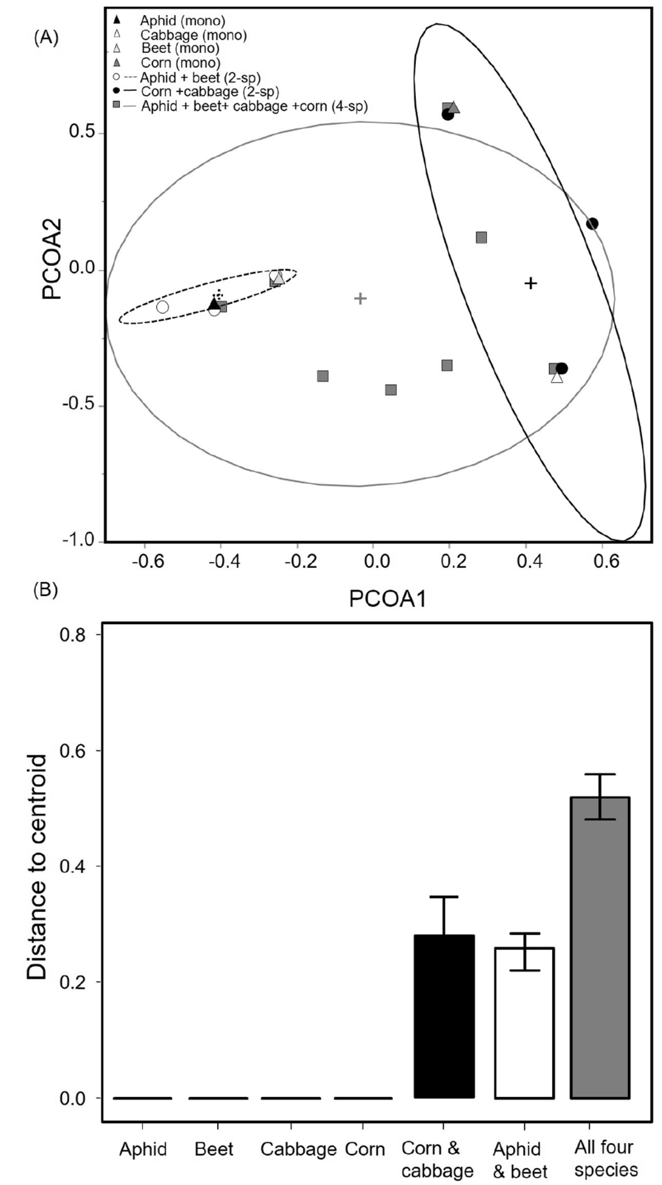

Fig. 2. Diet composition effects on OTU composition (A) and diet breadth (distance to centroid, B). Crosses represent centroids in A. Error bars are 1 ± SE from the treatment mean in B.

3. Results

3.1. *Laboratory feeding trial (Study 1)*

Our first question was to determine whether we were able to detect prey in four types of monotypic diets as well as diverse diets consisting of a mix of 2 or 4 species. We did not detect any prey in the control diet treatment and therefore this diet treatment was not used in the statistical analyses. For the remaining treatments, the average prey detection rate was 55.33% (Fig. 1). Prey detection in monotypic diets was highest (63.3%). Although detection was slightly lower in the diverse diets (2-species diet average 50%, 4-species diet 52.6%), there was no statistical difference in prey detectability across the three mixed diet treatments ($X^2 = 3.54$, df = 6, P = 0.73). Within an individual, we never detected all prey items in the 4-species mixed diet. In most cases within this treatment, only 1 prey item was detected (50% of individuals), and only a few individuals with 2 prey (20%) and 3-prey items (20%) detected were identified. For the 2-species diets, only 22% of the individual lady

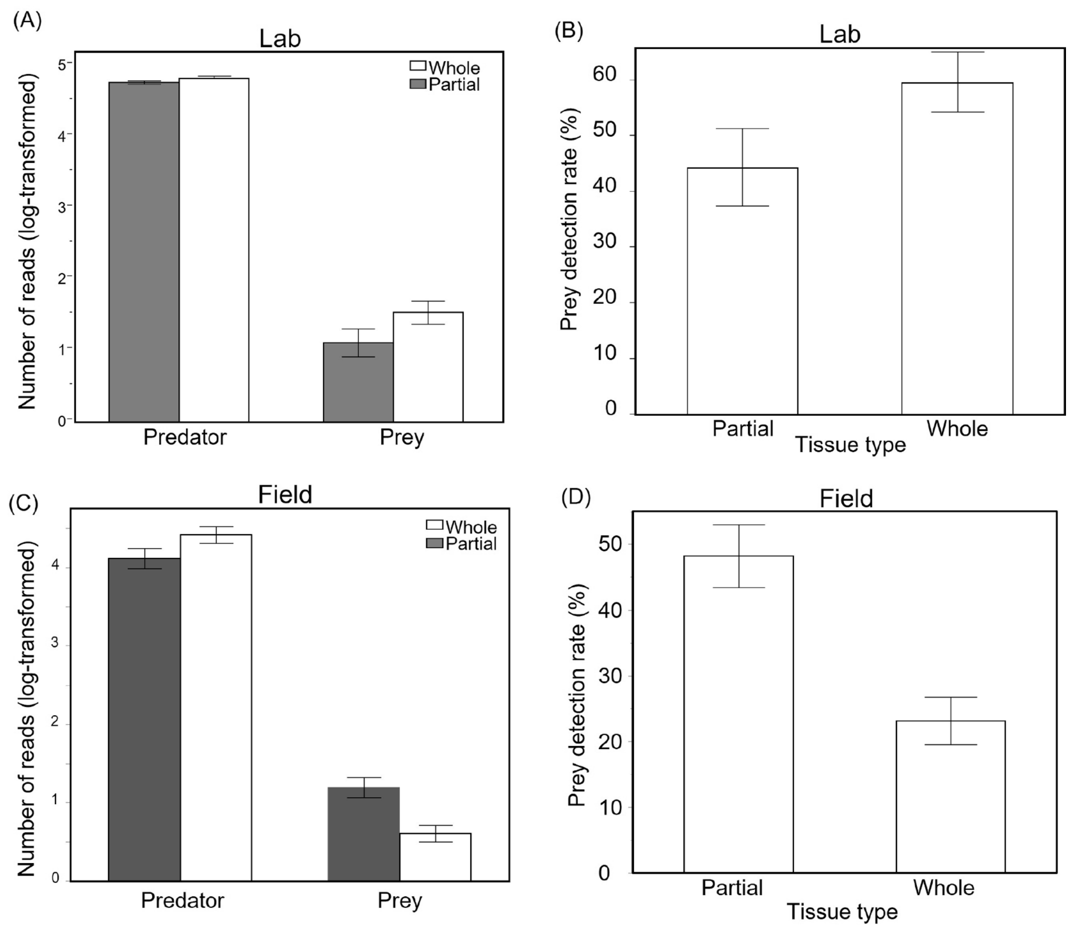

Fig. 3. The effects of partial and whole-body tissue types on the number of DNA reads (A,C) and prey detection rates (proportion of individuals with prey detected, B, D) for lady beetles used in the lab study (top panels) and field captured (bottom panels). Partial tissues for the lab study were dissected alimentary canals and regurgitant for the field study. Error bars are 1 ± SE from the treatment mean.

beetles had both prey species detected. In all cases, when prey was detected, the correct prey species was identified. The three diet treatments affected prey OTU community composition (perMANOVA, $F_{6,72} = 43.48$, $P = 0.001$, Fig. 2a) and breadth (betaDisp, $F_{6,66} = 8.57$, $P < 0.001$, Fig. 2b). As expected, monotypic diets and 2-species mixture diet treatments had lower breadth (distance to centroid) than the 4-species diet mixture. Lastly, there were no differences in the detectability of the each prey species in the 4-species diet treatment (74% of samples detected *T. ni*, 70% of *S. exigua*, 58% of *H. zea*, and 70% of *A. pisum*), suggesting that different digestion and decay rates of these prey species did not affect the likelihood of prey detection and prey community composition (Gagnon et al., 2011; Greenstone et al., 2014).

Our next question was to determine whether tissue type (whole body or alimentary canal) influenced the abundance of host reads detected or the ability to detect prey. In both tissues, the number of host ladybeetle reads was greater than the total number of prey reads (Fig. 3a) and the number of host ladybeetle reads was over two-orders of magnitude greater than prey reads. For both tissue types, the average number of host reads was 68,682 compared to 1796 when samples that had no prey detected were included in the analysis or 3345 when only samples that had prey detected were included. Tissue type affected abundance of reads derived from host ladybeetles where whole-body tissues typically yielded greater host ladybeetle reads compared to the alimentary canals ($X^2 = 4.29$, $df = 1$, $P = 0.038$). However, prey detection rates (percent of individuals with prey detected in a treatment) were not affected by tissue type ($X^2 = 2.07$, $df = 1$, $P = 0.14$, Fig. 3b) indicating that dissections of lady beetle guts are not needed to increase likelihood of prey detection.

Unexpectedly, we also detected the presence of *Dinocampus coccinellae* in our lab-reared specimens, a parasite of lady beetles. *Dinocampus* detection was low (11.0% of samples) and for those where *Dinocampus* was detected, tissue type did not affect detection rates ($X^2 = 0.47$, $df = 1$, $P = 0.492$, Fig. 4a). Likewise, *Dinocampus* presence did not affect prey detection rates ($X^2 = 1.27$, $df = 1$, $P = 0.259$, Fig. 4b) but generally, lady beetles without *Dinocampus* had a greater prey detection rates (58%) than lady beetle with *Dinocampus* (33%).

3.2. Field-captured specimens (Study 2)

The number of captured lady beetle species and species composition varied by habitat type. We captured three lady beetle species in corn (*Coleomegilla maculata*, *Cycloneda munda*, *Harmonia axyridis*), three lady beetle species in soybean (*H. axyridis* (majority), *Coccinella septempunctata*, *C. munda*), and six lady beetle species in grasslands (*C. munda*, *C. septempunctata*, *C. maculata*, *H. axyridis*, *Hippodamia convergens*, *Propylea quatuordecimpunctata*). Regurgitants had significantly higher numbers of DNA reads derived from prey DNA than whole body tissue samples ($X^2 = 3.6$, $df = 1$, $P = 0.05$, Fig. 1c) and prey detection rates were greater ($X^2 = 4.3$, $df = 1$, $P = 0.03$, Fig. 1d). The prey detection rate was two times higher in the regurgitant (48.2%) compared to extraction from the whole specimen (23.1%).

Across all four types of habitats, many lady beetles had no prey detected (Fig. 5) and the average prey detection rate for field caught specimens was 32% (Fig. 6a). For lady beetles with prey, we detected 1.77 OTUs on average (Fig. 5 and 6b). Most of the prey matches (Table 1) were to COI sequences from other (non-host) lady beetle

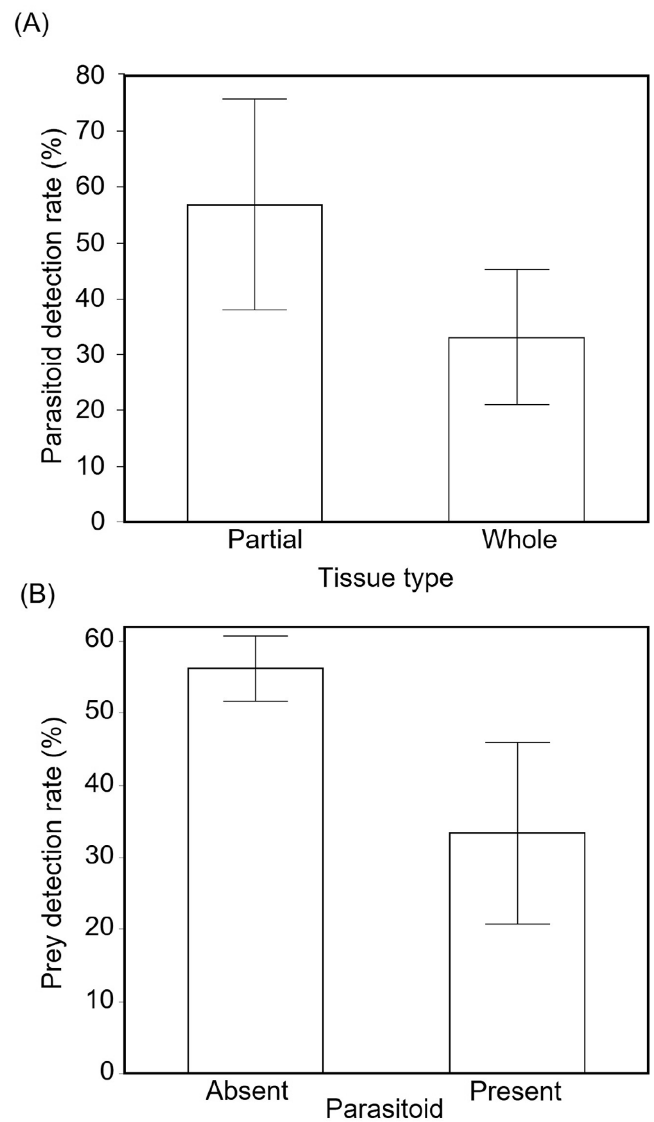

Fig. 4. Relationships between extraction methodology and parasitoid (A, top) and prey detection rates (B, bottom). Error bars are 1 ± SE from the treatment mean.

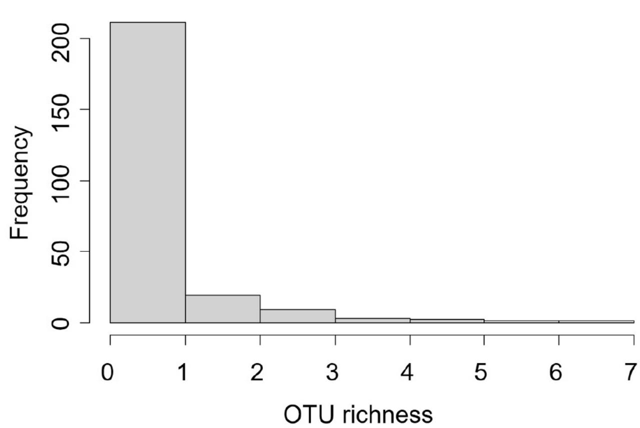

Fig. 5. OTU richness of lady beetles captured in all field specimens (soybean, corn, and tallgrass prairie fields). Most lady beetles did not have prey detected and the average number of OTU was 1.77.

species (23.65% of sample matches), followed by aphids (18.24%), flies (12.84%), other Hemiptera (12.8%), bees and non-parasitic wasps (8.78%), spiders (6.76%) and moths (4.05%) and other arthropods (12.84%). Most lady beetles were identified as true predators feeding on herbivorous insects (69.8%) while intraguild predation (i.e., feeding on other predators) was common (55.4%) in addition to IGP on other lady beetle species (23.65%).

3.2.1. *Individual-level prey detection and prey composition*

Prey detection rates for lady beetles varied with habitat type ($X^2 = 30.92$, df = 4, $P < 0.001$, Fig. 6a). This result was largely driven by lady beetles collected in urban environments where we did not detect any prey (with the exception of only 1 individual with 1 prey species detected) suggesting that areas near the urban structures were food desserts for the captured lady beetles. We, therefore, removed urban-caught specimens from the analyses. For the remaining individuals, the prey detection rates did not vary with habitat type; 41.4% of individuals had prey in grassland; 38.4% in corn, 25.5% in soybean. Additionally, OTU richness did not vary with habitat type ($X^2 = 0.64$, df = 2, P = 0.72, Fig. 6b). For the individuals with prey detected, average OTU richness across the three landscapes was 1.75 (grassland = 1.66, soybean = 1.9, corn = 1.90).

3.2.2. *Community-level prey detection*

Habitat type influenced OTU prey community composition, feeding strategies, and diet breadth.

We detected a total of 32, 30, and 11 OTUs for lady beetles captured in soybean, grassland, and corn respectively and only 1 OTU for an individual captured in the urban habitat. OTU composition of prey also varied by habitat type ($F_{2,82} = 7.37$, P = 0.001, Fig. 7a); prey OTU composition in soybean was very different from prey composition in corn and grassland. Soybean prey was mostly of comprised of aphids, flies, and spiders while for corn and grassland, the major prey was lady beetles, aphids/other hemipterans, and flies (Table 1). IGP for lady beetles also varied with habitat type ($X^2 = 19.43$, df = 2, $P < 0.01$); IGP was greatest in corn (81.8%), followed by grassland (47.6%) and soybean (13.3%). Diet breadth differed between habitat ($F_{2,80} = 3.61$, P = 0.03, Fig. 7b). Grasslands had greater breadth (distance to centroid) while corn has the smallest breadth.

We also found evidence of diet partitioning where different lady beetle species found in the same habitat had different OTU composition ($F_{5,82} = 1.44$, P = 0.015, Fig. 8a) and diet breadth ($F_{5,77} = 2.28$, P = 0.05, Fig. 8b). *Coleomegilla maculata* and *H. axrydis* had dissimilar composition from each other and *H. convergens* and *P. quatuordecimpunctata* had the narrowest diet breadth.

Finally, we found the parasitoid *Dinocampus* in our field specimens at higher detection rates (~32%) compared to those purchased for our laboratory study (11%). For our field collected specimens, the presence of *Dinocampus* did not affect the likelihood of prey detection ($X^2 = 0.125$, df = 1, P = 0.26). There was a marginally significant effect of habitat on *Dinocampus* detection rates ($X^2 = 4.85$, df = 2, P = 0.08, Fig. 9) where there was lower parasitoid detection in soybean compared to corn and grassland. We did not detect *Dinocampus* in the urban habitat.

4. Discussion

This study used a DNA *meta*-barcoding approach with HTS to identify prey species consumed by predatory lady beetles. We found that HTS -based diet analysis could successfully detect prey DNA in lady beetles to the species level, but not all prey species were detected in the diverse 4-species diet mixes. Prey detection in lab feeding trials was 55% and 32% in the field-caught specimens; these values were comparable to other studies (25.4% detection rates for insect in Sow et al. (2020), 44.2% in carabid beetles in Tiede et al. (2016), 40.8% in bats Zeale et al. (2011). In our controlled feeding experiments, we confirmed the identity of the

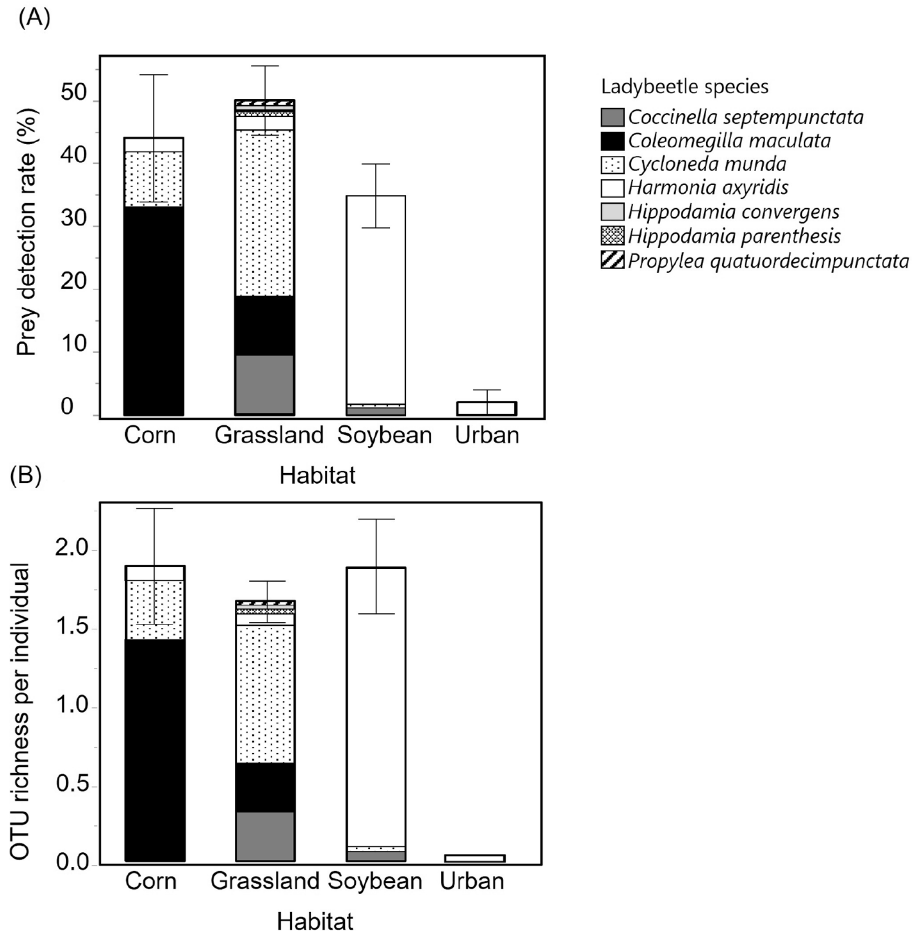

Fig. 6. Habitat differences in prey detection rate (A) and OTU richness (B) for different lady beetle species.

prey items consumed and that the composition of the diets were different from each other. We also found that niche breadth varied in predictable ways; monotypic diets had lower diet breadth whereas mixed diets had greater diet breadth. Therefore, we are confident that this approach can characterize and compare diet breadth and composition in the field in accurate ways.

While our prey detection rates were comparable to other studies, not all lady beetle individuals had prey DNA detected. Variability in digestion time, feeding preferences, and feeding rates might affect ability to detect prey DNA. Previous studies have found that the half-life of prey in insect guts varies with prey species and could explain why prey detection varies (Gagnon et al., 2011; Pompanon et al., 2012; Rondoni et al., 2018). In particular, previous work in lady beetles found that the DNA of herbivorous and predatory prey declined after 12 h and varied with prey species; herbivorous prey species declined faster than predatory prey species (Rondoni et al., 2018). In our lab-based feeding study, we used herbivorous prey only and prey extraction was conducted after 24 h to standardize feeding treatments and account for variations in feeding rates and prey preference between individuals. During this period, not all lady beetles may have fed on prey at the same times, and for individuals in the mixed diet treatments, not all lady beetles selected prey in the same order. This suggests that variability in feeding preferences and order could influence the detectability of prey in the guts of the lady beetles. While we did not watch lady beetle feed on prey items during the feeding period, most prey were damaged after the 24 h period or completely missing, suggesting that lady beetles at least partially fed on all prey items offered. However, future studies could track prey item consumed and/or collect samples at multiple timepoints after feeding to determine how long DNA from prey items remains detectable in the gut (e.g., 12 h and 24 h post exposure). In the field, the prey detection rate was 32%; a 40% reduction compared to lab conditions. Lower detection rates could be because lady beetles were fed in excess in the lab and assays were done within 24 hrs of feeding, while gaps between feeding events and collection date may be longer or more common in field-collected predators. Because HTS-based molecular gut content analyses are snap shots of prey use in the field, future studies should collect larger numbers of specimens and over longer periods of time to fully characterize the diet of lady beetles due to variability in feeding rates and prey type over the course of the field season.

We wanted to determine whether partial tissue samples would increase the likelihood of prey detection compared to whole body tissue. From a logistical standpoint, dissections of the alimentary canal can be time consuming and prone to DNA contamination during processing (Tiede et al., 2016). Furthermore, the use of blocking primers to reduce the amplification of DNA from the host can be problematic because they can obscure prey on closely related species and can underestimate possible IGP (Piñol et al., 2014). In our lab study, we found that performing PCR on the alimentary canal alone did not yield more prey DNA reads or increase prey detection rates compared to whole-body tissue suggesting that the additional step of gut removal is not needed. However, we did observe a difference in the detection rates for regurgitant versus whole body tissue with field-caught specimens. Regurgitant-only samples contained more prey reads and had higher rates of prey detection than whole-body specimens and the differences were stronger when comparing prey DNA in regurgitants versus whole-body specimens of the same individuals (paired *t*-test, $t = -2.6115$, $df = 58$, $P = 0.01$). Therefore, focusing analysis on the regurgitant rather than the whole insect may allow for better characterization of the prey

Table 1
Prey detection rates for major taxonomic prey groups of lady beetles captured in the field study in soybean, corn, tallgrass prairie, and urban habitats (N = 50 individuals per habitat type). Host OTUs were removed from the table. Other predators marked with asterisks. Numbers in parentheses represent the percentages of lady beetles that a particular prey item was detected relative to the total number of lady beetles in each landscape type that had detectable prey.

Taxa/species (OTU)	Corn	Grassland	Soybean	Urban
Lady beetles (23.6%)*				
Coccinella septempunctata (OTU3)	4.76%	1.43%	0.00%	0.00%
Cycloneda munda (OTU5)	0.00%	2.86%	0.00%	0.00%
Harmonia axyridis (OTU1)	42.86%	20.00%	0.00%	0.00%
Hippodamia convergens (OTU2)	4.76%	0.00%	7.02%	0.00%
Hippodamia glacialis (OTU33)	0.00%	1.43%	0.00%	0.00%
Propylaea quatuordecimpunctata (OTU7)	0.00%	2.86%	0.00%	0.00%
Aphids (18.24%)				
Acyrthosiphon pisum (OTU13)	0.00%	8.57%	0.00%	0.00%
Aphidius ervi (OTU71)	0.00%	0.00%	1.75%	0.00%
Aphis glycines (OTU17)	0.00%	0.00%	28.07%	0.00%
Schizaphis graminum (OTU28)	0.00%	1.43%	0.00%	0.00%
Sitobion sp (OTU66)	0.00%	0.00%	1.75%	0.00%
Uroleucon anomalae (OTU23)	0.00%	1.43%	0.00%	0.00%
Uroleucon sonchi (OTU86)	0.00%	1.43%	0.00%	0.00%
Flies (12.84%)				
Aedes trivittatus (OTU19)	4.76%	0.00%	3.51%	0.00%
Aedes vexans (OTU68)	0.00%	2.86%	0.00%	0.00%
Apallates coxendix (OTU65)	0.00%	1.43%	0.00%	0.00%
Aphidoletes aphidimyza (OTU50)	0.00%	0.00%	1.75%	0.00%
Cecidomyiidae (OTU21)	4.76%	0.00%	0.00%	0.00%
Drosophila simulans (OTU39)	0.00%	0.00%	1.75%	0.00%
Forcipomyia sp (OTU57)	0.00%	1.43%	0.00%	0.00%
Psychodidae (OTU53)	0.00%	1.43%	0.00%	0.00%
Rhopalomyia solidaginis (OTU81)	0.00%	0.00%	1.75%	0.00%
Simulium equinuum (OTU62)	0.00%	0.00%	1.75%	0.00%
Strongygaster triangulifera (OTU31)	0.00%	1.43%	0.00%	0.00%
Thienemanniella xena (OTU35)	0.00%	0.00%	0.00%	100.00%
Diptera (OTU69)	0.00%	0.00%	1.75%	0.00%
Diptera (OTU73)	4.76%	0.00%	0.00%	0.00%
Diptera (OTU40)	0.00%	1.43%	0.00%	0.00%
Diptera (OTU46)	4.76%	0.00%	0.00%	0.00%
Diptera (OTU47)	0.00%	0.00%	1.75%	0.00%
Other Hemiptera (12.8%)				
Daktulosphaira vitifoliae (OTU75)	0.00%	1.43%	0.00%	0.00%
Hemiptera (OTU16)	14.29%	20.00%	1.75%	0.00%
Other bees and wasps (8.78%)				
Polistes fuscatus (OTU49)	0.00%	1.43%	0.00%	0.00%
Hymenoptera (OTU78)	0.00%	1.43%	0.00%	0.00%
Hymenoptera (OTU24)	0.00%	0.00%	1.75%	0.00%
Hymenoptera (OTU27)	4.76%	1.43%	0.00%	0.00%
Hymenoptera (OTU32)	0.00%	7.14%	0.00%	0.00%
Hymenoptera (OTU41)	0.00%	1.43%	0.00%	0.00%
Hymenoptera (OTU55)	0.00%	0.00%	1.75%	0.00%
Hymenoptera (OTU59)	0.00%	2.86%	0.00%	0.00%
Spiders (6.76%)*				
Araneae (OTU72)	0.00%	1.43%	0.00%	0.00%
Araneae (OTU77)	0.00%	1.43%	0.00%	0.00%
Araneae (OTU34)	0.00%	0.00%	7.02%	0.00%
Araneae (OTU43)	0.00%	1.43%	0.00%	0.00%
Araneae (OTU45)	0.00%	0.00%	3.51%	0.00%
Araneae (OTU64)	0.00%	0.00%	1.75%	0.00%
Moths (4.05%)				
Grapholita interstinctana (OTU30)	0.00%	1.43%	0.00%	0.00%
Hypena scabra (OTU22)	0.00%	0.00%	3.51%	0.00%
Lepidoptera (OTU74)	0.00%	0.00%	1.75%	0.00%
Lepidoptera (OTU42)	4.76%	0.00%	0.00%	0.00%
Lepidoptera (OTU67)	0.00%	1.43%	0.00%	0.00%
Parasitic wasps (1.73%)				
Asaphes vulgaris (OTU38)	0.00%	0.00%	1.75%	0.00%
Lysiphlebus testaceipes (OTU70)	0.00%	0.00%	1.75%	0.00%
Trimorus sp (OTU20)	0.00%	0.00%	1.75%	0.00%
Mites (2.33%)				
Mesostigmata (OTU63)	0.00%	0.00%	1.75%	0.00%
Sarcoptiformes (OTU52)	4.76%	0.00%	0.00%	0.00%
Tarsonemidae (OTU44)	0.00%	0.00%	3.51%	0.00%
Caddisfly (1.35%)				
Oecetis inconspicua (OTU25)	0.00%	0.00%	3.51%	0.00%
Springtails (1.35%)				
Deuterosminthurus sp (OTU29)	0.00%	1.43%	0.00%	0.00%
Entomobryomorpha sp (OTU61)	0.00%	0.00%	1.75%	0.00%
Thrips (1.07%)				
Neohydatothrips sp (OTU36)	0.00%	0.00%	3.51%	0.00%
Thrips palmi (OTU76)	0.00%	0.00%	1.75%	0.00%
Ants (0.68%)*				
Lasius neoniger (OTU80)	0.00%	1.43%	0.00%	0.00%
Cicadas (0.68%)				
Burbunga gilmorei (OTU58)	0.00%	1.43%	0.00%	0.00%
Crickets (0.68%)				
Allonemobius allardi (OTU60)	0.00%	0.00%	1.75%	0.00%
Millepedes (0.68%)*				
Polydesmida (OTU48)	0.00%	0.00%	1.75%	0.00%

community. Previous work in ground beetles have found similar results of high detection rates with regurgitant (Tiede et al., 2016). However, because regurgitant only capture prey in the upper alimentary canal (foregut), further research is needed in identifying how much information is lost by not processing prey in different sections of the alimentary canal.

4.1. Trophic interactions

In addition to identifying prey use, characterizing feeding relationships using molecular approaches allows tests of hypotheses of biocontrol potential in natural settings. Theory suggests that when prey are limited, predators are more likely to be involved in IGP compared to true predation of the basal prey resource (Pimm and Lawton 1978; Schoenly et al., 1991; Kratina et al., 2012). Our findings support this hypothesis where lady beetles in annual crops such as corn had narrower diet breadth and greater rates of IGP (81.3%) suggesting that diet selection in these fields were less diverse than grasslands (13.3%). While we did not measure prey abundance and diversity in this study, we have found greater prey abundance and diversity in grasslands compared to annual agriculture such as corn in our previous studies (Liere et al., 2015; Fox et al., 2016; Kim et al., 2017) supporting the hypothesis of greater availability of resources in perennial systems. However, we found that lady beetles in monocultures of soybean had similar feeding characteristics as lady beetles in grasslands (i.e., wide diet breadth, greater predation rate, lower IGP (47.6%)), suggesting that pereniality *per se* may not always be a consistent predictor of diet diversity and resource availability. Furthermore, because lady beetles in corn were mostly intraguild predators compared to those in grassland and soybean, these findings suggest that lady beetles might be more effective natural

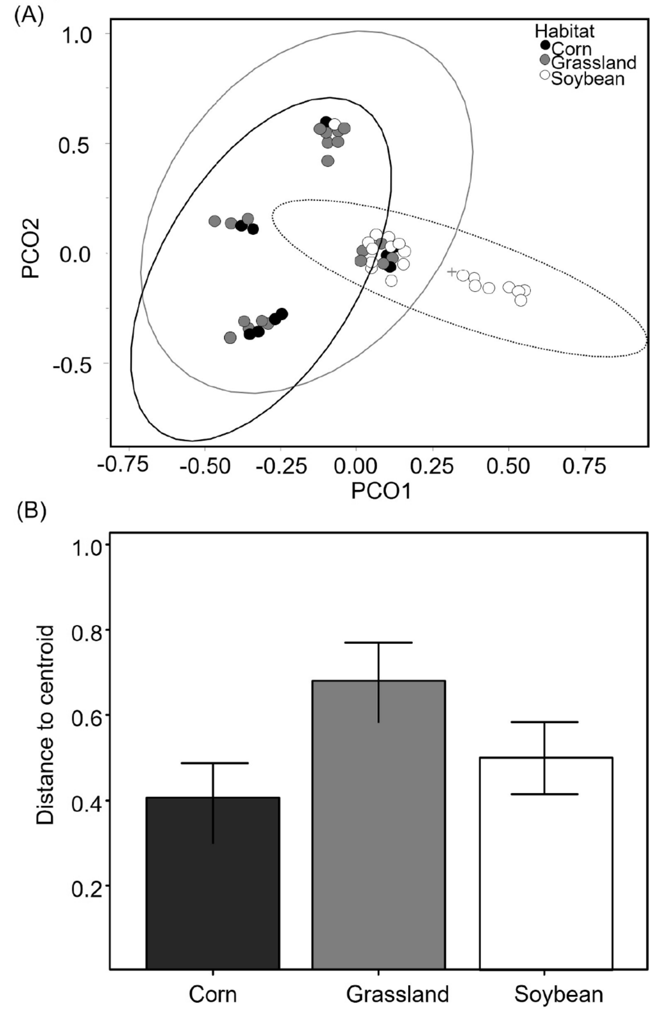

Fig. 7. OTU prey community composition (A) and diet breadth (distance from centroid, B) for lady beetles captured in corn, soybean, and grasslands.

enemies in soybean and grasslands compared to corn and that biocontrol potential varies with crop type and surrounding habitat type.

We found unexpected trophic interactions with soil-dwelling arthropods in our study. While lady beetles are considered foliar feeders, preferring soft-bodied foliar prey such as aphids, we found evidence of feeding on prey within the brown food web, including millipedes, collembola, and crickets. Previous studies (Hodek 1996; Weber and Lundgren 2009; Davidson and Evans 2010) have found that lady beetles can consume non-arthropod food (e.g., pollen and fungi) and non-preferred prey (e.g. thrips and collembola) but at lower frequencies. The consumption of non-preferred prey has been shown to increase lady beetle survival and fecundity during times of food scarcity but not affect flight (Hodek, 1996; Stowe et al., 2021b, 2021a). Our results suggest that lady beetles might be moving down plants to the leaf litter layer (rather than fly to new patches) to reach non-preferred prey when resources are in limited supply. Understanding the ecological conditions under which this strategy is adaptive warrants further investigation.

4.2. Other considerations

Feeding relationships can be determined by HTS but limitations exist and additional factors should be considered. For example, cannibalism rates cannot be detected since host and cannibalized prey DNA cannot be differentiated. While the prevalence of cannibalism in the field remains an open question, greater rates of cannibalism are likely to negatively affect biocontrol potential (Richardson et al., 2010; Rocca et al., 2017). Future studies can use protein markers to tag prey to determine cannibalism rates (González-Chang et al., 2016; Mansfield and Hagler 2016). In our study, we did not include primers that could detect non-animal prey such as plant and fungal material. Prior studies found that lady beetles can utilize these resources in the lab to enhance fitness, such as egg production and flight potential (Stowe et al., 2021a).

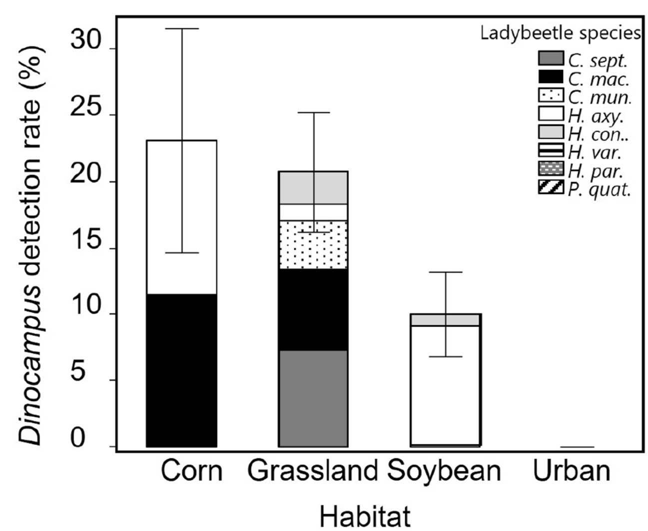

Fig. 9. Detection rates of *Dinocampus coccinellae* in different habitat types.

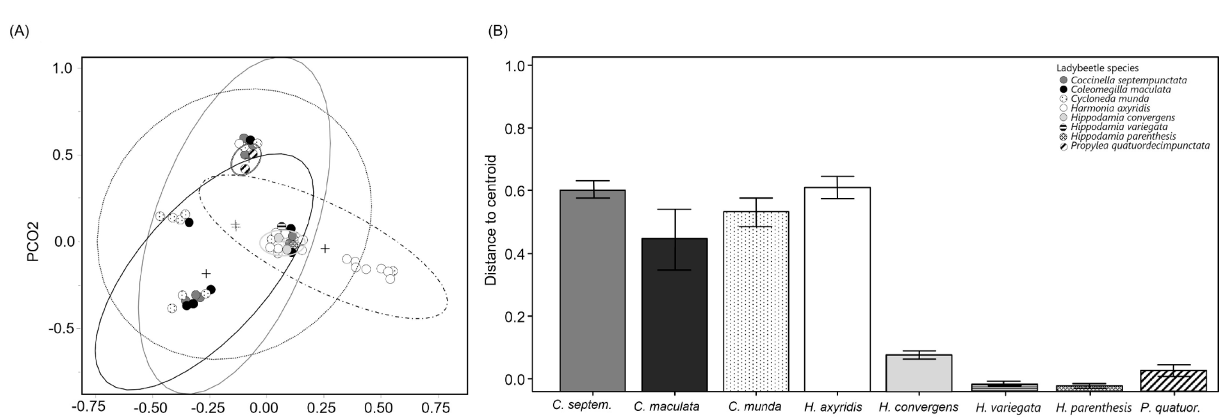

Fig. 8. OTU prey composition (A) and diet breadth (distance to centroid, B) as a function of lady beetle species.

Understanding the extent to which lady beetles utilize these non-prey resources is needed. Furthermore, in order to fully characterize predator–prey associations, many samples need to be collected from the field throughout the growing season as prey associations are likely to change with time (Roubinet et al., 2017). Therefore, understanding how resources use changes over time (and across space) will allow us to assess the efficacy of lady beetles as biocontrol agents in the field.

Finally, we found parasitism by *Dinocampus* in a small portion of our samples (11% of lab specimens, 32% of field caught specimens). Interestingly, we found *Dinocampus* DNA in the alimentary canal and regurgitant samples, even though *Dinocampus* is thought to feed on the fat bodies of lady beetles during larval development (Maure et al., 2011). The presence of *Dinocampus* DNA in the alimentary canal and regurgitant could be due to contamination of the samples during the dissection or regurgitating stages of sample preparation as surface sterilization occurred only after specimens were frozen and before DNA extraction. Another possibility could be that *Dinocampus* can feed on tissue in the alimentary canal of lady beetles. Although *Dinocampus* infection did not affect prey detection rates in either the lab or field studies, we did observe lower prey detection rates in ladybeetles that did not harbor *Dinocampus* suggesting that parasitism by this wasp may have some effect feeding activity. If *Dinocampus* larvae were feeding on gut tissue, this could affect feeding activity and possibly prey detection. Furthermore, we found marginally significant difference in *Dinocampus* detection rates between habitats with lower parasitoid detection in soybean compared to corn and grassland. Previous studies have found that the development of *Dinocampus* larvae to vary on lady beetles fed on different diets (Maure et al., 2011,2016) suggesting that the host (lady beetle) quality in soybean might be lower than lady beetle quality in corn and grassland. Further work in the role of parasitism by *Dinocampus* for lady beetle fitness, feeding and biocontrol is needed.

5. Conclusions

Diet analysis using HTS could successfully detect prey DNA in lady beetles to the species level. We found high levels of intraguild predation in some cropping systems (e.g., corn) while other habitats (e.g., prairie) exhibited lower levels of intraguild predation. These feeding patterns could be due to lower prey richness and diet breadth in corn compared to soybean and prairie. To our knowledge, this study is the first to use HTS to characterize the diet of lady beetles, an important group of biocontrol agents. This method can be used to test hypotheses about variability in feeding relationships and biocontrol potential in changing agricultural landscapes.

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Author contributions

T.N.K., B. J. S., and C. G. designed the research, T. N. K. and B. J. S. collected data and prepared lab specimens, Y. V. B., M. A. J., and E. D. S. performed bioinformatics, T. N. K. analyzed the data, T N. K. prepared the first draft of the paper, all authors reviewed and edited the paper. All authors have read and agreed to the published version of the manuscript.

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ARTICLE

Agroecosystems

Resource amount and discontinuity influence flight and reproduction in *Hippodamia convergens* (Coleoptera: Coccinellidae)

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Abstract

Industrial-scale agriculture creates a mosaic of large monocultures in the landscape, where seasonal cropping cycles generate discontinuous resource availability for insect predators both spatially and temporally. In this environment, selection will favor predator movement and reproductive behaviors that optimize the location and effective utilization of resource (prey) pulses that are both patchy and ephemeral in nature. Using a model system to study predator movement and reproduction, we tested how discontinuous periods of food resource access that mimic fluctuating resource populations (aphids) would influence flight behavior and reproduction of a highly mobile predator, *Hippodamia convergens* (convergent lady beetle), and possibly modify energetic trade-offs between these behaviors. Adult beetles were provided either short (3 h) or long (6 h) food pulses daily (continuous availability) or short (6 h) or long (12 h) food pulses every other day (discontinuous availability). We measured preoviposition period, fecundity, and fertility during an 18-day oviposition period, and female tethered flight activity (3 h) before and after the oviposition period. We found that discontinuous food access delayed the onset of oviposition in the high food quantity treatment; fewer females laid eggs overall, and 18-day fecundity was lower compared with continuous provision of the same food quantity. A longer preoviposition period was associated with fewer reproductive days and lower fitness. Flight distance and fecundity were negatively correlated, suggesting that energetic expenditure in flight can deplete energetic reserves otherwise used for subsequent reproduction. The negative effects of discontinuous resource access at fine temporal scales reveal how gaps in resource availability could influence lady beetle population dynamics and their ecosystem services within the agricultural landscape.

KEYWORDS

biological control, dispersal, ecological trade-offs, insect predators, temporal variability

INTRODUCTION

The negative effects of disturbance and resource fragmentation on insect biodiversity and ecosystem functions in agricultural landscapes are well documented (Angeler et al., 2016; Bianchi et al., 2009; Chaplin-Kramer et al., 2011; Haan et al., 2020; Samways, 1989; Tscharntke et al., 2012). Disturbances due to tillage, harvest, and chemical inputs can lead to direct insect mortality (Pearsons & Tooker, 2017; Tooker et al., 2020; van der Meer et al., 2020) or reduce habitat and food resources necessary for insect survival (Raven & Wagner, 2021; Wagner et al., 2021), thus decreasing ecosystem services such as biological control and pollination. Previous studies have examined the relationship between environmental disturbance and altered resource amount or quality (e.g., Karakoç et al., 2018; Ojima et al., 1994; Solbreck, 1995; Wagle & Gowda, 2018), but less information exists concerning the effects of variable access to resources, that is, resource discontinuity (Schellhorn et al., 2015; Spiesman et al., 2020; Welch & Harwood, 2014). Resource discontinuities created by disturbances can be spatial (i.e., rich resource patches are physically separated within resource matrices comprised largely of low resource regions) and/or temporal (i.e., rich resource patches are only ephemerally available) creating a landscape of unreliable resource availability in space and time. Discontinuity of resources can directly affect insect behavior and reproduction by reducing resource amount (Nicholson et al., 2021) with consequences for the population dynamics of many organisms (Holt, 2008; Yang et al., 2008) including insects (Ogilvie & Forrest, 2017). Furthermore, resource discontinuity can increase searching effort, making foraging more costly for adult predators (Forsman & Kivelä, 2021). These factors, among others, can combine to reduce the efficacy of insect-derived ecosystem services, including biological control (Schellhorn et al., 2015). Resource discontinuity is predicted to increase under climate change, as more frequent extreme weather events, including heat waves, droughts, and heavy precipitation events (Folland et al., 2002; Thornton et al., 2014) threaten to alter patterns of resource availability and stress insect communities, potentially diminishing the ecosystem services that they provide.

Insects that inhabit spatially and temporally variable resource environments face trade-offs between fitness-critical behaviors such as dispersal and reproduction. The ability of predators to track, or even anticipate, prey populations within annual crops can determine their survival and the effectiveness of their ecosystem services (Landis et al., 2000; Prasifka et al., 2004; Wissinger, 1997). For example, mobile predators may relocate to exploit alternative resources when the availability of a primary resource fluctuates capriciously (Landis et al., 2000; Wissinger, 1997), potentially incurring energetic trade-offs with other life history traits such as reproduction. Therefore, high mobility may be a prerequisite for success when generalist insect predators colonize agricultural environments, at least over short time frames (Wissinger, 1997; Xiao et al., 2017). However, flight is energetically costly, so excessive flight activity driven by food supply disruptions may diminish predator fitness and biological control services. Reproduction is also energy-intensive, and the allocation of energetic "capital" to flight may reduce the amount that can be allocated to reproduction in the future (Stearns, 1992). Considering these energetic trade-offs, any agricultural practices that minimize periods of resource scarcity within agricultural landscapes, that is, the use of cover crops, intercropping, or field crop complementarity, could reduce the energetic burden of local migrations for predators, leaving them with greater reserves to devote to foraging and reproduction when prey patches are finally encountered.

The present study addressed whether resource quantity and temporal availability would influence flight capacity and reproductive success in a highly mobile insect predator, the convergent lady beetle, *Hippodamia convergens* Guerin-Meneville (Coleoptera: Coccinellidae). Although it is impossible to fully reproduce the complexity of disturbance and resource variability in the agricultural environment, controlled laboratory assays permit isolation of key factors and provide insights into their potential effects on reproduction and movement under field conditions where a multiplicity of confounding factors often impede our ability to make clear inferences. The convergent lady beetle is one of the most abundant species of coccinellid on the North American High Plains where it is a key biological control agent of important cereal aphid pests such as greenbug, *Schizaphis graminum* Rondani (Rice & Wilde, 1988), Russian wheat aphid, *Diuraphis noxia* (Kurdjumov) (Nechols & Harvey, 1998), and sugarcane aphid, *Melanaphis sorghi* (Theobald) (Colares et al., 2015—formerly *Melanaphis sacchari* Zhentner, Nibouche et al., 2021). Although *H. convergens* can be considered a specialized aphid predator, as it relies heavily on aphids for reproduction (Michaud & Qureshi, 2006), both larvae and adults survive periods of aphid scarcity by exploiting a wide range of supplementary foods that include the immature stages of other insects and various plant-derived resources (Lundgren, 2009; Mercer et al., 2020; Stowe et al., 2021). On the High Plains, these beetles typically produce their first generation in wheat or alfalfa, the resulting adults migrating to summer crops and switching among prey types in response to their availability, ultimately reproducing in different crops than those that supported their development (e.g., Bastola et al., 2016; Tillman & Cottrell, 2012). Spatial and temporal disturbances inherent to large-scale farming operations can increase energy demands for *H. convergens* adults by increasing both the frequency and distance of flights required to track prey populations and maximize their reproductive success. *H. convergens* are strong flyers (Hagen, 1962; Jeffries et al., 2013) and will emigrate from early season crops to later season crops even before the latter have developed large prey populations (Prasifka et al., 2004). Therefore, this species has evolved specific adaptations to exploit seasonal patterns of changing resource availability across agricultural landscapes. However, these seasonal patterns of dispersal likely represent sizable energetic costs for the migrant adults that may manifest as trade-offs in reproductive success.

We hypothesized that *H. convergens* adults fed larger quantities of food would fly greater distances and achieve higher reproductive success than those fed lower amounts, without significant effects on egg fertility or timing of oviposition. Likewise, we hypothesized that sporadic (discontinuous) access to food would reduce flight distance and female fecundity, and delay onset of oviposition relative to more continuous access. We also hypothesized that the overall quantity of food would interact with its pattern of availability, with lower quantities resulting in more severe negative effects when availability is discontinuous. Finally, because of the many ways that food amount and continuity of access could affect flight behavior and reproduction, we used a structural equation model (SEM) framework (Figure 1), to disentangle how resource amount and continuity of access would affect the presumed energetic trade-off between flight activity and reproduction.

METHODS

Insect colony

Adult *H. convergens* beetles were collected from wheat fields at the Kansas State University Agricultural Research Center in Hays, KS, USA (38°51′32.1″ N, 99°20′07.7″ W) in June of 2020. Beetles (ca. 150) were placed in a 1-L glass mason jar covered with muslin netting and held at 24 ± 1°C, 50%–60% RH, and a photoperiod of 16:8 (L:D). Jars were filled with wax paper strips as harborage and provisioned with frozen eggs of *Ephestia kuehniella* every other day, with water provided on a cotton wick. Field-collected beetles are often parasitized by *Dinocampus coccinellae* Schrank (Hymenoptera: Braconidae), so the colony was inspected daily to remove any emerging wasps before they could complete pupation and emerge to attack other beetles in the colony.

A colony of greenbugs, *S. graminum*, was established from individuals collected from wheat at the same location as the beetle colony. The colony was reared on wheat seedlings grown in metal trays containing a soil/vermiculite/peat moss mixture (1/1/1). Trays were germinated in the greenhouse and infested in growth chambers under fluorescent lighting at 24 ± 1°C, 50%–60% RH, and a 14:10 (L:D) daylength. Wheat seedlings were infested by clipping infested wheat leaves from

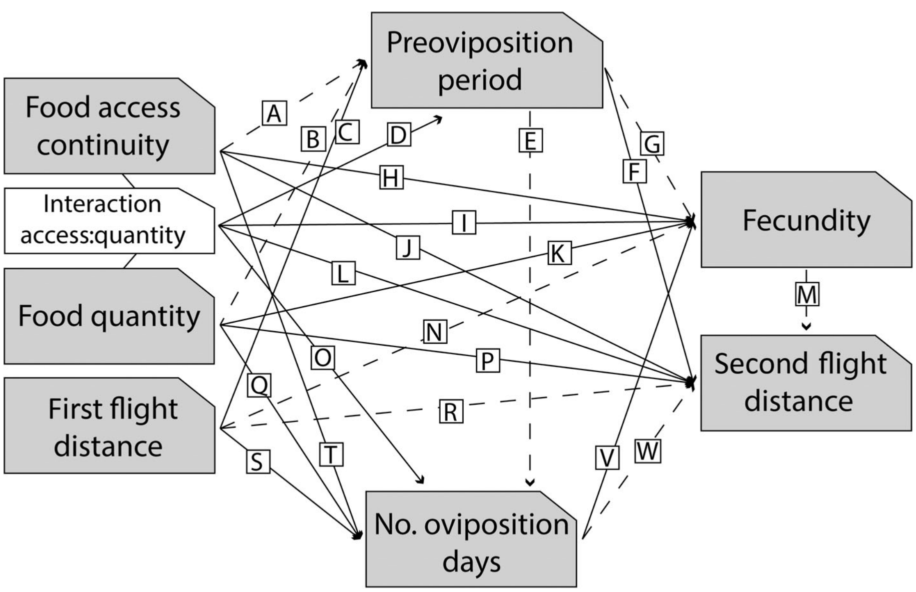

FIGURE 1 Hypothesis structural equation model: Arrows indicate directionality of hypothesized relationship, labeled with letters for identification. Solid lines denote positive influence and dashed lines indicate negative influence.

declining trays and distributing them across a new tray of seedlings at the two-leaf stage.

Experimental insects

Maternal females ($n = 12$) were removed from the stock colony and isolated in plastic Petri dishes (5.5 cm diameter) where they were provided greenbugs ad libitum to induce oviposition. After the fertility of each female was confirmed by observing the eclosion of several clutches, offspring from two successive days of oviposition were collected from each female. Eclosing neonates were allowed to disperse naturally from their egg clusters before they were isolated in Petri dishes (as above). This allowed teneral larvae time to harden their cuticles and consume their chorions, as occurs naturally. The maternity of each larvae was tracked so that progeny of each cluster could be distributed equally among treatments, and no siblings would be paired for reproduction.

All neonate larvae were reared under the same environmental conditions as the adult colony, with ad libitum access to frozen *E. kuehniella* eggs and water provided in a small square of sponge, both refreshed daily. Once larvae pupated, any remaining food was removed from the dishes and pupae were monitored daily until emergence, whereupon adults were sexed and weighed on an analytical balance (Mettler Toledo, AG285, Columbus, OH, USA) within 24 h. Pairs of non-sibling adults were established in ventilated snap-cap plexiglass vials (4.9 cm diameter × 9.0 cm height) where they remained for 12 days to permit maturation and mating. During this period, they were provisioned with frozen *E. kuehniella* eggs (ca. 5 mg), fresh foliage of wheat seedlings, pulverized bee pollen, diluted honey (1:2 honey:water) on a small cube of sponge, and water on a second cube. No aphids were provided during this maturation period to prevent oviposition prior to the flight assays and the imposition of treatments. All plant resources were refreshed daily, *E. kuehniella* eggs were provided every 3 days, water and diluted honey as required. This diet was designed to mimic patterns of food availability under field conditions when first-generation adults emigrate from natal fields and remain in reproductive diapause prior to encountering aphid populations that support their ovariole maturation and oviposition (Michaud & Qureshi, 2006).

Assays of flight behavior

A series of flight mills were constructed, modified from the instructions provided by Attisano et al. (2015), which allowed beetles to fly in a 10-cm-diameter circle. A small dot of magnetic paint (Rust-Oleum magnetic primer) was placed on the right elytra of each female beetle using a toothpick. After allowing 24 h for the paint to dry completely, each female was then magnetically attached to a flight mill arm via a small neodymium magnet. Flight tests were conducted in a climate-controlled growth chamber set to 24 ± 1°C and 50%–60% RH. Each beetle was attached to one end of a rotating arm with the opposite end passing through an infrared sensor to record the number and timing of revolutions. This information was collected via an Arduino MEGA 2560 REV3 single-board microcontroller and a terminal program (CoolTerm) for data collection. After flying for a total of 3 h, each female was returned to their container with their mate and assigned to one of four food access treatments, as described below. Female beetles were flown once just before beginning the reproductive diet, and again immediately after they completed the 18-day oviposition period, measured from their first day of oviposition, and the total distance flown by each female was recorded for both dates.

Food access treatments and experimental design

The experiment was constructed as a 2 × 2 factorial design, with varying resource amount (high vs. low) and resource access (continuous vs. discontinuous) to separate the effects of these two aspects of resource availability (quantity and period of access) on flight behavior and subsequent reproductive success. Mature lady beetle couples were randomly assigned to one of four food access treatments: (1) high amount, continuous availability, “HC” ($n = 21$ couples with access to 6 h of ad libitum food daily); (2) low amount, continuous availability, “LC” ($n = 25$ couples with access to 3 h of ad libitum food daily); (3) high amount, discontinuous availability, “HD” ($n = 22$ couples with access to 12 h of ad libitum food every other day); and (4) low amount, discontinuous availability, “LD” ($n = 26$ couples with access to 6 h of ad libitum food every other day). Food access treatments were designed to provide 24 h of cumulative food access in high food treatments and 12 h of cumulative food access in low food treatments during each 48-h period. The prey components comprised *S. graminum* aphids provided on excised wheat seedling leaves and frozen *E. kuehniella* eggs (ca. 5 mg), both refreshed at the start of each feeding period. Manipulation of available food biomass is difficult when aphids are used as prey because nymphal instars vary greatly in size and apterous adults continue to reproduce even when removed from host plants. Therefore, we opted to control periods of access to ad libitum food rather than trying to estimate the biomass of aphids or other foods consumed: an approach that has been successfully used in previous work (e.g., Vargas et al., 2013).

Reproduction

Following the 12-day maturation period, beetle pairs were supplied with greenbugs ad libitum on the excised leaves of their host plants to induce oocyte maturation in females, which requires 3–4 days (Michaud & Qureshi, 2006). Couples were checked everyday for oviposition and egg clutches were collected by simply moving adult beetles to a clean snap-cap vial. All egg clusters were held until eclosion under the same environmental conditions as the stock colony so that fecundity and egg fertility could be recorded for each beetle pair. Males were kept together with females throughout the observation period to ensure female fertility was maintained. Reproduction was recorded for 18 days for each couple, counted from the first oviposition day, a period long enough to capture any effect of treatment on reproductive success (Michaud, 2005) as fecundity declines rapidly in coccinellid females (Dixon & Agarwala, 2002). Females that failed to lay any eggs within 30 days after provision of the reproductive diet were recorded and excluded from analysis of reproductive performance data as including nonreproductive adults in measures of ovipositional timing and fecundity would skew treatment means and variance.

Statistical analysis

We analyzed treatment effects of resource continuity and amount on preoviposition period, fecundity, egg fertility, total oviposition days, and flight distance using separate two-way ANOVAs followed by Tukey's honestly significant difference for post hoc multiple comparisons. Preoviposition period was calculated for each female as the number of days from provision of the reproductive diet (i.e., *S. graminum* provided ad libitum) until the first clutch was laid. Oviposition days were tallied for each female as the number of days on which at least one egg was laid. Clutches were considered the total number of eggs laid by a female in 1 day. Fecundity was tallied as the total number of eggs laid, and egg fertility as the percentage of eggs hatching. Flight distance was calculated by counting the number of flight mill revolutions and multiplying by revolution circumference. All data passed tests for equality of variance (Levene's test) and normality (Shapiro–Wilk test) prior to the performance of ANOVA.

To examine the direct and indirect relationships between feeding treatment and fitness metrics (preoviposition period, fecundity, egg fertility, and oviposition days), and flight, we used a SEM framework. Experimental treatments were coded as 0 (low food quantity) and 1 (high food quantity) and resource access as 0 (discontinuous) and 1 (continuous). We predicted direct relationships between food quantity, food access continuity, the interaction between food access continuity and quantity, and reproduction timing and amount and post-reproductive flight distance (Figure 1, paths A, B, D, H, I, J, K, L, M, O, P, Q, and T). We also expected higher pre-reproductive flight distance, reproduction, and post-reproductive flight distance would each inversely influence later performance in these metrics (Figure 1, paths C, N, R, and S). Initial flight distance was included as a covariate in the SEM selection process because the initial flight distances may indicate intrinsic variation in flight capacity among females. Finally, we were interested in relationships between fitness and flight (Figure 1, paths E, F, G, M, V, and W), specifically, whether trade-offs existed between energy expended in flight effort and reproduction, which could diminish the availability of energetic reserves available for subsequent flight and/or reproductive effort.

Model selection was performed using maximum likelihood methods and linear relationships between variables were determined using bivariate scatterplots. We assessed the overall goodness of fit using Akaike information criterion (AIC) in a step-wise process of elimination. At each step, nonsignificant relationships were removed starting with higher order interaction terms the highest p values in the coefficient list. Instances where non-hypothesized direct relationships were significant or marginally significant, predictors were returned to the model starting from the lowest available p value. At each step, AIC was consulted and only steps that reduced ΔAIC >2 were retained. For models with similar AIC values ($\Delta <2$), the more parsimonious model was chosen (Burnham & Anderson, 2002; Grace, 2006). All analyses were conducted in R version 4.0.3 using the piecewise SEM package (Lefcheck, 2016).

RESULTS

A total of 300 larvae were reared to adulthood, with 12 deaths and 1 escape yielding 287 adults. The average time from oviposition to adult emergence was 23.0 ± 1.0 days. Females had greater fresh weight at emergence than males (20.6 ± 2.6 mg vs. 16.3 ± 1.8 mg, $F_{1,285} = 273.1$, $p < 0.005$). The reproductive performance experiment was conducted with non-sibling couples assigned to each of the four treatments (HC, $n = 21$; HD, $n = 22$; LC, $n = 25$; LD, $n = 26$). Initial flight distances flown by females showed a bimodal distribution (Figure 2), with most females flying only a short distance, and a subset exhibiting greater flight propensity, flying between 3.5 and 4 km in a 3-h test period.

Fewer females broke reproductive diapause in the LD treatment than in any other treatment ($\chi^2 = 10.47$, df $= 3$, $p = 0.015$). Among reproductive females, food quantity and access period interacted to influence pre-oviposition period ($F_{1,78} = 7.0$, $p = 0.037$). The HC treatment produced the shortest preoviposition period, and the HD and LD treatments the longest, with the LC treatment intermediate and not significantly different from other treatments (Figure 3). Fecundity was significantly increased by continuity of food access ($F_{1,78} = 19.4$, $p < 0.001$) and food quantity ($F_{1,78} = 9.0$, $p = 0.004$, Figure 4), but these two factors did not interact ($F_{1,78} = 0.102$, $p = 0.75$). The HC treatment produced the highest fecundity, followed by the HD treatment, with the LD treatment yielding the lowest, and the LC treatment intermediate between HD and LD. Egg fertility was affected by food quantity ($F_{1,78} = 20.2$, $p < 0.001$) but not by continuity of food access ($F_{1,78} = 1.3$, $p = 0.49$), with low food quantity reducing egg fertility relative to high (Figure 5). Post-oviposition flight distance was not significantly affected by food quantity ($F_{1,90} = 0.6$, $p = 0.65$) or continuity of food access ($F_{1,90} = 0.6$, $p = 0.45$), nor did the two factors interact significantly ($F_{1,90} = 0.6$, $p = 0.74$).

Our best fit SEM (Fisher's $C = 13.85$, df $= 12$, $p = 0.31$, Figure 6) revealed the various direct and indirect ways that food amount and continuity of access affected reproduction and flight. We found that both food amount (standardized path coefficient, $\beta_D = 0.26$, $p = 0.03$) and pre-reproductive flight distance ($\beta_L = 0.32$, $p = 0.005$) directly increased post-reproductive (second) flight distance ($R^2 = 0.23$). On the other hand, continuity of food access influenced post-reproductive flight distance only indirectly via its effects on preoviposition period and number of oviposition days (paths J → N → R). In particular, food

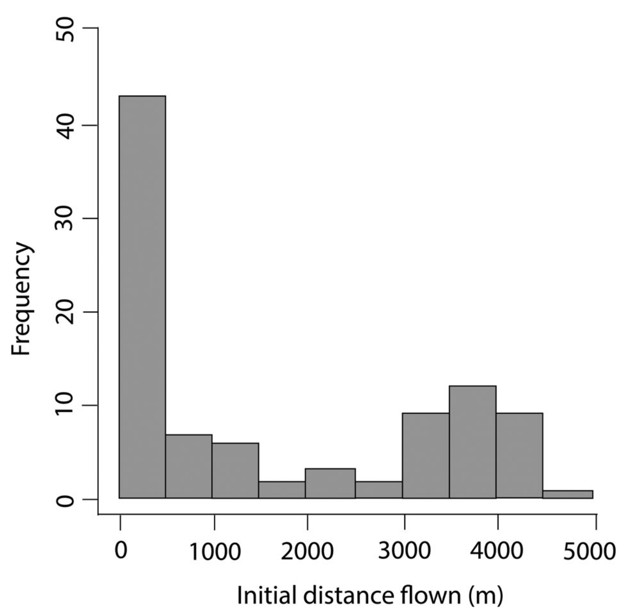

FIGURE 2 Frequency distribution of distances flown during first 3-h flights by pre-reproductive *Hippodamia convergens* females.

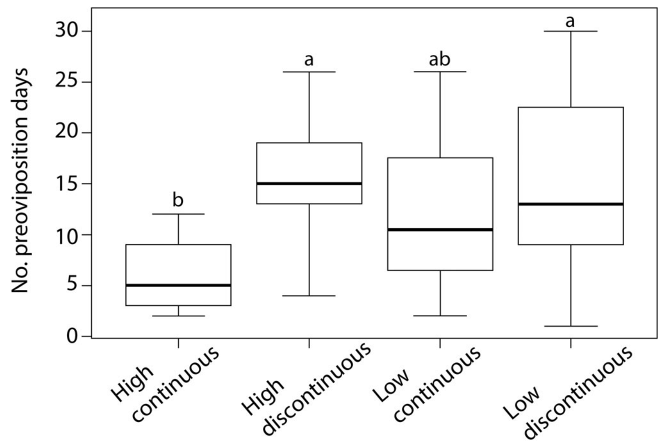

FIGURE 3 Median preoviposition periods, with interquartile range, of female *Hippodamia convergens* subjected to four different food supply treatments (high continuous = access to 6 h of ad libitum food daily; high discontinuous = access to 12 h of food every other day; low continuous = access to 3 h of ad libitum food daily; and low discontinuous = access to 6 h of food every other day). Treatments bearing the same letters were not significantly different (Tukey's test, α = 0.05).

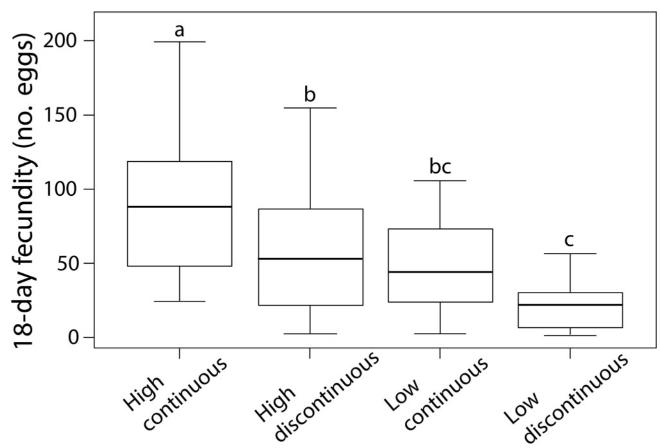

FIGURE 4 Median 18-day fecundities (number of eggs), with interquartile ranges, of female *Hippodamia convergens* subjected to four different food supply treatments (high continuous = access to 6 h of ad libitum food daily; high discontinuous = access to 12 h of food every other day; low continuous = access to 3 h of ad libitum food daily; and low discontinuous = access to 6 h of food every other day). Treatments bearing the same letters were not significantly different (Tukey's test, α = 0.05).

access continuity decreased the pre-ovipositional period ($\beta_J = -0.041$, $p < 0.001$), whereas greater pre-ovipositional period, in turn, decreased the number of oviposition days ($\beta_N = -0.45$, $p < 0.0001$). The number of oviposition days positively influenced 18-day fecundity ($\beta_F = 0.79$, $p < 0.0001$) but was negatively correlated with total post-reproductive flight distance ($\beta_R = -0.32$, $p = 0.011$ and $R^2 = 0.03$, $p = 0.036$, Figure 7), suggesting possible trade-offs between flight and fecundity.

Although both food quantity and access affected fecundity and flight, the magnitude of the effect of food quantity on fecundity ($\Sigma\beta_{\text{quant-fecund}} = 0.316$, Table 1) and flight ($\Sigma\beta_{\text{quant-flight}} = 0.132$) was more than twice that of food access ($\Sigma\beta_{\text{access-fecund}} = 0.145$, $\Sigma\beta_{\text{access-flight}} = -0.05$) for our specific levels of variance. Food quantity also affected fecundity (range $\beta_{\text{fecund}} = 0.14$–$0.61$, $R^2 = 0.77$) more than it affected flight distance (range $\beta_{\text{flight}} = -0.05$ to 0.132, $R^2 = 0.23$).

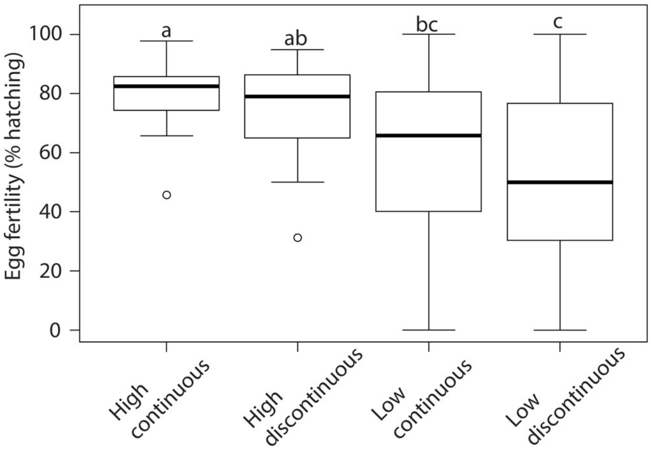

FIGURE 5 Median egg fertility (% eggs hatching), with interquartile range, of female *Hippodamia convergens* subjected to four different food supply treatments (high continuous = access to 6 h of ad libitum food daily; high discontinuous = access to 12 h of food every other day; low continuous = access to 3 h of ad libitum food daily; and low discontinuous = access to 6 h of food every other day). Treatments bearing the same letters were not significantly different (Tukey's test, α = 0.05). Open circles represent outliers.

DISCUSSION

Both higher food quantity and higher continuity of access increased female fitness via effects on oviposition behavior. Fewer females became reproductive in the LD treatment than in any other treatment, suggesting that resource quantity in this treatment was close to the lower limit for reproduction in this species. Higher food quantity increased the number of oviposition days, whereas greater food continuity accelerated the onset of

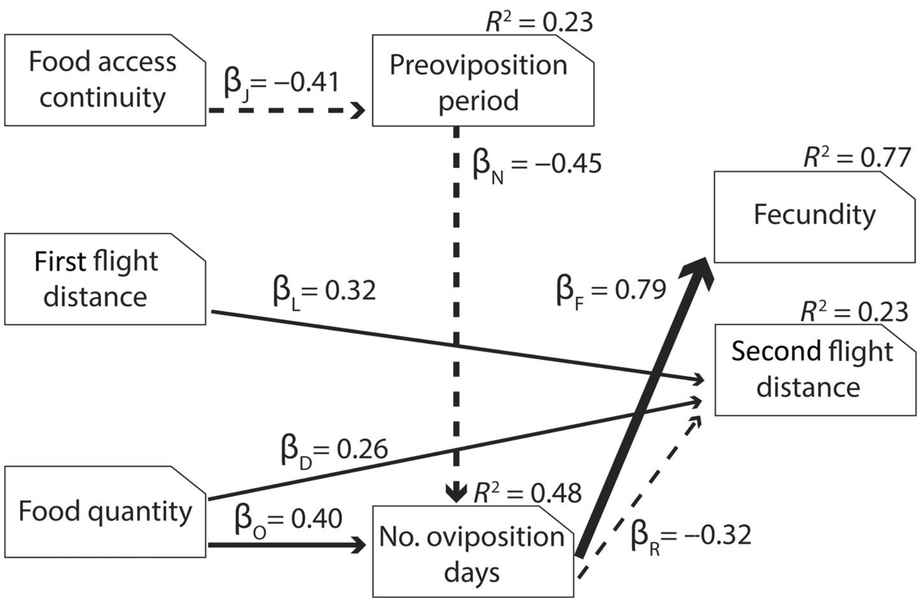

FIGURE 6 Structural equation model of the effects of food treatments (food access continuity and food quantity) on flight performance and reproduction of female *Hippodamia convergens*. Solid arrows represent positive causal relationships and dashed arrows represent negative causal relationships. Values are standardized path coefficients (β) with line sizes scaled to illustrate standardized coefficients. Coefficient of determination (R^2) values indicate the proportion of the variation explained by the model. Fecundity is the number of eggs laid per female in 18 days of oviposition. Preoviposition period is the number of days from provision of the reproductive diet (greenbugs) to first oviposition. No. oviposition days is the number of days on which clutches were laid in the 18-day observation period. Flight distance is the total distance flown (in meters) during a 3-h period on a tethered flight mill. Model fit: Fisher's $C = 13.85$, df = 12, $p = 0.31$.

oviposition. These results supported our initial hypothesis that both continuity of food access and food quantity affect female fitness. According to our piecewise SEM, the quantity of resource provided affected the oviposition amount (i.e., number of oviposition days), whereas continuity of resource access affected the timing of oviposition onset (i.e., preoviposition period). Food resources have clear bottom-up effects on insect predators (Agrawal et al., 1999; Alomar & Weidenmann, 1996; Polis & Winemiller, 1996), but past work has focused mostly on resource quantity and quality, whereas the effects of access continuity on insect predators has received less study (Rosenheim, 2001). The SEM indicated that resource quantity has stronger effects on both flight distance and reproductive performance compared with resource continuity for the levels examined here, although both predictors were influential. Discontinuity of resources may alter population persistence (Fahrig, 2002; Gibson et al., 2013), erect barriers to movement between prey patches (Henle et al., 2004), or create "bottlenecks" or "interruptions" that delay reproduction or diminish population fitness (Schellhorn et al., 2015). A delay in onset of reproduction can slow population growth without necessarily affecting individual fecundity (Houston & McNamara, 1999; Millon et al., 2010). However, we examined only two levels of resource discontinuity and amount in the present study, and a complete understanding of their potential effects on predator population dynamics would require examination of a wider range of variation and combinations of these factors.

Onset of oviposition was delayed by resource discontinuity, especially when food quantity was high. Whereas HC beetles were the first to begin laying eggs, HD beetles were the last, and LC and LD females began laying eggs sooner than HD females. The greatest difference in timing of oviposition onset occurred in high food treatments, whereas low food treatments were intermediate. The delayed onset of oviposition observed in the HD treatment relative to the LD treatment suggests that the physiological impact of food supply interruptions on oocyte maturation is greater when females are acclimated to higher prey densities (greater food quantity) and would suggest that oviposition may begin earlier under conditions of resource discontinuity when the overall supply of prey is limited. However, the life history impacts of resource quantity and continuity of access are likely to vary with the amount of variance in each component, so results would likely differ according to both the amplitude and periodicity of food access cycles. Delayed reproduction in response to environmental variability and resource discontinuity has been documented in many insects (e.g., Johnson et al., 2016) and other animals (owls in Millon et al., 2010, and bats in Nurul-Ain et al., 2017). Delayed or reduced reproduction can extend lifespan (Holliday, 1989; Michaud & Qureshi, 2006; Speakman & Mitchell, 2011) but also carries fitness costs (Clutton-Brock, 1984; Viallefont et al., 1995). In *H. convergens*, long periods of reproductive diapause in summer extend lifespan, but reduce lifetime fecundity (Michaud & Qureshi, 2006). All organisms must balance current reproductive effort against the potential benefits of longer survival and future reproductive success under (possibly) more favorable conditions (Hadley et al., 2007;

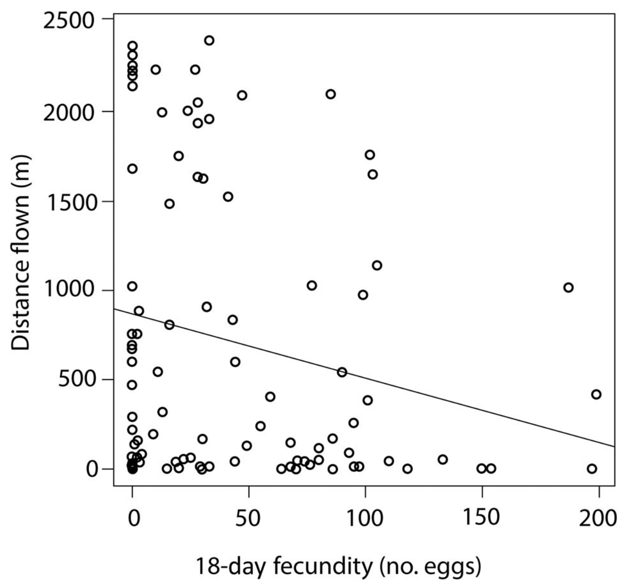

FIGURE 7 Linear regression of post-reproductive flight distances covered by *Hippodamia convergens* females on their 18-day fecundities. Equation: $y = -7.26x + 1744.2$. $R^2 = 0.036$, $p = 0.036$.

TABLE 1 The direct, indirect, and total effects of food quantity and continuity on fecundity and flight.

	Fecundity			Flight		
Variable	Indirect	Direct	Total	Indirect	Direct	Total
Quantity	0.32	…	0.32	−0.13	0.26	0.13
Continuity	0.15	…	0.15	−0.06	…	−0.06

Note: Indirect effects were calculated as the product of structural equation model β coefficients while the total effects were the sum of the direct and indirect effects. Ellipses (…) represent no direct effects observed.

Roff, 1993; Stearns, 1992; Williams, 1966). Although delayed onset of oviposition reduced the initial 18-day period of fecundity measured in our study, discontinuous access to high prey densities may signal that better oviposition opportunities lie ahead, and so represent an adaptive response that averts excessive premature egg production. Measurement of female reproductive performance over a longer period might elucidate longer-term impacts of these treatments on lifetime reproductive success.

Egg fertility declined in low food quantity treatments, but was not affected by continuity of access. This result could reflect reduced egg or sperm viability due to nutrient limitation. Although differences in food quantity or quality do not always affect egg fertility in *H. convergens* (e.g., Stowe et al., 2021; Vargas et al., 2012a), effects can appear over longer observational periods, as egg fertility declines as a function of maternal age in both very large and very small females, and eggs get progressively smaller with age in the case of the latter (Vargas et al., 2012a, 2012b). Any reduction in egg fertility may also improve the survival of siblings, given that cannibalism of infertile eggs within hatching clusters provides a valuable food source for eclosing larvae within hatching clusters (Michaud & Grant, 2004; Osawa, 1992). Although it is unclear if a fraction of each cluster may be left intentionally unfertilized to serve as "trophic eggs" (e.g., Perry & Roitberg, 2005), sibling egg cannibalism does occur in *H. convergens* (Bayoumy & Michaud, 2015) and could serve to raise maternal fitness when prey density is low.

Studies on the energetic trade-offs associated with flight and fitness have historically focused on investment in wing development in species with wing polymorphism (e.g., Harrison, 1980; Roff, 1986; Zera & Denno, 1997) and very few have examined those associated with flight effort expended by individuals (but see Guerra & Pollack, 2009). Although flight behavior is complex and comprises multiple related metrics such as bout length, distance, and flight propensity, in the present study, we used total flight distance as a metric for energy expenditure, with the expectation that trade-offs would be manifest in reproductive effort (amount of oviposition). The SEM revealed that the initial distance flown by pre-reproductive females was negatively correlated with their fecundity during the observation period, and that post-reproductive flight distance decreased with an increasing number of reproductive days, rather than with increased fecundity per se. The relationship between reproductive effort and flight capacity persisted even when considered in isolation, as the total distance flown was negatively correlated with fecundity. These findings are generally consistent with our hypothesis of an energetic trade-off between flight activity and reproductive effort. Longer, or more frequent, flights will also expose insects to increased predation risk, exhaustion, and physical damage (Bonte et al., 2012) while reducing the energy reserves needed for survival and reproduction (Guerra & Pollack, 2009; Schmid-Hempel et al., 1985). Interestingly, pre-reproductive flight and post-reproductive flight distance were positively correlated. Although this finding contradicts our hypothesis that greater initial flight distance would decrease subsequent flight distance, it is consistent with our inference that substantial variation exists among females with respect to intrinsic flight propensity, as environmental uncertainty in the agricultural landscape would likely counter any normalizing selection around some universally optimum mean. We also suspect that the experimental flight period provided in the experiment was insufficiently demanding to produce any negative effect on later flight activity. Many females remained actively flying at the end of the 3-h flight period, suggesting that energy reserves were not exhausted. A flight duration of approximately 60 min appears typical for large coccinellid species (Maes et al., 2014), although some *H. convergens* adults (ca. 10%) may fly for as long as 12 h in a single bout (Rankin & Rankin, 1980). Future investigations of possible trade-offs between flight and reproduction in this species should increase flight periods or induce repeated bouts of flight to increase energetic expenditure.

Agricultural systems present high levels of resource variability for predators and pests due to harvest cycles and other human disturbances. Conservation practices have primarily focused on increasing resource amount and diversity to support insect communities within agroecosystems (Power, 2010). More recently, continuity of access to resources has been increasingly recognized as important to the efficacy of natural biological control and other ecosystem services (Angeler et al., 2016; Cateau et al., 2018; Cohen & Crowder, 2017; Egli et al., 2020; Iuliano & Gratton, 2020; Kennedy et al., 2016; Schellhorn et al., 2015; Spiesman et al., 2020). A more even distribution of supplemental resources across the landscape would complement efforts to increase the amount and quality of these resources. More continuous availability of food resources has been shown to decrease emigration in planthoppers (Denno et al., 1991) and certain pollinators (Nicholson et al., 2021). Our results reveal that more continuous feeding by predatory beetles leads to earlier oviposition and more oviposition days. Increased resource continuity could be provided by the judicial planting of cover crops, management of non-crop habitat, or even crop field arrangement in the landscape, to materially improve the effectiveness of natural enemies, independent of the absolute amount of resources provided. (Bianchi et al., 2006; Landis et al., 2000; Rayl et al., 2018; Rusch et al., 2010; Tscharntke et al., 2005). We conclude that spatial and temporal continuity of resources across the landscape should be considered in concert with absolute resource amounts in order to conserve populations of generalist insect predators like *H. convergens* and the biological control services they provide in agriculture.

This study illustrates how the interactive and complex nature of resource access (i.e., amount and access variability) may alter various aspects of *H. convergens* behavior and fitness. Complex behavioral and fitness trade-offs exist between various resource needs in insect predators, both with respect to resource amount and resource access continuity. The various ways that resources influence natural enemy responses provide opportunity for improvements to land management success. An understanding of these effects could provide novel and nuanced ways to support crucial ecosystem services. Biological control agents benefit from increased resource availability, and as sustainable agricultural systems are expected to increasingly rely on biological control services, we might increase these benefits by considering resource availability in time and space. Conservation of native natural enemies, especially generalist insect predators like *H. convergens*, might be substantially improved by increasing resource continuity across the landscape and throughout the year, resulting in better control of the pests that threaten agricultural ecosystems. Improving resource continuity on the landscape must be considered in concert with resource amount to maximize the survival and population growth of generalist insect predators like *H. convergens*.

AUTHOR CONTRIBUTIONS

All authors conceived of the study and designed the experiment collectively. Hannah E. Stowe performed the experiments and collected the data. Hannah E. Stowe and Tania N. Kim analyzed the data and conducted model selection. All authors contributed equally to writing the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data (Stowe et al., 2022) are available from Dryad: https://doi.org/10.5061/dryad.7m0cfxpx6.

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Attraction, mobility, and preference by *Lasioderma serricorne* (Coleoptera: Ptinidae) to microbially-mediated volatile emissions by two species of fungi in stored grain

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Insects and microbes are known to interact in a variety of ways at food facilities, compounding damage. However, little research has explicated how specific common fungal species affect the behavior of the cosmopolitan secondary stored product pest, *Lasioderma serricorne*. Enhanced knowledge about attraction to microbially-produced volatile organic compounds (MVOCs) may be used to manipulate insect behavior. *Aspergillus flavus* and *Fusarium verticillioides* are two common, widespread pre- and postharvest fungi on small cereals that produce aflatoxins and fumonisins, respectively, while directly competing with each other for nutrients. Our goals were to (1) characterize the volatile emissions from grain inoculated by *A. flavus* or *F. verticillioides* derived from the cuticle of *L. serricorne* compared to uninoculated and sanitized grain, and (2) understand how MVOCs from each fungal species affects mobility, attraction, and preference by *L. serricorne*. Headspace collection revealed that the *F. verticillioides*- and *A. flavus*-inoculated grain produced significantly different volatiles compared to sanitized grain or the positive control. Changes in MVOC emissions affected close-range foraging during an Ethovision movement assay, with a greater frequency of entering and spending time in a small zone with kernels inoculated with *A. flavus* compared to other treatments. In the release-recapture assay, MVOCs were found to be attractive to *L. serricorne* at longer distances in commercial pitfall traps. There was no preference shown among semiochemical stimuli in a still-air, four-way olfactometer. Overall, our study suggests that MVOCs are important for close- and long-range orientation of *L. serricorne* during foraging, and that MVOCs may have the potential for inclusion in behaviorally-based tactics for this species.

Insects inflict over $100 billion in economic losses to commodities after harvest[1], while microbes, especially fungi, may endanger the safety of human through the production of mycotoxins[2,3]. Insects and microbes may interact in a variety of ways at food facilities, compounding damage and threatening food security. Although insects and microbes may be found together in food facilities, they are often not discussed together even though microbes can alter the grain in environment in ways that are beneficial to stored product pests[4]. For example, the presence of microbes increased progeny production of *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) and simultaneously elevated the humidity and temperature of the grain mass, thereby creating favorable growth conditions for themselves and *S. oryzae*. *Fusarium verticillioides* likewise was found to increase the progeny of *Sitophilus zeamais* (Coleoptera: Curculionidae)[5]. Hubert et al.[6] found that the abundance of bacteria in spent growth medium was positively correlated with mite fitness.

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In addition to altering the abiotic environment, microbes produce microbial volatile organic compounds (MVOCs, hereafter), which may act as insect pheromones or food kairomones that mediate key behaviors including attraction, repulsion, mating, foraging, dispersal, and aggregation[7]. In a recent metanalysis, Ponce et al.[4] found that insect behavioral responses (e.g., attraction, repellency, or net neutrality) to MVOCs are complex and can vary by microbial taxa and community composition, abiotic environment, insect species, life history (internal-infesting or external-infesting stored product insects). Steiner et al.[8] found the stored product parasitoid, *Lariophagus distinguendus* Förster (Hymenoptera: Pteromalidae), avoided the fungal volatile 1-octen-3-ol. Van Winkle et al.[9] found that tempering grain to different moisture levels and incubating for 9–28 days at 30 °C significantly changed the blend of volatiles and microbial communities, which showed attraction at moderate levels to *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae), but not the secondary pest, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). Ponce et al.[10] found that MVOCs affected the close-range foraging of an internal-infesting pest, *S. oryzae*, after inoculation of grain with different life stages (e.g., sexual vs. asexual) of *Aspergillus flavus*. An important knowledge gap that needs to be addressed is how and whether fungal MVOCs impact behavior of external-infesting stored product pests.

One important external-infesting pest is the cigarette beetle, *Lasioderma serricorne* (F.) (Coleoptera: Ptinidae). This is a cosmopolitan pest globally in and around grain storage and food processing facilities that has the unique ability to detoxify plant secondary metabolites, including nicotine and caffeine, through interactions with its midgut symbionts[11]. Contrary to its common name, *L. serricorne* has one of the most diverse host breadths behind *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae)[11], feeding on 222 different dried plant and animal products and reproducing on 49 commodities[12]. The injury inflicted by *L. serricorne* is regularly severe, and includes reduced market value from direct feeding, quality decreases from excrement and exuvia, and loss of business from consumer complaints[11,13]. *Lasioderma serricorne* has been proposed as a new model species to evaluate symbioses with microorganisms[14], and certainly its interaction with the microbiome of grain pathogens should be included under this umbrella. This species is highly mobile, able to disperse readily by flight during most of the year in Mediterranean climates[15] and possesses strong walking and climbing capabilities[16]. There have also been promising preliminary reports showing control of *L. serricorne* may be possible with semiochemical-based strategies[17–19]. Prior work in Japan isolated 17 species of molds and 5 species of yeasts from the cuticles of *L. serricorne*, including *Aspergillus*, *Arthrinium*, *Eurotium*, *Fusarium*, and *Penicillium*[20]. However, the ecological role of these microbes and how they affect *L. serricorne* behavior is poorly understood.

Pathogens that colonize raw grain and finished products also threaten commodities and can have significant negative impacts on both human and animal health[21–23]. In addition, off-odors in raw and finished commodities have also been linked to microbial contamination[24]. Common fungal species that contaminate grain and commodities post-harvest typically include those from the genera *Aspergillus*, *Fusarium*, *Penicillium*, and *Alternaria*, among others. However, prior work has frequently found that *Aspergillus* spp. are the most common genera in flour production, with 35% of 1258 samples belonging to this genus from various food facilities in Europe, and a significant portion (10%) belonging to *Fusarium* spp. as well[25]. Likewise, *Aspergillus*, *Penicillium* and *Fusarium* spp. are the main micro-organisms involved in contamination of maize[26]. Specifically, *A. flavus* and *F. verticillioides* were most frequently isolated from Brazilian maize, and have a high proportion of strains that produce aflatoxins and fumonisins[27]. *A. flavus* and *F. verticillioides* are direct competitors on grains, and *A. flavus* is expected to outcompete other *Fusarium* spp. in small cereals under climate change going forward, with documented higher incidence during particularly warm, dry years[28,29]. Thus, in this work, we focus on these two genera, including *A. flavus*, a widespread post-harvest plant pathogen[30], which causes significant economic losses after harvest[2] that is known to produce the carcinogenic secondary metabolite, aflatoxin B_1[31]. Although much progress has been made in reducing aflatoxin contamination, it remains a sporadic problem for corn stored in the southern United States and it is routinely detected in stored commodities at markets in developing countries[32]. The other focus of this study, and a common fungal contaminant of cereals is *Fusarium verticillioides*, which may attack wheat either pre- or post-harvest[33]. Under most years in Italy, contamination of maize with *F. verticillioides* is a concern, which can produce fumonisins[28]. Maize from smallholder farms in Vietnam were found to be predominately contaminated with *F. verticillioides* and fumonisin B_1, with traditional harvest and postharvest practices correlating to higher contamination[34]. This is more broadly a problem for smallholders around the world, with *A. flavus* and *F. verticillioides* implicated in aflatoxin and fumonisin production, including in Africa[35]. Despite the high frequency of these fungal pathogens in postharvest environments, little work has been done to investigate headspace volatiles of grain contaminated by either microbe and the behavioral impacts on stored product insects associated with commodities.

One alternative suite of tactics in IPM to combat stored product pests are semiochemical-mediated, behaviorally-based tactics (reviewed recently in Morrison et al.[36]) that use volatile cues to manipulate behavior in ways that are conducive to protecting commodities from insect colonization. Effective attractants and/or repellents are required for the success of these IPM tactics. One class of stimuli that may be broadly attractive to stored product insects are MVOCs, partially because of the evolutionary history of these taxa that involved using animal caches of food before the advent of agriculture. One particular application of MVOCs may be in attract-and-kill, whereby insects are attracted to a spatially-circumscribed area by a long-range attractant treated with an insecticide and then removed from the foraging population (e.g.,[37–40]). Recent work has found that attract-and-kill based interception traps placed on the perimeter of food facilities were successful at preventing progeny production when they included long-lasting insecticide netting, capturing over 3000 individuals in weekly 48-h deployments in 2 years[39]. However, a challenge in deploying this tactic at food facilities is competing with existing volatile stimuli, including nearby food resources, insects emitting aggregation or sex pheromones, and sites of spillage. Nonetheless, the incorporation of MVOCs into lures for attract-and-kill tactics may make these volatile sources unique among the backdrop of odors at food facilities and support delivering improved and unique IPM tools in the food environment to stakeholders.

Thus, our goals were to (1) characterize the volatile emissions from grain inoculated by *Aspergillus flavus* or *Fusarium verticillioides* derived from the cuticle of *L. serricorne* compared to uninoculated and sanitized grain, and (2) understand how MVOCs from each fungal species affects mobility, attraction, and preference by *L. serricorne*. We hypothesized that headspace blends will be unique among our fungal treatments, and that this will lead to differences in preference and mobility by *L. serricorne*.

Results

Fungal morphotype sequencing. After running a BLASTn search against the nt database, the top ten matches for the ITS consensus sequences from the AF morphotype were all with isolates of *Aspergillus*, with nine of them *A. flavus* and one of them *A. niger* with 99% sequence identity and 97–98% query coverage (E ≈ 0). Among the top 10 matches for the ITS sequence of the FS morphotype, 90% were to *F. verticillioides*, while one match was to *Fusarium annulatum*. There was 99% sequence identity with the matches over 43% query coverage (E ≈ 0) for the FS morphotype.

Four-way olfactometer. The presence of the four stimuli did not significantly affect the percentage of adult *L. serricorne* choosing a treatment side in the four-way olfactometer ($\chi^2 = 3.53$; df = 3; $P = 0.32$; Fig. 1). Overall, *L. serricorne* chose UV-sanitized grain 30% of the time, while on average, they chose *Fusarium*-inoculated grain 21% of the time.

The semiochemical treatments in the four-way olfactometer had no significant effect on the time to decision by adult *L. serricorne* (ANOVA: $F = 0.22$; df = 3,174; $P = 0.88$). On average, decisions were made in 88–96 s by *L. serricorne* for the *Fusarium*-inoculated grain and *A. flavus*-inoculated grain, respectively.

Movement assay overall measures. The semiochemical treatments significantly affected the distance moved by adult *L. serricorne* over the 30-min observation periods (ANOVA: $F = 541$; df = 4, 86; $P < 0.0001$). Adult *L. serricorne* moved two to fourfold less when exposed to a grain kernel of one of the four semiochemical treatments compared to the negative control (Tukey HSD, Fig. 2). Adults moved numerically the least when exposed to *A. flavus*-inoculated grain kernels.

While velocity moved by *L. serricorne* showed the same numerical pattern in response to semiochemical treatments, it did not differ significantly (ANOVA: $F = 1.28$; df = 4, 86; $P = 0.28$). Overall, *L. serricorne* velocity

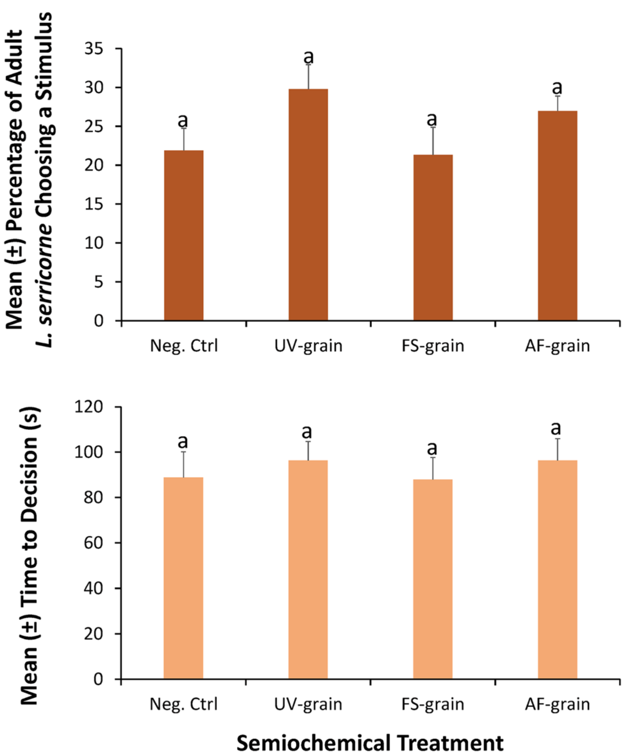

Figure 1. Preference shown in the four-way, still-air olfactometer using the mean percentage of *L. serricorne* adults choosing a stimulus (± SE) after given a 4-min opportunity (top panel) and their mean time to decision (± SE, s; bottom panel) based on the semiochemical treatment to which they were exposed in the laboratory at the USDA-ARS Center for Grain and Animal Health Research in Manhattan, KS. A total of n = 200 adults replicate were tested in this assay. Bars with shared letters are not significantly different from each other (Tukey HSD, α = 0.05). Neg. Ctrl, negative control; UV-grain, UV-sanitized grain; P. Ctrl, uninoculated grain; FS-grain, *F. verticillioides*-inoculated grain; AF-grain, *A. flavus*-inoculated grain.

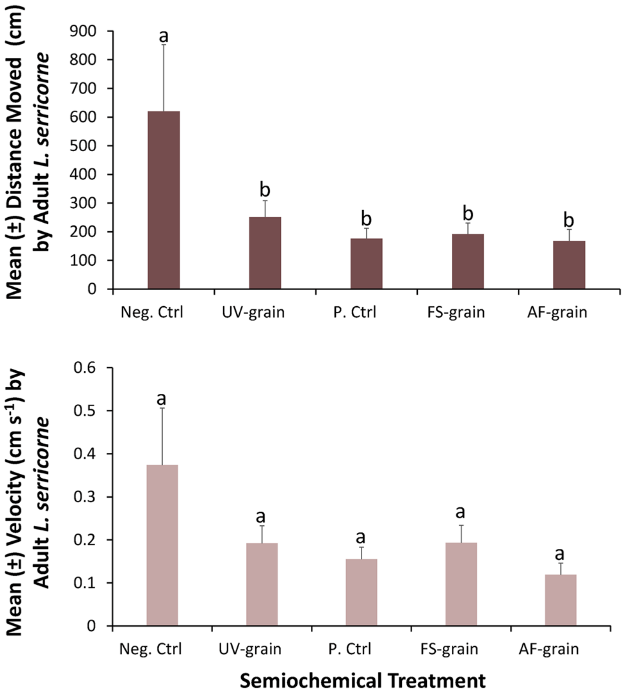

Figure 2. Metrics from the movement assay assessing the effects of MVOCs on mean distance moved (± SE, top panel) and the mean instantaneous velocity (± SE, bottom panel) by adult *L. serricorne* over a period of 30 min using a network camera coupled with Ethovision in the laboratory at the USDA-ARS Center for Grain and Animal Health Research in Manhattan, KS. There were a total of n = 20 replicates per treatment, translating to 50 h of data in this assay. Bars with shared letters are not significantly different from each other (Tukey HSD, $\alpha = 0.05$). Neg. Ctrl, negative control; UV-grain, UV-sanitized grain; P. Ctrl, uninoculated grain; FS-grain, *F. verticillioides*-inoculated grain; AF-grain, *A. flavus*-inoculated grain.

was numerically decreased by two to threefold when exposed to a grain kernel of one of the four semiochemical treatments compared to the negative control (Fig. 2). The mean velocity ranged from 0.12 to 0.37 cm s^{-1} for the *A. flavus*-inoculated grain and negative control, respectively.

Movement assay in kernel zones. The semiochemical treatments significantly affected close-range foraging by altering the frequency by which an adult *L. serricorne* entered each kernel zone (MANOVA: $F = 11.8$; df = 4, 86; $P < 0.0001$; Fig. 3). The semiochemical treatments significantly affected the frequency by which an adult *L. serricorne* entered the control kernel zone, which lacked stimuli (ANOVA: $F = 3.81$; df = 4, 86; $P < 0.01$). Adult *L. serricorne* were two to threefold less likely to enter the control kernel zone in the *A. flavus*-inoculated grain and *Fusarium*-inoculated grain arenas compared to the negative control or UV-sanitized grain arenas (Fig. 3). Furthermore, the frequency of adult *L. serricorne* entering treatment kernel zones was significantly affected by the semiochemical treatment present (ANOVA: $F = 6.01$; df = 4, 86; $P < 0.001$). There were two to threefold more entries by adult *L. serricorne* to the treatment kernel zone containing the *A. flavus*-inoculated grain than to treatment kernel zones with uninoculated grain or no grain at all (Tukey HSD, Fig. 3). Importantly, *L. serricorne* entered treatment kernel zones with *A. flavus*-inoculated grain significantly more often than the associated control kernel zones (Post-hoc: $t = 2.50$; df = 19; $P < 0.05$; Fig. 3).

The semiochemical treatment significantly affected the cumulative duration spent in each kernel zone or arena half by adult *L. serricorne* (MANOVA: $F = 51.9$; df = 4,86; $P < 0.0001$). Particularly, the presence of the semiochemical treatments significantly affected the duration spent in both the control kernel zone (ANOVA: $F = 3.19$; df = 4,86; $P < 0.05$) and treatment kernel zone (ANOVA: $F = 50.9$; df = 4,86; $P < 0.0001$). The mean (± SE) cumulative duration spent in the control kernel zone was short and ranged from 3 (± 1.1) to 8 (± 3.0) s over the 30-min period. By contrast, *L. serricorne* spent 6–17-fold more time in the treatment kernel zone containing *Fusarium*-inoculated grain and *A. flavus*-inoculated grain, respectively, compared to the treatment kernel zone in the negative control. Adult *L. serricorne* spent the most time in the treatment kernel zone for UV-sanitized grain and *A. flavus*-inoculated grain, which was approximately 40 ± 28 and 119 ± 73 s, respectively. Importantly, *L. serricorne* spent significantly more time in the treatment kernel zone than the control kernel zone for *A. flavus*-inoculated grain (Post-hoc: $t = 2.14$; df = 19; $P < 0.05$).

The semiochemical treatments significantly affected the latency with which adult *L. serricorne* found each kernel zone or arena half (MANOVA: $F = 44.7$; df = 4, 86; $P < 0.0001$). For example, the semiochemical treatments significantly affected the latency to finding and entering the control kernel zone (ANOVA: $F = 7.86$; df = 4, 86; $P < 0.0001$) as well as the treatment kernel zone (ANOVA: $F = 3.63$; df = 4, 86; $P < 0.01$). Adult *L. serricorne* were two to threefold faster in finding *Fusarium*-inoculated grain control kernel zones than ones for UV-sanitized

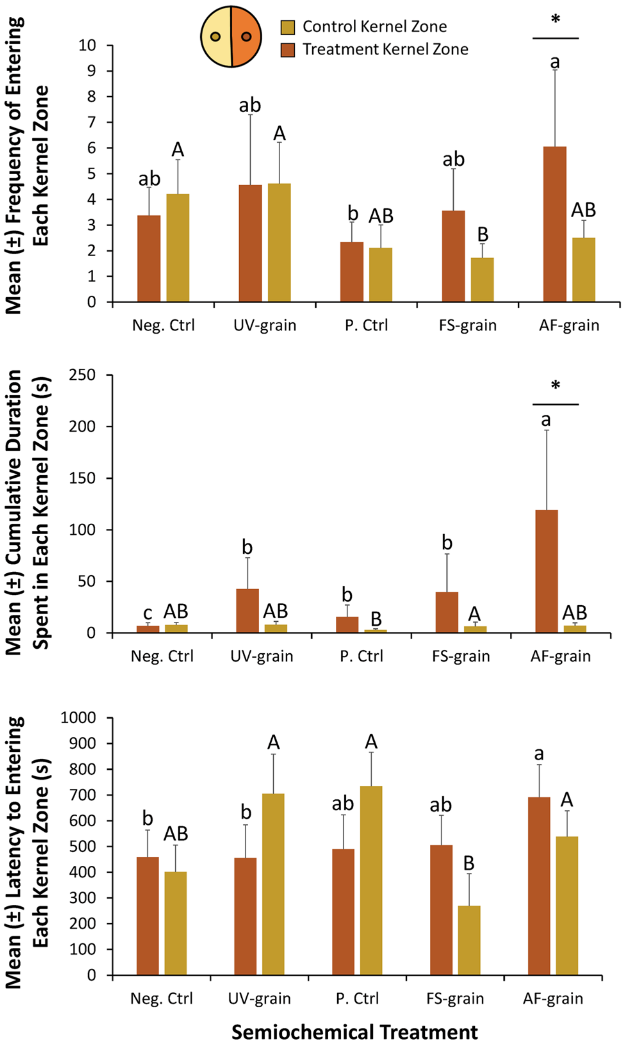

Figure 3. Treatment (dark orange bars) and control (dark yellow bars) kernel zone metrics from the movement assay, including determining how MVOCs affect the mean (± SE) frequency of entering each kernel zone (top panel), the cumulative duration spent in each kernel zone (middle panel), and the latency to finding and entering each kernel zone (bottom panel) by adult *L. serricorne* for a period of 30 min using video-tracking with a network camera coupled with Ethovision Software in the laboratory at the USDA-ARS Center for Grain and Animal Health Research in Manhattan, KS. The treatment kernel zone contains a single hard winter wheat kernel, while the control kernel zone does not contain any stimuli (neg. ctrl). There was a total of n = 20 replicate adults tested per treatment, translating to 50 h of data in this assay. Upper case letters represent multiple comparisons among levels of the control kernel zones only, while lower case letters represent multiple comparisons among levels of the treatment kernel zones only. Bars with shared letters are not significantly different from each other (Tukey HSD, α = 0.05). Asterisks (*) indicate post-hoc comparisons using paired t-tests (with Bonferroni correction), and only significant results have been displayed on applicable bars. Neg. Ctrl, negative control; UV-grain, UV-sanitized grain; P. Ctrl, uninoculated grain; FS-grain, *F. verticillioides*-inoculated grain; AF-grain, *A. flavus*-inoculated grain.

grain or uninoculated grain (Fig. 3). All semiochemical treatments in treatment kernel zones had a similar latency to being found and entered by *L. serricorne* except *A. flavus*-inoculated grain, which resulted in 1.5-fold higher latency than the other treatments.

Movement assay in arena halves. The frequency by which an adult *L. serricorne* entered the control half varied significantly among the semiochemical treatments (ANOVA: $F = 5.21$; df = 4, 86; $P < 0.001$). Adults entered control halves rarely, on average between 2 and 5 times over the 30 min period, corresponding to the *Fusarium*-inoculated grain and UV-sanitized grain treatment respectively (Fig. 4). Moreover, the frequency of entering the treatment half by an adult *L. serricorne* was significantly affected by the semiochemical treatment (ANOVA: $F = 6.30$; df = 4, 86; $P < 0.001$). There were each about twofold less frequent entries by adult *L. serricorne* to treatment halves with *A. flavus*-inoculated and *Fusarium*-inoculated grain compared to the negative control.

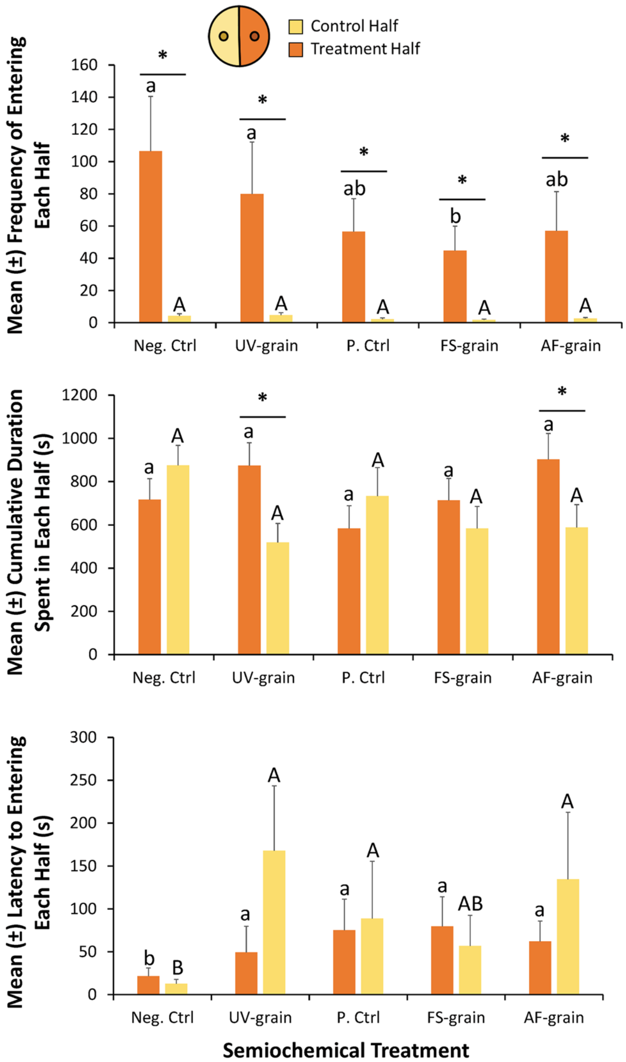

Figure 4. Treatment (orange bars) and control (yellow bars) zone metrics from the movement assay, including determining how MVOCs affect the mean (± SE) frequency of entering each zone (top panel), the cumulative duration spent in each zone (middle panel), and the latency to finding and entering each zone (bottom panel) by adult *L. serricorne* for a period of 30 min using video-tracking with a network camera coupled with Ethovision Software in the laboratory at the USDA-ARS Center for Grain and Animal Health Research in Manhattan, KS. The treatment zone contains a single hard winter wheat kernel, while the control zone does not contain any stimuli (neg. ctrl). There was a total of n = 20 replicate adults tested per treatment, translating to 50 h of data in this assay. Upper case letters represent multiple comparisons among levels of the control zones only, while lower case letters represent multiple comparisons among levels of the treatment zones only. Bars with shared letters are not significantly different from each other (Tukey HSD, α = 0.05). Asterisks (*) indicate post-hoc comparisons using paired t-tests (with Bonferroni correction), and only significant results have been displayed on applicable bars. Neg. Ctrl, negative control; UV-grain, UV-sanitized grain; P. Ctrl, uninoculated grain; FS-grain, *F. verticillioides*-inoculated grain; AF-grain, *A. flavus*-inoculated grain.

Importantly, each semiochemical treatment in the treatment half resulted in significantly more entries than the corresponding control half (Post-hoc paired t-test, Bonferroni correction; Fig. 4).

By contrast, the semiochemical treatments did not significantly affect the cumulative duration spent in both control half (ANOVA: $F = 1.12$; df = 4,86; $P = 0.35$) nor in the treatment half (ANOVA: $F = 0.81$; df = 4,86; $P = 0.52$). Adult *L. serricorne* spent a range of 519–875 s in the control halves, on average, associated with the UV-sanitized and negative control treatment, respectively. Adults spent an average duration ranging from 584 to 902 s in the treatment halves, which corresponded to the uninoculated- and *A. flavus*-inoculated grain, respectively. Importantly, the cumulative duration spent in the *A. flavus*-inoculated treatment half was significantly more than the control half (Post-hoc: $t = 2.01$; df = 19; $P < 0.05$), as was the cumulative duration spent in the UV-sanitized treatment half compared to the control half ($t = 2.56$; df = 19; $P < 0.01$).

The semiochemical treatments significantly affected the latency to entering the control half (ANOVA: $F = 28.1$; df = 4,86; $P < 0.0001$) and treatment half (ANOVA: $F = 13.7$; df = 4,86; $P < 0.0001$) of the arenas. It took *L. serricorne* adults 4–13-fold longer to find control halves associated with UV-sanitized, uninoculated-, AF-inoculated grain, and *Fusarium*-inoculated grain semiochemical treatments compared to the negative control (Fig. 4). By contrast, *L. serricorne* were three to fourfold faster in finding the treatment halves associated with *Fusarium*-inoculated and *A. flavus*-inoculated grain compared to the negative control. Numerically, *L. serricorne* adults were over threefold faster in finding UV-sanitized treatment halves compared to control halves, while adults were twofold faster in finding *A. flavus*-inoculated treatment halves compared to control halves.

Release-recapture assay. In total, the percent of adults recaptured out of 800 was 41%, ranging between 26 and 51%. The attractant in the trap significantly affected recapture of *L. serricorne* in commercially-available pitfall traps (ANOVA: $F = 7.11$; df = 4,35; $P < 0.001$; Fig. 5). In fact, the greatest recapture was in traps baited with UV-sanitized, uninoculated-, and *A. flavus*-inoculated grain, which each captured approximately twofold more *L. serricorne* than the negative control. Traps baited with *Fusarium*-inoculated grain captured an intermediate percentage of *L. serricorne*.

Headspace volatiles collected from inoculated grain. In total, 44 compounds were tentatively identified from the semiochemical treatments (Table 1). The uninoculated grain had the fewest number of compounds (e.g. just 15), while the UV-sanitized grain, *A. flavus*-inoculated grain, and *Fusarium*-inoculated grain each had 23, 26, and 25 compounds in its headspace, respectively (Table 1). There was some overlap, but significant differentiation among treatments in volatile composition (ANOSIM: $R = 0.305$; Perm = 1000; $P < 0.0001$; Fig. 6). Many of the compounds occurring in the *A. flavus*- and *Fusarium*-inoculated grain were unique compared to the uninoculated- or UV-sanitized grain (Table 1), including 2-(1-propenyl)-6-methylphenol-butanoic acid, cyclopropanecarboxylic acid, and octane for *A. flavus*-inoculated grain and 2-epi-α-cadrene, (1*R*,4*S*,5*S*)-1,8-dimethyl-4-(prop-1-en-2-yl)spiro[4.5]dec-7-ene, and 4-ethyl-1,2-dimethoxy-benzene for *Fusarium*-inoculated grain. Finally, there was significantly higher total emissions by *Fusarium*-inoculated grain compared to the uninoculated grain ($t = 47.7$; df = 86; $P < 0.0001$), UV-sanitized grain ($t = 42.9$; df = 86; $P < 0.0001$), and *A. flavus*-inoculated grain ($t = 47.1$; df = 86; $P < 0.0001$), with 5-, 4-, and 5-fold higher emissions, respectively (Fig. 7).

Discussion

Overall, this is the first study to examine in an in-depth manner how specific fungal species affect the behavior of adult *L. serricorne*, particularly how MVOCs from fungi may be used as foraging cues to manipulate insect attraction, mobility, and preference in the post-harvest environment. We included multiple behavioral assays (e.g., four-way olfactometer for preference, video-tracking to evaluate close-range foraging decisions, and a release-recapture assay to determine long-distance attraction) all to ultimately characterize how *L. serricorne* responds to MVOCs and interacts with microbes. Here, we show that inoculating grain with known plant pathogens has direct implications on the MVOCs produced, and in turn, this affects the behavior of an external-infesting stored product pest, *L. serricorne*. We found that this is true at both close-range and at distance. This may be a result of the fact that *L. serricorne* evolved from a lineage of insects that likely infested animal caches[4,36], which are hypothesized to preferentially be attracted to MVOCs as a reliable host cue. MVOCs may be reliable cues in this situation because animals often forget or do not use many of their caches[41], leaving them to mold in temperate environments. It may be possible *L. serrcorne* responds specifically to *A. flavus* due to its prevalent frequency in flour[25], and its propensity especially for xeric conditions[28], which is a propensity shared by *L. serricorne*[11]. By contrast, *F. verticillioides* prefers more moist or humid environments. While there is abundant data that there are close symbioses between *L. serricorne* and *Symbiotaphrina*, there is no support for this for *A. flavus* and *F.*

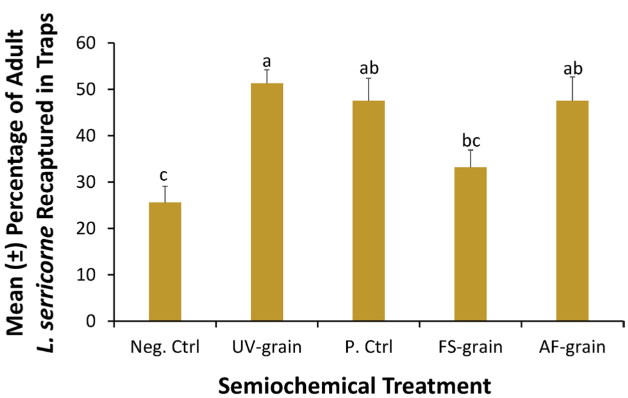

Figure 5. The mean percentage of adult *L. serricorne* recaptured in the release-recapture assay in response to MVOCs and grain used as lures in commercially-available monitoring pitfall traps, with a total of 20 adult, mixed-sex *L. serricorne* released in large plastic bins and placed in a large, walk-in environmental chamber under constant abiotic conditions and given 24 h to respond at the USDA-ARS Center for Grain and Animal Health Research in Manhattan, KS for a total of n = 8 replicate releases per treatment. Bars with shared letters are not significantly different from each other (Tukey HSD, α = 0.05).

Compound	RT	P. ctrl		UV-grain		*A. flavus*-grain		*Fusarium*-grain	
		Mean ± SE	% of Total	Mean ± SE	% of Total	Mean ± SE	% of Total	Mean ± SE	% of Total
4-Ethylbenzamide	4.26	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0.17 ± 0.11	6
cis-1-Butyl-2-methylcyclopropane	4.38	0.10 ± 0.06	18	0.03 ± 0.01	4	0.04 ± 0.02	6	0.01 ± 0.02	0
3-Octene, (E)[a]	4.38	0.00 ± 0.00	0	0.02 ± 0.01	3	0.02 ± 0.02	3	0.00 ± 0.00	0
4-Heptanone[a]	4.45	0.01 ± 0.01	2	0.02 ± 0.01	2	0.01 ± 0.01	2	0.03 ± 0.04	1
Octane[a]	4.46	0.00 ± 0.00	0	0.00 ± 0.00	0	0.02 ± 0.03	3	0.05 ± 0.07	2
2,4-Dimethyl-1-heptene	4.91	0.01 ± 0.01	3	0.05 ± 0.02	7	0.02 ± 0.02	4	0.03 ± 0.03	1
2-Pentanone, 4-hydroxy-4-methyl	4.92	0.03 ± 0.02	6	0.05 ± 0.04	6	0.03 ± 0.04	5	0.00 ± 0.00	0
3-Hexanone, 2-methyl[a]	5.26	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0.03 ± 0.04	1
Pyrazole-1-methanol	5.63	0.00 ± 0.00	0	0.03 ± 0.02	3	0.01 ± 0.01	1	0.00 ± 0.00	0
Mesitylene[a]	6.62	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0.06 ± 0.07	2
Pentane, 2,2,3,3-tetramethyl	7.09	0.03 ± 0.01	6	0.06 ± 0.02	8	0.02 ± 0.02	4	0.04 ± 0.03	1
Octane, 3,4,5,6-tetramethyl	7.15	0.04 ± 0.01	7	0.03 ± 0.01	4	0.05 ± 0.03	9	0.13 ± 0.13	4
Hexanoic acid, 2-ethyl-, methyl ester	7.57	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0.12 ± 0.06	4
Ether, hexyl pentyl	7.89	0.00 ± 0.00	0	0.01 ± 0.01	1	0.01 ± 0.01	1	0.00 ± 0.00	0
3-Hydroxy-3-methylvaleric acid	7.89	0.01 ± 0.01	2	0.00 ± 0.00	0	0.00 ± 0.00	0	0.06 ± 0.05	2
Benzene, 1,4-diethyl	7.89	0.00 ± 0.00	0	0.01 ± 0.01	2	0.00 ± 0.00	0	0.00 ± 0.00	0
Phenol, 3,4,5-trimethyl	8.13	0.00 ± 0.00	0	0.01 ± 0.01	2	0.01 ± 0.01	1	0.15 ± 0.05	5
Ethanol, 2-(octyloxy)	8.14	0.01 ± 0.01	1	0.00 ± 0.00	0	0.01 ± 0.01	1	0.00 ± 0.00	0
1-Undecene, 7-methyl[a]	8.20	0.00 ± 0.00	0	0.01 ± 0.01	1	0.02 ± 0.01	3	0.00 ± 0.00	0
1-Dodecanol[a]	8.21	0.00 ± 0.00	0	0.01 ± 0.01	1	0.01 ± 0.01	2	0.00 ± 0.00	0
Undecane[a]	8.41	0.04 ± 0.03	7	0.03 ± 0.03	4	0.02 ± 0.03	3	0.01 ± 0.01	0
Nonanal[a]	8.51	0.06 ± 0.04	11	0.06 ± 0.05	7	0.03 ± 0.03	5	0.00 ± 0.00	0
Cyclopropanecarboxylic acid	8.52	0.00 ± 0.00	0	0.00 ± 0.00	0	0.02 ± 0.03	3	0.25 ± 0.26	8
5-Methylthiopyridin-2-ol	9.17	0.04 ± 0.03	8	0.03 ± 0.02	4	0.00 ± 0.00	0	0.03 ± 0.04	1
1-Hexanol, 5-methyl-2-(1-methylethyl)	10.54	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.01	1	0.00 ± 0.00	0
4-Phenyl-2-butanol[a]	10.65	0.00 ± 0.00	0	0.03 ± 0.03	3	0.00 ± 0.00	0	0.00 ± 0.00	0
Benzene, 1,3-bis(1,1-dimethylethyl)-	10.78	0.09 ± 0.03	16	0.16 ± 0.05	20	0.13 ± 0.04	22	0.11 ± 0.06	4
Ethanone, 1-(4-ethylphenyl)-	10.96	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00	0
Butanoic acid, 2-(1-Propenyl)-6-methylphenol-	11.04	0.00 ± 0.00	0	0.00 ± 0.00	0	0.02 ± 0.02	4	0.00 ± 0.00	0
m-Ethylacetophenone-[a]	11.25	0.00 ± 0.00	0	0.08 ± 0.07	10	0.00 ± 0.00	0	0.00 ± 0.00	0
2,3,6-Trimethylhept-3-en-1-ol -	11.54	0.00 ± 0.00	0	0.00 ± 0.00	0	0.01 ± 0.01	2	0.00 ± 0.00	0
Ketone, methyl 2,2,3-trimethylcyclopentyl	11.66	0.00 ± 0.00	0	0.00 ± 0.00	1	0.01 ± 0.01	1	0.00 ± 0.00	0
Dodecane, 4-methyl-	11.67	0.02 ± 0.01	3	0.00 ± 0.00	0	0.01 ± 0.01	2	0.00 ± 0.00	0
Benzene, 4-ethyl-1,2-dimethoxy-	11.68	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0.49 ± 0.31	16
.alpha.-Cubebene	13.22	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0.08 ± 0.05	3
Germacrene D	13.37	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0.16 ± 0.08	5
(1R,4S,5S)-1,8-Dimethyl-4-(prop-1-en-2-yl)spiro[4.5]dec-7-ene	13.82	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0.40 ± 0.15	13
Di-epi-.alpha.-cedrene	15.89	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0.35 ± 0.17	12
Benzimidazole, 2-ethyl-1-propyl-	18.42	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0.03 ± 0.02	1
Hexanedioic acid, bis(2-ethylhexyl) ester	23.11	0.04 ± 0.04	7	0.03 ± 0.03	4	0.03 ± 0.05	5	0.11 ± 0.12	4
Total emissions (ng per h per g grain)		0.6 ± 0.01	100	0.8 ± 0.01	100	0.58 ± 0.01	100	3.0 ± 0.05	100

Table 1. Summary of headspace volatiles emitted (in ng h^{-1} g^{-1} grain) from grain that was uninoculated (P. ctrl), surface sanitized with UV-radiation for 10-min (UV-grain), or inoculated with *A. flavus* or *Fusarium* spp. for periods of 3 h using a push headspace collection system. There were n = 7 replicates per treatment, and mean emission rates per compound are portrayed. [a]Compound identity confirmed by co-injecting technical standard onto same column.

verticillioides, which we isolated from the beetle cuticle. It is more likely, these fungi were already present on the grain, and *L. serricorne* acted as a physical vector, though certainly the microbial community has been known to create microclimates in grain and have an effect on the insect community under some conditions (Ponce and Morrison, unpublished data). Together, this presents a convincing case for the importance of insect-microbe interactions on the foraging ecology of *L. serricorne*. Below, we go over each of the main results in turn.

We found close-range foraging by *L. serricorne* is affected by grain and MVOCs. Altogether, the volatile emissions from inoculated grain by the fungal morphotype significantly affects the foraging decisions by *L. serricorne* both when in close-proximity and at a distance to the inoculated grain as compared to uninoculated and sanitized grain. In fact, this supports prior work showing that there are a variety of ecological and behavioral

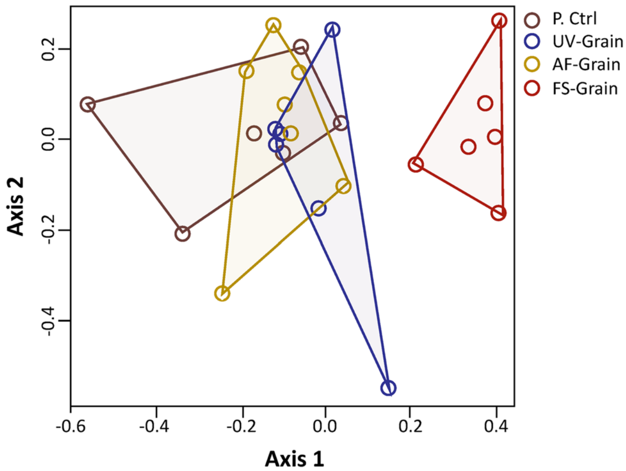

Figure 6. Non-metric multi-dimensional scaling ordination plot based on pairwise Bray–Curtis similarity index calculated between each combination of volatile emissions (ng h^{-1} g^{-1} grain) by samples after headspace collection for 3 h from 20 g of grain that was UV sanitized for 10 min (blue circles), inoculated with *A. flavus* (yellow circles), inoculated with *Fusarium* spp (red circles), or uninoculated (brown circles). There were n = 7 replicates per semiochemical treatment. Polygons represent convex hulls drawn between samples and are color coded similarly to treatments. There were a total of n = 1000 permutations, and stress was < 0.10, enabling adequate interpretation. UV-grain, UV-sanitized grain; P. Ctrl, uninoculated grain; FS-grain, *F. verticillioides*-inoculated grain; AF-grain, *A. flavus*-inoculated grain.

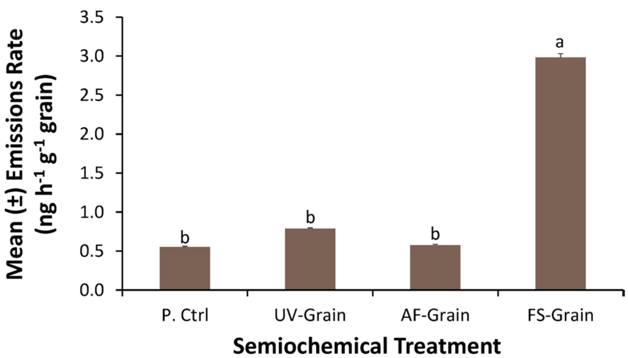

Figure 7. Mean emissions rate from the semiochemical treatments (in ng h^{-1} g^{-1} grain) during headspace collection in 500-mL capacity containers after adsorption to Porapaq-Q volatile collection trap, then elution with 150 µL dichloromethane. Bars with shared letters are not significantly different from each other (Tukey HSD, $\alpha = 0.05$). UV-grain, UV-sanitized grain; P. Ctrl, uninoculated grain; FS-grain, *F. verticillioides*-inoculated grain; AF-grain, *A. flavus*-inoculated grain.

responses by insects to volatile emissions from microbes[7]. At close-range, *L. serricorne* used volatiles from *Aspergillus flavus*-inoculated grain for foraging decisions, and this was reflected in some differentiation in the volatile composition from the *A. flavus* treatment compared to the other treatments. Similarly, another stored product pest, *S. oryzae*, oriented to MVOCs from *A. flavus*-inoculated grain at close-ranges, but preferred to use only grain volatiles at a distance[10]. Interestingly, our results also showed that grain without microbes (i.e. UV-sanitized grain) was also an attractive source for *L. serricorne* at close-range and a distance. In particular, our work showed that the presence of UV-sanitized and *A. flavus*-inoculated grain increased the cumulative duration spent in and the frequency of entering each of those kernel zones. Once *L. serricorne* entered the UV-sanitized or microbial-inoculated kernel zones, their movement was arrested. In the experiment, mixed-sex adults were used because of difficulty distinguishing sexes as adults. However, males and females may be under different selection pressures. For example, males may be more motivated to disperse, and as such may seek advantages conferred from microbial partners to use intact grain that they may not otherwise have access to (mentioned as a hypothesis in Ponce et al.[4]). By contrast, females may be under selective pressure to find optimal oviposition locations that would be free from microbial contamination for progeny. Follow-up work could find support for this hypothesis by sexing *L. serricorne* when they are pupae, then repeating the experiment to determine if there are differences in response to these two stimuli that break down along sex-based lines. In terms of food kairomones that affect foraging, volatiles from *Capsicum* products were found to be significantly more attractive to *L. serricorne* than products without *Capsicum* (e.g., cracked wheat, corn, tobacco, mineral oil, grape seed oil, rolled oats, coriander seeds, and sesame oil)[42]. In prior work, the fungal volatile, 1-octen-3-ol was a strong attractant for the stored product beetle, *Ahaversus advena* (Waltl) (Coleoptera: Silvanidae)[43] supporting the contention that stored product insects use MVOCs in foraging decisions. Overall, our work confirms that *L. serricorne response* to MVOCs is microbe species-dependent, and volatiles from *A. flavus*-inoculated grain elicit a behavioral response at close- and long-range by the insect.

Here, we found that adult *L. serricorne* moved two to fourfold less and their velocity was numerically decreased by 2–threefold when exposed to a grain kernel of one of the four semiochemical treatments compared to the negative control. This suggests that one of the key processes happening at close range in response to the grain is arrestment, demonstrated by slower and less movement of *L. serricorne* in response to the semiochemicals. Prior work has documented the sublethal effects of insecticide, dispersal, and foraging using distance moved and velocity as surrogates of movement[44] that can be mediated by good and poor sanitation[45]. However, prior work has rarely evaluated response to MVOCs using velocity or distance moved. Nevertheless, Lizarraga[46] found a numeric decrease in the distance moved and velocity when *S. oryzae* were exposed to the uninoculated grain, but this was not significantly different from the negative control or any other treatment. McFarlane[47] used Ethovision and found an important stored product pest, the red flour beetle, *T. castaneum* most often spent time in a wind tunnel adjacent to an empty arena instead of one with a stimulus containing the fungus, *Aspergillus tubingensis*. Most other work with Ethovision has been on the sublethal effects of insecticides[48–52] demonstrating an overlooked tool in understanding the effect of MVOCs on the behavioral and chemical ecology of stored product pests.

While prior studies have directly assessed response to pure fungal volatiles bought from a supplier or synthesized[43,53–55], our study links inoculation of grain by specific microbes with changes in MVOC headspace profiles and determined the implications for *L. serricorne* behavior. We found a total of 44 tentatively identified, but distinct principal compounds in the headspace emissions from clean and fungal-inoculated grain. There were unique cues in the fungal-inoculated grain, but fungal identity, and by consequence, compound identity appears to matter for behavioral response by *L. serricorne*. *Fusarium verticillioides*-inoculated grain was significantly different than the other treatments, yet it did not elicit the same change in behavior as the *A. flavus*-inoculated grain, which showed more overlap with the other treatments. Thus, minor compounds uniquely emitted by or emitted in unique ratios by *A. flavus*, but not present in the other treatments (Table 1), may be important for the behavioral response of *L. serricorne*. For example, both 5-methyl-2-(1-methylethyl)-1-hexanol and 2-(1-propenyl)-6-methylphenol-butanoic acid were only present in *A. flavus*-inoculated grain and not in any other treatment. Interestingly, Ponce et al.[4] found that 3-octanone, 1-octen-3-ol, octane, 3-methyl-1-butanol and many others were the most commonly tested MVOCs for behavioral effects against stored-product arthropods, and we found a subset of these in the headspace emissions here. Similarly, Sinha[56] found 3-methyl-1-butanol, 1-octen-3-ol, and 3-octanone were the most common compounds isolated in experimental grain bins, which supports some of the headspace volatiles emitted from grain in our experiment. In addition, many of the compounds occurring in the *A. flavus*- and *Fusarium*-inoculated grain were unique compared to the uninoculated- or UV-sanitized grain (Table 1), including 2-(1-propenyl)-6-methylphenol-butanoic acid, cyclopropanecarboxylic acid, and octane for *A. flavus*-inoculated grain and 2-epi-α-cadrene, a long-chain alkene, and 4-ethyl-1,2-dimethoxy-benzene for *Fusarium*-inoculated grain. Overall, the total emission rate for volatiles produced by *Fusarium*-inoculated grain is three to fivefold several magnitudes higher, than the uninoculated grain. While overall volatile emissions among fungal-inoculated grain were similar, the identity of the volatiles varied. This may lead to the differences observed insect behavior. In the future, complex blends of MVOCs emitted by common fungi should be evaluated against stored product insects using gas chromatography coupled with electroantennography (GC-EAD) in order to determine which volatiles are perceived by individuals. Further investigation of the MVOCs on foraging behavior of stored product insects may lead to promising candidate compounds that are behaviorally-active to manipulate pest populations in management tactics at food facilities[36,57].

No preference was found by *L. serricorne* among the semiochemical stimuli including UV-sanitized grain, uninoculated grain, *F. verticillioides*-inoculated grain, *A. flavus*-inoculated grain and the negative control using the four-way olfactometer. This could be because there may have been a thigmotactic reaction to the glass substrate by *L. serricorne*. Other stored product species have been documented to exhibit thigmotactic responses to netting, for example[58], though this has not been documented for *L. serricorne*. Additionally, it is possible that while some of the treatments have attractant or arresting properties as individual bouquets, complex blends admixing in the headspace of the release chamber may result in no preference among each other. Prior work has confirmed that volatiles are able to quickly and adequately diffuse by Brownian motion over the allotted trial period in the assay[10]. Thus, performance of this assay in the biosafety cabinet did not prevent volatiles from reaching test subjects, and was a valid test of the odor treatments. Prior work found that the invasive stored product dermestid *Trogoderma granarium* Everts (Coleoptera: Dermestidae) showed attraction to pheromonal and kairomonal stimuli that were not perfectly congruous with the results from work in related preference assays for the same stimuli[59]. Gerken et al.[60] found that even with the same species (e.g., *T. castaneum*), one could obtain different results using multiple behavioral assays, and suggested that one must carefully select the assays and consider the actual variables being tested. However, the lack of a positive response in only one out of the multiple assays to headspace by *A. flavus* in this study suggests that there is a relatively robust response to the mVOCs from this microbial species by *L. serricorne*.

In the future, research should be prioritized to (1) address which compounds identified in Table 1 are actually detected by *L. serricorne* through GC-EAD, (2) follow-up on GC-EAD results with behavioral tests to determine whether the compounds are attractive, repellent, or neutral, and (3) formulate new lures with the most attractive compounds to improve monitoring and management programs. This will help narrow the behaviorally-active components of the headspace blends to those that are being detected by the insect. Overall, this study has enhanced our understanding of how microbially-produced volatiles organic compounds from postharvest fungal grain pathogens affect the behavior of *L. serricorne*. Increasingly, it is apparently essential to manage microbes and insects simultaneously in the postharvest agricultural supply to ensure that our planet can feed its growing population.

Materials and methods

Source insects. Adult cigarette beetles, *Lasioderma serricorne* (F.) (Coleoptera: Ptinidae), were used from insect colonies that were originally collected in 2012 from a rice mill in Arkansas and continuously maintained on a diet of 95% bleached wheat flour and 5% brewer's yeast with oats sprinkled on top and a moistened, crumpled towel added. Colonies were kept at the United States Department of Agriculture Agricultural Research Service's (USDA-ARS) Center for Grain and Animal Health Research facility in Manhattan, KS. Insects were routinely subcultured by sieving (No. 30 sieve, 594 × 594 μm mesh, W.S. Tyler Co., Cleveland, OH, USA) 150 mixed-sex *L. serricorne* adults from diet mixtures, transferring them into new mason jars (950-ml capacity) filled two-thirds of the way to the top with diet, and then placed inside environmental chambers under constant conditions at 27.5 °C, 65% RH, and 14:10 L:D photoperiod. Individuals used in bioassays described below were never tested more than once.

Fungal morphotype culturing. To initially isolate the two fungal morphotypes, *L. serricorne* were allowed to disperse on agar. A total of 32 g of potato dextrose agar (Merck, Darmstadt, Germany) was mixed with 900 ml of deionized water in a 1000-mL glass media bottle with a magnetic stirring rod placed inside the bottle. The agar solution was autoclaved (533LS, Getinge, Rochester, NY, USA) for 30 min and then stirred on a hot plate to cool down for 20 min. Before pouring the potato agar solution into petri dishes (100 × 15), the biosafety cabinet (75 × 73 × 95 cm L:H:W, #302381101, Labconco, Kansas City, MO, USA) was sanitized with 70% ethanol and exposed to UV light for 10 min. A total of 36 petri dishes with potato dextrose agar solution were left in the biosafety cabinet to solidify overnight.

A single *L. serricorne* was introduced into to a single PDA dish and microbial growth was visualized after 3 and 5 d. Each PDA plate was sealed with parafilm, and stored in an environmental chamber under constant conditions of 30 °C, 60% RH, and 14:10 L:D photoperiod. Transfer of *L. serricorne* from containers to agar was performed inside the biosafety cabinet to prevent contamination. Pictures of the agar dishes and corresponding microbial growth were acquired using a DSLR camera (EOS 7D Mark II, Canon, Tokyo, Japan) mounted to 3D imaging StackShot (CogniSys, Inc., Traverse City, MI, USA) equipped with a dual flash (MT-26EX-RT, Canon, Tokyo, Japan). Light was diffused using a partially cut frosted plastic jar (15.2 × 7.6 cm D:H) making a total of 12 replicates. From the fungal isolation, two fungal morphotypes were selected by close examination of microbial characteristics such as spore shape, size, and color. Morphologically, *A. flavus* often appears yellowish or green in pigment, while *F. graminearum* often appears white (Fig. 8). This was used to generate the two primary morphotypes for the rest of the assays in the experiments below, and to generate sequences for the morphotypes.

Treatments. To study how MVOCs affected *L. serricorne* behavior, the two morphotypes identified above were introduced to grain and compared to uninoculated and UV-sanitized grain in various behavioral assays. The uninoculated grain treatment (e.g., P. ctrl hereafter) consisted of 10 g of hard winter wheat at a 10.6% moisture content taken directly from cold storage (Table 2). UV-sanitized grain was as above but treated with ultraviolet radiation for 10 min in the biosafety cabinet (75 × 73 × 95 cm L:H:W, #302381101, Labconco, Kansas City, MO, USA) to surface sterilize. Grain inoculated by specific fungal species included *Fusarium*-inoculated

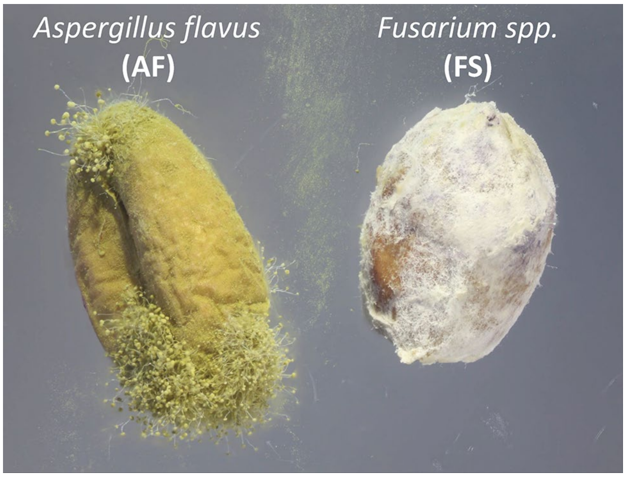

Figure 8. Habitus images of morphotype 1 (left, *A. flavus*-inoculated grain) and morphotype 2 (right, *F. verticillioides*-inoculated grain) at ×10 magnification (SMZ-18, Nikon Inc., Tokyo, Japan) on grain after inoculation procedure.

Abbrev.	Treatments	Amount in assay			
		4-way Olfact	Ethovision	Headspace	Release-recapture
Neg. Ctrl	Negative control	0	0	0	0
P. Ctrl	Positive control (uninoculated)	–	1 kernel	20 g	10 g
AF-grain	*Aspergillus flavus*-inoculated	10 g	1 kernel	20 g	10 g
FS-grain	Fusarium *verticillioides*-inoculated	10 g	1 kernel	20 g	10 g
UV-grain	UV-surface sanitized for 10 min	10 g	1 kernel	20 g	10 g

Table 2. List of semiochemical treatments and the amounts used in each assay during the study.

grain (e.g. *F. verticillioides*, determined by sequencing, see below: FS, hereafter) and *A. flavus*-inoculated grain (AF, hereafter) (Fig. 8). In order to develop uniform, inoculated cultures on grain, our process was modelled on mushroom farming techniques with boiling[61]. To prepare the *Fusarium*-inoculated grain, 600 g of grain was transferred to a stainless-steel pot filled with water and placed on a hot plate at 100 °C. Once boiling for 15 min, the water was drained and the grain was evenly spread out on sterile wipes (38.1 × 42.5 cm, 3 ply, Tech wipes, Skilcraft, NIB, Alexandria, VA) and dried inside a laminar flow hood (ca. 3 h). Afterwards, grain was evenly divided (~300 g) and placed in 2 separate autoclaved mason jars (950-mL capacity). A single hole was pierced through each lid and lined with a cotton ball. The jars were then sealed with aluminum foil and were autoclaved (533LS, Getinge, Rochester, NY, USA) for 30 min. To inoculate with *Fusarium* spp. or *A. flavus*, a 3-inch agar plug of fungi was placed into each jar containing the grain. The plug had been colonized and growing without contamination in a petri dish for 7–10 days in an environmental chamber set to 30 °C, 60% RH, and 14:10 L:D photoperiod (Supplementary Fig. 2). Jars with freshly prepared *Fusarium*-inoculated grain sat at room temperature for roughly 10–15 days or until the fungi evenly covered as much of the grain as possible. Grain was never used more than once for each replicate of every trial in each assay experiment to prevent cross contamination.

Microbial morphotype sequencing. To identify microbes associated with the cuticle of *L. serricorne*, fungal morphotypes were isolated from cuticles and cultured for the purpose of sequencing. Pure isolations from the two morphotypes were made by excising a 1 × 1 cm agar plug of fungi and subculturing it onto a new potato dextrose agar dish, sealed with parafilm to obtain a pure culture. DNA was extracted from pure cultures after 7 days of growth in an environmental chamber set to 30 °C, 60% RH, and 14:10 L:D photoperiod using the Qui*ck*-DNA Fecal/Soil Microbe Miniprep Kit (D6010, Zymo Research Corp, Irvine, CA, USA). Concentration of DNA and quality were assessed using the Take 3 Assay on a microplate reader (Gen5™, BioTek Instruments, Winooski, VT, USA) before performing the PCR.

Polymerase chain reaction (PCR) was used to amplify the internal transcribed spacer (ITS) region for both morphologically identified fungi using primer sets: ITS4 5′-TCCTCCGCTTATTGATATGC-3′ and ITS5 5′-GGA AGTAAAAGTCGTAACAAGG-3′[62]. For the morphologically identified *Fusarium* sample, the translation elongation factor 1 alpha (TEF1α) region and the RNA polymerase II subunit (RBP2) region were also amplified to facilitate species levels identification. The following primer pairs were used for TEF1α and RBP1, respectively: EF1 (5’-ATGGGTAAGGARGACAAGAC-3’)/EF2 (5’-GGARGTACCAGTSATCATGTT-3’) and RBP2-5F2 (5’-GGGGWGAYCAGAAGAAGGC-3’)/RPB2-7cR (5’-CCCATRGCTTGYTTRCCCAT-3’)[63–65]. Each reaction contained 1.0 µL extracted DNA, 1.0 µL of each primer (10 µM), 9.5 µL of nuclease free water, and 12.5 µL of master mix containing 50 units/mL of Taq DNA polymerase master mix (Hot Start Taq 2X Master Mix, Promega, Madison, WI, USA). Briefly, the PCR program consisted of 2 min of initial denaturation at 95 °C, followed by 30 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 1.5 min, and a final extension at 72 °C for 5 min (S1000 Thermal Cycler, Bio-Rad, Hercules, CA, USA). PCR products were treated with ExoSAP-it prior to sequencing following the manufacturer’s protocol (ThermoFisher Scientific, Waltham, MA, USA) Amplicons were sequenced bidirectionally on an ABI 3730XL instrument (Eurofins Scientific, Brussels, Belgium), and the resulting sequences were quality-filtered and aligned using Geneious Prime 2021.0.3 (Biomatters Ltd, Auckland, New Zealand). The consensus sequences were searched against NCBI’s nucleotide database (nt) using the BLASTn algorithm[66]. In order to circumvent taxonomic misassignments, the ITS consensus sequences were also checked against the UNITE Database using the Ribosomal Database Project Classifier algorithm[67]. The consensus sequence for the ITS sequence of *A. flavus* was submitted to GenBank under OM490684 while the ITS, TEF1α, and RBP2 sequences from *F. verticillioides* were deposited under OM460744, OM542207, and OM542208.

Four-way olfactometer. In order to assess preference by *L. serricorne* among the stimuli, a still-air four-way olfactometer was used. This device (Sigma Scientific, LLC., Micanopy, FL, USA) consisted of a cylindrical central release glass chamber (9.0 × 12.1 cm D: H) with exit ports (2.5 × 3.5 cm D:L) at each cardinal direction connecting the central chamber to four identical glass chambers (7.0 × 11.5 cm D:H) where 10 g of each odor source was placed (Table 2). The bottom of the release arena was acid-etched to provide a surface over which insects could easily crawl. Each of the four outer chambers were spaced at a 90° angle from each other. Inert septa buffered and sealed each component. A removable glass lid with a hole was used to cover the top of the main central glass chamber to allow for airflow, but also prevent insects from exiting. A single *L. serricorne* was released into the middle of the central arena for each bioassay and given a maximum of 4 min to make a decision to choose one of the stimuli. A choice was considered to have been made when the individual traveled 2.54 cm into the adjoining 1-way exit port that branched off to the odor chamber. After every 5 replicates, the entire olfactometer was rotated 90° to control for positional effects. After every 15 replicates, the whole apparatus was washed first with methanol followed by hexane, and a new, independent set of each treatment was used, with the grain replaced. The time to decision and choice of odor treatment were recorded and an individual insect was never tested more than once. Non-responsive (NR) insects were also recorded, but excluded from the final statistical analysis. The assay was conducted in a biosafety cabinet to maintain a constant flow of air and odor. A total of n = 200 replicate individuals were tested.

Movement assay. A movement assay was used to assess if fungal inoculated grain impacted close-range foraging behavior of *L. serricorne*. This was accomplished through the use of video-tracking with a network camera (GigE Network Camera, Basler AG, Germany), suspended using a pole and clamp 80 cm above the test arenas, coupled with Ethovision Software (Noldus Inc., Leesburg, VA, USA). The test arenas consisted of five petri dishes (100 × 15 mm) with 85 mm diameter filter paper adhered to the bottom of the petri dish with double sided tape, on an artist's lightbox, each containing a single *L. serricorne* and a single treatment kernel (Table 2). Each test arena was halved vertically, with the left half designated for the control half (e.g., no kernel) and the right half for the treatment half. Embedded within each half of the arenas were 1.16 cm diameter control kernel zones or treatment kernel zones, located midway and halfway on each respective half (Supplemental Fig. 1). These were coded as hidden zones in Ethovision and the smaller treatment kernel zones were where the grain treatments were placed, consisting of a single kernel of each treatment listed in Table 2. A single *L. serricorne* was released at the midpoint in the center of each arena. Trials lasted for a period of 30 min. The Ethovison software automatically measured several variables including the total distance moved, the cumulative duration of time spent in each half/kernel zone, the frequency of entry to each half/kernel zone, and latency to entering the half/kernel zone. The location of each treatment was randomized between each replicate to control any positional bias. There was a total of n = 20 replicates per treatment. No petri dish, filter paper, or adult was ever used more than once.

Release-recapture assay. A trapping assay was utilized to determine how microbial fungal volatiles impact the capture by *L. serricorne* in monitoring traps at a longer distance. In a large, walk-in environmental chamber (5 × 6 × 2 m, L:W:H), plastic bins (86.3 × 30.5 × 39.4 cm L:H:W) were set up to perform the experiment under constant environmental conditions (28 °C, 59% RH, 14:10 L:D). A ring of polytetrafluoroethylene (Millipore Sigma, Burlington, MA, USA) around the top and bottom of the plastic bins was added to prevent insects from climbing out of the bins. The bottom of the bins was roughened with sandpaper to provide traction. The traps consisted of commercially-available pitfall traps (Storgard, Trece, Adair, OK, USA) with two connectible pieces[68,69], containing a central well where the treatments were added as bait. Traps were baited with treatments in Table 2 and placed in one corner inside the bins. A total of 20 adult, mixed-sex *L. serricorne* were released in the corner diagonal and on the opposite side from where the trap was placed. The positions of traps were randomized in a different corner inside the bin to reduce any positional bias between each replicate. The insects were given 24 h to respond to stimuli. Afterwards, traps were collected, and the number of insects recaptured was recorded. A total of n = 8 replicate releases were performed per treatment.

MVOC headspace collection. To determine how microbial colonization impacted volatile emissions from grain, headspace collections were performed on fungal-inoculated, uninoculated, and UV-treated grain. The headspace volatile collection apparatus consisted of four 500-ml glass chambers, each with an air inlet and outlet. Central air was first purified, then regulated with a flow meter to 1 L/m, after which it entered the headspace chamber via PTFE tubing and was collected onto a volatile collection trap (VCT). Each glass chamber was secured during headspace volatile collection with a plastic lid and a PTFE-faced butyl septum. After each use, chambers were washed with methanol first followed by hexane inside a laminar fume hood. The VCT consisted of an angled drip-tip collection point borosilicate glass tube with a mesh (Stainless Steel #316 screen), packed with 20 mg of PoraPak-Q™ chemical absorbent held in place with a borosilicate glass wool plug, and followed by a PTFE Teflon™ compression seal, which was used to collect and concentrate MVOCs over 3 h periods from 20 g of each treatment (Table 2). Background volatiles were also collected from empty glass chambers as a negative control. Volatiles were eluted from VCTs inside the fume hood with 150 µL of dichloromethane, which was pushed through with N_2 gas into labeled 2 mL GC vials with 150-µL glass inserts with PTFE polymer feet and magnetic seal caps. Using a microsyringe (2-µl capacity, Hamilton Co., Reno, NV, USA), 1 µL of internal standard (190.5 ng tetradecane) was added to each sample. Between each use, the syringe was washed with 2 µL of dichloromethane in triplicate to avoid cross contamination between samples. All headspace samples were sealed with Teflon tape and stored at − 13 °C until GC–MS analysis below. For reuse of VCT, traps were washed in triplicate with 700 µL of dichloromethane, which was pushed through with N_2 gas.

MVOC GC–MS analysis. All headspace collection sample extracts were run on an Agilent 7890B gas chromatograph (GC) equipped with an Agilent Durabond HP-5 column (30 m length, 0.250 mm diameter and 0.25 µm film thickness) with He as the carrier gas at a constant 1.2 mL/min flow and 40 cm/s velocity. This was coupled with a single-quadrupole Agilent 5997B mass spectrometer (MS). The compounds were separated by auto-injecting 1 µl of each sample under split mode into the GC–MS at room temperature (approximately 23 °C). The flow was split in a 15:1 ratio with a split flow rate of 18 ml/min. The GC program consisted of 40 °C for 1 min followed by 10 °C/min increases to 300 °C and then held for 26.5 min. After a solvent delay of 3 min, mass ranges between 50 and 550 atomic mass units were scanned. Compounds were tentatively identified by comparison of spectral data with those from the NIST 17 library and by GC retention index[70]. Using the ratio of the peak area for the internal standard to the peak area for the other compounds in the headspace, the emission rates of samples was normalized in ng of volatile per g of grain, per μl of solvent, and per h of collection were calculated.

Statistical analysis. In order to analyze the quantitative data from the vectoring assay, the mean greyscale value was used as the response variable in a linear model. Semiochemical treatment (Table 2) was included as a fixed, explanatory variable. Residuals were inspected to validate assumptions of normality and homoscedasticity. If these assumptions were not met, log -transformed data were used, which then satisfied both assumptions. Upon a significant result of the model, Tukey HSD was used for multiple comparisons and implemented using the function *ghlt* from the package *multcomp* in R Software. R software[71] was used for this and all other tests, with $\alpha = 0.05$ except where noted.

To analyze the data from the four-way olfactometer, a Chi-squared test was performed with a Bonferroni adjustment to the α-threshold. The null hypothesis assumed that there was an equal probability that an insect would choose one of the four sides.

To analyze how grain and microbial volatiles affect the mobility and foraging of *L. serricorne* during video-tracking, a generalized linear model based on a Gaussian distribution with a log-link function was used. In particular, separate models were used for the distance moved, and instantaneous velocity as response variables, while the explanatory variable included the odor treatment included in each test arena (e.g. from Table 2). To detect behavioral differences in this assay, the frequency of entering each zone, cumulative duration spent in each zone, and latency to finding each zone were each analyzed using a multivariate analysis of variance (MANOVA) with the semiochemical treatments as a fixed explanatory variable. Upon a significant overall result, sequential ANOVAs were performed for each variable. For responses with significant ANOVAs, multiple comparisons were performed with Tukey HSD. Post-hoc t-tests were performed as a contrast between the two means of treatment vs. control zone and treatment vs. control kernel zone to assess differences within a semiochemical treatment with a Bonferroni-corrected alpha-threshold for multiple testing.

To analyze the data from the release-recapture assay, a generalized linear model based on a Poisson distribution and log link function was used. The response was the number of *L. serricorne* recaptured in traps for a particular stimulus. The explanatory variable included the odor treatments from Table 2. Overdispersion was not found to be a problem. Upon a significant result from the model, multiple comparisons in a generalized linear model framework were implemented using the function *ghlt* (e.g. Tukey HSD) from the package *multcomp* in R Software.

To characterize the change in headspace volatiles with inoculation of fungi in grain, raw peak areas were extracted from the gas chromatograms using MSD ChemStation v2.00 software (Agilent Technologies, Inc., Santa Clara, CA, USA). The emission rate was calculated on a ng per gram of grain weight and per hour basis by using the ratio of the peak area for tetradecane to each headspace volatile. Background volatiles found in the negative control without grain were discarded from the other samples, since these represent transient background volatiles. Pairwise Bray–Curtis similarities were calculated between each headspace sample, and non-metric multi- dimensional scaling (NMDS) was used to visualize the differences in volatile profiles among treatments. A total of $n = 1000$ permutations were used for the ordination procedure. Stress values for the NMDS procedure were < 0.1, indicating that good interpretation was possible. An analysis of similarity (ANOSIM) was used to determine significant differences for headspace volatile profiles among odor treatment categories. ANOSIM calculates an R statistic that can vary from 1 to -1, with values above zero interpreted as greater dissimilarity between treatments than within and values below zero interpreted as greater dissimilarity within treatments than between treatments. A total of $n = 1000$ permutations were performed for the test. For all multivariate statistics, the R Package *vegan* was used[72]. To assess differences in the complexity of the headspace blends (e.g. number of compounds per treatment), pairwise chi-square tests were performed among treatments with the null hypothesis of an equal number of compounds identified from each treatment. To determine differences in overall quantitative emissions of volatiles, pairwise t-tests among means were performed using a Bonferroni-correction to the P-value.

Ethics declaration. These studies adhered to the highest US and international standards for scientific integrity. No humans or vertebrate animals were used in these experiments. This BSL-2 research is covered and approved through the Institutional Biosafety Committee at Kansas State University under approved permit IBC#1437 through 3/17/2023.

Data availability

The datasets generated for this study can be found in the Ag Data Commons Repository managed by the U.S. Department of Agriculture at: Ponce, Marco A.; Sierra, Petra; Maille, Jacqueline; Kim, Tania N.; Scully, Erin D.; Morrison, William R. Data from Attraction, mobility, and preference by *Lasioderma serricorne* (F.) (Coleoptera: Ptinidae) to microbially-mediated volatile emissions by two species of fungi in stored grain. Ag Data Commons. https://doi.org/10.15482/USDA.ADC/1528422. Accessed 2023-01-12.

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Author contributions
M.P., P.S., and W.R.M. conceived of the experiments. M.P. and P.S. collected the data for the behavioral experiments, chemical data, and isolated the DNA, while J.M.M. and E.D.S. consulted on molecular methods, and J.M.M. cleaned up the DNA and prepped for sequencing. W.R.M. obtained funding for supplies and approval for permits. All authors read, edited, and approved the final manuscript.

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Competing interests
The authors declare no competing interests.

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Insect-Microbial Interaction

Grain Inoculated with Different Growth Stages of the Fungus, *Aspergillus flavus*, Affect the Close-Range Foraging Behavior by a Primary Stored Product Pest, *Sitophilus oryzae* (Coleoptera: Curculionidae)

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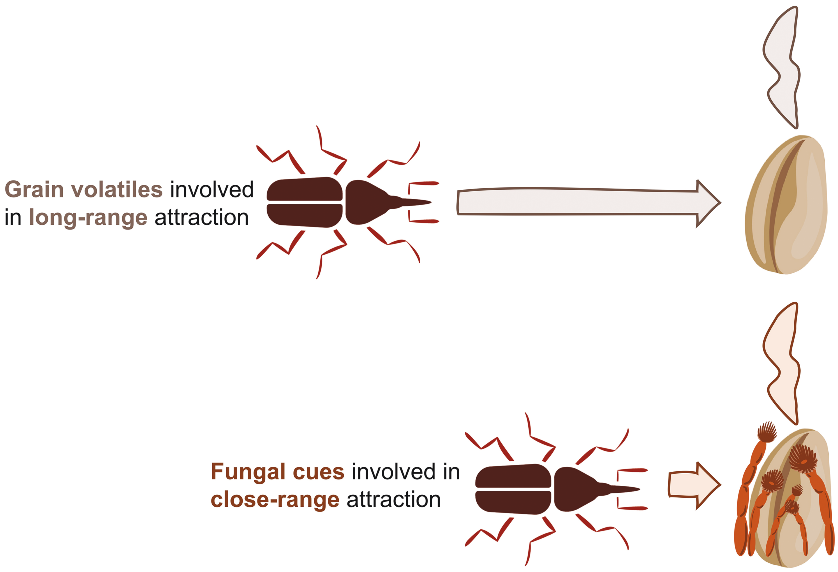
Abstract

Although some research has investigated the interactions among stored product insects and microbes, little research has examined how specific fungal life stages affect volatile emissions in grain and linked it to the behavior of *Sitophilus oryzae*, the cosmopolitan rice weevil. Thus, our goals were to 1) isolate, culture, and identify two fungal life stages of *Aspergillus flavus*, 2) characterize the volatile emissions from grain inoculated by each fungal morphotype, and 3) understand how microbially-produced volatile organic compounds (MVOCs) from each fungal morphotype affect foraging, attraction, and preference by *S. oryzae*. We hypothesized that the headspace blends would be unique among our treatments and that this will lead to preferential mobility by *S. oryzae* among treatments. Using headspace collection coupled with GC-MS, we found the sexual life stage of *A. flavus* had the most unique emissions of MVOCs compared to the other semiochemical treatments. This translated to a higher interaction with kernels containing grain with the *A. flavus* sexual life stage, as well as a higher cumulative time spent in those zones by *S. oryzae* in a video-tracking assay in comparison to the asexual life stage. While fungal cues were important for foraging at close-range, the release-recapture assay indicated that grain volatiles were more important for attraction at longer distances. There was no significant preference between grain and MVOCs in a four-way olfactometer. Overall, this study enhances our understanding of how fungal cues affect the close and longer range foraging ecology of a primarily stored product insect.

Key words: microbial volatiles, rice weevil, taxis, Ethovision, semiochemicals

Graphical Abstract



Insects and microbes represent the two largest threats to the post-harvest supply chain exceeding US$100 billion losses annually worldwide (Wacker 2018). Controlling these pests after harvest represents an easier method of meeting food production targets than growing more food, because one must only prevent the loss of already existing products during the post-harvest supply chain. Key stored product pathogens after harvest include fungi belonging to *Aspergillus*, *Fusarium*, *Penicillium*, and *Alternaria* spp. (Mannaa and Kim 2017; Barney et al. 1995). Some strains of these fungal species naturally produce mycotoxins, which are compounds secreted by hyphae that can contaminate the food supply for animals (Magan and Olsen 2004). Different mycotoxins may be produced by different taxa and life stages (Sulyok et al. 2010). For example, aflatoxins are a family of toxins produced by a variety of species, including *Aspergillus flavus* (Klich, 2007). In agriculture, mycotoxin contamination is responsible for approximately US$1 billion in losses (Amaike and Keller 2011). The presence of aflatoxin has been linked to numerous health issues in humans and livestock (Hubert et al. 2018; Fleurat-Lessard 2017). As a result, microbes intimately affect the post-harvest supply chain and the health of end consumers and animals.

A widespread and common fungal pathogen of stored grain is *Aspergillus flavus* Link (Eurotiales: Trichocomaceae) (Tang et al. 2018) (Supp Fig. 1 [online only]). This microbe causes significant economic losses after harvest (e.g., Magan et al. 2003) as a result of mycotoxin contamination and associated health hazards. *Aspergillus flavus* can be identified by its morphology, which varies depending on sexual stage. For example, asexual stages of infestation will be white and green, with visible spores, and as it vegetatively grows, old areas appear darker green. The asexual fungus produces hyphae, which is a filamentous structure that secretes enzymes to break down nutrients (Horn et al. 2009). By contrast, the sexual stages are whitish in coloration and produce spores through meiosis. It can produce spores asexually or through sexual reproduction, depending on environmental conditions. Prior work has shown that different microbial growth stages (e.g., sexual vs. asexual) of other fungal species may result in the production of different microbially-produced volatile organic compounds (MVOCs) that differ in both abundance and composition (Misztal et al. 2018). However, this has never been evaluated for *A. flavus*.

Simultaneously, post-harvest pest management is made more complicated by insect infestation. Among the most pernicious insect pests of stored bulk grains is *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), the rice weevil. This species is a cosmopolitan primary pest (Hagstrum and Subramanyam 2006) and a full generation generally requires 32 d at 17.2°C. While bin aeration can be used to help slow development of this species (Arthur et al. 2020), this often fails in warmer climates (Morrison et al. 2020a, 2021a), and the most common remedial action is fumigation with phosphine (e.g., Navarro 2006). The overuse of this fumigant has caused an increase in insecticide resistance (e.g., Konemann et al. 2017, Nayak et al. 2020). Additionally, there is increasing consumer demand for little or no insecticide inputs throughout the supply chain (Batte et al. 2007). As a result, *S. oryzae* is an economically important pest whose management requires the evaluation of alternative pest management tactics to minimize losses while reducing insecticide use. Unfortunately, little is known about how *S. oryzae* interacts with the microbial community in grain (Supp Fig. 2 [online only]), with no published study investigating the complex bouquets of microbial volatiles and how those may affect behavior (e.g., Ponce et al. 2021).

One broad class of alternative tactics to potentially target *S. oryzae* includes semiochemical-mediated, behaviorally-based management, whereby pest or natural enemy behavior is manipulated from a distance for improved integrated pest management (e.g., Morrison et al. 2021b). This approach exploits the fact that insects have highly developed, sophisticated chemosensory systems (Sánchez-García et al. 2017), which allow them to navigate an environment filled with odors. In food facilities, MVOCs may be especially advantageous cues to manipulate agricultural (Beck and Vannette 2017) or stored product insects' behavior (Ponce et al. 2021). For example, because food facilities try to eliminate any areas of microbial contamination, and are extremely dry, MVOCs may be relatively unique signals amongst a large backdrop of otherwise ubiquitous food and grain cues (Morrison et al. 2021b). In addition, it is assumed that before the advent of agriculture, stored product insects evolved to seek out and exploit caches of food by a diversity of animals, including insects and small mammals (Linsley 1944). However, with imprecise spatial memory by animals that may lose track of up to a third of caches (MacDonald 1997), fungal colonization of seeds seems to have been likely associated with caches used by current-day stored product insects. As a result, it is possible that there will be a conserved behavioral response to some fungal cues by stored product insects across taxa (Davis et al. 2013b; Phelan and Lin 1991; Pierce et al. 1991). However, there is a relative lack of knowledge of insect-microbe interactions in the post-harvest supply chain compared to other groups associated with plants (reviewed in Biere and Bennett 2013). Among the stored product insects, especially *S. oryzae* has been neglected in outsized proportion to its importance whereas the related *S. zeamais* was generally found to be repelled by MVOCs (Usseglio et al. 2017) and *S. granarius* was either repelled or there was a net neutral behavioral effect (as systematically reviewed in Ponce et al. 2021).

While there has generally been less work done in the post-harvest supply chain, there is at least some evidence suggesting that MVOCs modulate the behavior of stored product insects (Vanhaelen et al. 1978). Some microbes produce volatiles that may attract stored product insects. In a few studies, *Aspergillus flavus* was found to be the preferred egg-laying substrate for both *Typhaea stercorea* L. (Coleoptera: Mycetophagidae), the hairy fungus beetle (Tsai et al. 2007) as well as *Amyelois transitella* Walker (Lepidoptera: Pyralidae), the navel orange worm (Beck and Higbee 2015). With a more comprehensive understanding of these insect-microbe interactions, we could potentially use targeted MVOCs in behaviorally-based tactics. Thus, the goal of this study was to elucidate the behavioral response of *S. oryzae* to emissions of asexual or sexual strains of *A. flavus*, specifically focusing on 1) preference, 2) close-range movement, and 3) the ability to attract from a distance. We also identified each fungal morphotype using DNA sequencing and used GC-MS to link behavioral changes to altered headspace volatile emissions from asexual and sexual *A. flavus* life stages.

Materials and Methods

Experimental Insects and Source of Grain

Beetles used in this study were obtained from stock colonies kept in the laboratory of the USDA Agricultural Research Service's (ARS) Center for Grain and Animal Health Research (CGAHR) situated in Manhattan, Kansas, USA. Colonies of *S. oryzae* (field-derived strain Hudson, Kansas in 2012) were reared on organic whole kernel wheat after tempering it to 15% grain moisture from 10.6% moisture. Colonies with 4- to 8-wk-old adults were kept in 800-ml mason jars (8.5 D × 17 cm H) and stored in an environmental chamber (136VL, Percival Instruments, Perry, IA) set at constant conditions (27.5°C, 60% RH, and 14:10 L:D). Whole wheat grain used for colonies and inoculations below were obtained from a local Kansas farmer and held in cold storage (~4°C) at the USDA-ARS Center for Grain and Animal Health Research (CGAHR) for ~3 yr until needed for experiments.

Fungal Isolation, Culturing, and Identification

Sets of 25 (100 × 15 mm) agar petri dishes were prepared by mixing 34 g of potato dextrose agar (Neogen, Lansing, MI) with 800 ml of deionized water in a 1 liter glass media container. A sterile stirring magnet was placed inside the container to mix the solution for 10 min. Afterward, the agar solution was placed in the autoclave (Model 533LS, Getinge, Wayne, NJ) for 30 min to steam-sterilize the solution. Autoclave tape was placed on all glass to confirm success in the sterilization process. Subsequently, the agar solution was stirred for 20 min on a hot plate. Agar was poured into petri dishes (100 × 10 mm) within a certified biosafety cabinet (Labconco, Purifier Biosafety Cabinet. Kansas City, KS) after interior surfaces were sanitized with 70% Ethanol and 10 min of exposure to high intensity UV light. The agar solution was allowed to solidify over 24 hr.

Wheat kernels from grain stores held in cold storage (e.g., 4°C) at the USDA-ARS CGAHR were placed on the potato dextrose starch agar plates with four kernels placed equidistantly from each other in the four quadrants of a plate and incubated at 30°C and 65% RH to promote fungal growth and development. After 7 d, fungi were excised, then subcultured from individual isolates onto a new plate to obtain a pure culture. The two fungal morphotypes were initially identified by close examination of their morphological characteristics such as spore shape, size, and color as well as the morphology of hyphae using a high-powered dissecting microscope (SMZ18 Nikon Inc., Tokyo, Japan). Morphotype designations M1 (e.g., morphotype 1) and M2 (eg., morphotype 2) were initially assigned to each fungal life stage through this process.

Fungal identification was confirmed with DNA isolation and the use of polymerase chain reaction (PCR). Both fungal morphotypes (the asexual stage of *A. flavus* or M1 and the sexual stage of *A. flavus* or M2) were removed from infested wheat kernels by scrapping the mycelium (in excess) from a pure culture on a Petri dish into microcentrifuge tubes with a sterilized dissecting needle. DNA was extracted using the Norgen Biotek Corp. plant/fungal DNA isolation kit (E5038, Ontario, Canada,). The ITS region of the fungal DNA was then amplified using the primer ITS4 5'-TCCTCCGCTTATTGATATGC-3' and ITS5 5'-GGAAGTAAAAGTCGTAACAAGG-3' (White et al. 1990) for asexual *A. flavus* and 5'-AACTCCCAAACCCCTGTGAACATA-3' and 5'-TTTAACGGCGTGGCCGC-3' for sexual *A. flavus*. Each reaction consisted of 1 µl of extracted DNA, 1 µl of each primer (10 µM), 9.5 µl of nuclease-free water, and 12.5 µl of master mix containing 50 units/ml of Taq DNA polymerase (Hot Start Taq 2X Master Mix, Promega, Madison, WI) in a proprietary reaction buffer (pH 8.5). The PCR program consisted of 2 min of initial denaturation at 95°C, followed by 30 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1.5 min. Afterward, a final extension at 72°C for 5 min was performed, then PCR products were held chilled at 4°C. A total of 5 µl of PCR products were mixed with 2 µl of ExoSAP-it (ThermoFisher Scientific, Waltham, MA) for DNA clean-up, then placed in the thermal cycler at 37°C for 15 min, and ramped to 80°C for 15 min. Finally, the amplicons were sent for bidirectional sequencing on an ABI 3730XL instrument (Eurofins Scientific, Brussels, Belgium), and the resulting sequences were quality-filtered and aligned using Sequencher (v. 5.4.6, Gene Codes, Ann Arbor, MI). The consensus sequences were searched against NCBI's nucleotide database (nt) using the BLASTn algorithm (Altschul et al. 1997). To circumvent taxonomic misassignments, the consensus sequences were also checked against Michigan State's Ribosomal Database Project (RDP) that searches the UNITE Database (Wang et al. 2007). The consensus sequences were submitted to GenBank under the access numbers OK361788-OK361791.

Treatments

In the assays described below, we analyzed the behavioral response of *S. oryzae* to five different blends of semiochemicals found and introduced in wheat (Table 1). Briefly, these included no stimuli (negative control), UV-sanitized grain, clean grain from storage (unmanipulated, positive control), as well as grain from storage inoculated with fungal morphotype 1 (M1, identified as the asexual life stage of *Aspergillus flavus*) and fungal morphotype 2 (M2, identified as the sexual life stage of *A. flavus*). Fresh samples of

Table 1. List of semiochemical treatments and their amounts used in each assay during experiments at the USDA-ARS Center for Grain and Animal Health Research in Manhattan, KS from June to Sept. 2020

		Amount in Assay						
Abbreviations	Semiochemical Treatment	Olfactometer	Ethovision	Release-Recapture	Headspace Collection	DNA Identification	Function	Source
-Ctrl	Negative Control	Empty	Empty	Empty	Empty	Empty		Unbaited control
M1	Morphotype 1 (identified as asexual *Aspergillus flavus*)	10 g	1 kernel	10 g	20 g	10 g	Food & one microbial stimulus	300 g wheat kernels inoculated with a petri dish containing a pure culture morphotype 1 and incubated for 7 d
M2	Morphotype 2 (identified as sexual *Aspergillus flavus*)	10 g	1 kernel	10 g	20 g	10 g	Food & microbial stimulus	300 g wheat kernels inoculated with a petri dish containing a pure culture of morphotype 2 and incubated for 7 d
UV	Sanitized grain	10 g	1 kernel	10 g	20 g	10 g	Food stimulus without microbes	300 g of wheat kernels sanitized with UV light for 10 min in pan
+Ctrl	Positive control (unsanitized)	10 g	1 kernel	10 g	20 g	10 g	Food stimulus with ambient microbes	Wheat kernels directly from wheat source in laboratory

semiochemicals were used for each day of testing for each assay. In order to prevent cross-contamination, 300 g of grain (tempered to 15% grain moisture) was initially sanitized using UV for 20 min. This procedure was done before inoculating grain with either morphotype 1 or 2. The 300 g of grain was kept in a sanitized mason jar (8.5 D × 17 cm H). To inoculate grain with the two different morphologies, we scraped an entire isolation from a petri dish into the 300 g of grain. Each isolation was ~1 wk old and completely colonized by the given morphotype. After inoculation, each treatment was placed in an environmental chamber (136VL, Percival Instruments, Perry, IA) set at constant conditions (30°C, 65% RH, and 14:10 L:D). This procedure was the same for both morphologies and was done every 2 wk to ensure fresh treatments for each experimental assay.

Four-Way Still-Air Olfactometer

To evaluate the preference among the various grain and fungal volatiles by *S. oryzae* a four-way still-air olfactometer was employed (Fig. 1). The apparatus consisted of a central release chamber of glass (12.1 cm height × 9 cm diameter) with the bottom etched by acid to provide a roughened surface over which insects could walk. There were four passageways (3.5 cm long × 2.5 cm diameter) spaced at 90° angles connecting the main release chamber at ground level to four odor chambers. The odor chambers (11.5 cm height × 7 cm diameter) also had a single passageway (5.0 cm length × 1.5 cm diameter) protruding from the main chamber whose end was covered with a porous glass barrier that allowed diffusion of volatiles but prevented entry by insects in the first round of testing, and which had two equally spaced small holes in the second round of testing to ensure volatiles were not blocked. The odor and release chambers were connected with a glass tube (7.5 cm length × 2.2 diameter) attached on both sides with a PTFE (polytetrafluoroethylene) screw and septum. The final distance between the release and the odor chambers was 8.9 cm, and the apparatus was held in place by PTFE parts designed to fit the release and odor chambers. The following treatments were compared to assess preference by adding 10 g of each into one of the four odor chambers: positive control (clean,

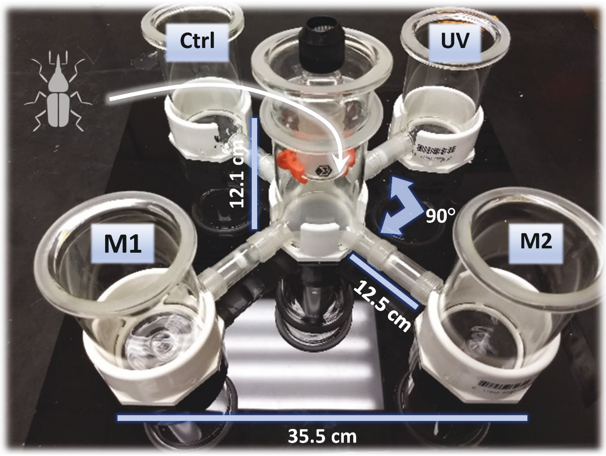


Fig. 1. Experimental 4-way, still-air olfactometer assay. A total 10 g of each treatment were placed in each odor source chamber, then a single *Sitophilus oryzae* was placed in the central release chamber and given 2 min to make a decision. A decision was considered to have been made when the individual walked 2.54 cm toward an arm.

unsanitized grain), grain inoculated with morphotype 1 (asexual *Aspergillus flavus*), grain inoculated with morphotype 2 (sexual *A. flavus*), and UV-sterilized grain (Table 1). The position of the treatments was rotated every five replicates. Insects were individually introduced into the central release chamber and given 3 min to make a choice. A choice was considered to have been made when the insect left the release chamber and traveled 2.54 cm up one of the adjoining passages. Nonresponders were excluded from the final data analysis, as is common practice in behavioral research (e.g., Morrison et al. 2020b, Wilkins et al. 2020). A total of n = 240 replicate insects were evaluated in each round of testing. Bioassays were conducted in a BSL2 biosafety cabinet (Labconco, Kansas City, MO) to prevent diffusion of ambient odors or landmarks from affecting results. Every ten replicates, all glassware, including connectors, screw caps, and septa, were washed first with methanol, then hexane, and allowed to dry before reuse. The side chosen by an individual, and the time it took to make a decision were recorded.

To analyze the data from the four-way olfactometer, a generalized linear model based on a Poisson distribution and log link function was used. The response was the counts of insects choosing a particular stimulus, with date of testing used as a random blocking variable. The explanatory variable included the odor treatments from Table 1. Overdispersion was checked and found not to be a problem. Upon a significant result from the model, multiple comparisons in a generalized linear model framework were implemented using the function *ghlt* (e.g., Tukey HSD) from the package *multcomp* in R Software (Hothorn et al. 2008). R software (R Core Team 2019) was used for this and all other tests, with α = 0.05 except where noted.

Ethovision

To examine how grain and microbial volatiles affect the mobility and orientation of *S. oryzae* at close range, we used video-tracking coupled with Ethovision software v.14.0 (Noldus, Inc., Leesburg, VA: Noldus et al. 2002; Supp Fig. 3 [online only]). This system has been used in the past for analyzing the mobility of stored product insects (Morrison et al. 2018, 2019b; Wilkins et al. 2020). A network video camera (GigE, Basler AG, Ahrensburg, Germany) was suspended using a pole and clamp 80 cm above test arenas, and the movement of five individuals was simultaneously livestreamed to an adjacent computer. Arenas consisted of Petri dishes (VWR Petri dishes, 100 × 15 mm) with an 85-mm filter paper (Grade 1, Whatman, GE Healthcare, Chicago, IL) adhered to the bottom using double-sided sticky tape. A thin ring of PTFE was used to coat the vertical surface of the test arena to prevent insect escape. In Ethovision, a total of four zones were created, including two halves of the petri dish (treatment half vs. control half), and two smaller zones nested in the middle of each half (e.g., treatment kernel zone, and control kernel zone) (Supp Fig. 3 [online only]; Fig. 2). Smaller kernel zones were 1.16 cm diameter. Control zones lacked stimuli, while treatment zones contained a single kernel of one of the treatments listed in Table 1. Treatment halves were compared to controls halves, and the position of treatments was randomized between replicates. A single insect was introduced into an arena and its mobility was tracked for a period of 30 min. Several variables were tracked, including the cumulative or total distance moved (cm), instantaneous velocity (cm/s), the number of times that an insect enters each kernel zone, the cumulative time spent in the kernel zone (treatment vs. control), the time it took to first enter the kernel zone (s) (treatment vs. control), and the amount of time spent on each side of test arena (treatment vs. control). A total of n = 15 replicate individuals were tested per treatment.

To analyze how grain and microbial volatiles affected the mobility and orientation of the *S. oryzae* during video-tracking, a generalized linear model based on a Gaussian distribution with a log-link function was used. In particular, separate models were used for the distance moved, and instantaneous velocity as response variables, while the explanatory variable included the odor treatment in each test arena (Table 1). An aggregate variable was used consisting of the frequency of interaction with the kernel, latency to finding the kernel, the cumulative time spent in the each half of the petri dish and each kernel zone. A multivariate analysis of variance (MANOVA) was then performed on the aggregate response variable using the semiochemical treatments as a fixed explanatory variable. Upon a significant overall result, sequential ANOVAs for each response variable were performed. For responses with significant ANOVAs, multiple comparisons were performed with Tukey Honestly Significantly Difference (HSD). Post-hoc *t*-tests were performed as a contrast between the two means of treatment versus control zone and treatment versus control kernel zone to assess differences within a semiochemical treatment with a Bonferroni-corrected alpha-threshold for multiple testing.

Small Release-Recapture Trapping Assay

To determine whether microbial volatiles may improve capture of *S. oryzae*, a trapping assay was conducted using plastic bins (Sterilite, Townsend, MA: 87.9 × 47.6 × 32.1 cm, L:W:H; Supp Fig. 4A and B [online only]). The bottom surface of the plastic bin was roughened with sandpaper to allow for easy movement by insects, but a ring

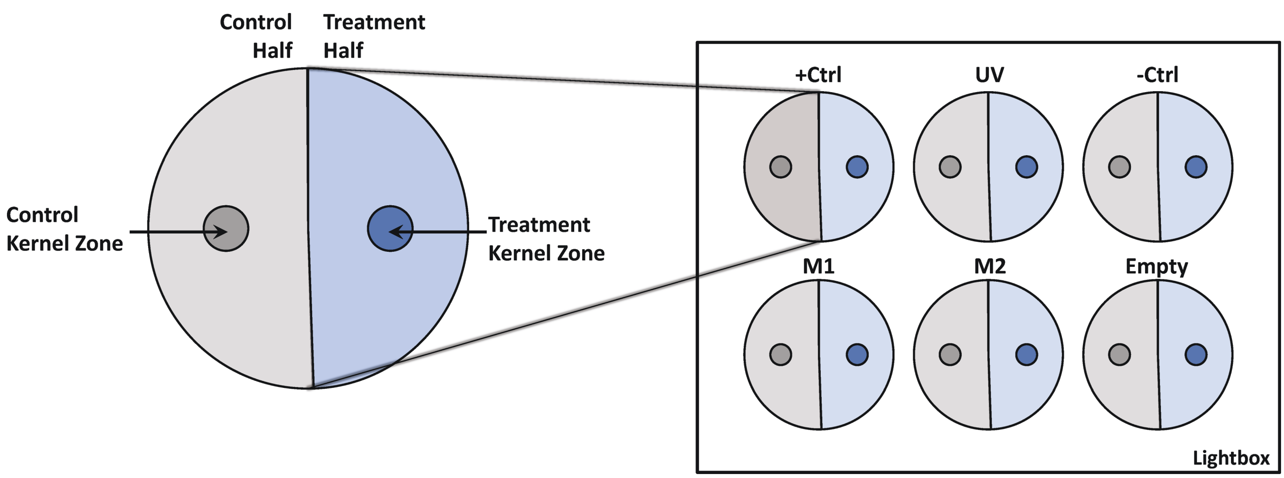


Fig. 2. Schematic of test arenas (each 100 × 15 mm D:H) for the video-tracking assay coupled with Ethovision with insects released at the mid-point in the center of the arena. On the right is a representation of the light box containing the six test arenas on a lightbox being recorded by the network camera. On the left, one of the arenas is blown up to illustrate how the zones are coded in Ethovision. Blue areas represent treatment areas of the test arena, including the half of the test arena in which the treatment was added (light blue, treatment half) and specifically where a single grain kernel corresponding to the treatment (dark blue, treatment kernel zone) was placed. The grey areas represent the corresponding parts of the other half of the arena considered controls where no stimuli were placed. Ethovision recorded the time spent by an individual insect on each half of the arena, in each kernel zone, and frequency of entering each of the four zones. The location of treatments was randomized between each replicate. For color, please check online version of article.

of polytetrafluoroethylene (PTFE) was placed at a height of 7.6 cm from the bottom on the vertical surfaces to prevent insects from escaping. A total of 20 *S. oryzae* were released in one corner of the bin. Diagonally across from the release point, a commercially-available pitfall trap (Dome Trap, Trécé, Inc., Adair, OK) was added. The pitfall trap consisted of two connectable pieces. The bottom half of the trap was rough along the ascending edges to create traction for the rice weevils, but a ring of PTFE was added halfway down on the vertical surface of the central well to prevent captured insects from escaping. The traps were baited with 10 g of one of the treatments in Table 1. The bins were located in a 5 × 6 × 2 m L:W:H walk-in environmental chamber (Percival Instruments, Dallas County, IA) under constant conditions (25°C, 65% RH, 14:10 L:D). On a given round of release, two replicates of each treatment were performed simultaneously, and further replicates were accumulated over time. In total, there were n = 16 replicates for each treatment. The released *S. oryzae* were given 24 hr to respond to the stimuli. The traps were then collected and the number of insects in each trap was recorded.

Large Release-Recapture Trapping Assay

Because there was a concern with saturation of the small bin with the volatile stimuli, a large release-recapture assay was conducted to measure long-distance attraction to each semiochemical treatment in a larger space (Supp Fig. 4C [online only]). A 5 × 6 × 2 m L:W:H walk-in environmental chamber (Percival Instruments, Dallas County, IA) under constant conditions (25°C, 65% RH, 14:10 L:D) was used for this experiment. The chamber floor was covered with white paper that was taped down to easily locate *S. oryzae* and allow free movement. A strip of PTFE was also placed 25 cm above the floor of the chamber to discourage insects from climbing on the walls. The same pitfall traps (as above) (Dome Trap, Trécé, Inc., Adair, OK) were baited with 10 g of one of the treatments in Table 1 and placed equidistantly around the perimeter of the chamber. After each round of release, trap position for each treatment was rotated to reduce any positional bias. In each replicate, all five semiochemical treatments were used and 100 *S. oryzae* were released per replicate, with a total of n = 10 replicates. The released *S. oryzae* were given 24 hr to respond to the stimuli. The traps were then collected and the number of insects in each trap was recorded.

To evaluate whether microbial volatiles may affect attraction to traps in the small and large release-recapture assays, generalized linear model based on a Poisson distribution above was used with number of *S. oryzae* recaptured as the response variable and the explanatory variable consisting of the full range of treatments listed in Table 1.

Headspace Volatile Collection

To analyze how microbial inoculation changes the volatile blends emitted by grain, headspace collection using a push-only setup was used (Supp Fig. 5 [online only]). A total of 20 g of each of the odor treatments above (Table 1) was placed into a screw top headspace chamber (500 ml capacity; 10.2 × 12.7 cm D: H). Central air was scrubbed of background volatiles using an activated carbon filter, and flow was split into four lines and regulated using flow meters (Volatile Collection Systems, Gainesville, FL). Chemically inert PTFE tubes were used to connect each piece of the system. A drip tip borosilicate glass tube packed with 20 mg of absorbent Poropaq-Q (Volatile Collection Systems, Gainesville, FL) was added to the output point of the headspace chamber to adsorb volatiles with a stainless-steel screen (No. 316), borosilicate glass wool, and PTFE compression seal bracketing the adsorbent material. Volatiles were collected for 3 hr, then volatiles were eluted with 150 µl of dichloromethane into 2-ml GC vials with 200-µl inserts (Phenomenex, Torrance, CA) with the solvent pushed through by inert N_2 gas. After extraction, 1 µl of tetradecane (190.5 ng at 99% purity, MilliporeSigma, Burlington, MA) was added to each of the labeled vials using a microsyringe (7000 Series Modified Microliter Syringe, Hamilton Company, Inc., Reno, NV) and was used as an internal standard for quantification of compounds. Between uses, all volatile collection traps were washed with 700 µl of dichloromethane in triplicate. All volatile collection vials were sealed with PTFE tape and stored at –4°C until GC-MS analysis, using standard methodology (Morrison et al. 2016b). Between each replicate, all glassware was washed first with methanol, then hexane, and baked in a convection drying oven for 5 min before reuse. There was a total of n = 7 replicates per treatment.

Gas Chromatography Coupled with Mass Spectrometry

All headspace sample extracts were run on an Agilent 7890B gas chromatograph (GC) equipped with an Agilent Durabond HP-5 column (30 m length, 0.250 mm diameter, and 0.25 µm film thickness) with He as the carrier gas at a constant 5 ml/min flow and 39 cm/s velocity, which was coupled with an Agilent 5997B mass spectrometer (MS) single quadrupole detector. The compounds were separated by injecting 1 µl of each sample at an inlet temperature of 250°C under split mode into the machine at a 15:1 ratio. The oven program began at 60°C for 1 min followed by 10°C/min ramping to 300°C over 26.5 min, and subsequently held for 4 min at 300°C. After a solvent delay of 4 min, mass ranges between 50 and 550 atomic mass units were scanned. Preliminary identification of compounds from representative chromatograms were obtained by comparing sample spectral data with the NIST 17 library, by GC retention index (Adams 2007) and calculated Kovats indices for compounds (Girard, 1996).

To characterize the presence of headspace volatiles from the extracts, raw peak areas were extracted from the gas chromatograms using MSD ChemStation v2.00 software (Agilent Technologies, Inc., Santa Clara, CA). The emissions rate was calculated on a ng per gram of grain weight and per hour basis by using the ratio of the peak area for tetradecane to each headspace volatile. Background volatiles found in the negative control without grain were discarded from the other samples, since these represent transient background volatiles in the general vicinity of headspace collection but are not informative of differences among the treatments. In addition, volatiles only identified in a single sample were discarded, because they are not informative of similarities between groups of treatments. Pairwise Bray-Curtis similarities were calculated between each headspace sample, and nonmetric multi-dimensional scaling (NMDS) was used to visualize the differences in volatile emissions among treatments. A total of n = 1,000 permutations were used for the ordination procedure. Stress values for the NMDS procedure were <0.1, indicating that good interpretation was possible. An analysis of similarity (ANOSIM) was used to determine significant differences for headspace volatiles among odor treatment categories. ANOSIM calculates an R statistic that can vary from 1 to –1, with values above zero interpreted as greater dissimilarity between treatments than within and values below zero interpreted as greater dissimilarity within treatments than between treatments. A total of n = 1,000 permutations were performed for the test. For all multivariate statistics, the R Package *vegan* was used (Oksanen et al. 2022). To assess differences in the complexity of the headspace blends (e.g., number of compounds per treatment), pairwise chi-square tests were performed among treatments with the null hypothesis of an equal number of compounds identified from each treatment. To determine differences in overall quantitative emissions of volatiles, pairwise *t*-tests among means were performed using a Bonferroni-correction to the *P*-value.

Results

Four-Way Olfactometer Assay

There were no significant differences between the four semiochemical stimuli (χ^2 = 2.9; df = 3; *P* = 0.39; Fig. 3A) when tested in the four-way olfactometer with fused porous glass barriers. *Sitophilus oryzae* chose each side 22 ± 2–27 ± 3% (means ± SE) of the time, but there was no significant preference among the positive control, UV, M1, and M2 sides. The side that *S. oryzae* also had chosen had no significant effect on the time to decision (χ^2 = 2.65; df = 3; *P* = 0.45). In each case, it took *S. oryzae* an average of 60 ± 8–76 ± 7 s to choose a stimulus.

Additionally, there were also no significant differences between the semiochemical stimuli (χ^2 = 5.97; df = 3; *P* = 0.11; Fig. 3B) when tested in the four-way olfactometer with fused porous glass barriers containing holes. *Sitophilus oryzae* chose each side 18 ± 5–35 ± 6% (means ± SE) of the time, but there was no significant preference among the positive control, negative control, UV, and M1 sides. The side that *S. oryzae* also had chosen had no significant effect on the time to decision (χ^2 = 6.87; df = 3; *P* = 0.07). In each case, it took *S. oryzae* an average of 44 ± 5 – 72 ± 13 s to choose a stimulus.

Ethovision Assay

The semiochemical treatments did not significantly affect the instantaneous velocity and the distance moved of *S. oryzae* (MANOVA: Approx. *F* = 1.5; df = 4, 69; *P* > 0.22; Fig. 4A and B). The mean (±SE) distance moved by *S. oryzae* ranged between 200 (±28) cm when exposed to the positive control to 317 (±44) cm in the negative control. By contrast, the semiochemical treatments significantly increased the frequency with which *S. oryzae* entered the kernel zones (MANOVA: Approx. *F* = 5.2; df = 4, 69; *P* < 0.01; Fig. 5). However, the frequency of entrances to the control kernel zone, which lacked stimuli, were not significantly different among the treatments (ANOVA: *F* = 1.5; df = 4, 69; *P* = 0.29), but frequency of entrances by *S. oryzae* were significantly affected in the treatment kernel zone by the stimuli present (*F* = 5.0; df = 4, 69; *P* < 0.01). In particular, there were almost 2-fold and greater than 3-fold more entries into the treatment kernel zone for M2 compared to the negative control and positive control, respectively (Tukey HSD).

The semiochemical treatments did not significantly affect the cumulative duration that *S. oryzae* spent on each half of the arena (MANOVA: Approx. *F* = 1.7; df = 4, 69; *P* = 0.16; Fig. 6A). Individuals spent an average of 732–964 s and 821–947 s on halves

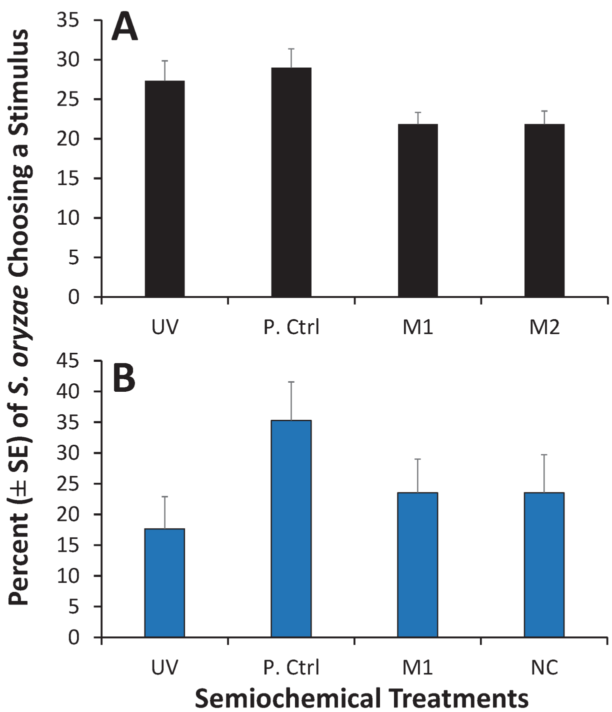


Fig. 3. Percent of *S. oryzae* choosing different semiochemical treatments in a four-way olfactometer assay from a set up with A) fused porous glass barriers, or B) holes in fused porous glass barriers between headspace chamber and release chamber. A total of *n* = 240 replicate insects were tested and a single *S. oryzae* was used per replicate and round of assay. Bars represent standard error bars for groups of 10 insects. There were no significant differences among the semiochemicals treatments (χ2test, Bonferroni correction), thus letters have not been added to bars. Abbreviations: UV—UV-sanitized grain, P. Ctrl—clean grain from storage, M1- grain from storage inoculated with asexual *A. flavus*, M2—grain from storage inoculated with sexual *A. flavus*.

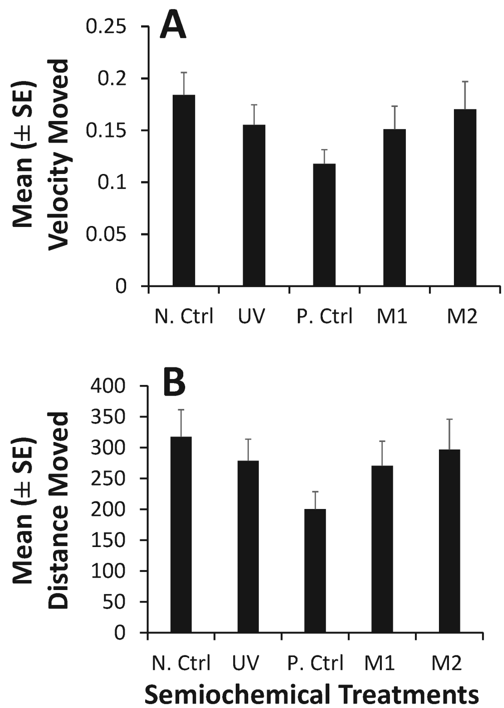


Fig. 4. Movement parameters from the Ethovision assay, including **A)** the mean (± SE) instantaneous velocity and **B)** mean (±SE) distance moved by *S. oryzae* in whole arenas over 30 min periods, depending on semiochemicals treatment. Each bar represents the mean for *n* = 15 replicate tested insects. The semiochemical treatments did not significantly affect either variable (Tukey HSD, α = 0.05). Abbreviations: N. Ctrl—negative control (no stimuli), UV—UV-sanitized grain, P. Ctrl—clean grain from storage, M1—grain from storage inoculated with asexual *A. flavus*, M2—grain from storage inoculated with sexual *A. flavus*.

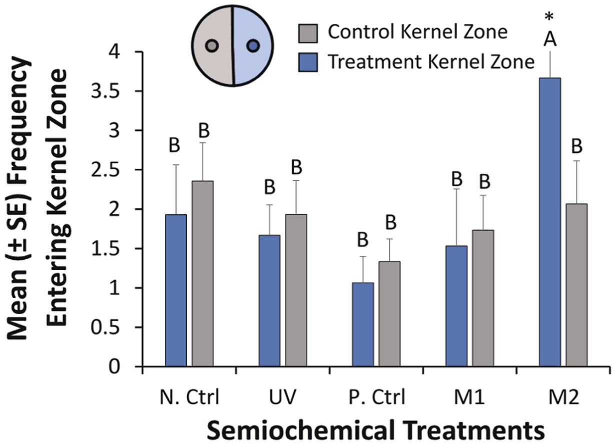


Fig. 5. Mean frequency of *S. oryzae* entering the smaller kernel zone over a 30-min trial period in the Ethovision assay. The dark gray kernel zone represents the zone with no treatment (negative control), while the dark blue kernel zone represents the zone with the semiochemical treatment. A total of n = 15 replicates insects were tested. Letters represent comparisons among the frequency of *S. oryzae* entering a treatment or control kernel zone with the different semiochemical treatments. Bars with shared letters are not significantly different from each other (Tukey HSD, α = 0.05). *Sitophilus oryzae* entered the treatment kernel zone containing M2 significantly more times than the kernel zones containing the rest of the semiochemicals treatments or none at all. (*) Abbreviations: N. Ctrl—negative control (no stimuli), UV—UV-sanitized grain, P. Ctrl—clean grain from storage, M1—grain from storage inoculated with asexual *A. flavus*, M2—grain from storage inoculated with sexual *A. flavus*. For color interpretation, please check online version of article.

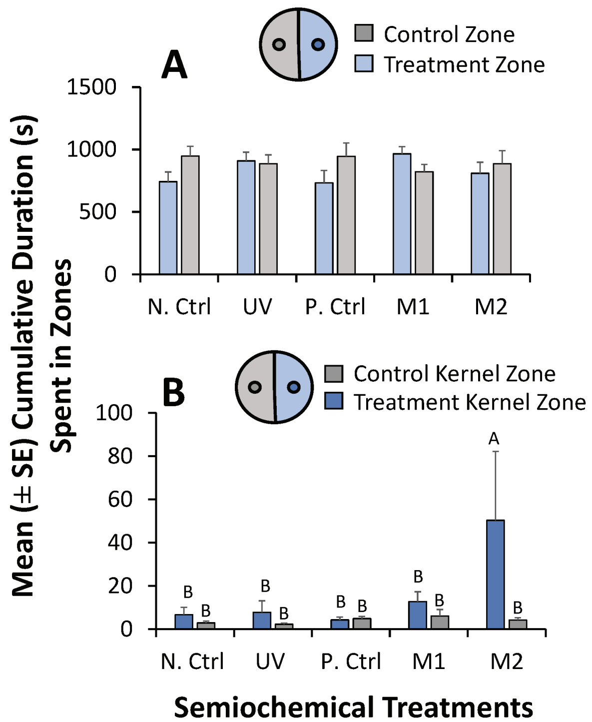


Fig. 6. Mean cumulative duration (±SE, s) spent in each treatment (blue) and control (gray) **A)** zones (lighter shades) and **B)** kernel zones (darker shades) in 30-min trials during the Ethovision assay. A total of n = 15 replicates insects were tested per treatment. The semiochemical treatments did not significantly affect the duration that *S. oryzae* spent on each half of the test arena (e.g., zone) (MANOVA), however it did affect the time spent in the smaller kernel zones. The kernel zone containing M2 resulted in *S. oryzae* spending a significantly greater duration of their time in compared to the other treatments or the equivalent control kernel zone. Bars with shared letters are not significantly different from each other (Tukey HSD, α = 0.05). Letters are not displayed in (**A**) because none of the bars are significantly different from each other. Abbreviations: N. Ctrl—negative control (no stimuli), UV—UV-sanitized grain, P. Ctrl—clean grain from storage, M1—grain from storage inoculated with asexual *A. flavus*, M2—grain from storage inoculated with sexual *A. flavus*. For color interpretation, please check online version of article.

of the arena with stimuli and without stimuli, respectively. By contrast, the semiochemical treatments significantly affected the time spent in the smaller kernel zones (MANOVA: Approx. F = 32.9; df = 4, 69; P < 0.0001; Fig. 6B). In particular, the semiochemical treatments significantly affected the time spent in the kernel zones with stimuli (F = 32.3; df = 4, 69; P < 0.0001), but not the kernel zones lacking stimuli (F = 1.43; df = 4, 69; P = 0.23). In the kernel zones, *S. oryzae* spent 12-fold more time in the M2 treatment than the corresponding treatment zone for the negative control, and 4–8-fold more time in the M2 treatment than in the kernel zones for the other semiochemical treatments (Tukey HSD, Fig. 6B).

Release-Recapture Assays

In the small release recapture assay, traps baited with one of the four semiochemical treatments showed a significantly increased mean percent recapture of *S. oryzae* compared to the negative control (χ^2 = 62.2; df = 4; P < 0.0001; Fig. 7A). In particular, traps baited with UV-sterilized grain, the positive control, grain incubated with M1, or grain incubated with M2 had 19–23% greater captures than the negative control. Similarly, in the large release-recapture assay, semiochemical lure significantly affected recapture of *S. oryzae* (χ^2 = 10.1; df = 4; P < 0.05; Fig. 7B), with 6–7-fold greater recapture in traps baited with one of the four semiochemical stimuli compared to the negative control lacking a stimulus. The four semiochemical treatments were not significantly different among one another (Tukey HSD, Fig. 7).

Headspace Assay

Overall, 35 compounds were tentatively identified in headspace. The headspace blends were significantly more complex for positive control (χ^2 = 4.00; df = 1; P < 0.05), asexual *A. flavus* (M1)-inoculated (χ^2 = 9.80; df = 1; P < 0.001), and sexual *A. flavus* (M2)-inoculated grain (χ^2 = 14.3; df = 1; P < 0.001) compared to UV-sterilized grain (Table 2). A total of 12 compounds were present in the UV-sterilized grain, with a single compound, 1,4-diethyl-benzene comprising 89% of the total emissions (Table 2). The positive control with untreated grain, by contrast, had almost 2-fold more compounds (e.g., 22), but with similar main components; together, 1,4- and 1,2-diethyl-benzene comprised 50% of the headspace emissions. The fungal-inoculated grain exhibited even more complex emission patterns, but mostly among the minor components. For example, asexual *A. flavus* (M1)-inoculated grain contained 22 compounds, with 1,4-diethyl-benzene and 1,2-diethyl-benzene comprising 87.3% of total emissions, whereas 20 compounds comprised the remaining 13%. Likewise, sexual *A. flavus* (M2)-inoculated grain contained 22 compounds, with just three compounds (e.g., 1,4-diethyl-benzene; 1,2-diethyl-benzene; (Z)-9-octadecenamide; Table 2) comprising 77% of emissions. Interestingly, fungal-inoculated grain also emitted quantitatively more volatiles. For instance, asexual *A. flavus* (M1)-inoculated grain emitted a 5–6-fold greater amount of volatiles than UV-sterilized (t = 17.0; df = 12; P < 0.0001) and the positive control grain (t = 16.9; df = 12; P < 0.0001). Likewise, sexual *A.*

flavus (M2)-inoculated grain emitted an 6–7-fold greater amount of volatiles than UV-sterilized (t = 30.3; df = 12; P < 0.0001) and the positive control grain (t = 31.1; df = 12; P < 0.0001). Overall, while there was significant overlap in the composition of the treatments, they were significantly different from each other (R = 0.200; Perm = 1000; P < 0.001; Stress < 0.10; Fig. 8), suggesting that inoculation with fungi affected composition of volatile emissions from the grain.

Discussion

For the first time in stored products, we specifically manipulated the fungal community present on the grain to determine how it affects the behavioral response by the primary pest, *S. oryzae*. Our tests explicitly tested different aspects of behavior, including preference (e.g., 4-way olfactometer), close-range foraging decisions (Ethovision), and long-distance attraction (release-recapture assays), contributing to a fuller understand of the behavioral response by *S. orzyae*. Overall, we have shown that manipulating the fungal community resulted in altered emissions of headspace volatiles, and shifting fungal cues affected the foraging decisions of *S. oryzae* when in close proximity to grain inoculated with the sexual strain of *A. flavus* but not at a distance. At close proximity, this suggests that the behavioral response by *S. oryzae* may be prompted by either gustatory or odorant receptors, and future work should attempt to identify which are the primary modes of chemoreception. It appears MVOCs may not be as important for long-distance foraging to *S. oryzae* as they are to other insects (Davis et al. 2013a). In particular, our work adds to the evidence that stored product insects use fungal cues at close-range to inform foraging decisions. For instance, Van Winkle et al. (2021) incubated grain for various periods after tempering to specific grain moistures and found grain with moderate fungal colonization was more attractive to the primary pest, lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae) than to the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). Interestingly, we found through the Ethovision trials that, at close range, fungal cues by particular growth stages of fungi in grain could affect foraging decisions. In particular, *S. oryzae* was more likely to approach and stay in close proximity to grain kernels with MVOCs from the sexual life stage of *A. flavus* but not the asexual life stage-inoculated grain. This indicates that the fungal cues from the sexual life stage of *A. flavus* had an arresting effect on the behavior of *S. oryzae*. Arrestment is a behavioral process that involves retention at a resource of interest (Blaauw et al. 2017). However, the presence of fungal cues from sexual *A. flavus* did not significantly alter the overall distance moved or velocity of *S. oryzae;* grain volatiles were the more important determinant for these overall measures of movement. Our study corresponds with prior research that found close-range repellency by *S. oryzae* for volatiles emitted by *Fusarium* spp. (Selitskaya et al. 2014) as well stored product insects showing behavioral changes to grain with moderate incubation with complex fungal communities (Van Winkle et al. 2021). Our study suggests that once *S. oryzae* reaches a food source, food quality such as microbial infestation may become more important in controlling foraging decisions at a local scale than other types of factors.

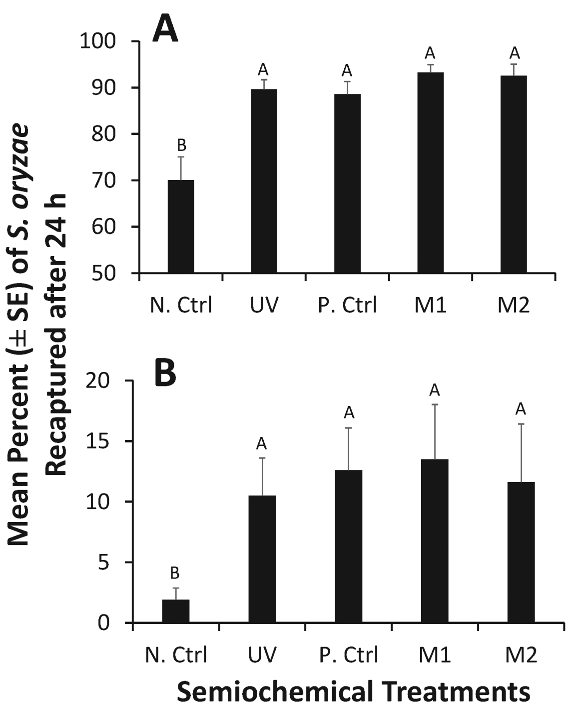


Fig. 7. Mean percent (±SE) *S. oryzae* recaptured in the **A)** small and **B)** large release-recapture assay in commercial pitfall traps baited with a given semiochemicals treatment after 24 hr under constant environmental conditions (25°C, 65% RH, 14:10 L:D). In the small version, 20 *S. oryzae* were released in smaller bins for each replicate, with a total of n = 7 replicates per treatment. In the large version, 100 *S. oryzae* were released in each replicate, for a total of n = 10 replicates. In both cases, traps containing grain showed a significant increase in captures of *S. oryzae* compared to the negative control, but MVOCs did not affect capture. Bars with shared letters are not significantly different from each other (Tukey HSD, α = 0.05). Abbreviations: N. Ctrl—negative control (no stimuli), UV—UV-sanitized grain, P. Ctrl—clean grain from storage, M1—grain from storage inoculated with asexual *A. flavus*, M2—grain from storage inoculated with sexual *A. flavus*.

In the release-recapture assay, we used commercially available pitfall traps to assess long-distance attraction to MVOCs and grain. *Sitophilus oryzae* did not show elevated long-distance attraction to grain inoculated with the asexual or sexual life stage of *A. flavus* compared to the positive control or UV-sanitized grain. When initially using the smaller release-recapture assays, it was possible that volatile emissions from traps saturated the small release arenas; a larger version of the test was employed. However, the results from the smoke and odor tests indicated the bins were not saturated. Both the small and large release-recapture assay indicated the same results, in which the presence of grain was more important than whether grain had been inoculated with either fungal growth stage for recapture of *S. oryzae* and were likely a successful test of long-distance attraction to grain odors relative to the size of the insect considering that *S. oryzae* is only 2 mm long and cannot fly, while traps were placed 879 and 3000 mm away. Indeed, prior research has found that *S. oryzae* are attracted to a range of grain odors, including maltol, vanillin, and valeraldehyde (Phillips et al. 1993). As a consequence, the volatiles produced by the sexual life stage of *A. flavus*-inoculated grain may be necessary but not sufficient without additional long-range stimuli for use in behaviorally-based management methods. This is because behaviorally-based management methods require attractive long-range stimuli for successful implementation at food facilities. For example, the stimuli for attract-and-kill and push-pull tactics often need to attract pest populations from a longer distance away than the immediate vicinity of where the commodity is being protected (e.g., Cox 2004; Morrison et al. 2016, 2019a), and be able to retain the pest until it can be removed from the foraging population (Morrison et al. 2021b).

The goal of the four-way olfactometer assay was to assess preference by *S. oryzae* to four different semiochemical treatments,

Table 2. Mean emission rates (ng g^{-1} h^{-1}) and percent contribution to total emissions of tentatively identified compounds in headspace from UV-sterilized grain, untreated grain (positive control), asexual *Aspergillus flavus*(M1)- inoculated grain, and sexual *A. flavus*(M2)-inoculated grain from samples (n = 7 replicates per treatment). Compounds were tentatively identified based on mass spectra of each compound compared to the reference library NIST17. Emissions from fungal-inoculated grain were significantly greater and more complex compared to UV-sterilized or untreated grain

		UV-Sterilized Grain		Positive Control Grain		Asexual *A. flavus* (M1)-Inoculated Grain		Sexual *A. flavus* (M2)-Inoculated Grain	
Compound	Retention Time	Mean ± SE	% of Total	Mean ± SE	% of Total	Mean ± SE	% of Total	Mean ± SE	% of Total
Pivalaldehyde, semicarbazone	4.735	0.0 ± 0.0	0.0	0.1 ± 0.1	0.8	0 ± 0	0.0	0 ± 0	0.0
2-Butenal	4.788	0.0 ± 0.0	0.0	0.3 ± 0.1	2.4	0 ± 0	0.0	0 ± 0	0.0
2,4-Dimethyl-1-heptene	4.792	0.0 ± 0.0	0.0	0 ± 0	0	0.8 ± 0.2	1.2	0.1 ± 0.1	0.1
2-Pentanone, 4-hydroxy-4-methyl	4.802	0.2 ± 0.2	1.4	0.7 ± 0.5	5.6	0.2 ± 0.2	0.3	0.5 ± 0.3	0.6
Benzene, propyl	6.292	0.0 ± 0.0	0.0	0.1 ± 0.1	0.8	0.4 ± 0.3	0.6	0.3 ± 0.1	0.4
1-Octen-3-ol	6.516	0.0 ± 0.0	0.0	0 ± 0	0	0.5 ± 0.3	0.8	3.5 ± 2	4.1
Butanal	6.525	0.0 ± 0.0	0.0	0 ± 0	0	0.6 ± 0.4	0.9	0 ± 0	0.0
4,5-Dichloro-1,3-dioxolan-2-one	6.628	0.0 ± 0.0	0.0	0.2 ± 0.1	1.6	0 ± 0	0.0	0 ± 0	0.0
3-Octanone	6.648	0.0 ± 0.0	0.0	0 ± 0	0	1.8 ± 0.3	2.7	6.2 ± 3.6	7.3
Decane	6.823	0.0 ± 0.0	0.0	0.2 ± 0.2	1.6	0.2 ± 0.2	0.3	0 ± 0	0.0
Mesitylene	6.869	0.1 ± 0.1	0.6	0.2 ± 0.2	1.6	0.2 ± 0.2	0.3	1.3 ± 1.1	1.5
Benzene, 1,2,4-trimethyl-	7.319	0.0 ± 0.0	0.0	0.2 ± 0.2	1.6	0.1 ± 0.1	0.2	0 ± 0	0.0
D-Limonene	7.364	0.1 ± 0.1	0.7	0.1 ± 0.1	0.8	0 ± 0	0.0	0.3 ± 0.2	0.4
Benzeneethanol, beta-ethyl	7.541	0.0 ± 0.0	0.0	0 ± 0	0	0 ± 0	0.0	0.2 ± 0.1	0.2
Benzene, 1,4-diethyl	7.659	10.1 ± 10.1	88.9	4.8 ± 4.8	38.4	26.1 ± 18.9	39.4	18.6 ± 5.8	22.0
Benzene, 1,2-diethyl	7.759	0.1 ± 0.1	1.3	1.4 ± 0.6	11.2	31.7 ± 22.5	47.9	37.4 ± 11	44.3
1,3,8-p-Menthatriene	7.86	0.0 ± 0.0	0.0	0 ± 0	0	0.5 ± 0.3	0.8	0.4 ± 0.2	0.5
4-Dichloromethyl-2[[2-[1-methyl-2-pyrrolidinyl] ethyl]amino-6-Trichloromethylpyrimidine	8.271	0.0 ± 0.0	0.0	0.2 ± 0.1	1.6	0.1 ± 0.1	0.2	0.9 ± 0.8	1.1
Benzene, (2-methyl-1-propenyl)-	8.279	0.0 ± 0.0	0.0	0.6 ± 0.4	4.8	0.2 ± 0.2	0.3	0 ± 0	0.0
1-Phenyl-1-butene	8.282	0.0 ± 0.0	0.0	0 ± 0	0	0.2 ± 0.1	0.3	0.1 ± 0.1	0.1
Linalool	8.314	0.2 ± 0.2	1.6	0.1 ± 0.1	0.8	0 ± 0	0.0	0.5 ± 0.3	0.6
Nonanal	8.37	0.1 ± 0.1	1.3	0.7 ± 0.3	5.6	0.4 ± 0.2	0.6	0.7 ± 0.6	0.8
2-Thiophenecarboxylic acid, 5-nonyl-	9.044	0.0 ± 0.0	0.0	0.2 ± 0.1	1.6	0 ± 0	0.0	0.4 ± 0.4	0.5
Dichloroacetaldehyde	9.735	0.0 ± 0.0	0.0	0.1 ± 0.1	0.8	0 ± 0	0.0	0 ± 0	0.0
Linalyl acetate	10.558	0.0 ± 0.0	0.0	0 ± 0	0	0 ± 0	0.0	0.2 ± 0.2	0.2
Beta-Ocimene	10.561	0.2 ± 0.2	1.4	0.1 ± 0.1	0.8	0 ± 0	0.0	0 ± 0	0.0
2-Thiophenecarboxylic acid	10.59	0.1 ± 0.1	0.8	0.8 ± 0.5	6.4	0.7 ± 0.3	1.1	2.6 ± 2.5	3.1
1-Pent-3-ynylcyclopenta-1,3-diene	10.653	0.1 ± 0.1	0.8	0.2 ± 0.1	1.6	0.3 ± 0.2	0.5	0.3 ± 0.2	0.4
1,5,6,7-Tetramethylbicyclo[3.2.0]hepta-2,6-diene	10.822	0.0 ± 0.0	0.0	0 ± 0	0	0.1 ± 0.1	0.2	0.4 ± 0.2	0.5
Ethanone, 1-(4-ethylphenyl)	11.108	0.0 ± 0.0	0.0	0 ± 0	0	0.5 ± 0.4	0.8	0.6 ± 0.3	0.7
Butyl citrate	21.812	0.0 ± 0.0	0.0	0 ± 0	0	0.1 ± 0.1	0.2	0 ± 0	0.0
1-Methyl-4-phenyl-5-Thioxo-1,2,4-triazolidin-3-one	23.957	0.1 ± 0.1	0.7	0.6 ± 0.3	4.8	0 ± 0	0.0	0 ± 0	0.0
9-Octadecenamide, (Z-)	26.311	0.1 ± 0.1	0.5	0.6 ± 0.3	4.8	0.5 ± 0.3	0.8	9 ± 8.6	10.7
Total		**11.4 ± 0.2**	**100**	**12.5 ± 0.3**	**100**	**66.2 ± 1.4**	**100**	**84.5 ± 0.7**	**100**

including our positive control (unaltered grain), UV-sterilized grain, asexual (M1), and sexual *A. flavus* (M2)-inoculated grain. We found that *S. oryzae* did not prefer either fungal growth stage more than the positive control grain or UV-sanitized grain. Each odor source was preferred equally by *S. oryzae*. The olfactometer contained crystalized glass semi-permeable barriers, which was intended to prevent dispersal of insects, but may have hindered volatile diffusion over the short periods used here for testing *S. oryzae*. As a result, a follow-up version of this experiment was performed, wherein two small holes were pierced in each of the glass barriers, but again there was no significant preference among the negative control (no grain), positive control (unaltered grain), UV-sterilized grain, and asexual (M1) *A. flavus*-inoculated grain. There are four possible alternative hypotheses for this behavior. It could be that the glass olfactometer elicits a thigmotactic response by *S. oryzae* that disrupts its olfaction. Domingue et al. (2021) demonstrated the thigmotactic arrestment of the stored product quarantine threat, khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae) when it came into contact with control netting (even without insecticide), which disrupted orientation to semiochemicals. A second hypothesis is that *S. oryzae* may have needed air movement to exhibit a preference to the semiochemical treatments, which would not have been provided in the still-air olfactometer. Prior work has shown that air movement improves insect response by allowing the dispersal of semiochemicals and increasing the effectiveness of commercially-available traps (Campbell 2012, Jian 2019, Sajeewani et al. 2020), which is more realistic for food facilities where air flow and eddies are common. However, this appears unlikely, since many *S. oryzae* chose a stimulus quickly. A third explanation is that the time provided in the

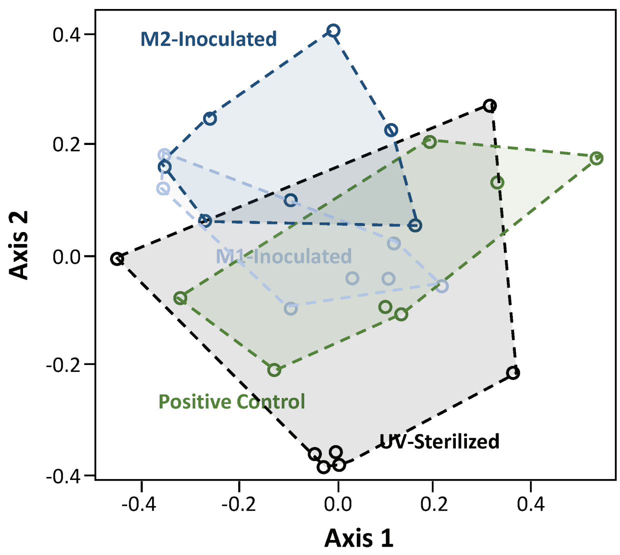


Fig. 8. Nonmetric multidimensional scaling ordination of headspace samples based on the pairwise Bray–Curtis similarities between emissions rates (ngg^{-1} h^{-1}) by compounds in each sample. There were a total of $n = 7$ replicates per treatment. While there is substantial overlap among some treatments, the treatments were significantly different from each other. There were $n = 1{,}000$ permutations for the ordination, and stress = 0.09, indicating reasonable interpretation was possible. Abbreviations: UV—UV-sanitized grain, P. Ctrl—clean grain from storage, M1—grain from storage inoculated with asexual *A. flavus*, M2—grain from storage inoculated with sexual *A. flavus*.

trial may have been insufficient for diffusion of volatiles. This also appears highly unlikely given the supplementary data showing adequate diffusion of volatiles (see Supplementary Materials [online only]). Finally, it could be that gustatory receptors are involved and close proximity is required for *S. oryzae* to differentiate among the signals, as in the Ethovision trial. This latter hypothesis appears to be the most likely given the whole of the dataset.

We confirmed through smoke and odor tests that volatiles diffused sufficiently in the assay, but did not saturate arenas (Supplemental Methods and Results [online only]). For example, we observed smoke exiting Ethovision arenas, and never forming a uniform, saturated layer of volatiles in the arenas. While observers were able to smell grain on the far side of the Ethovision arenas after 15 and 30 min, this was only for about 80% of arenas. This suggests volatiles do diffuse, but do not saturate the arenas. In the four-way olfactometer, it took grain volatiles longer to diffuse than smoke volatiles, likely due to their lower emission rates. In the release-recapture bins, we were able to detect smoke volatiles after about 24 s, but could not detect grain volatiles after 10 min or 24 hr. However, we did detect grain odors directly outside of the pitfall trap, suggesting that grain odors do diffuse, but do not saturate the small bins. In each assay, it appears likely that a volatile gradient was formed to which *S. oryzae* could react to behaviorally.

The most common grain headspace volatiles found in our study were diethyl benzenes across all grain treatments regardless of fungal presence. Various benzene compounds have commonly been isolated from headspace emitted by both whole wheat and milled grain in prior work (Maeda et al. 2008). The key differences among the treatments seem to be more in the presence and complexity of minor components, evidenced by the increased complexity of the fungal-inoculated grain. For instance, over half the total tentatively identified compounds were found only in the fungal-inoculated grain. Among these, key volatiles associated with fungi in prior studies included, but were not limited to, 3-octanone, 1-octen-3-ol, butanal, limonene, butyl citrate, octane, and many others (Borjesson et al. 1992; reviewed in Jelen and Wasowicz 1998; Ponce et al. 2021). It is possible that stored product insects such as *S. oryzae* respond to more complex emissions at close range, especially considering that increased complexity of volatile blends may result in elevated attraction across a range of other insects (Szendrei and Rodriguez-Saona 2010). At low concentrations, other stored product insects such as *T. castaneum* exhibited the strongest antennal responses to (*Z*)-3-hexen-1-ol, hexenal, 2-heptanone, 3-octen-2-one, and ethyl hexanoate (Balakrishnan et al. 2017). Van Winkle et al. (2021) found that 3-methyl-1-butanol, 2-methyl-1-butanol, and octane were the most common fungal volatiles produced after grain was incubated and tempered for extended periods to higher grain moisture, while Sinha et al. (1987) found 3-methyl-1-butanol, 1-octen-3-ol, and 3-octanone were the most common in experimental grain bins. These and related volatiles may provide potential targets for use in behaviorally-based management tactics.

We have found that the asexual stage (M1) and sexual stage (M2) of *A. flavus* produce different MVOCs. Misztal et al. (2018) found that when developing fungi go through different stages of cellular respiration (e.g., different life stages of microbes), this had a significant effect on not only the amount of MVOCs produced but also their identity. Additional research suggests that *Tuber* spp., a symbiotic fungus found in plant roots, releases specific volatiles during its different life stages to attract or repel insects (Splivallo et al. 2011). *Leiodes cinnamomea* (L.) (Coleoptera: Leiodidae) is a pest that is only attracted to unripe truffles from a distance, indicating that volatile cues are only produced by unripe truffles (Splivallo et al. 2011). Similarly, we analyzed how different life stages of *A. flavus* (asexual vs. sexual) can modulate the behavior of *S. oryzae*. We saw that grain inoculated with the sexual *A. flavus* (M2) affected the close-range foraging decisions of *S. oryzae*, while the asexual *A. flavus* (M1) did not. While prior work characterized grain sources that were likely not homogenous communities of fungi (Van Winkle et al. 2021), we determined here that M1 was primarily asexual *A. flavus*, while M2 was primarily sexual *A. flavus*.

Overall, our in-depth behavioral analysis has revealed that *S. oryzae* likely uses grain volatiles at a distance but may prefer to use other cues of host quality at close-range. Our study also demonstrated that postharvest grain constitutes a complex ecosystem of fungal stages, each of which is emitting a unique blend of volatiles that may have contrasting behavioral effects.. Future work should assess antennal response via gas chromatography coupled with electroantennographic detection (GC-EAD) to understand which volatiles identified here are actually detected by *S. oryzae*. This may also help narrow the possible behaviorally-active components of the headspace blends to those that are being detected by the insect. Furthermore, the life stages of other fungi during testing (e.g., sexual vs. asexual) may impact volatile emissions (Misztal et al. 2018), but whether these changes are primarily detected by gustatory or odorant receptors of *S. oryzae* should be specifically tested. Differing temperatures during insect orientation and response may influence plume reach of volatiles as well as chemosensory and neurophysiological processes in the insect and so may air movement (Sajeewani et al. 2020), so in the future, this should be explicitly considered. Finally, specific behaviorally-active volatiles should be individually tested in order to determine whether they could be used in behaviorally-based management strategies (e.g., Wilkins et al. 2021). Overall, this study has substantively enhanced our understanding of how MVOCs affect the behavior of *S. oryzae*. Further research into this area on related stored product species and specific headspace compounds will undoubtedly help us improve management, while ensuring that our planet can feed its growing population.

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Data Availability

(dataset) Ponce, Marco A.; Lizarraga, Sandra; Bruce, Alexander; Kim, Tania N.; Morrison, III William R. Data from Grain inoculated with different growth stages of the fungus, Aspergillus flavus, affect the close-range foraging behavior by a primary stored product pest, Sitophilus oryzae (Coleoptera: Curculionidae). Ag Data Commons. https://data.nal.usda.gov/dataset/data-grain-inoculated-different-growth-stages-fungus-aspergillus-flavus-affect-close-range-foraging-behavior-primary-stored-product-pest-sitophilus-oryzae-coleoptera-curculionidae-3. Accessed 2022-05-11.

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

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Title: Plant size, latitude, and phylogeny explain within-population variability in herbivory

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Abstract: Interactions between plants and herbivores are critical in most ecosystems, but the strength of these interactions is highly variable. Theory predicts that the amount of variation in a system can strongly alter ecological and evolutionary processes. Sparse data, however, limit our understanding of what influences the magnitude of interaction variability at macroecological and macroevolutionary scales. Using standardized surveys of plant–herbivore interactions for 503 plant species at 790 sites across 116° of latitude, we show that variability in herbivory within plant populations increases with latitude, decreases with plant size, and is phylogenetically structured. Moreover, these patterns were stronger than the ones for mean herbivory. Our results indicate that differences in the variability of herbivory are central to how plant–herbivore biology varies across macroscale gradients.

One-Sentence Summary: The level of variability in herbivory is a key feature differentiating plant–herbivore systems at macroscales.

Plant–herbivore interactions, which involve more than half of macroscopic biodiversity and 90% of its biomass (*1*), are believed to shape macroscale biological patterns and processes, such as biodiversity gradients, biomass distributions, community structure, plant species coexistence, and trait evolution (*2*–*4*). Biologists have studied the role of herbivory at macroscales by quantifying how the mean herbivore damage level covaries with latitude, biome, functional traits, and phylogeny (*5*–*7*). However, macroscale patterns have not always matched expectations. For example, despite the paradigm that herbivore pressure increases towards the equator owing to more benign environmental conditions, empirical patterns have been weak or inconsistent (*8*–*10*). Similarly, despite the expectation that closely related plant species should face similar pressures from herbivores, phylogenetic signal in mean herbivore damage is often undetectable or restricted to certain groups (*5*, *11*). We suggest that our understanding of macroscale patterns in herbivory can be improved by considering patterns in the magnitude of variability in herbivory, rather than means only.

Variability is a hallmark of plant-herbivore interactions (*12*). Within populations, patterns in damage are often highly skewed, with most plant individuals receiving very low levels of damage and a few plants receiving high levels (*13*). Although there are limited data on the drivers and consequences of this variability, theory indicates that within-species variation in traits or interactions can be as important as the mean for biological processes ranging from population viability to evolutionary dynamics (*14*, *15*). For example, spatial variability can stabilize plant–herbivore dynamics by giving plants refuges from overexploitation (*16*), increase the importance of competition among herbivores (*17*), maintain diversity by facilitating the evolutionary coexistence of alternative strategies (*18*), and drive disease dynamics by causing superspreading events (*19*). Variation in damage among plant individuals also indicates the potential pattern of selection by herbivores, which drives plant defense evolution (*20*). Despite the central role that variability likely plays in the ecology and evolution of plants and herbivores, macroscale patterns in the variability of this key interaction remain uncharacterized. Here we propose and test three hypotheses for patterns in the magnitude of variation in herbivore damage among individuals within plant populations.

First, we hypothesize that herbivory variability increases with distance from the equator, owing to shorter growing seasons and less stable abiotic conditions at higher latitudes reducing time available for herbivores to move and feed. A latitudinal gradient in the variability of herbivory could help explain how herbivores have influenced global patterns of plant evolution, despite the weak latitudinal gradient in mean herbivory (*21*, *22*). Second, we hypothesize that herbivory is more variable among small plants than large plants because small individuals are more likely to escape herbivory entirely or receive substantial damage in a single event, owing to their small size. In contrast, large individuals are more likely to receive damage levels close to the population mean because their large size averages over within-plant spatial variation in herbivore pressure, reducing the probability of extreme values. If supported, this pattern would expand our understanding of long-studied differences in chemical defenses between trees and herbs (*17*), potentially explaining why trees produce higher concentrations of defensive compounds (*24*). Third, we hypothesize that variability in herbivory is phylogenetically structured, with more closely related plants displaying more similar levels of variability. This pattern, which has been documented for mean herbivory (*5*), would indicate that variability is influenced by species-level traits, and is not simply random as it has often been treated.

To characterize macroscale patterns in population-level mean and variability in herbivory, 127 research teams in 34 countries used a standardized protocol to sample plants and quantify aboveground herbivore damage for 790 populations of 503 species in 135 families. This sample comprised more than 50,000 plant individuals distributed across six continents and 116° of latitude. Past macroscale studies that have focused on differences in means typically examined relatively few individuals per population (*5*). In contrast, we sampled 60 individuals per population, allowing us to analyze patterns in population-level variability. For each plant individual, we recorded plant size (height for most species or canopy diameter for prostrate species) and visually estimated the proportion of leaf tissue damaged by invertebrate and vertebrate herbivores. We quantified the variability in herbivory among individuals within populations using the Gini coefficient, a commonly used scale-invariant metric that ranges from 0–1 (perfectly even to perfectly uneven) (*25*). We tested our hypotheses by quantifying associations between each macroscale factor and the Gini coefficient or mean herbivory using Bayesian phylogenetic beta regressions.

Overall, within-population variation in herbivore damage was very high (mean Gini coefficient = 0.61; 95% CI: 0.40–0.78; Fig. 1). On average, the most-damaged individual in each plant population had herbivory on 34.2% (32.4–36.0%) of its leaf area, while 27.9% (25.9–29.9%) of individuals completely or essentially escaped herbivory (< 0.5% damage). Indeed, half of the damage in each population was concentrated on 11.3% (10.7–11.9%) of its individuals on average. The level of variation within populations also varied significantly across populations and species, with the Gini coefficient ranging from 0.03, an almost perfectly even distribution of damage, to 1.0, a perfectly uneven distribution with all damage on one plant (Fig. 1B–C).

Geographic patterns of variability

We found strong support for the latitudinal variability gradient hypothesis (Fig. 2A–B). Variation was lowest at the equator (Gini = 0.51 [0.33–0.69]) and increased towards 70° N/S (Gini = 0.70 [0.54–0.84], R^2 = 5%, p_p = 1.0, BF = 2.0e4). Mean herbivory, in contrast, declined with latitude, from 8.0% (4.1–12.3%) at the equator to 2.9% (1.4–4.7%) at 70° N/S; this relationship was less predictable than the one for the Gini coefficient (R^2 = 2%, p_p = 1.0, BF = 2.9e4, Figs. 2C and S1–S2, Tables S1–S3). Thus, plants at higher latitudes, which have shorter growing seasons and lower temperatures (Supplementary Materials) receive less herbivory on average, and that herbivory is concentrated on fewer individuals. This result could conceivably be an artifact of the Gini coefficient, which while normalized by the mean can nevertheless be correlated with it. We therefore repeated our analysis with mean herbivory included as a covariate. The estimated latitudinal variability gradient was still strongly positive, though lower in magnitude, with a 20% (6–38%) increase in the Gini coefficient from the equator to 70° N/S (R^2 = 23%, p_p = 1.0, BF = 14.5, Fig. S3). This relationship captured differences among biomes: higher latitude and higher elevation biomes had higher Gini coefficients and lower mean herbivory (Fig. 2D, Fig. S4). While there was a negative correlation between the mean and Gini coefficient among biomes ($\boldsymbol{\rho}$ = -0.68 [-0.95 – -0.10]), there were also large differences in the Gini coefficient between biomes with similar mean herbivory. This suggests that interaction variability could be a fundamental characteristic differentiating biological systems across macroscales.

Debate over the contribution of herbivory to global patterns of plant evolution has been contentious (*3*, *6*, *8*, *10*, *21*, *22*). Our data are the strongest evidence to date of a meaningful, although noisy, latitudinal decline in mean levels of herbivore damage. They also show that herbivory becomes more variable with increasing latitude. This pattern may explain how herbivores contribute to the latitudinal gradient in plant diversity despite the noisy mean herbivory gradient (*8*, *21*). Herbivory may speed plant evolution at low latitudes not just by being more intense on average, but also by being more consistently important within a plant population. Indeed, theory predicts that the relationship between the strength of antagonistic interactions and the intensity of selection is concave-down (saturating) at low mean interaction strengths (*26*), meaning that the variability at high latitudes, where mean herbivory is low, should erode selection via nonlinear averaging (*14*), depending on the empirical relationship between herbivore damage and plant fitness. Our finding is also consistent with the hypothesis that inducible defenses are more common among temperate than tropical plants (*27*, *28*) since greater variation in herbivory is predicted to select for inducibility (*29*). In addition to seasonality and climate, other mechanisms for the latitudinal variability gradient could include greater predation pressure on herbivores at low latitudes (*3*) supressing localized outbreaks and high tropical herbivore diversity and specialization (*30*) evening out damage patterns across plant individuals. More generally, our results confirm the long-standing view that biotic interactions are more consistent in the tropics, perhaps owing to longer growing seasons or greater species diversity and specialization (*3*).

Variability and plant size

We also found strong support for the plant size hypothesis. Populations of larger individuals exhibit less variability in herbivory among individuals. A 2 m increase in mean plant size (from 0.05–2.05 m, encompassing ~90% of our populations) resulted in a 32.7% (20.6–44.7%) decrease in the Gini coefficient (from 0.70 [0.54–0.85] to 0.47 [0.29–0.66], R^2 = 13.3%, p_p = 1.0, BF = 4.6e7, Figs. 3A and S5). This relationship held even after accounting for the decline in plant size with increasing latitude and differences in plant abundance (Tables S4–S5) (*31*). Woody species, which averaged 4.1 times larger than herbs in our dataset, had 10.9% (2.9–19.1%) lower Gini coefficients than herbaceous species (0.56 [0.37–0.76] vs. 0.63 [0.44–0.81], BF = 4.25); the overall variance explained by growth form, including climber and graminoid categories, was low (R^2 = 2.8%, Figs. 3B and S7), suggesting that mean size is a more important determinant of herbivory patterns than growth form. Mean herbivory, in contrast, was unrelated to mean size or growth form (Figs. S6 and S8).

We posit that higher among-plant variability in herbivory on small plants results from the law of large numbers, which tells us that processes that involve more random events tend to lead to values closer to the overall mean. In other words, large plants, which have a greater number of potential herbivory events, essentially average over within-plant variability in herbivory and receive values closer to the population mean on average. Small plants, in contrast, are more likely to escape herbivory entirely or be severely damaged by a few events, resulting in high variability. A key implication of this phenomenon is that larger species (and larger stages within species) should experience selection for high concentrations of constitutive defenses or tolerance. Smaller species (and stages), in contrast, should experience selection for inducible defenses and low concentrations of metabolically cheap toxins to save resources in the absence of herbivory and repel herbivores when encountered. This dichotomy in defense evolution has been the focus of decades of research on differences in defenses between trees and herbs (*23*) and across ontogenetic stages (*32*). Whereas previous work has invoked complex biological explanations for these differences, such as how “apparent” plants are to herbivores (*23*), our results suggest patterns are more simply explained by the statistical consequences of mean plant size.

Phylogenetic patterns of variability

Finally, we tested the hypothesis that variability in herbivory is phylogenetically structured. The Gini coefficient exhibited significant phylogenetic signal (Pagel's $\lambda = 0.51$ [0.45–0.52], $P < 0.001$), indicating that more closely related species display more similar variability (Figs. 4 and S9). Mean herbivory, in contrast, did not show meaningful phylogenetic signal ($\lambda = 0.07$ [0.06–0.08], $P = 1.0$). These results were robust to tree topology and species sampling (Supplementary Materials). Our findings suggest that the mean damage level across species changes relatively rapidly in response to evolutionarily labile plant traits, whereas the variability is more strongly determined by traits that are phylogenetically conserved. Indeed, traits thought to influence the amount of herbivore damage, such as chemical defenses, diverge as plants escape their herbivores by evolving novel defenses (*2*, *33*), whereas characteristics such as geographic location and plant size, which we find relate to variability, tend to be less labile. High variability in some families (e.g., Apocynaceae and Plantaginaceae) invites further investigation and could help reveal drivers of these conserved patterns. To examine macroevolutionary patterns, we fit Brownian motion and Ornstein-Uhlenbeck models to test for differences in rates of evolution and the strength of stabilizing selection. The best-fitting models included optima for variability and mean herbivory in tropical vs. temperate systems and woody vs. herbaceous growth forms (Tables S6–S7), indicating that the evolution of variability in herbivory seems driven by conserved plant traits and therefore is a biologically informative feature rather than random noise.

Conclusion

The assumption that plant–herbivore interactions are highly variable has long dominated ecology and evolution, with foundational works on "variable plants and herbivores" (*12*) and theory exploring the consequences of variable herbivory (*29*). Our data confirm this assumption but also reveal a pattern that has not been previously documented: strong differentiation across systems in the level of variability itself. Variation in herbivory covaried with factors central to the ecology and evolution of plant–herbivore interactions such as latitude, biome, plant size, and phylogeny. These macroscale patterns were often stronger than patterns for mean herbivory levels. This suggests that the level of variability could be important for driving differences in plant–herbivore biology around the planet, between species with different traits, and across phylogeny. While the importance of variability in interactions has been recognized by a few fields, such as epidemiology (*19*), the central role of interaction variability in shaping macroscale patterns of life on Earth has been underappreciated. Our global dataset is evidence for the ubiquity and predictability of variability in one biotic interaction and highlights the promise of further explorations of the causes and consequences of interaction variability.

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Competing interests: Authors declare that they have no competing interests.

Data and materials availability: The dataset generated and analyzed in the current study is available at Data Dryad (34). Our code is archived at Zenodo (35).

Supplementary Materials

Herbivory Variability Network Authors

Materials and Methods

Supplementary Text

Figs. S1 to S9

Tables S1 to S7

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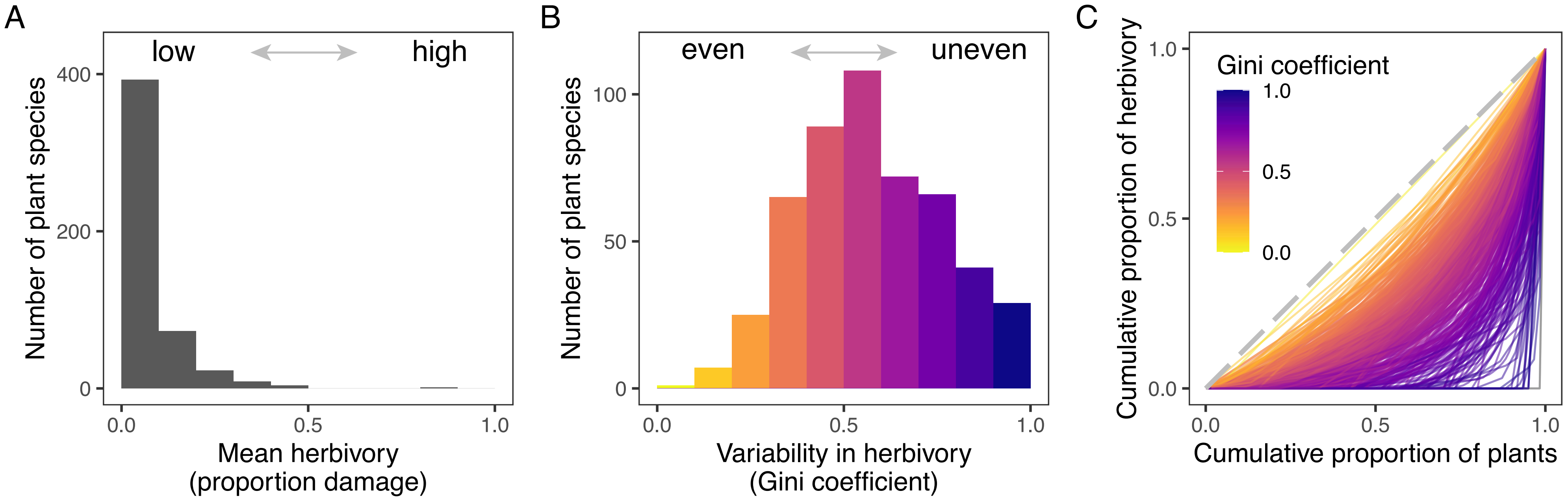
Fig. 1. Mean and variability in plant-herbivore interactions. (**A**) Histogram of the number of plant species with different mean proportion leaf area damaged by herbivores. (**B**) Histogram of the Gini coefficient values for all plant species in our dataset. (**C**) Lorenz curves from all 790 population surveys in our dataset. Each curve shows the cumulative proportion of herbivory across the cumulative proportion of plants, ordered by increasing herbivory, for one plant population. Curves closer to the 1:1 line (gray dashes) indicate more even distributions. Lorenz curves form the basis for the calculation of the Gini coefficient of inequality, which ranges from 0 (a perfectly even distribution) to 1 (a perfectly uneven distribution). Curves are colored by their Gini coefficient (as in 1b). Sample sizes are 790 surveys of 503 plant species.

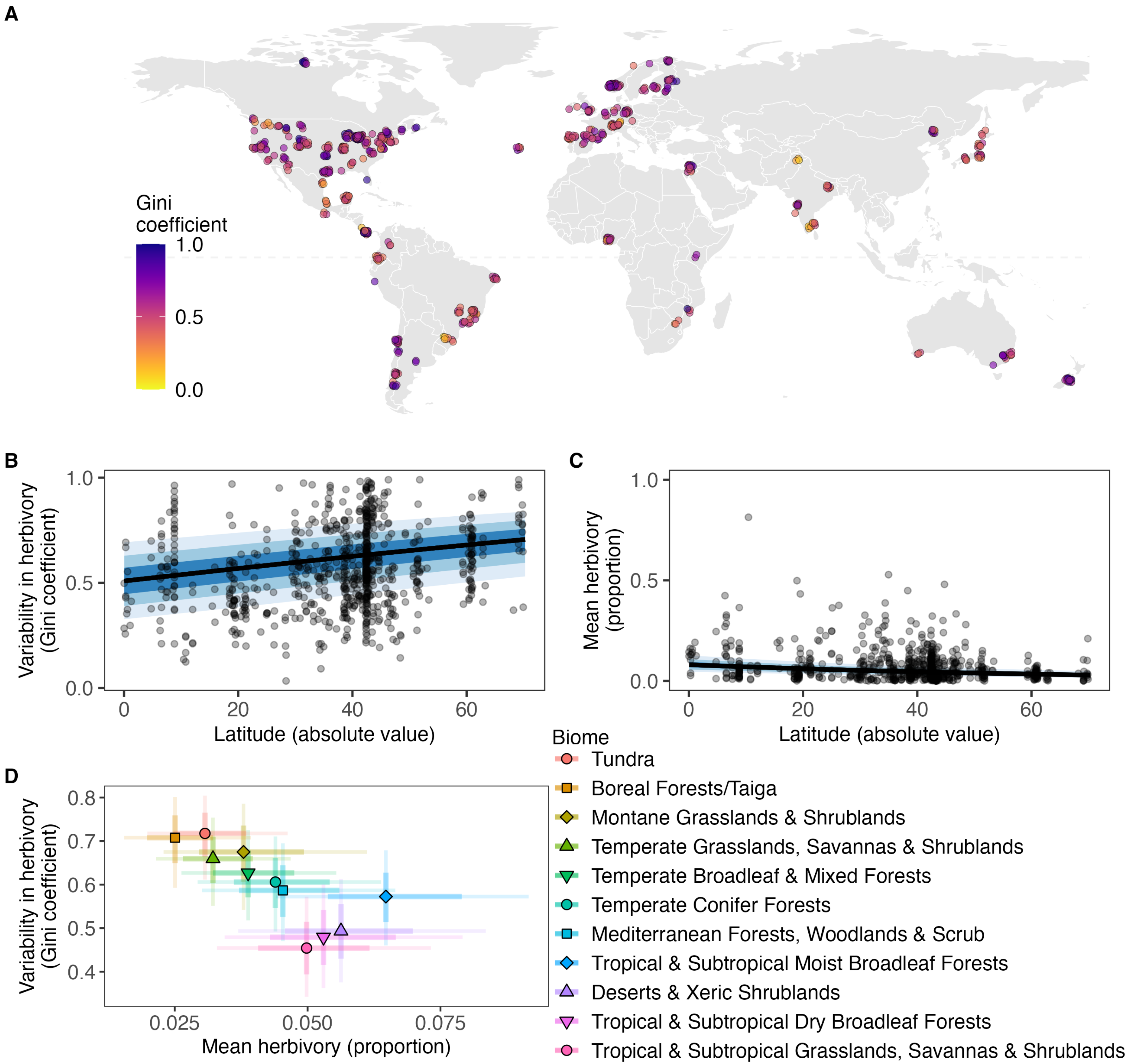
Fig. 2. Global patterns of variability in herbivory within plant populations. (**A**) The geographic distribution of our sampling sites, colored by variability in herbivory among individuals within populations (Gini coefficient). Points are slightly jittered for visibility. (**B**–**C**) Variability in herbivory increased and mean herbivory decreased with latitude across our sampling extent. Lines show predicted means and 50, 80, and 95% credible intervals from Bayesian phylogenetic beta regressions. (**D**) The 11 biomes in our study can be characterized by their mean and variability in herbivory. Herbivory variability and mean showed an inverse relationship across biomes ($\boldsymbol{\rho}$ = -0.67 [-0.94 – -0.08]), but there were also differences in variability between biomes with similar means. Error bars show 50 and 80% credible regions. Sample size is 790 surveys of 503 species. Legend in (D) is ordered by Gini coefficient.

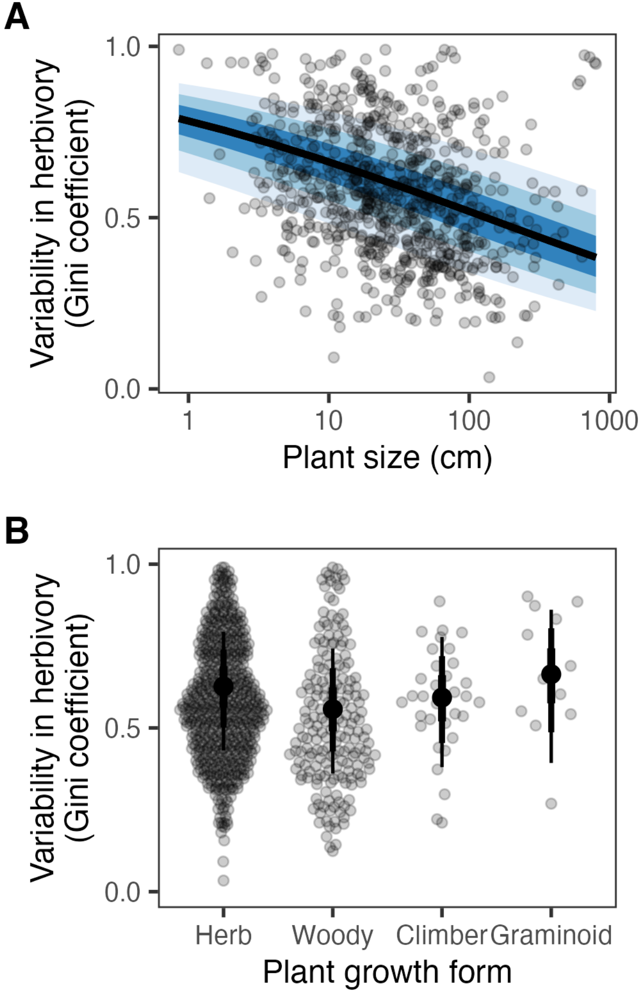
Fig. 3. Plant size shapes variability in herbivory. (**A**) Variability in herbivory among individuals within populations declines with the average size (height or canopy diameter for prostrate species) of plants in the population (R^2 = 13.3%, p_p = 1.0, BF = 4.6e7; 735 surveys of 472 species). (**B**) Variability in herbivory, however, is only weakly related to plant growth form (R^2 = 2.8%), with woody plants having 10.9% (2.9–19.1%) lower Gini coefficients than herbaceous species (790 surveys of 503 species). Lines, shaded regions, and large points show predicted means and 50, 80, and 95% credible intervals from phylogenetic Bayesian beta regressions. Each small grey point is one survey.

Fig. 4. Phylogenetic patterns of mean and variability in herbivory. Variability in herbivory among plants within populations (Gini coefficient) show greater phylogenetic signal (Pagel's λ = 0.51 [0.45–0.52], $P < 0.001$) than mean herbivory levels (Pagel's λ = 0.07 [0.06–0.08], $P > 0.1$). For clarity, this tree includes only the 240 species from the 11 best-represented plant families (≥ 8 species per family). Our analyses included all 503 species in the dataset (see Fig. S9 for the full tree).

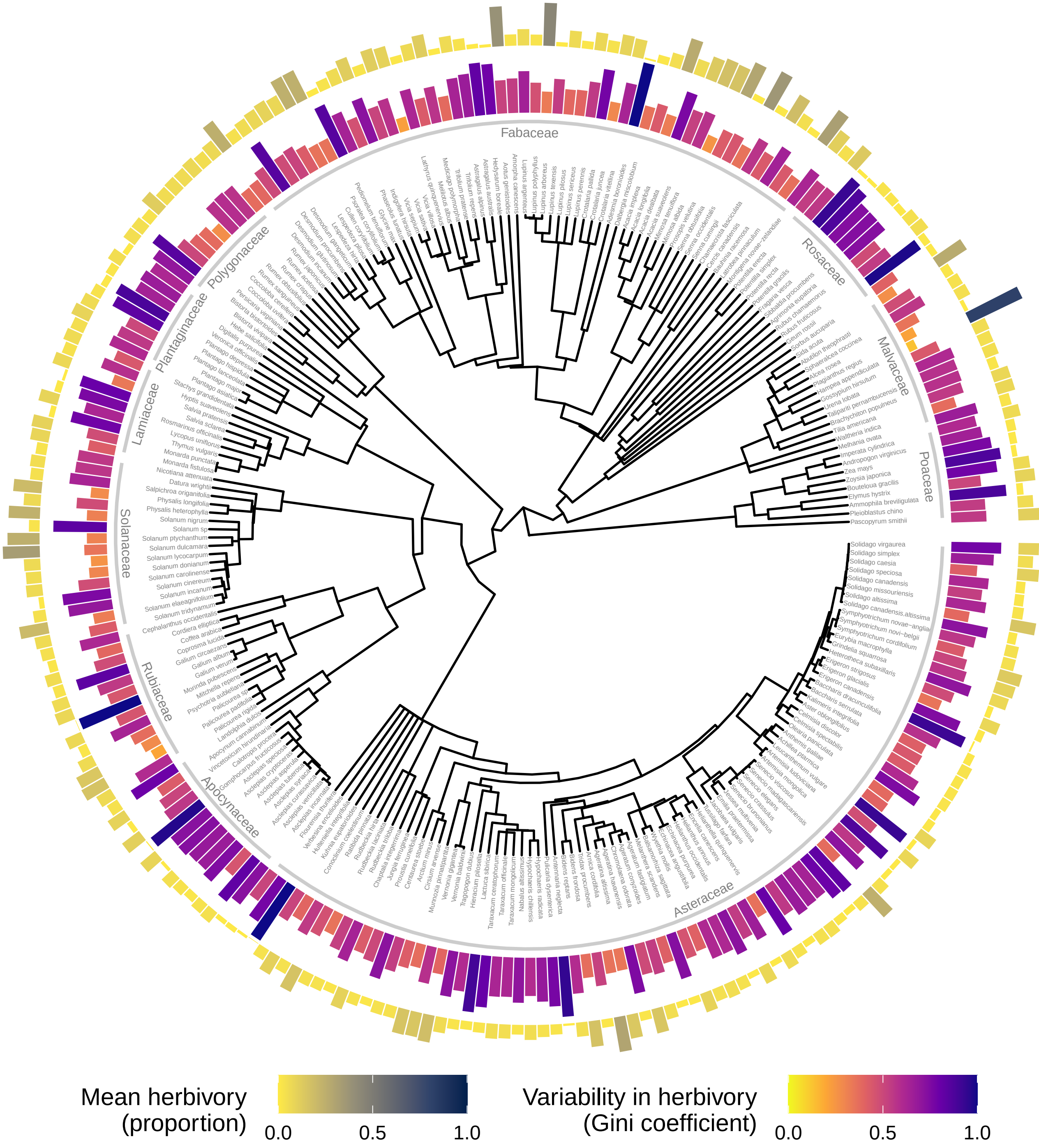


A
low
high
400
200
0
0.0
0.5
1.0
Number of plant species
Mean herbivory
(proportion damage)
B
even
uneven
100
50
0
0.0
0.5
1.0
Number of plant species
Variability in herbivory
(Gini coefficient)
C
Gini coefficient
1.0
0.5
0.0
1.0
0.5
0.0
0.0
0.5
1.0
Cumulative proportion of herbivory
Cumulative proportion of plants


A
Gini coefficient
1.0
0.5
0.0
B
Variability in herbivory (Gini coefficient)
1.0
0.5
0.0
0
20
40
60
Latitude (absolute value)
C
Mean herbivory (proportion)
1.0
0.5
0.0
0
20
40
60
Latitude (absolute value)
D
Variability in herbivory (Gini coefficient)
0.8
0.7
0.6
0.5
0.4
0.025
0.050
0.075
Mean herbivory (proportion)
Biome
Tundra
Boreal Forests/Taiga
Montane Grasslands & Shrublands
Temperate Grasslands, Savannas & Shrublands
Temperate Broadleaf & Mixed Forests
Temperate Conifer Forests
Mediterranean Forests, Woodlands & Scrub
Tropical & Subtropical Moist Broadleaf Forests
Deserts & Xeric Shrublands
Tropical & Subtropical Dry Broadleaf Forests
Tropical & Subtropical Grasslands, Savannas & Shrublands



A
Variability in herbivory (Gini coefficient)
1.0
0.5
0.0
1
10
100
1000
Plant size (cm)
B
Variability in herbivory (Gini coefficient)
1.0
0.5
0.0
Herb
Woody
Climber
Graminoid
Plant growth form


Fabaceae
Rosaceae
Malvaceae
Poaceae
Asteraceae
Apocynaceae
Rubiaceae
Solanaceae
Lamiaceae
Plantaginaceae
Polygonaceae
Mean herbivory
(proportion)
0.0
0.5
1.0
Variability in herbivory
(Gini coefficient)
0.0
0.5
1.0

Science
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Supplementary Materials for

Plant size, latitude, and phylogeny explain variability in global herbivory

Herbivory Variability Network

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Materials and Methods

Species and site selection

The Herbivory Variability Network (HerbVar) is a research coordination network of researchers from 34 countries that aims to better understand the role of variability in the ecology and evolution of plant–herbivore interactions. We maximized the geographic and phylogenetic breath of our dataset for this paper by prioritizing sampling new sites and species from families and clades not yet sampled by us. Using those goals, 127 research teams selected species and sites with which they had familiarity, allowing expert assessment. This effort resulted in 790 population-level surveys encompassing 503 plant species from 135 plant families across 34 countries and six continents. Across the 135 plant families, we sampled a median of 2 species per family, though we had five families with more than 20 species (Asteraceae, Fabaceae, Plantaginaceae, Polygonaceae, and Solanaceae). Of the 503 plant species, 415 (83%) were surveyed once, but three plant species (*Plantago lanceolata*, *P. major*, and *Taraxacum officinale*) were surveyed more than 10 times each. Separate surveys of the same species were grouped in analyses via random effects (see below).

Field surveys

For each species and site, members surveyed herbivory and other ecological variables using a standardized protocol developed collaboratively by the network. Whereas past macroscale herbivory studies had small sample sizes within species and populations, our protocol established a target of 60 individuals within each population survey, facilitating robust estimation of variation and other patterns within populations. The full protocol can be found in the Supplementary Text (below) or at The Herbivory Variability Network's website (https://herbvar.org). In brief, for each survey, we recorded the geographic coordinates of the site's origin and used randomized transect and subtransect distances to select 30 individuals and each of their nearest conspecific neighbors, for a total of 60 individuals sampled. For small populations (< 90 individuals), we exhaustively surveyed all individuals in the population, rather than randomly selecting 30 individuals. Surveys in our final database had an average of 66 (±2 SE) individuals per population, with a mode of 60, though some surveys had as few as 21 or as many as 869 individuals.

For each of the plant individuals within a survey, we quantified aboveground herbivory by visually estimating the proportion of surface area of leaves and other photosynthetic tissue damaged by herbivores. We included all visible herbivory, including invertebrate and vertebrate damage and chewing damage, mining, and visible sucking damage. We standardized visual estimates of herbivory across researchers by disseminating a detailed guide to visually estimating herbivory, providing a printable template that researchers could take to the field, and by having researchers undertake online training before going to the field including the ZAX Herbivory Trainer (*36*). For individuals under 2 m tall, we visually estimated herbivory by examining all aboveground tissue. Because this would not be feasible for individuals over 2 m tall, we randomly sampled 30 leaves per plant, estimated proportion herbivory on each leaf, and averaged those values to estimate whole plant proportion herbivory. Finally, we recorded the size of each individual by recording the linear dimension that best represented the size of individuals of that species, height for most species but canopy diameter for others (e.g., prostrate species). Our final dataset included plants with an average size (height or diameter) of 0.85–799.8 cm. All data were uploaded to a repository hosted by Michigan State University, where a data team checked the data for consistency, integrated them into the database, and prepared them for analysis.

Response variables

Our analyses focused on two response variables, the amount of variation in herbivory among individuals within a population and the mean herbivory within a population. We summarized variation in herbivory across individuals within populations by calculating the sample-size corrected Gini coefficient of variation in proportion aboveground herbivory among individuals in each population using R package DescTools (*37*). The Gini coefficient is a widely used metric that represents the level of variation or unevenness of a distribution of a variable among units. It is analogous to the more widely known coefficient of variation except calculated with L-moments instead of conventional moments, making it more robust to outliers and more reliable at small sample sizes (*38*). The Gini coefficient has been used extensively in ecology, including recent work describing the distribution of abundances and changes in abundances among species within communities (*39*, *40*) and the distribution of size hierarchies in plant populations (*41*). Although it is normalized by the mean, the Gini coefficient, all else equal, typically displays a negative relationship with the mean, like the coefficient of variation. Thus, in addition to models quantifying total variation in the Gini coefficient, we also accounted for this relationship and asked if variation in the Gini coefficient could be related to variation in the mean by adding mean herbivory to models as a covariate.

Our second response variable, mean herbivory, allowed us to ask what factors influence the average or relative total herbivory across individuals and to examine how they complement or differ from factors that influence variability. For mean herbivory, we averaged the proportion of aboveground herbivory across all individuals surveyed in a population. An alternative metric for describing the center of a distribution is the median. In our dataset, mean herbivory and median herbivory, however, had a correlation of 0.964 across species. Given this high correlation, we present the mean to enable comparison with past studies, which all report patterns in mean herbivory.

Site- and species-level predictors

In addition to latitude, we used the geographic coordinates of each site to extract the site's biome type using a 2017 global assessment of biomes (*42*). Our surveys spanned 11 biomes. Nine of those contained at least 25 surveys: desert and xeric shrublands; Mediterranean forests, woodlands and scrub; temperate broadleaf and mixed forests; temperate conifer forests; temperate grasslands, savannas and shrublands; tropical & subtropical dry broadleaf forests; tropical and subtropical grasslands, savannas and shrublands; tropical and subtropical moist broadleaf forests; and tundra. Two biomes, boreal forests/taiga and montane grasslands and shrublands, contained nine and eight surveys, respectively.

In our data set, latitude is strongly correlated with many temperature variables from the bioclim database (mean annual temperature: $r = -0.50$, $p < 0.001$; temperature seasonality: $r = 0.63$, $p < 0.001$; annual temperature range: $r = 0.55$, $p < 0.001$), and to a lesser degree with precipitation (mean annual precip: $r = -0.22$, $p < 0.001$; precipitation seasonality: $r = -0.32$, $p < 0.001$).

For each plant species in our dataset, we determined growth form by recording them in the field or extracting them from the literature (*43–45*). Plant species were grouped into one of four categories: herb/forb (306 species); woody shrub/tree (157); climber (using other plants for physical support, 29 species); and graminoid (grasses and sedges, 11 species). Climbers included both herbaceous vines and woody lianas. Species that could span multiple categories were placed in the best-fitting category based on species descriptions in the literature.

<u>Statistical modeling</u>

We modeled our response variables – mean herbivory and variability in herbivory among plant individuals within a population – as a function of our predictors using Bayesian phylogenetic generalized linear mixed models in R in the brms package, which uses Hamiltonian Monte Carlo estimation via the Stan platform (*46–48*). We used a beta response distribution because it is well suited to represent variables on the 0–1 interval (*49*). We accounted for correlations among surveys of the same species and phylogenetic correlations among plant species by including random effects for plant species and phylogeny, based on a phylogenetic covariance matrix built using a phylogenetic tree of our species (see below for phylogenetic methods). We modeled differences across biomes by using the Gini coefficient and mean herbivory as response variables together in a single multivariate (multi-response) model, allowing us to estimate the correlation between the Gini coefficient and mean herbivory across biomes.

Because the beta distribution is undefined for 1 and three surveys had Gini coefficient values of 1, we truncated those values to 0.99 (the next highest Gini coefficient value was 0.985). This can be thought of as representing the limits to our ability to detect extremes and allows the use of beta models, thereby avoiding the need for zero/one hurdle models, which we feel is justified because we do not think that zero or one values arose from fundamentally separate processes from values close to zero or one (*49*). We log transformed plant size before using it as a predictor.

Models ran across at least seven chains for at least 40,000 iterations total, using the first half of each chain as a warm up. We assessed runs by ensuring all Rhat values were < 1.03, and visually checked fits via posterior predictive checks (*50*). We used weakly informative priors on all parameters: N(0, 2) for slopes and intercepts, gamma(1, 0.05) for the φ dispersion parameter of the beta distribution, and half-Cauchy(0, 1) for the standard deviations associated with the random effects.

For each model, we report effect sizes, 95% credible intervals (CIs), and marginal Bayesian R^2 values in the main text and parameter posteriors, 95% CIs, posterior predictive checks, and diagnostics in Figs. S1–S8. Marginal Bayesian R^2 values represent the percent of the variance in the response explained by the population-level parameters (fixed effects). For each of our directional hypotheses, we also report the proportion of the posterior on the hypothesized side of zero (p_p) and the Bayes factor (BF) estimated with the Savage-Dickey density ratio, restricting the prior and posterior to the hypothesized side of zero. Values of p_p closer to one indicate stronger support. BF values greater than one can be interpreted as evidence against the null, with higher values indicating stronger evidence.

When examining latitudinal gradients, we first asked whether the relationship between latitude and herbivory (mean herbivory or variability in herbivory) differed between the Northern and Southern Hemispheres by testing for an interaction between latitude and hemisphere. Neither the Gini coefficient nor the mean showed a significant interaction with hemisphere (Tables S1–S2). Moreover, Gini coefficients and means were similar on average in the Northern and Southern Hemispheres ($Gini_{Northern}$ = 0.58 [0.22–0.95], $Gini_{Southern}$ = 0.54 [0.21–0.90]; $mean_{Northern}$ = 0.070 [0.00–0.29], $mean_{Southern}$ = 0.070 [0.00–0.29]). Because of the similarity of these patterns north and south of the equator, we used the absolute value of latitude (degrees from equator) as our predictor variable for latitude in all analyses. We examined the potential for latitudinal differences in plant abundance and plant size to drive the latitudinal gradient in herbivory variability by re-fitting our latitudinal model of the Gini coefficient with either focal plant abundance (percent cover) as a covariate (Table S3) or mean plant size as a covariate (Table S4). We also examined the potential for differences in focal plant abundance to drive the plant size–variability relationship by re-fitting our plant size model with focal plant abundance (percent cover) as a covariate (Table S5).

Patterns of herbivory mean and variability across the plant tree of life

We generated a phylogenetic tree for the 503 plant species in the dataset using R package V.PhyloMaker2 (*51*). This method uses the most recent dated phylogenies for both seed- and spore-bearing plants to infer the largest dated mega-tree of vascular plants available (*52*, *53*). This megatree was then pruned to match a list of provided taxa. If taxa were missing from the megatree they were bound to the node of a congener or, if no congeners were present, to the ½ or upper ⅓ point of the family branch (V.PhyloMaker2 scenario S3). In our dataset, 338 species were present in the megatree, and 165 required binding. Of bound species, 134 had a congener in the megatree, and were thus bound at the genus level; the remaining 31 species did not have a congener in the megatree and were bound at the family level.

We estimated phylogenetic signal in the Gini coefficient and mean herbivory using Pagel's λ in R package phytools (*54*). Both the Gini coefficient and mean herbivory were logit-transformed prior to analysis (*49*). To account for uncertainty in tree inference, we estimated phylogenetic signal for mean and variability in herbivory in a distribution of 1000 trees with different placement of missing taxa. For each tree, missing taxa were bound to a random node at or below the corresponding genus or family-level node in R package V.PhyloMaker2 (scenario S2) (*51*). We report the mean and 95% CI for λ across this distribution of trees, as well as percent of trees with significant phylogenetic signal. We also tested the sensitivity of λ to species sampling effects in two ways. First, we ensured that sparse sampling within some families was not driving our results by re-running our analyses on a tree pruned to families with $\geq$ 8 species (11 families and 240 species). Second, we quantified phylogenetic signal after resampling trees 1000 times with random exclusion of 10–50% of species using R package sensiPhy (*55*) (see Supplementary Text below).

We also fit different macroevolutionary models to our data to explore whether the evolution of herbivory (Gini coefficient and mean) could be modeled as driven by plant growth form (herbaceous vs. woody; woody includes woody shrubs and trees, but vines were not considered) or biome affinity (temperate vs. tropical; temperate: latitude $\leq$23°; tropical: latitude >23°). The subset of dataset for this analysis had 306 herbaceous species and 157 woody species and 638 temperate surveys and 152 tropical surveys We implemented models that considered herbivory evolving under a Brownian Motion dynamic in which the rate of evolution (σ^2) parameter was shared across trait states (BM1 model) or allowed to vary depending on the state of the trait being examined (BMS); that is, whether herbivory evolved at different rates in herbaceous and woody (or temperate and tropical) taxa. We also examined models to explicitly evaluate whether (variability or mean) herbivory is evolving under a regime that pulls with strength α towards one or many evolutionary optima (parameter θ), known collectively as Ornstein-Uhlenbeck models. Of these, we only considered OU1 models (single evolutionary optimum θ while keeping rate and strength of pull towards the optimum as constant), and OUM models, which allow for herbivory to evolve towards different optima, depending on the state of the trait (i.e, herbaceous vs. woody, or tropical vs. temperate). We did not consider models that allow multiple rates or strengths of pull towards the optima because they did not produce reliable parameter estimates for our data. To implement these models, mean herbivory data were logit-transformed.

For each plant character trait, we mapped its evolution onto a phylogeny using continuous-time reversible Markov models (*56*), evaluated models of evolution, and iterated this process across 100 phylogenies to account for uncertainty in both trait mapping and phylogeny estimation. Stochastic maps of trait evolution were generated with the *make.simmap* function in the 'phytools' R package (*54*). Plant phylogenies were derived from a megatree (*52*) with the 'V.Phylomaker2' R package (*51*). Because some taxa were not present in the megatree, we bound missing tips to randomly selected nodes in respective genera or families with each iteration (V.PhyloMaker2 scenario 2).

All models of evolution were implemented using the 'OUwie' package in R (*57*). The algorithm was set to "invert" for which all models converged and reached a reliable solution. To compare models, we used the average BIC across the 100 iterations described above (Tables S6 and S7).

Sample size sensitivity analyses

While our target sample size within each survey was 60 plant individuals, some surveys had fewer than 60 individuals due to logistical constraints. Other surveys reached more than 60 surveys. We ensured that our results were not influenced by differences in within-survey sample size using a sampling procedure and rerunning our analyses. First, we excluded 14 of our 790 surveys with fewer than 30 individuals. Next we sampled 30 plant indivudals from each survey without replacement. We repeated this 100 times, giving us 100 replicate datasets with 30 plant individuals per survey. Finally, we repated our analyses for each of these 100 replictate datasets, including calculations of mean herbivory and the Gini coefficient, phylogenetic generalized linear mixed models, and other phylogenetic analyses. We present the results of this sensivity analysis in the Supplementary Text.

Gini asymmetry coefficient

Whereas the Gini coefficient describes the amount of variation or uneveness of the distribution of a variable among units within a population, the Gini asymmetry coefficient, a metric designed to supplement the Gini coefficient describes the contribution of individuals with low or high values to the observed Gini coefficient value. The Gini asymmetry coefficient is thus an additional descriptor of the shape of variation among individuals within a population. When the Gini asymmetry coefficient is less than one, it indicates that a distroportionately high number of individuals with low values contributes to observed unevenness. When the Gini asymmetry coefficient is greater than one, it indicates that individuals with disproportionately high herbivory contribute most to uneveness. Finally, when the Gini asymmetry coefficient is close to one, it indicates that individuals with low values and individuals with high values contribute similarly to observed unevenness and that the Lorenz curve (Fig. 1C) is symmetric.We calculated the Gini asymmetry coefficient using the ineq package in R and examined its relationship with latitude and plant size using Bayesian phylogenetic linear mixed models. We present these results in the Supplementary Text (below).

Supplementary Text

Field survey protocol

A protocol for quantifying variability in plant–herbivore interactions
HerbVar: A collaborative network studying global patterns of variability in herbivory

1. Motivation:
Published studies and personal observations suggest the distribution of herbivore feeding damage among individual plants within a population is often highly skewed such that most plants experience relatively low levels of damage, and a small fraction of plants experience disproportionately high levels of damage. Theory suggests that such variability can have dramatic ecological and evolutionary consequences. For example, variability among plants can lead overall herbivore population size to be greater or less than expected based on average plant quality and asymmetric fitness surfaces can lead to over-investment in defensive traits. Surprisingly, despite the theoretical importance and potential generality of variability in herbivory, it has received little empirical attention, limiting our fundamental understanding of how plants and herbivores interact.

We are forming a global collaboration to quantify the distribution of herbivory for diverse plant species in multiple ecosystems across the world. The goal of this work is (1) to assess if variability in herbivory is indeed a common feature of plant–herbivore interactions, and (2) to examine how the amount of variability and skew varies with key ecological and evolutionary factors. Quantifying general patterns in the distribution of herbivore damage within populations would be a major contribution to our fundamental understanding of herbivory. In addition, identifying the factors that relate with variability in herbivory would provide the field with a new paradigm for describing plant–herbivore interactions and allow us to generate novel hypotheses about the ecology and evolution of plant–herbivore interactions.

2. Project goals:
1. Quantify the within-population distribution of plant damage and herbivore density across many systems
2. Quantify how within-population distributions of damage and herbivore density differs across
    a. Plant species
    b. Plant functional traits (from literature)
    c. Latitude
    d. Plant ecology (e.g., rarity)
    e. Herbivore species
    f. Herbivore functional groups
    g. Ecosystem type
    h. And many other potential factors (e.g., seasonality, precipitation...)

3. Overview:
Below, we provide a straight-forward and broadly applicable protocol to achieve these goals. This is the Primary HerbVar Survey Protocol. In brief, 30 randomly-selected plant individuals in a site (~population) are surveyed for herbivore damage and (possibly) herbivore abundance. Data

are also collected on the nearest conspecific neighbor of each plant (for a total of N = 60 plants). These methods yield estimates of variability, skew, and spatial patterns (e.g., autocorrelation) in herbivore damage.

The HerbVar Primary Survey Protocol is designed to work for many common plant growth forms and contexts, so we expect most surveys to use this protocol. The primary protocol, however, will not work for every plant growth form or context, so HerbVar has multiple alternative survey protocols. Alternative protocols can be found in the shared Drive in the “Alternative protocols” folder. These include protocols for surveying plants with low density or abundance, mature trees, cacti and other succulents, reproductive (flower/fruit/seed) damage, and vertebrate browsing damage, as well as an optional insect sampling protocol. If the primary protocol is not feasible for a species or site, then we suggest one of these alternative protocols. If none of these alternative protocols fits the situation, then collaborators may deviate from the primary protocol. We trust collaborators to decide how to adapt the primary protocol in ways that works for their systems. We suggest, however, that collaborators strive to follow the spirit of the protocol below: randomly select at least 30 plants from a site and census them and their nearest neighbors for herbivory and herbivore data. For a dataset to be usable in the overall study, it will have to be comparable to data collected using this protocol. Collaborators who deviate from the HerbVar protocols should carefully record their methods.

The primary protocol works best for sites with at least ~90 plant individuals, such that it makes sense to sample individuals randomly. If your site has fewer than ~90 individuals of your plant species, then please consider comprehensively censusing all individuals within the site as suggested in our document on surveying low-density/low-abundance sites. A comprehensive census, when feasible, would be even better than the protocol below. If plants are far enough apart, please take GPS coordinates for each plant. If a comprehensive census is not feasible, then please modify the primary protocol or the low-density/low-abundance guidelines to work efficiently with your species and site. Please reach out to the HerbVar coordinators if you have questions or want to check that your modifications will lead to adequate data.

4. The Primary HerbVar Survey Protocol:
There is a template data sheet for this protocol, and example of a completed datasheet in the HerbVar shared Google Drive

- Pick a plant species (see “6. Guidelines for selecting plant species” below)
- Pick a site (see “7. Delineating a site” below for advice)
- Pick a time to sample (see “8. When to Sample” below for advice)
- Calculate a ‘custom’ radius for circular quadrats. We developed the following method to create quadrat sizes specific to each plant species and site, given that plant size and density vary immensely. This approach seeks an optimal, intermediate quadrat size that balances the costs associated with a small quadrat size (many empty quadrats) and a large quadrat size (quadrats that require counting many plant individuals).
    - Estimate mean density of plants per square meter by counting the number of plants in 1 m^2 at 10 random locations within the site; calculate mean density (D)
    - Use D to calculate a circular quadrat radius (r) that would on average contain 4 plants:
        - $r = \sqrt{4/(\pi D)}$

- Lay a transect through the middle of the site
    - Record GPS coordinates of origin, length (m), and compass direction (degrees) of transect (need to pick a coordinate system and precision)
- Select center points of circular quadrats. Randomly select 40+ points in the site by selecting pairs of random numbers. One random number represents distance along the transect (0–length of transect); the other represents distance left or right of the transect (left=negative, 0=center, right=positive). These are the center points of quadrats.

For each quadrat:

- Locate a quadrat center point using transect and measuring tape or stick
- Count and record the number of focal plants within r meters of the center point (a circular quadrat)
- Record other quadrat level data:
    - Percent cover of focal plant (ignore non-focal species)
    - Percent cover of all non-focal plant species (ignore focal species)
        - These 2 percent covers could total more than 100% if they overlap
        - If surveying understory plants, ignore forest canopy when estimating percent cover
- If the circular quadrat has 0 plants, record a zero and continue to the next quadrat

If the circular quadrat has > 0 plants:

- Randomly choose 1 of the plants within the quadrat to survey
    - A quicker alternative would be to choose the plant closest to the quadrat center. But this is recommended only if you think it will produce an unbiased sample of plants from your site. Be careful about over-representing large and/or isolated plants (which will be closer to more points relative to small plants in crowded patches).
- Data to record for each selected plant (1 per quadrat):
    - Plant life stage: seedling, vegetative, reproductive
    - Plant size, use judgement to pick best measure for your species
        - E.g., standing plant height (ground to tallest living part), stem length, foliage diameter, stem diameter
    - Herbivore damage (see Damage estimation training document) in 3 ways:
        - (1) Presence/absence of leaf damage: *If a plant has ~60 leaves or less in total*, please record the total number of leaves on the plant, and the number of those leaves that have damage (count leaf as damaged if it has > 0.5% herbivory). *If a plant has more than ~60 leaves*, record presence/absence of herbivory on 60 randomly (arbitrarily) chosen leaves and please note you stopped at 60.
            - If plants have reproductive parts (flowers/fruits/seeds) that could have been damaged by herbivores, please see the HerbVar Flower/Fruit/Seed Damage Protocol. This is optional, but encouraged.
        - (2) Estimated percent damage on 10 randomly (arbitrarily) chosen leaves
            - One estimate per leaf (for a total of 10 estimates)
            - Ideally, chosen leaves will be representative of all leaves (e.g., sample young and old leaves in proportion to frequency on plant)

                - For leaves with herbivore-built leaf shelters (rolls and ties), please carefully peer into or open shelters to estimate damaged area and count resident herbivores
            - (3) Estimated percent damage across the whole plant, optionally also breaking apart damage by type or even species of herbivore if possible (e.g., sucking damage versus chewing damage, add columns as needed)
                - E.g., If a plant has 4 equally-sized leaves and 2 of those leaves are 50% eaten, then whole plant has 25% herbivory
                - But take leaf size into account when leaves vary in size
        - Presence of plant diseases
        - Number of leaf mines and galls per plant (= herbivory + herbivores).
            - If there is reason to believe that galls or mines have accumulated through multiple years (e.g. stem galls on woody perennials), please note this
            - If there are too many mines or galls to count individually, estimate the number per plant by tallying the number per module (e.g. stem, branch) and multiplying by number of modules
        - Optional: abundance of other externally-feeding herbivores (standardized approach; see Herbivore sampling protocol to decide if/how to collect these data)
        - Distance to nearest conspecific neighbor (where the nearest neighbor is the plant with the closest aboveground tissue to any aboveground tissue on the focal plant)

- Data to record for the first nearest conspecific neighbor of selected plant:
    - All the same data as focal plant except nothing for neighbor's neighbor
- Continue visiting the randomly selected points until ≥ 30 focal plants and 30 nearest neighbors have been surveyed

5. Methods notes:
- Modifications of this protocol may be necessary to adapt it to different systems (see "3. Overview" above). If this protocol won't work for your system, please first consult our alternative protocols (see page 2 above and Alternative protocols folder). If our alternative protocols do not solve the issues, then you may adapt the primary protocol as needed. Whatever you do, please record methods carefully and strive to follow the spirit of the protocol and produce comparable data.
- In our experience, 1 survey (of 1 site of 1 plant species) takes 2 well-trained undergraduates 2-8 hours to complete using the methods above (after a species and site have already been selected). This is in old fields, prairies, and deciduous forests in Michigan. Could take longer in other systems.
- We select 40 quadrat center points (instead of 30) so that we have extra points ready in case some quadrats are empty. If you predict that many quadrats will be empty (e.g., in a very spatially clumped population of plants), then select more points (e.g., 60 points). (Remember the goal is to have 30 focal plants sampled).
- Sometimes, especially in small populations, a focal plant ends up being another focal plant's neighbor. This is fine. Just note and keep going. If you have time, you can add an extra focal plant at the end (but this isn't totally necessary).

- For clonal plants, we have been calling stems “plant individuals” if they are not connected aboveground. When looking for aboveground connections, we clear away detritus, but we do not dig or move soil.
- Please see our Damage estimation training document for guidelines on how to estimate herbivore damage. Here are two tips:
    - Sometimes discerning herbivore damage from physical damage (e.g., wind, trampling) is tricky. We do the best we can. We look at things like how jagged the cut edges are and if they travel past the missing area into the remaining leaf tissue (which would suggest the damage may have been physical).
    - Another challenge is old damage that occurred when leaves were still expanding. This could potentially make area removed seem larger than it was. If we suspect something like this happened, then we try to bend the leaf back into shape to see if it seems like the missing area expanded over time.
- We will accept surveys that only assess damage and do not identify herbivores. This will allow people without insect ID skills to participate in the study.

6. Guidelines for picking plant species:
We are hoping for a broad sampling of plant species, so data on any plant species will be valuable. However, we have developed a sampling plan structured around 1) gathering data for as many plant families as possible; 2) in-depth sampling of plant species within five focal families (Apocynaceae, Asteraceae, Fabaceae, Rubiaceae, and Solanaceae); and 3) sampling of three globally-distributed taxa: *Taraxacum officinale* (dandelion), *Plantago lanceolata* (narrowleaf plantain), *Plantago major* (broadleaf plantain). You can read more about our sampling plan on our website (https://herbvar.org/protocols.html; “”HerbVar species selection plan”).

Thus, contributed surveys would ideally include one new family that is not currently in the database, one species from a focal family, and one survey of a focal species. Additional surveys would be the collaborator’s choice and could include re-sampling the same species through time or across a gradient. While this stratified sampling approach is preferred, all plant populations are of interest and collaborators are welcome to select plants based on criteria that make sense to them (familiarity with taxa, location & feasibility, etc). Also, feel free to re-sample species that have already been sampled. It will be interesting to have estimates of how consistent our data are within species. But once a species has been surveyed 2-3 times, it’s probably preferable to survey a new species.

We have charts in tabs in the Completed surveys document that are constantly updating to indicate gaps in sampling. In addition to the guidelines above, other features of a plant species that would make it a valuable addition to the dataset include:
- Occurs in a novel ecosystem
- Possesses a novel or underrepresented growth form, life history, or other set of traits

Other species selection notes:
- We have been surveying both native and non-native plant species.
- We are interested in agricultural and other cultivated plants and have already sampled a handful. When surveying cultivated plants, make sure the plants have been free of insecticides for an ecologically meaningful time before your survey.

7. Delineating a site:

We realize that defining the ‘edges’ of a site can be subjective and not easy. We search for an area where a given plant species occurs at a high enough density to easily select 30 focal plants and 30 unique neighbors with our method. This is usually a relatively dense patch. Walk around and see if you see the density drop off to well below the mean density that is used to calculate radius size. This is usually quite simple, e.g., when we walk out from the center of a “site” and don’t see any individuals of the focal species within 5 m, we decide we’re at the edge of a patch. In some systems, delineating a single, sampleable population simply might not be possible (e.g., where a species covers a vast area). In these cases, collaborators should simply do their best to select a reasonable, representative area to sample.

Pagel's lambda sensitivity analyses

Pagel's λ was significantly greater than zero for all 1000 trees with randomized placement of missing taxa for the Gini coefficient (μ [95% CI] = 0.48 [0.45–0.52], $P < 0.001$) and never significant for mean herbivory (μ [95% CI] = 0.069 [0.06–0.08], $P > 0.1$), suggesting our results are not sensitive to uncertainty in tree topology.

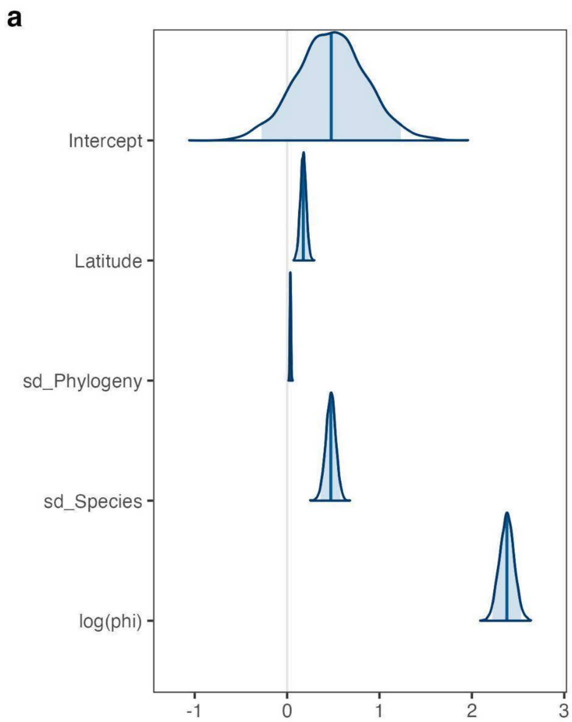
Our results were also robust to species sampling effects. As with the full tree, the subtree with only families with ≥ 8 species (11 families and 240 species) displayed significant phylogenetic signal for the Gini coefficient ($\lambda = 0.21$, $P = 0.049$) but not for mean herbivory ($\lambda = 0.057$, $P = 0.21$). Moreover, these results were not highly sensitive to the random removal of species from the tree and dataset. We found significant phylogenetic signal for the Gini coefficient in 100% of 1000 simulations with random removal of 10% of species, in 99% of simulations with random removal of 20% of species, in 91% of simulations with random removal of 30% of species, and in 75% of simulations with random removal of 40% of species. For mean herbivory, phylogenetic signal was non-significant in > 98% of all simulations for 10, 20, 30, and 40% random species removal.

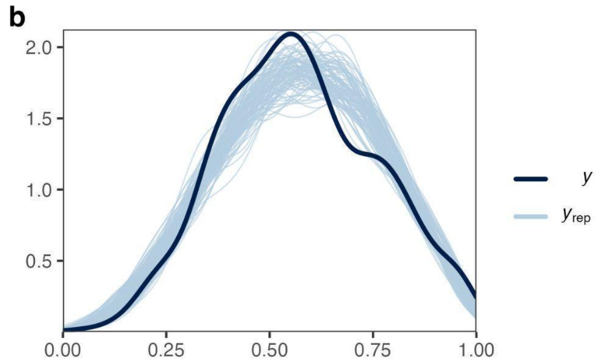
Sample size sensitivity analyses

The results of the analyses on the datasets subsampled to have 30 plant individuals per survey were essentially identical to the results on the full dataset presented in the main text. The model-estimated mean Gini coefficient averaged across the 100 subsampled datasets was 0.60 [95% CI = 0.57–0.64], which is nearly identical to the estimate from the full dataset (0.61). Similarly, the mean slope for the latitudinal gradient of herbivory variability across the subsampled datasets was 0.17 [0.14–0.19], which is very close to the slope estimated using the full dataset (0.18). Moreover, 100% of the subsampled datasets yielded latitudinal slopes with credible intervals that did not overlap zero. The estimated relationship between plant size and the Gini coefficient was also highly similar between the subsampled dataset (-0.26 [-0.24–-0.28]) and full data set (-0.26), and all subsampled data sets yielded slopes with credible intervals that did not overlap zero. These results indicate that our results were not influenced by differences in within-survey sample sizes across surveys.

Gini asymmetry coefficient

The model-estimated mean Gini asymmetry coefficient was slightly less than one on average but had a 95% credible interval that overlapped one (0.94 [0.89–1.02]). Gini asymmetry coefficient values close to one indicate that a disproportionately high number of indivduals with low values and individuals with disproportionately high values both contributed similarly to the observed Gini coefficient value. This result therefore suggests that observed evenness is a function of both a high number of plant individuals that escape herbivory and a number of plants that receive disproportionately high herbivory. However, the fact that most of the credible interval was slightly below one suggests that on average observed Gini coefficients are driven slightly more by a disproportionately high number of plants that escape herbivory than they are by plants that receive very high herbivory. When we modeled the Gini asymmetry coefficient as a function of latitude or plant size, we found flat relationships. This included a slope of 0.00 (-0.01–0.02) for latitude and 0.01 (-0.01–0.02) for plant size. These results indicate that the shape of the variability may be less predictable than the amount of variability itself.



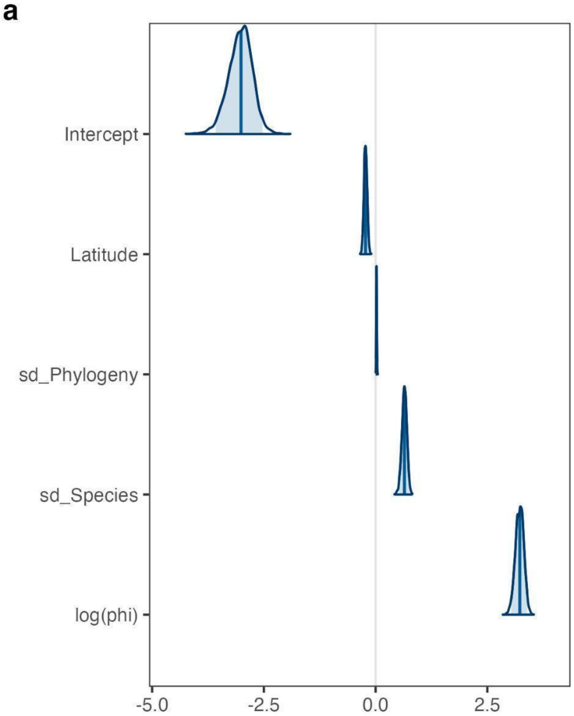


c

Parameter	Rhat	Bulk ESS	Tail ESS
Intercept	1.000	18,888	23,463
Latitude	1.000	28,298	32,093
sd_Phylogeny	1.001	5,227	12,206
sd_Species	1.001	5,291	9,154
phi	1.001	9,481	17,442

Fig. S1. Summary of the model of the relationship between the Gini coefficient of herbivory and latitude. (**a**) Posterior distributions of parameters from this phylogenetic beta regression. Latitude is absolute value and scaled. sd_Phylogeny is the standard deviation of the phylogenetic random effect. sd_Species is the standard deviation of the random effect grouping surveys (populations) by species. Phi is the precision parameter. The shaded regions show the 95% probability mass. (**b)** and (**c**) Posterior predictive check with 100 draws and diagnostics table from the same model. ESS is effective sample size. Sample size is 790 surveys of 503 species.

a



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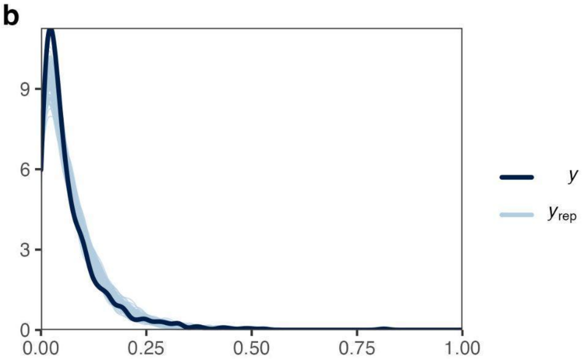
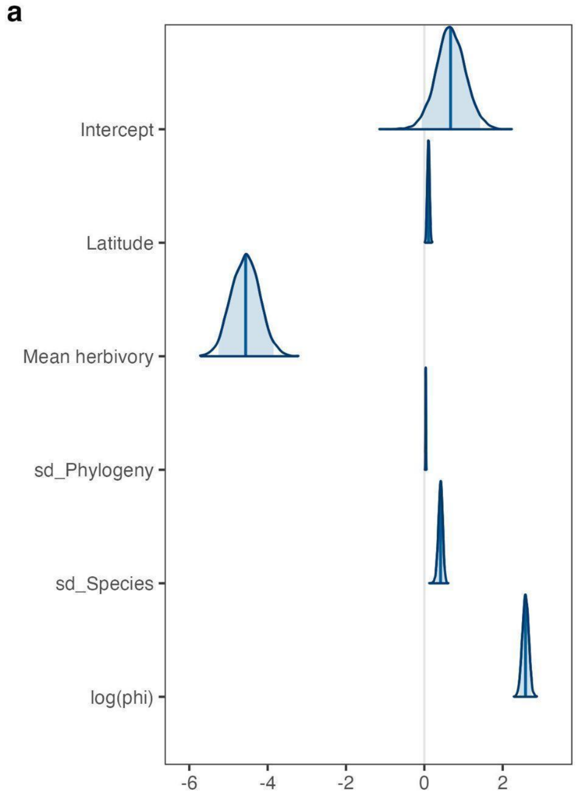
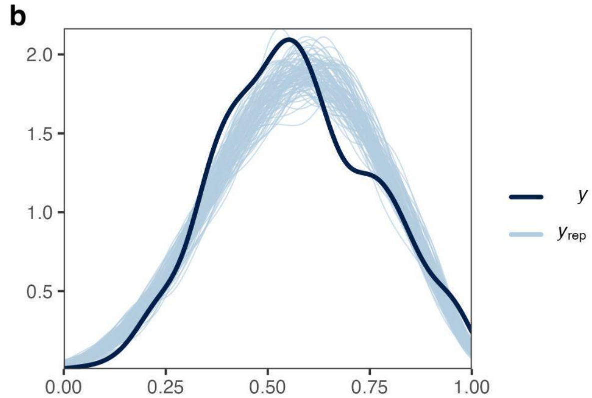


c

Parameter	Rhat	Bulk ESS	Tail ESS
Intercept	1.001	11,270	11,741
Latitude	1.000	17,197	15,910
sd_Phylogeny	1.002	2,541	4,725
sd_Species	1.001	3,504	7,036
phi	1.001	4,813	9,309

Fig. S2. Summary of the model of the relationship between mean herbivory and latitude. (**a**) Posterior distributions of parameters from this phylogenetic beta regression. Latitude is absolute value and scaled. sd_Phylogeny is the standard deviation of the phylogenetic random effect. sd_Species is the standard deviation of the random effect grouping surveys (populations) by species. Phi is the precision parameter. The shaded regions show the 95% probability mass. (**b)** and (**c**) Posterior predictive check with 100 draws and diagnostics table from the same model. ESS is effective sample size. Sample size is 790 surveys of 503 species.

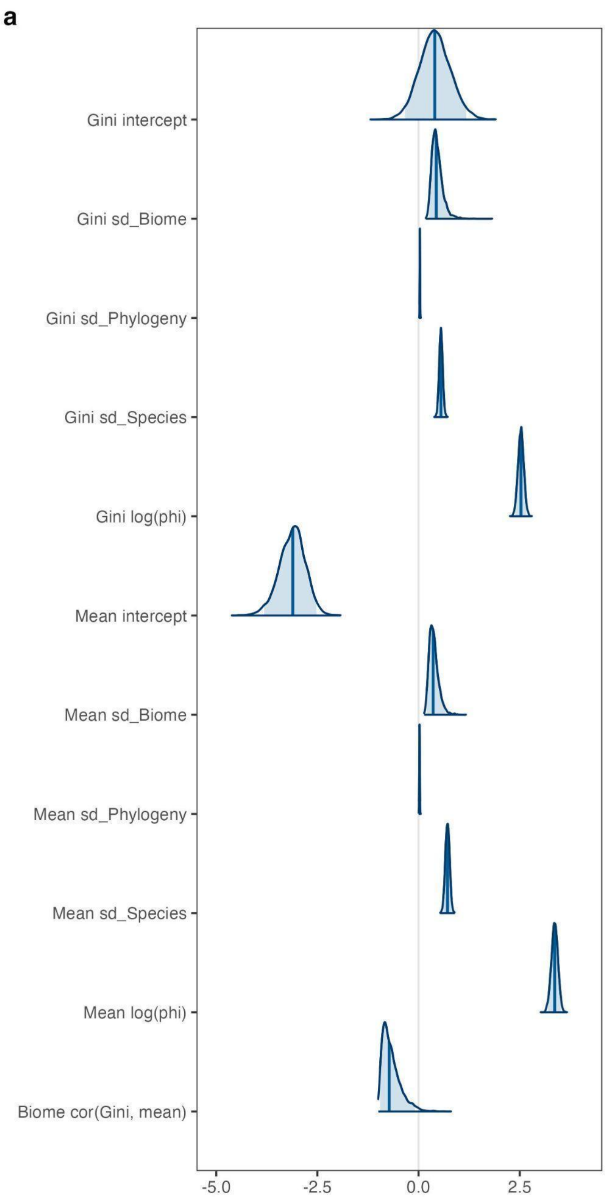


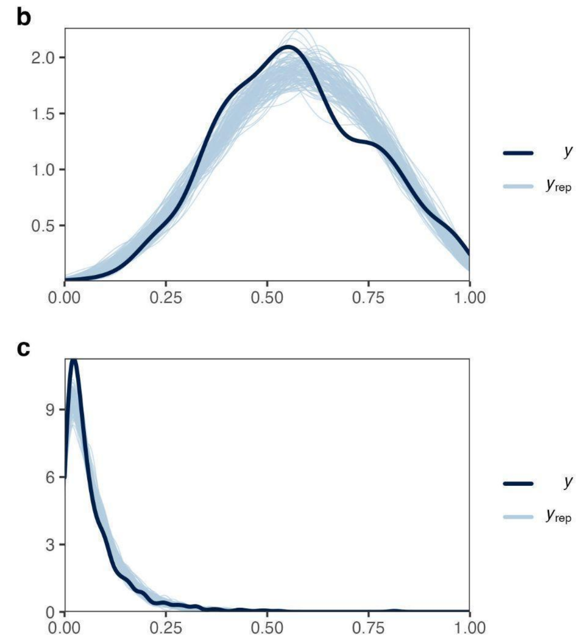


c

Parameter	Rhat	Bulk ESS	Tail ESS
Intercept	1.000	9,798	12,708
Latitude	1.001	18,245	17,557
Mean herbivory	1.000	22,086	17,295
sd_Phylogeny	1.002	2,786	5,645
sd_Species	1.004	2,431	3,902
phi	1.002	4,857	7,968

Fig. S3. Summary of the model of the relationship between the Gini coefficient of herbivory and latitude with mean herbivory as a covariate. (**a**) Posterior distributions of parameters from this phylogenetic beta regression. Latitude is absolute value and scaled. sd_Phylogeny is the standard deviation of the phylogenetic random effect. sd_Species is the standard deviation of the random effect grouping surveys (populations) by species. Phi is the precision parameter. The shaded regions show the 95% probability mass. (**b)** and (**c**) Posterior predictive check with 100 draws and diagnostics table from the same model. ESS is effective sample size. Sample size is 790 surveys of 503 species.



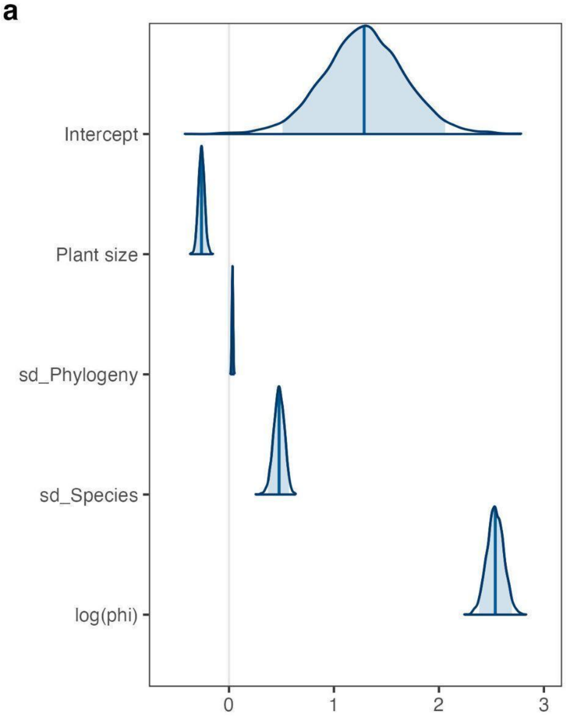


d

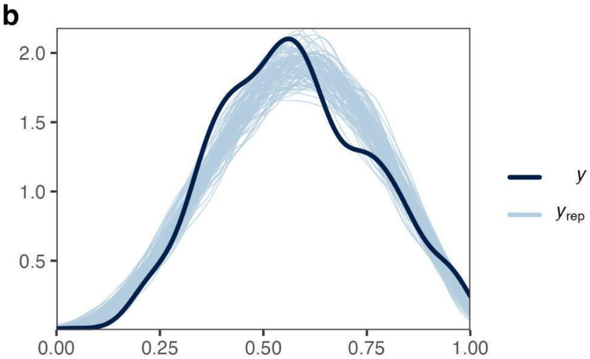
Parameter	Rhat	Bulk ESS	Tail ESS
Gini intercept	1.001	13,736	18,959
Gini sd_Biome	1.001	11,693	19,216
Gini sd_Phylogeny	1.002	4,735	9,488
Gini sd_Species	1.001	8,082	16,838
Gini log(phi)	1.000	18,858	28,639
Mean intercept	1.001	13,150	17,971
Mean sd_Biome	1.000	13,931	22,306
Mean sd_Phylogeny	1.003	3,605	4,010
Mean sd_Species	1.001	6,816	15,363
Mean log(phi)	1.000	15,652	24,738
Biome cor(Gini, mean)	1.001	14,323	21,812

Fig. S4. Summary of the model of the relationship between the Gini coefficient of herbivory and mean herbivory as responses and biome as a random grouping variable. (**a**) Posterior distributions of parameters from this multivariate phylogenetic beta regression. sd_Phylogeny is the standard deviation of the phylogenetic random effect. sd_Species is the standard deviation of the random effect grouping surveys (populations) by species. Biome cor(Gini, mean) is the estimated correlation between the Gini coefficient and mean herbivory across biomes. Phi is the precision parameter. The shaded regions show the 95% probability mass. (**b)**, (**c**), and (**d)**, Posterior predictive check with 100 draws and diagnostics table from the same model. ESS is effective sample size. Sample size is 790 surveys of 503 species.

a



b

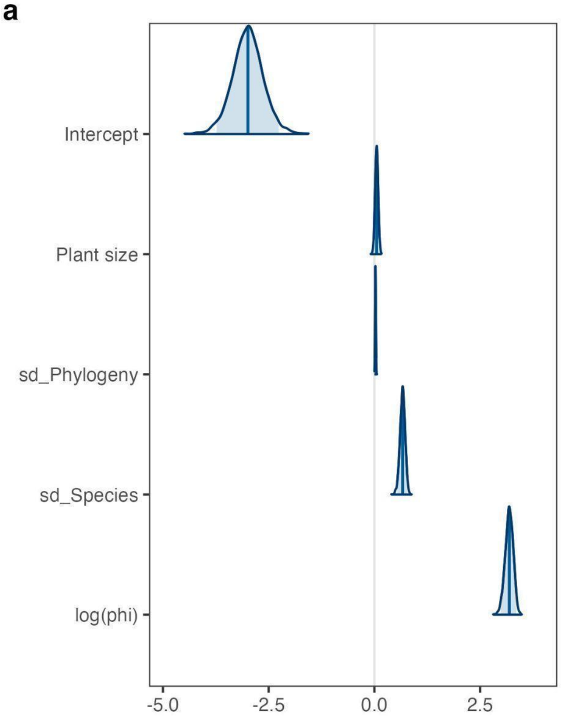


c

Parameter	Rhat	Bulk ESS	Tail ESS
Intercept	1.000	23,765	25,288
Plant size	1.000	32,430	31,688
sd_Phylogeny	1.001	5,331	9,953
sd_Species	1.001	5,427	11,142
phi	1.000	11,431	21,091

Fig. S5. Summary of the model of the relationship between the Gini coefficient of herbivory and plant size. (**a**) Posterior distributions of parameters from this phylogenetic beta regression. Plant size is log transformed plant diameter. sd_Phylogeny is the standard deviation of the phylogenetic random effect. sd_Species is the standard deviation of the random effect grouping surveys (populations) by species. Phi is the precision parameter. The shaded regions show the 95% probability mass. (**b)** and (**c**) Posterior predictive check with 100 draws and diagnostics table from the same model. ESS is effective sample size. Sample size is 735 surveys of 472 species.

a



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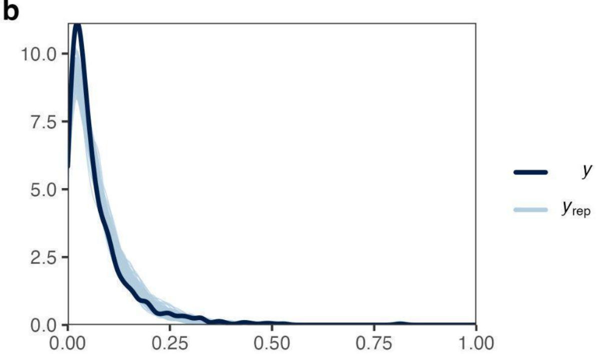
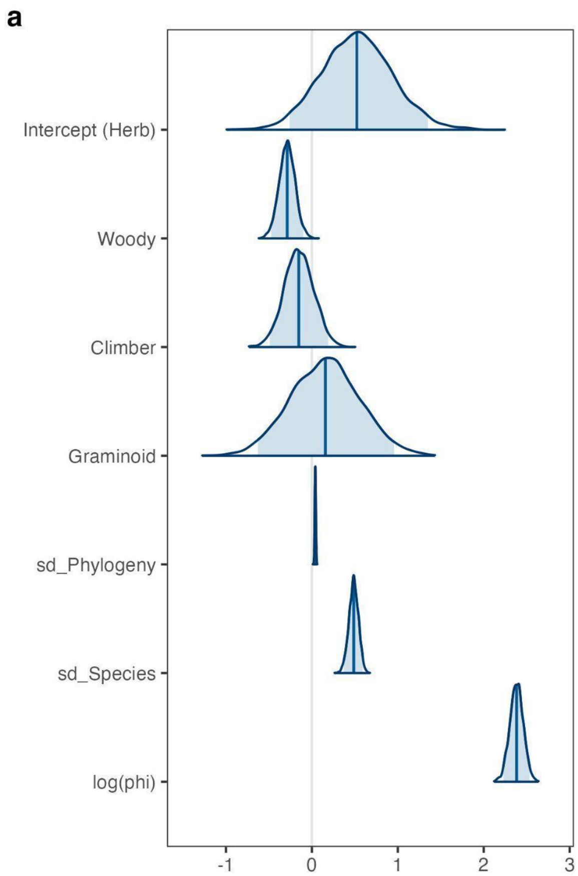
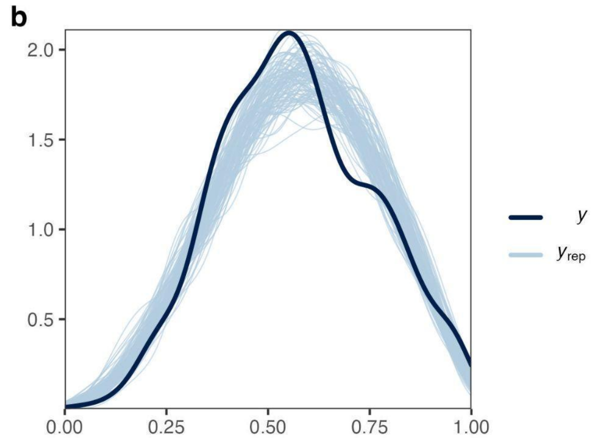


c

Parameter	Rhat	Bulk ESS	Tail ESS
Intercept	1.000	12,205	11,539
Plant size	1.001	14,462	15,647
sd_Phylogeny	1.012	1,561	2,867
sd_Species	1.009	2,755	5,908
phi	1.004	4,719	7,508

Fig. S6. Summary of the model of the relationship between mean herbivory and plant size. (**a**) Posterior distributions of parameters from this phylogenetic beta regression. Plant size is log transformed plant diameter. sd_Phylogeny is the standard deviation of the phylogenetic random effect. sd_Species is the standard deviation of the random effect grouping surveys (populations) by species. Phi is the precision parameter. The shaded regions show the 95% probability mass. (**b)** and (**c**) Posterior predictive check with 100 draws and diagnostics table from the same model. ESS is effective sample size. Sample size is 735 surveys of 472 plant species.

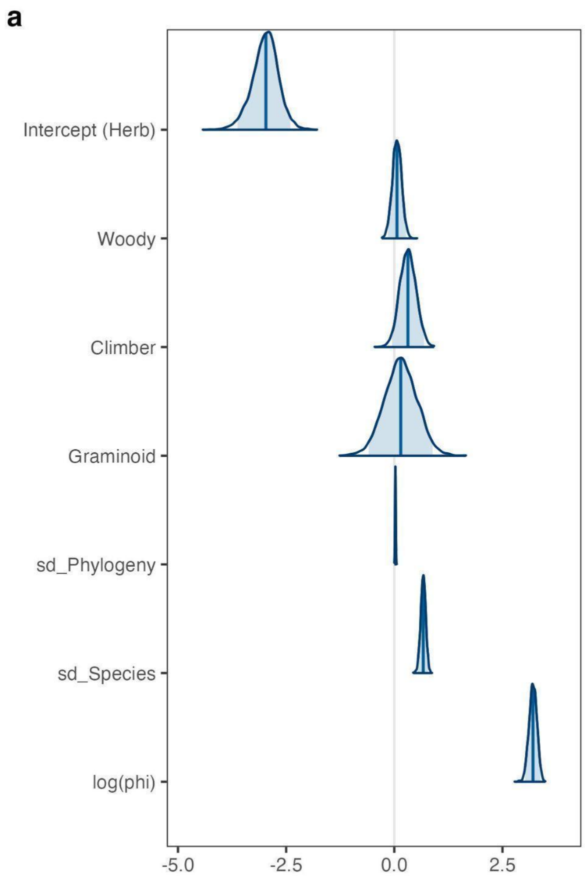


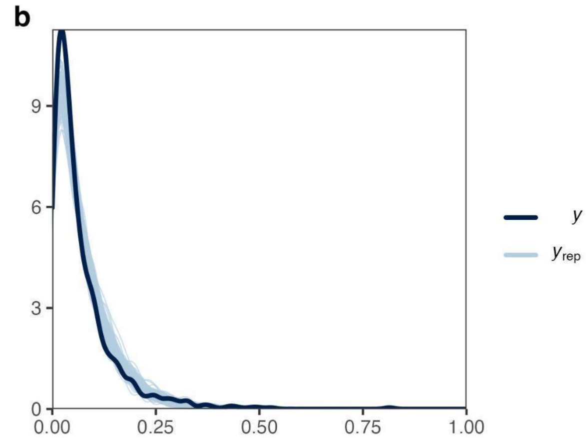


c

Parameter	Rhat	Bulk ESS	Tail ESS
Intercept (Herb)	1.001	14,678	14,739
Woody	1.000	16,870	15,694
Climber	1.000	20,807	16,111
Graminoid	1.001	20,160	14,921
sd_Phylogeny	1.004	3,187	5,688
sd_Species	1.003	3,158	6,254
phi	1.002	5,703	10,734

Fig. S7. Summary of the model of the relationship between the Gini coefficient of herbivory and plant growth form. (**a**) Posterior distributions of parameters from this phylogenetic beta regression. The intercept is the predicted mean for herbs, whereas parameters for other growth forms are differences from herbs. sd_Phylogeny is the standard deviation of the phylogenetic random effect. sd_Species is the standard deviation of the random effect grouping surveys (populations) by species. Phi is the precision parameter. The shaded regions show the 95% probability mass. (**b)** and (**c**) Posterior predictive check with 100 draws and diagnostics table from the same model. ESS is effective sample size. Sample size is 790 surveys of 503 species.





c

Parameter	Rhat	Bulk ESS	Tail ESS
Intercept (Herb)	1.000	10,847	11,173
Woody	1.001	8,808	13,878
Climber	1.000	11,087	14,045
Graminoid	1.000	14,012	14,424
sd_Phylogeny	1.005	1,890	2,663
sd_Species	1.002	3,080	5,338
phi	1.001	4,599	7,242

Fig. S8. Summary of the model of the relationship between mean herbivory and plant growth form. (**a**) Posterior distributions of parameters from this phylogenetic beta regression. The intercept is the predicted mean for herbs, whereas parameters for other growth forms are differences from herbs. sd_Phylogeny is the standard deviation of the phylogenetic random effect. sd_Species is the standard deviation of the random effect grouping surveys (populations) by species. Phi is the precision parameter. The shaded regions show the 95% probability mass. (**b)** and (**c**) Posterior predictive check with 100 draws and diagnostics table from the same model. ESS is effective sample size. Sample size is 790 surveys of 503 species.

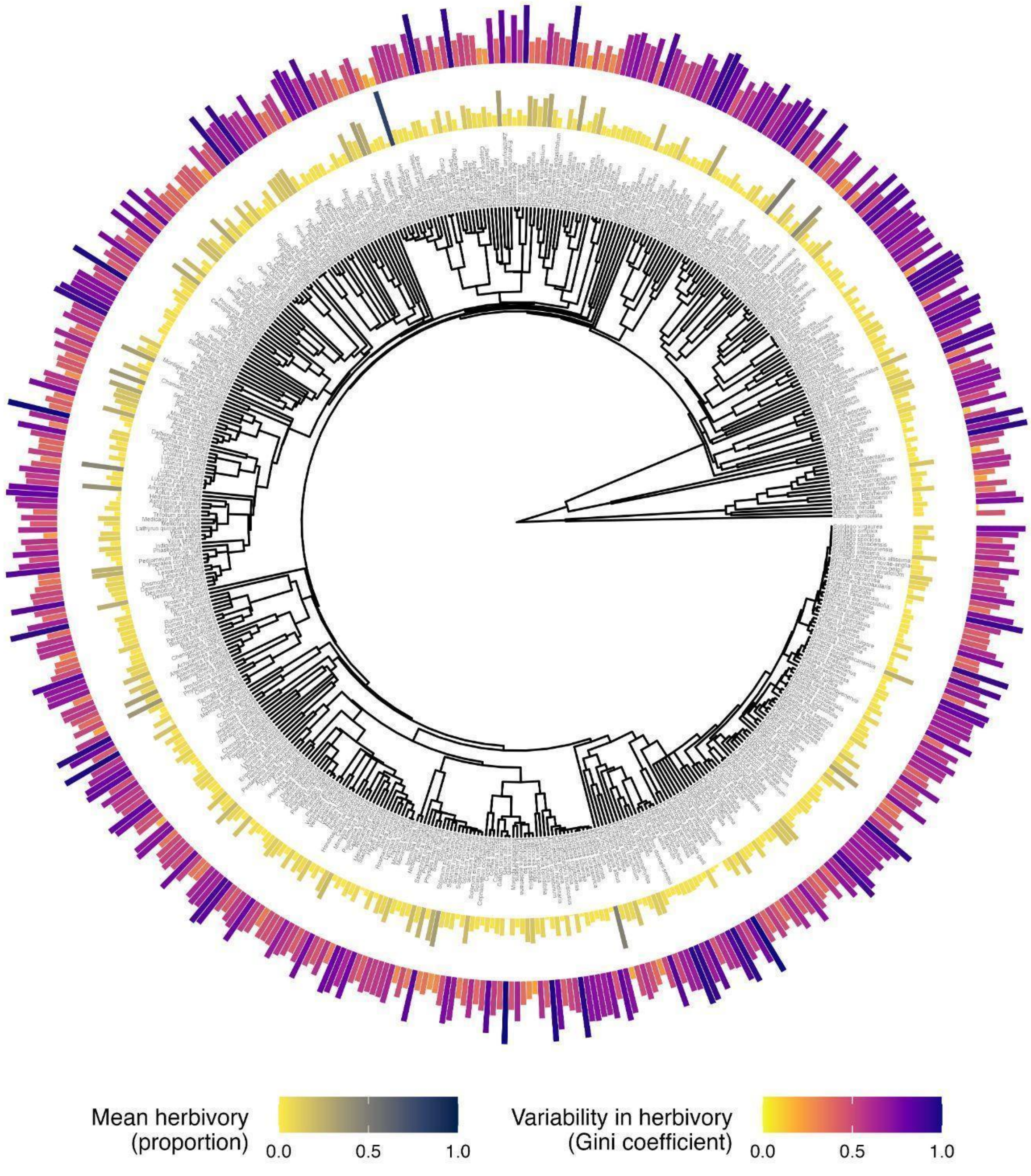


Fig. S9. The phylogeny of all 503 species in the dataset. Levels of variation in herbivory among plants within populations (Gini coefficient) show greater phylogenetic signal ($\lambda = 0.51$ [0.45–0.51], $p < 0.001$) than mean herbivory levels ($\lambda_{Mean} = 0.07$ [0.06–0.08], $p > 0.1$). This figure is as Fig. 4 in the main text except with all species shown, whereas Fig. 4 omits less well represented families for clarity.

Table S1. Summary of the model of the relationship between the Gini coefficient of herbivory and latitude and its interaction with hemisphere. As described in the methods, we started our latitudinal analyses by fitting models that allowed for different latitudinal slopes in the Northern Hemisphere (652 surveys) and Southern Hemisphere (138 surveys). The model is a phylogenetic beta regression. Latitude is absolute value and scaled. Hemi. interxn (S) is the interaction parameter describing the difference in slope between the N and S Hemispheres. sd_Phylogeny is the standard deviation of the phylogenetic random effect. sd_Species is the standard deviation of the random effect grouping surveys (populations) by species. L95CI and U95CI are the lower and upper bounds of the 95% credible interval. ESS is effective sample size. Sample size is 790 surveys of 503 species. The 95% credible interval for the interaction parameter overlapped zero and had a Bayes factor of 0.23, suggesting support for the hypothesis of similar latidudinal slopes between hemispheres. We therefore focused our analyses on models with one latitudinal gradient slope (with the absolute value of latitude) and presented those models in the main text.

Parameter	Estimate	SE	L95CI	U95CI	Rhat	Bulk ESS	Tail ESS
Intercept	0.53	0.38	-0.20	1.30	1.000	10,502	12,057
Latitude (N)	0.15	0.04	0.07	0.22	1.000	14,276	15,467
Hemi. interxn (S)	0.14	0.08	-0.01	0.30	1.000	14,577	15,595
sd_Phylogeny	0.04	0.01	0.02	0.05	1.002	3,031	5,680
sd_Species	0.48	0.06	0.36	0.58	1.001	2,841	4,656
phi	10.90	0.88	9.26	12.66	1.000	5,075	8,998

Table S2. Summary of the model of the relationship between mean herbivory and latitude and its interaction with hemisphere. The model is a phylogenetic beta regression. Latitude is absolute value and scaled. Hemi. interxn (S) is the interaction parameter describing the difference in slope between the N and S Hemispheres. sd_Phylogeny is the standard deviation of the phylogenetic random effect. sd_Species is the standard deviation of the random effect grouping surveys (populations) by species. L95CI and U95CI are the lower and upper bounds of the 95% credible interval. ESS is effective sample size. Sample size is 790 surveys of 503 species. The 95% credible interval for the interaction parameter overlapped zero, and we therefore focused our analyses on models with one latitudinal gradient slope (with the absolute value of latitude) and presented those models in the main text.

Parameter	Estimate	SE	L95CI	U95CI	Rhat	Bulk ESS	Tail ESS
Intercept	-3.02	0.28	-3.61	-2.50	1.000	9,765	10,399
Latitude (N)	-0.23	0.04	-0.31	-0.15	1.000	12,823	13,802
Hemi. interxn (S)	0.01	0.08	-0.16	0.18	1.000	13,668	14,650
sd_Phylogeny	0.02	0.01	0.01	0.04	1.002	2,207	3,823
sd_Species	0.64	0.06	0.52	0.75	1.001	3,517	6,230
phi	25.16	2.32	20.66	29.76	1.001	4,611	8,233

Table S3. Summary of the model of the Gini coefficient as a function of latitude and focal plant abundance. We tested the potential for differences in plant abundances to influence the observed latitudinal gradient in herbivory variability by re-fitting our Gini coefficient latitudinal gradient model (Fig. 2 and fig. S1) with focal plant abundance (percent cover) as a covariate, thus estimating the marginal effect of latitude conditional on plant abundance. The parameter for latitude in this model was still strongly positive (p_p = 1.0, BF = 1.9e2). This suggests that the association between latitude and herbivory variability is not explained by differences in plant abundance. Latitude is absolute value and scaled. sd_Phylogeny is the standard deviation of the phylogenetic random effect. sd_Species is the standard deviation of the random effect grouping surveys (populations) by species. L95CI and U95CI are the lower and upper bounds of the 95% credible interval. ESS is effective sample size. Sample size is 643 surveys of 410 species.

Parameter	Estimate	SE	L95CI	U95CI	Rhat	Bulk ESS	Tail ESS
Intercept	1.06	0.38	0.36	1.83	1.000	29,684	25,802
Latitude	0.15	0.03	0.09	0.22	1.000	47,968	33,646
Plant abundance	-0.01	0.00	-0.01	-0.01	1.000	51,968	35,503
sd_Phylogeny	0.03	0.01	0.02	0.04	1.002	5,504	10,570
sd_Species	0.49	0.06	0.37	0.61	1.003	6,638	12,927
phi	11.13	1.00	9.26	13.18	1.002	11,126	19,847

Table S4. Summary of the model of the Gini coefficient as a function of plant size and latitude. Our finding that the Gini coefficient increases with plant size could be explained by the decline in plant size with increasing latitude (ϱ = -0.35 in our dataset) and the negative relationship between latitude and the Gini coefficient. In other words, latitude could conceivably have been the driver of the negative Gini coefficient–plant size association we found. We accounted for this association by fitting a phylogenetic beta regression model of the Gini coefficient with both plant size and latitude as predictors, thus estimating the marginal effect of each predictor conditional on the other predictor. The parameter for plant size in this model was still strongly negative (p_p = 1.0, BF = 2.7e6). This suggests that the association between plant size and herbivory variability is not explained by the negative effect of latitude on plant size and is consistent with the plant size hypothesis. Likewise, the parameter for latitude was still positive with plant size in the model (p_p = 1.0, BF = 3.9). Plant size is log transformed plant diameter. Latitude is absolute value and scaled. sd_Phylogeny is the standard deviation of the phylogenetic random effect. sd_Species is the standard deviation of the random effect grouping surveys (populations) by species. L95CI and U95CI are the lower and upper bounds of the 95% credible interval. ESS is effective sample size. Sample size is 735 surveys of 472 species.

Parameter	Estimate	SE	L95CI	U95CI	Rhat	Bulk ESS	Tail ESS
Intercept	1.18	0.36	0.47	1.90	1.000	29,091	26,788
Plant size	-0.24	0.03	-0.30	-0.18	1.000	36,299	31,797
Latitude	0.10	0.03	0.04	0.16	1.000	38,588	33,626
sd_Phylogeny	0.03	0.01	0.02	0.04	1.000	6,305	11,931
sd_Species	0.47	0.05	0.37	0.57	1.001	6,534	11,734
phi	12.70	1.03	10.76	14.83	1.001	11,679	19,389

Table S5. Summary of the model of the Gini coefficient as a function of plant size and focal plant abundance. We tested the potential for differences in plant abundances to influence the observed relationship between plant size and herbivory variability by re-fitting our plant size model (Fig. 3A and fig. S5) with focal plant abundance (percent cover) as a covariate, thus estimating the marginal effect of plant size conditional on plant abundance. The parameter for plant size in this model was still strongly negative (p_p = 1.0, BF = 1.8e6). This suggests that the association between plant size and herbivory variability is not explained by differences in plant abundance. Plant size is on a log scale. Plant abundance is percent cover of the focal plant species. sd_Phylogeny is the standard deviation of the phylogenetic random effect. sd_Species is the standard deviation of the random effect grouping surveys (populations) by species. L95CI and U95CI are the lower and upper bounds of the 95% credible interval. ESS is effective sample size. Sample size is 621 surveys of 397 species.

Parameter	Estimate	SE	L95CI	U95CI	Rhat	Bulk ESS	Tail ESS
Intercept	1.66	0.38	0.96	2.45	1.000	59,406	51,306
Plant size	-0.25	0.03	-0.31	-0.18	1.000	84,664	67,984
Plant abundance	-0.01	0.00	-0.01	0.00	1.000	92,280	70,625
sd_Phylogeny	0.02	0.01	0.01	0.04	1.001	9,658	14,592
sd_Species	0.45	0.06	0.33	0.57	1.001	11,438	20,234
phi	11.30	1.01	9.41	13.37	1.001	20,917	35,989

Table S6. Parameter estimates of models of evolution of variability in herbivory (Gini) and mean herbivory (logit-transformed) as a function of plant growth form. Means and standard deviations of parameter estimates across 100 phylogenetic trees are provided. Models considered herbivory evolving under Brownian Motion where the rate of evolution (σ^2) was shared across trait states (BM1 model) or allowed to vary depending on the trait (herbaceous vs. woody) (BMS). We also examined Ornstein-Uhlenbeck models (OU1) that considered herbivory evolving under a regime with a single evolutionary optimum θ while keeping rate (σ^2) and strength of pull towards an evolutionary optimum (α) constant, and OUM models, which allow for herbivory to evolve towards different optima, depending on the trait (herbaceous vs. woody). Stochastic maps of trait evolution were generated with *make.simmap* in 'phytools'. Models were fit using the 'OUwie' package in R. To compare models, we used the average BIC across 100 trees. Variability in herbivory across herbaceous and woody taxa (Gini) is best modeled as evolving with constant rate and strength of selection towards different optima (OUM model ΔBIC = 4.5 lower than OU1) so that the optimum is higher in herbaceous taxa (θ= 0.6) compared to woody ones (θ= 0.52). In contrast, the best model for mean herbivory suggests that herbaceous and woody taxa are evolving under a selection regime with a constant rate and strength of selection towards a single optimum (OU1).

			Herbaceous						Woody					
Response	**model**	**BIC**	**α**	**α_{SD}**	**σ^2**	**σ^2_{SD}**	**θ**	**θ_{SE}**	**α**	**α_{SD}**	**σ^2**	**σ^2_{SD}**	**θ**	**θ_{SE}**
Variability in herbivory (Gini)	OUM	-105.0	0.91	0.00	0.09	0.01	0.60	0.01	0.91	0.00	0.09	0.01	0.52	0.02
	OU1	-97.0	0.91	0.00	0.09	0.01	0.57	0.01	0.91	0.00	0.09	0.01	0.57	0.01
	BMS	959.4	NA	NA	0.03	0.02	0.63	1.19	NA	NA	0.01	0.01	0.63	1.19
	BM1	1021.1	NA	NA	0.02	0.01	0.61	1.37	NA	NA	0.02	0.01	0.61	1.37
Mean herbivory (logit)	OU1	1797.8	0.91	0.0003	5.34	1.18	-3.20	0.08	0.91	0.0003	5.34	1.18	-3.20	0.08
	OUM	1802.3	0.91	0.0003	5.32	1.18	-3.27	0.10	0.91	0.0003	5.32	1.18	-3.06	0.14
	BMS	2980.7	NA	NA	2.42	1.46	-3.86	10.63	NA	NA	0.35	0.06	-3.86	10.63
	BM1	3089.2	NA	NA	1.98	1.20	-3.80	12.76	NA	NA	1.98	1.20	-3.80	12.76

Table S7. Parameter estimates of models of evolution of variability in herbivory (Gini) and mean herbivory (logit-transformed) as a function of plant biome affinity. Temperate: ≤23° latitude; tropical: >23° latitude. Means and standard deviation of parameter estimates across 100 phylogenetic trees are provided. Models considered herbivory evolving under Brownian Motion where the rate of evolution (σ^2) was shared across trait states (BM1 model) or allowed to vary depending on the state of the trait (temperate vs. tropical) being examined (BMS). We also examined Ornstein-Uhlenbeck models (OU1) that considered herbivory evolving under a regime with a single evolutionary optimum θ while keeping rate (σ^2) and strength of pull towards an evolutionary optimum (α) constant, and OUM models, which allow for herbivory to evolve towards different optima, depending on the state of the trait (i.e, temperate vs. tropical). Stochastic maps of trait evolution were generated with the *make.simmap* function in the 'phytools' R package. All models were implemented using the 'OUwie' package in R. To compare models, we used the average BIC across the 100 trees described above. Both mean and variability in herbivory evolve under regimes with constant rates and strength of selection towards slightly different evolutionary optima (OUM). Temperate species had a higher optimum for variability (θ=0.6) compared to tropical species (θ=0.5), whereas for mean proportion of herbivory temperate species had a lower optimum (θ=0.036 back-transformed) than tropical species (θ=0.060 back-transformed).

			Temperate						Tropical					
Response	**model**	**BIC**	**α**	**α_{SD}**	**σ^2**	**σ^2_{SD}**	**θ**	**θ_{SE}**	**α**	**α_{SD}**	**σ^2**	**σ^2_{SD}**	**θ**	**θ_{SE}**
Variability in herbivory (Gini)	OUM	-118.0	0.91	0.00	0.09	0.02	0.60	0.01	0.91	0.00	0.09	0.02	0.50	0.02
	OU1	-106.1	0.91	0.00	0.09	0.02	0.57	0.01	0.91	0.00	0.09	0.02	0.57	0.01
	BMS	1037.4	NA	NA	0.03	0.02	0.60	1.07	NA	NA	0.00	0.00	0.60	1.07
	BS1	1130.9	NA	NA	0.02	0.02	0.61	1.39	NA	NA	0.02	0.02	0.61	1.39
Mean herbivory (logit)	OUM	1945.3	0.91	0.0003	5.21	1.85	-3.30	0.09	0.91	0.0003	5.21	1.85	-2.76	0.16
	OU1	1948.3	0.91	0.0003	5.29	1.84	-3.17	0.08	0.91	0.0003	5.29	1.84	-3.17	0.08
	BMS	3240.2	NA	NA	2.25	2.14	-3.85	9.47	NA	NA	0.12	0.04	-3.85	9.47
	BS1	3370.4	NA	NA	2.02	1.97	-3.80	12.73	NA	NA	2.02	1.97	-3.80	12.73