

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.

ARTICLE INFO

Keywords:

Diet analyses
Biological control
Next generation sequencing
Intraguild predation
Metabarcoding
Omnivory

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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<https://doi.org/10.1016/j.biocontrol.2022.104920>

Received 17 June 2021; Received in revised form 31 January 2022; Accepted 6 April 2022

Available online 9 April 2022

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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 (*Acyrtosiphon 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 soufflé cups, 1 lady beetle, and an excess of prey given lady beetle consumption 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 LC01490/COI-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 LC01490: 5'-GGTCAACAAATCATAAAGATATTGG-3' (Folmer et al., 1994) and COI-CFMRa: 5'-GGWACTAATCAATTC-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

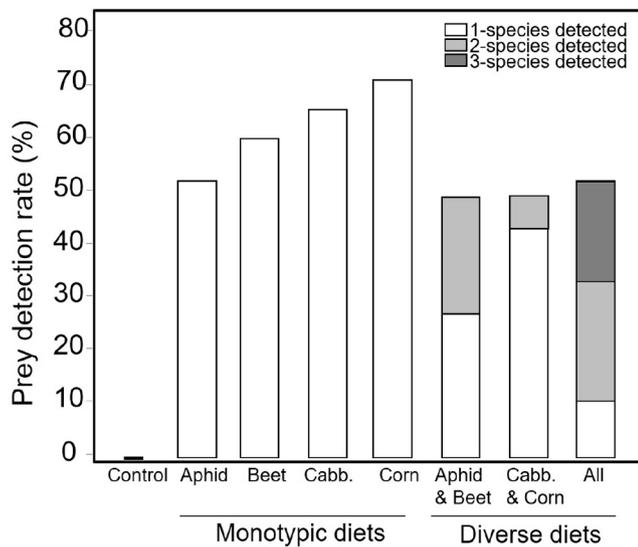


Fig. 1. Diet treatment effects on prey detection rates (i.e., the number of lady beetle individuals with prey items detected). Monotypic diets include only 1 species of prey offered to the lady beetles (aphids, beet armyworm, cabbage looper larvae, or corn earworm larvae). Diverse diets include two-species mixtures (aphids and beet armyworm mix or cabbage looper 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).

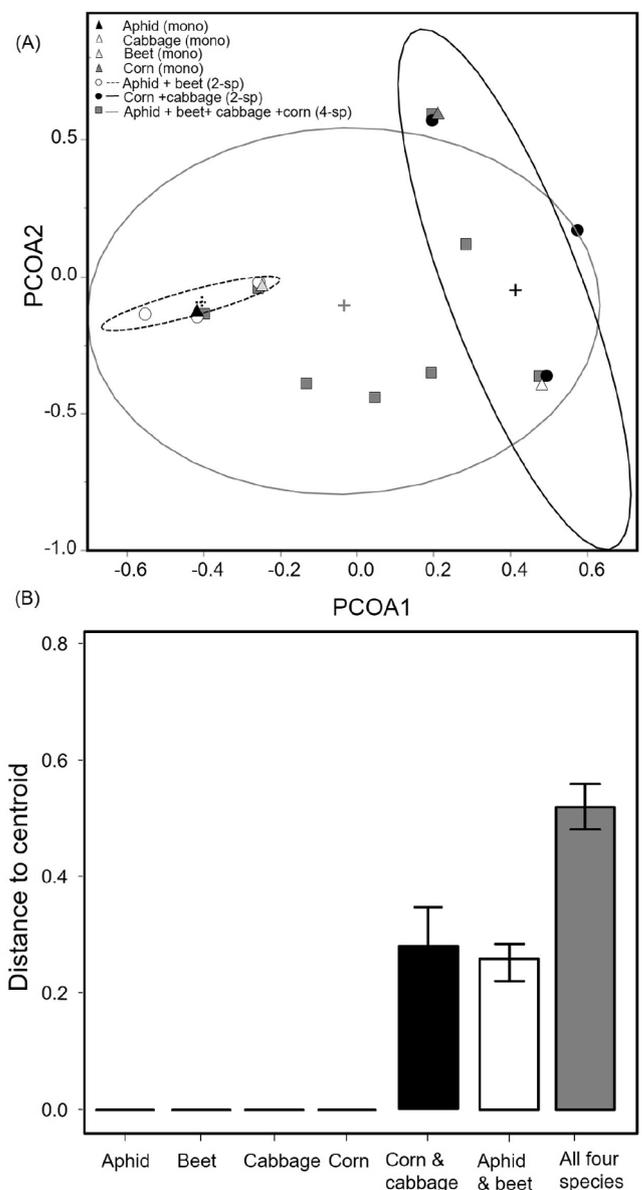


Fig. 2. Diet composition effects on OTU composition (A) and diet breadth (distance to centroid, B). Crosses represent centroids in A. Error bars are $1 \pm SE$ from the treatment mean in B.

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

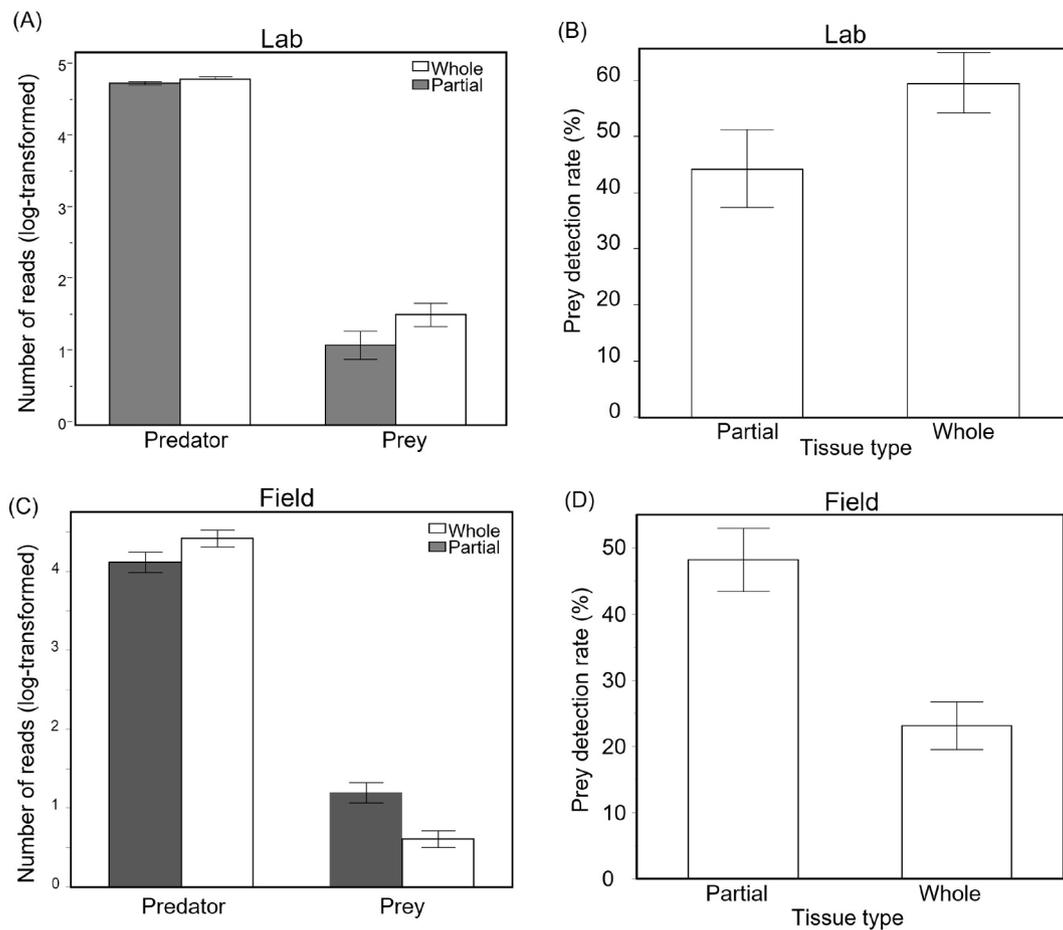


Fig. 3. The effects of partial and whole-body tissue types on the number of DNA reads (A,C) and prey detection rates (proportion of individuals with prey detected, B, D) for lady beetles used in the lab study (top panels) and field captured (bottom panels). Partial tissues for the lab study were dissected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

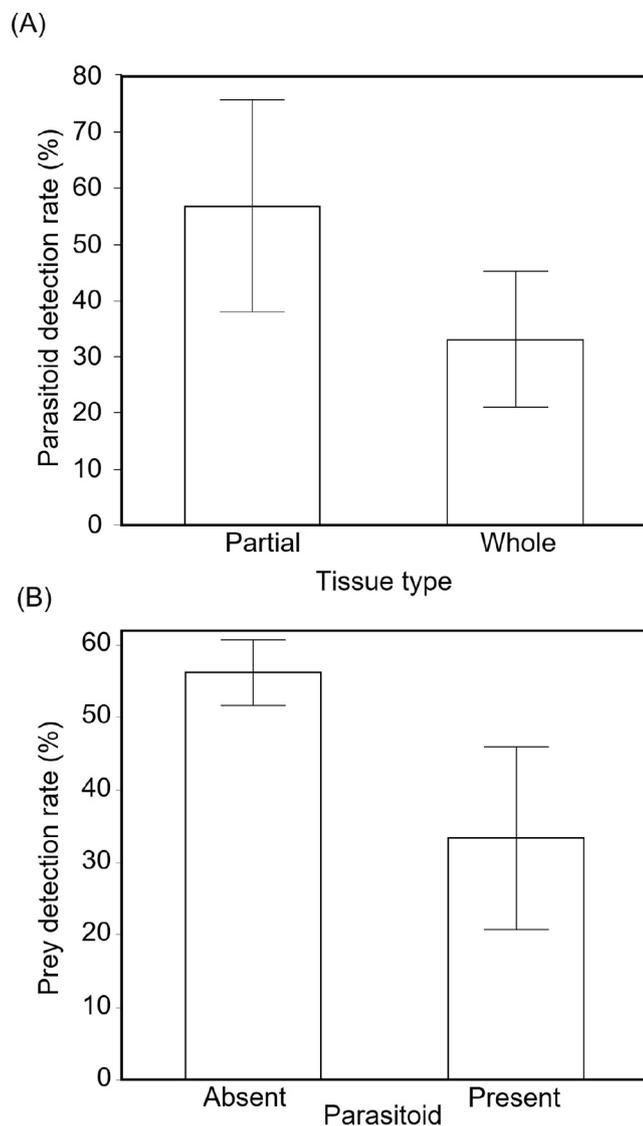


Fig. 4. Relationships between extraction methodology and parasitoid (A, top) and prey detection rates (B, bottom). Error bars are $1 \pm SE$ from the treatment mean.

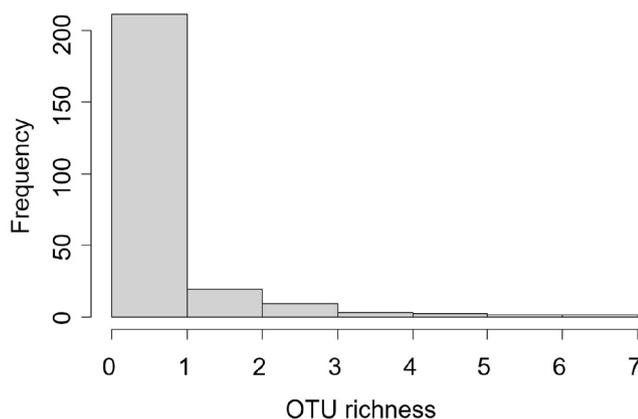


Fig. 5. OTU richness of lady beetles captured in all field specimens (soybean, corn, and tallgrass prairie fields). Most lady beetles did not have prey detected and the average number of OTU was 1.77.

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 deserts 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

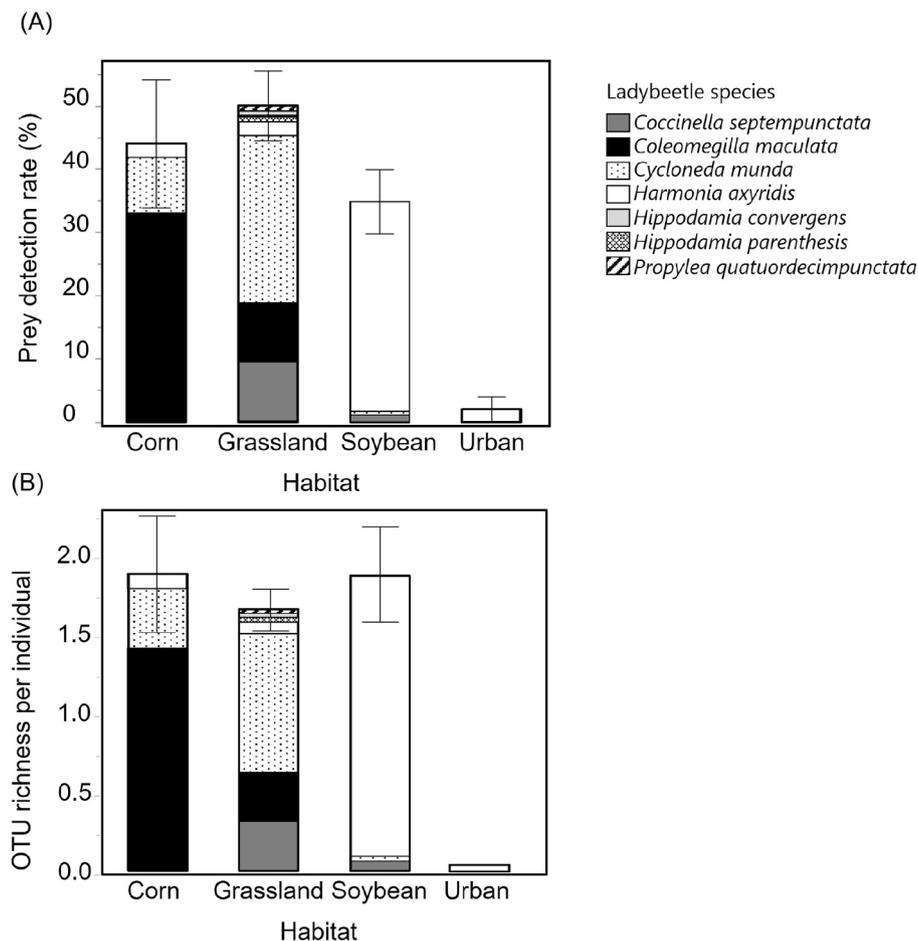


Fig. 6. Habitat differences in prey detection rate (A) and OTU richness (B) for different lady beetle species.

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 snapshots 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%)*				
<i>Coccinella septempunctata</i> (OTU3)	4.76%	1.43%	0.00%	0.00%
<i>Cycloneda munda</i> (OTU5)	0.00%	2.86%	0.00%	0.00%
<i>Harmonia axyridis</i> (OTU1)	42.86%	20.00%	0.00%	0.00%
<i>Hippodamia convergens</i> (OTU2)	4.76%	0.00%	7.02%	0.00%
<i>Hippodamia glacialis</i> (OTU33)	0.00%	1.43%	0.00%	0.00%
<i>Propylaea quatuordecimpunctata</i> (OTU7)	0.00%	2.86%	0.00%	0.00%
Aphids (18.24%)				
<i>Acyrtosiphon pisum</i> (OTU13)	0.00%	8.57%	0.00%	0.00%
<i>Aphidius ervi</i> (OTU71)	0.00%	0.00%	1.75%	0.00%
<i>Aphis glycines</i> (OTU17)	0.00%	0.00%	28.07%	0.00%
<i>Schizaphis graminum</i> (OTU28)	0.00%	1.43%	0.00%	0.00%
<i>Sitobion</i> sp (OTU66)	0.00%	0.00%	1.75%	0.00%
<i>Uroleucon anomalae</i> (OTU23)	0.00%	1.43%	0.00%	0.00%
<i>Uroleucon sonchi</i> (OTU86)	0.00%	1.43%	0.00%	0.00%
Flies (12.84%)				
<i>Aedes trivittatus</i> (OTU19)	4.76%	0.00%	3.51%	0.00%
<i>Aedes vexans</i> (OTU68)	0.00%	2.86%	0.00%	0.00%
<i>Apallates coxendix</i> (OTU65)	0.00%	1.43%	0.00%	0.00%
<i>Aphidoletes aphidimyza</i> (OTU50)	0.00%	0.00%	1.75%	0.00%
Cecidomyiidae (OTU21)	4.76%	0.00%	0.00%	0.00%
<i>Drosophila simulans</i> (OTU39)	0.00%	0.00%	1.75%	0.00%
<i>Forcipomyia</i> sp (OTU57)	0.00%	1.43%	0.00%	0.00%
Psychodidae (OTU53)	0.00%	1.43%	0.00%	0.00%
<i>Rhopalomyia solidaginis</i> (OTU81)	0.00%	0.00%	1.75%	0.00%
<i>Simulium equinum</i> (OTU62)	0.00%	0.00%	1.75%	0.00%
<i>Strongygaster triangulifera</i> (OTU31)	0.00%	1.43%	0.00%	0.00%
<i>Thienemanniella xena</i> (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%)				
<i>Daktulosphaira vitifoliae</i> (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%)				
<i>Polistes fuscatus</i> (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%)				
<i>Grapholita interstinctana</i> (OTU30)	0.00%	1.43%	0.00%	0.00%
<i>Hypona scabra</i> (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%

Table 1 (continued)

Taxa/species (OTU)	Corn	Grassland	Soybean	Urban
Lepidoptera (OTU67)	0.00%	1.43%	0.00%	0.00%
Parasitic wasps (1.73%)				
<i>Asaphes vulgaris</i> (OTU38)	0.00%	0.00%	1.75%	0.00%
<i>Lysiphlebus testaceipes</i> (OTU70)	0.00%	0.00%	1.75%	0.00%
<i>Trimorus</i> 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%)				
<i>Oecetis inconspicua</i> (OTU25)	0.00%	0.00%	3.51%	0.00%
Springtails (1.35%)				
<i>Deuterostminthurus</i> sp (OTU29)	0.00%	1.43%	0.00%	0.00%
<i>Entomobryomorpha</i> sp (OTU61)	0.00%	0.00%	1.75%	0.00%
Thrips (1.07%)				
<i>Neohydatothrips</i> sp (OTU36)	0.00%	0.00%	3.51%	0.00%
<i>Thrips palmi</i> (OTU76)	0.00%	0.00%	1.75%	0.00%
Ants (0.68%)*				
<i>Lasius neoniger</i> (OTU80)	0.00%	1.43%	0.00%	0.00%
Cicadas (0.68%)				
<i>Burbunga gilmorei</i> (OTU58)	0.00%	1.43%	0.00%	0.00%
Crickets (0.68%)				
<i>Allonemobius allardi</i> (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 perenniality *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

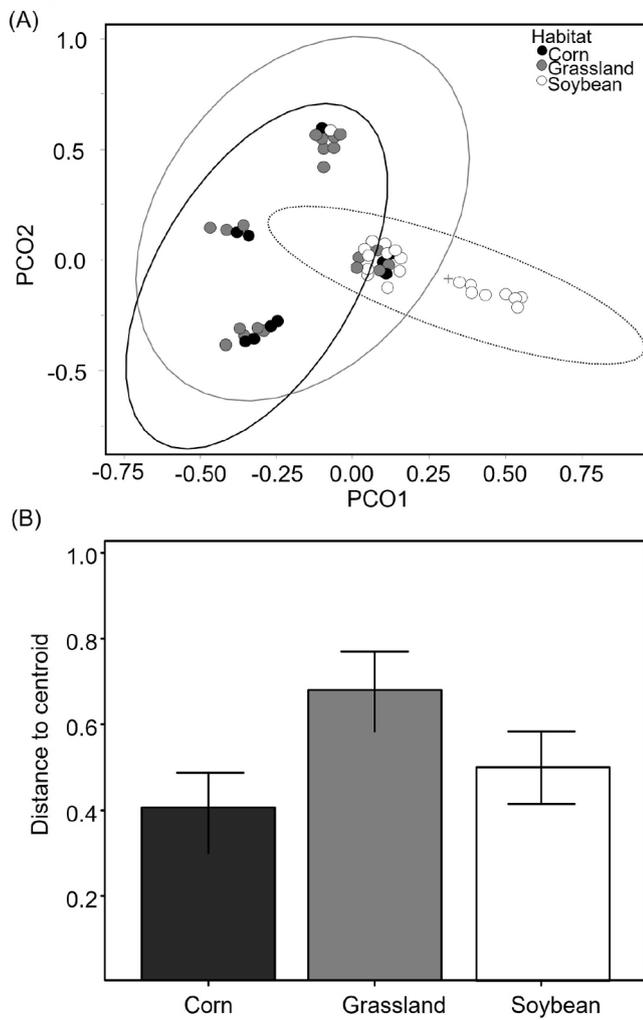


Fig. 7. OTU prey community composition (A) and diet breadth (distance from centroid, B) for lady beetles captured in corn, soybean, and grasslands.

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).

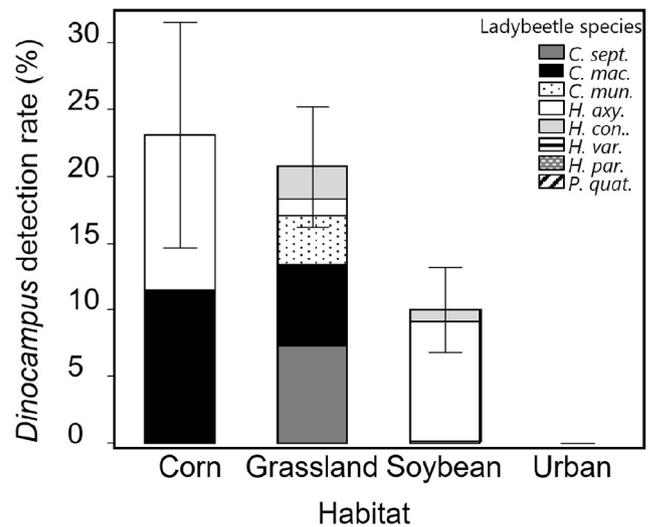


Fig. 9. Detection rates of *Dinocampus coccinellae* in different habitat types.

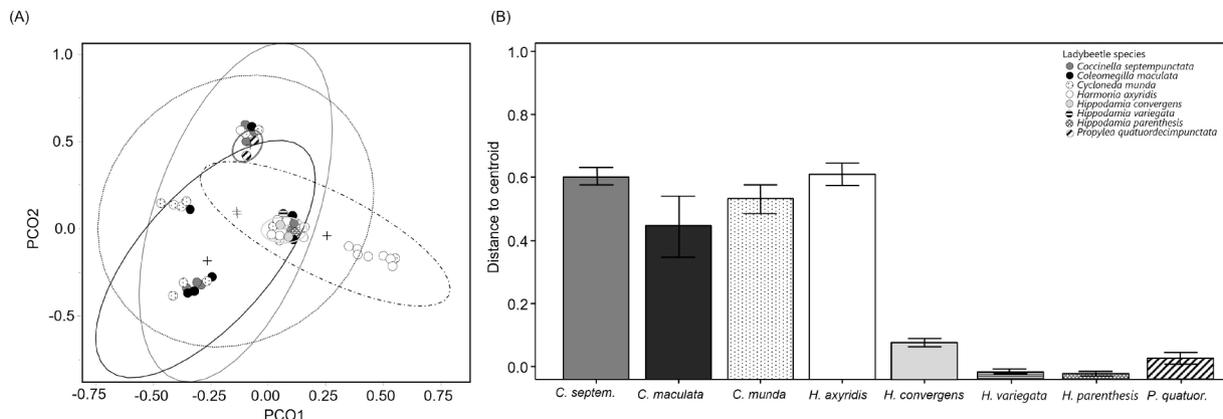


Fig. 8. OTU prey composition (A) and diet breadth (distance to centroid, B) as a function of lady beetle species.

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.

Acknowledgements

We thank the anonymous reviewers for providing feedback on earlier versions of the manuscript. We thank David Cavalier and the University of Wisconsin Biotechnology Center DNA Sequencing Facility for providing sequencing services. This research was funded by the Department of Energy (DOE) Great Lakes Bioenergy Research Center (Office of Science DE-FC02-07ER64494 and DOE Office of the Biomass Program, Office of Energy Efficiency and Renewable Energy DE-AC05-76RL01830) and USDA Grant 2018-67013-28060. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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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