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Short Communication

A molecular analysis to assess codling moth *Cydia pomonella* L. (Lepidoptera: Tortricidae) predation by orchard birds

Anna M. Mangan[a,b,*], Antoinette J. Piaggio[a], Matthew W. Hopken[a,c], Scott J. Werner[a], Liba Pejchar[d]

[a] United States Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, National Wildlife Research Center, 4101 LaPorte Ave., Fort Collins, CO 80521, United States
[b] Graduate Degree Program in Ecology, 1021 Campus Delivery, Colorado State University, Fort Collins, CO 80523, United States
[c] Department of Microbiology, Immunology, and Pathology, 1682 Campus Delivery, Colorado State University, Fort Collins, CO 85223, United States
[d] Department of Fish, Wildlife, and Conservation Biology, 1474 Campus Delivery, Colorado State University, Fort Collins, CO 80523, United States

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ABSTRACT

The codling moth *Cydia pomonella* L. (Lepidoptera: Tortricidae) is a major economic pest in organic apple orchards. Observational methods, prey removal experiments and correlative experiments with exclosures or nest boxes have demonstrated that birds contribute to the removal of this insect pest. However, the majority of research conducted in the last several decades has taken place outside of the United States and methods for studying biological pest control have advanced dramatically and now include molecular techniques. We conducted a proof-of-concept study to test a DNA-based approach to detect *C. pomonella* prey in the diets of birds occupying organic apple orchards. We tested published Tortricidae primers, polymerase chain reaction (PCR) and sequencing for detection of *C. pomonella* in avian feces. We also tested the quality of DNA isolated and purified from fecal samples using two DNA extraction kits. Finally, we field-tested this tool to identify the presence or absence of *C. pomonella* in the laboratory and from field samples. *C. pomonella* DNA was amplified in less than 1% of field samples and was successfully sequenced in 0.5%. A single species, the brown-headed cowbird *Molothrus ater* (Boddaert), had fecal samples positive for *C. pomonella* DNA. While our results do not provide evidence that birds play a strong role in the control of *C. pomonella* in western Colorado organic apple orchards, the approach we present is a new tool for understanding bird-mediated ecosystem services, avian feeding ecology, and supporting management decisions for sustainable agricultural practices and farmland biodiversity.

1. Introduction

Organic farmers have limited management options for controlling insect pests (National Organic Program, 2018). Their challenge is to satisfy increasing demands for environmentally friendly food, yet keep pests below thresholds required for such production systems to be economically viable. While the field of economic ornithology dates back to the late 19th century (Whelan et al., 2015), investigation of the potential value of birds in controlling pests has reemerged more recently for specialty crops such as coffee (Perfecto et al., 2004) and wine grapes (Jedlicka et al., 2011). In another specialty crop, organic apples, the most damaging pest is the codling moth *Cydia pomonella* L. (Lepidoptera: Tortricidae), whose larvae burrow into the fruit during development (Solomon et al., 1976).

Since the early 1900's, predator-prey studies of birds and *C. pomonella* using observational methods, prey removal experiments, or correlative experiments with exclosures or nest boxes have demonstrated that birds contribute to the removal of this insect pest (e.g., McAtee, 1912; Mols and Visser, 2002; Solomon et al., 1976). However, the majority of economic ornithology research conducted in the last several decades has taken place outside of the United States. Furthermore, tools for studying biological pest control have advanced dramatically as well. In situ observation of *C. pomonella* in bird diet is labor intensive and often impractical while morphological identification of invertebrates in stomach contents or fecal samples produces coarse and incomplete taxonomical results due to the challenges in identifying easily digestible

* Corresponding author at: United States Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, National Wildlife Research Center, 4101 LaPorte Ave., Fort Collins, CO 80521, United States.
E-mail addresses: Anna.Mangan@aphis.usda.gov (A.M. Mangan), Toni.J.Piaggio@aphis.usda.gov (A.J. Piaggio), Matt.W.Hopken@aphis.usda.gov (M.W. Hopken), Scott.J.Werner@aphis.usda.gov (S.J. Werner), Liba.Pejchar@colostate.edu (L. Pejchar).

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or larval prey (Jedlicka et al., 2013; Symondson, 2002). Molecular (DNA) detection of prey items from feces can be a less invasive, non-destructive alternative that has the potential to be a more accurate indicator of species interactions (Symondson, 2002). While DNA-based methods for analyzing bird droppings are not novel (e.g. Jedlicka et al., 2013; Karp et al., 2014), they have not previously been applied to investigate bird predation of *C. pomonella*.

To fill these knowledge gaps we conducted a proof-of-concept study to test a DNA-based approach to detect *C. pomonella* prey in the diets of birds occupying organic apple orchards. Our objectives were to: (1) test published Tortricidae primers, polymerase chain reaction (PCR) and sequencing for detection of *C. pomonella* in avian feces, (2) test the quality of DNA isolated and purified from fecal samples from two DNA extraction kits and (3) identify the presence or absence of *C. pomonella* in fecal samples collected from the avian community occupying organic apple orchards in western Colorado.

2. Methods

2.1. Sample collection

We contacted all 16 apple farmers in Delta County, Colorado with publicly available contact information and the participating five orchards were selected based on three criteria: (1) observations of farmers of birds and *C. pomonella* in the orchard, (2) minimal use of pesticides (i.e., USDA organic), and (3) willingness to participate (Mangan et al., 2017). We set mist nets in apple blocks within the orchards (Fig. 1) to capture birds and collect fecal samples (Federal Fish and Wildlife Permit number MB019065-0; NWRC QA 2286). Mist netting occurred throughout the apple growing season (May–September) in the interior and along the edges of nine apple blocks in 2014 and 12 blocks in 2015 for a total of 65 mornings (Appendix Table A.1). Following standard mist netting protocols (The North American Banding Council, 2001), nets were opened ~30 min before sunrise and closed by ~10:00 a.m. Captured birds were individually placed in clean cotton bags and subsequently processed to identify species and basic morphological measurements. Immediately after processing, birds were released and bags were checked for fecal material. Using spatulas cleaned with ethanol, fecal samples were transferred into either 99.5% ethanol or 70% isopropyl alcohol (Appendix Table A.1) and were stored frozen (−20 °C at the field site and −80 °C upon return to the laboratory) until they were extracted in May–June 2016 (Oehm et al., 2011). Bags were cleaned with a 70% bleach solution, washed and dried to prevent contamination among captured birds. For use as positive controls, adult *C. pomonella* were collected from pheromone traps in Hotchkiss, Colorado and stored frozen in 99.5% ethanol until DNA extraction. Avian feces known to be free of *C. pomonella* (hereafter target-free) were obtained from captive birds in an outdoor animal research facility at the National Wildlife Research Center (NWRC) with no access to overwintering pupae in December 2015, a time of year when adult moths are not present. Samples were frozen at −80 °C immediately after collection.

2.2. DNA extraction

We tested two DNA extraction kits, the QIAamp® DNA Stool Mini Kit (Qiagen, Germany) and the DNeasy® *mericon* Food Kit (Qiagen), to determine an optimal DNA isolation and purification method for our avian fecal (low-quality, low-quantity DNA) samples. Target-free avian fecal samples were thawed and vortexed briefly (i.e., 5 s) for homogenization. Approximately 1 cm^2 of feces was removed and the manufacturer's 'Isolation of DNA from Stool for Human DNA Analysis', and 'Standard Protocol (200 mg)' protocols for the stool and food kits, respectively, were followed with isolation automated on a QIAcube (Qiagen). Modifications to the stool kit protocol also included an initial incubation in Buffer ASL (stool lysis buffer) for 1 h and increased centrifuge times to completely pelletize particles. Before beginning the standard food kit protocol, feces were added to 1 mL of Food Lysis Buffer with a 5 mm stainless steel bead and were disrupted in a TissueLyser LT (Qiagen) for six minutes at 30 Hz. An extraction blank was included in all extraction sets to monitor contamination. Field samples were extracted in the same manner using only the optimal extraction kit (see Results). Extractions were purified using OneStep™ PCR Inhibitor Removal Kits (Zymo Research, Irvine CA) prior to PCR to reduce PCR inhibition caused by uric acid present in avian feces (Jedlicka et al., 2013).

Adult *C. pomonella* DNA was extracted from one specimen using a DNeasy® Blood and Tissue Kit (Qiagen) by crushing two dry *C.*

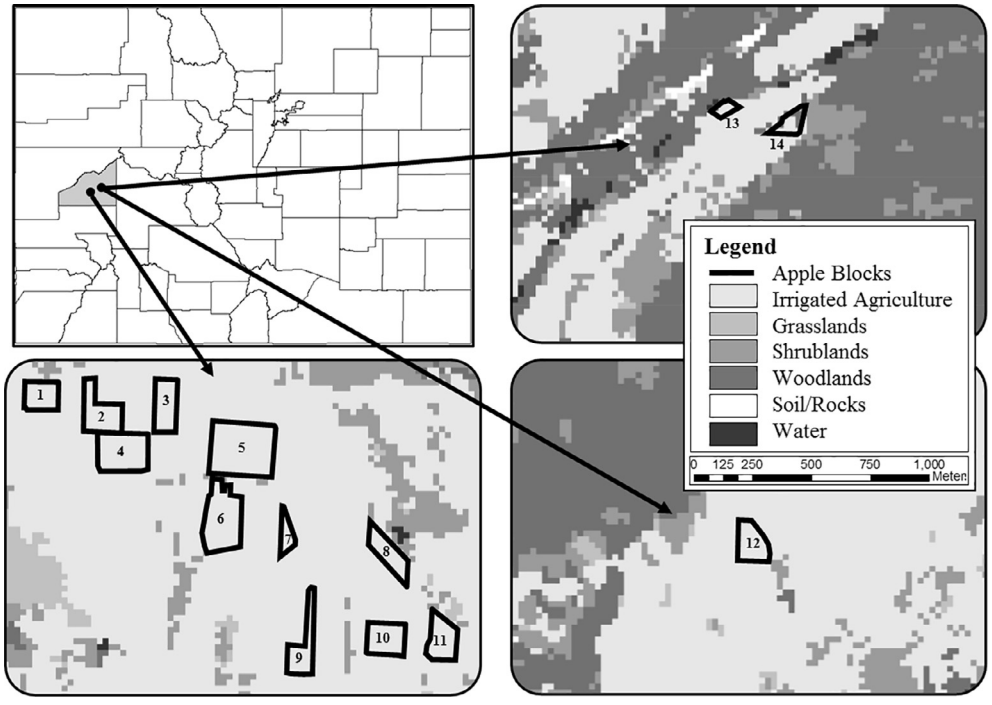

Fig. 1. Study Area. Study site locations in Delta County, Colorado 38°51′00″N, 107°45′00″W (grey-shaded polygon on Colorado map, top left). Numbered polygons denote apple blocks studied within the five participating fruit orchards in Hotchkiss (bottom left) and Paonia, Colorado (right). Land cover vegetation classes are illustrated in grey scale at a resolution of 25 m (Simpson et al., 2013). 'Irrigated Agriculture' includes fruit orchards, vegetable crops, hops and hay. Roads, property boundaries, etc. are not included to protect the anonymity of collaborating farmers.

pomonella legs and an abdomen segment in 180 µL ATL buffer and 20 µL proteinase K (Gilligan et al., 2014), and following the manufacturer's protocol with part of the extraction automated on a QIAcube (Qiagen). DNA recovery was quantified using a Qubit® 2.0 Fluorometer (Invitrogen) with Qubit™ dsDNA Assay Kits (Invitrogen). All laboratory work was conducted at a facility where DNA extractions were processed in a laboratory space dedicated to non-invasive samples, and PCR and post-PCR procedures were carried out in separate laboratories to limit contamination.

2.3. PCR amplification and DNA sequencing

Using published Tortricidae primers designed for *C. pomonella* (C.pom-F 5′-AATTTCAAGCAGAATCGTT-3′ and C.pom-R 5′-TTAACAG CTCCTAAAATA-3′) (Boreau de Roincé et al., 2012) labeled with 6-FAM on the 5′ end of the C.pom-F, a 153 base pair (bp) segment of mitochondrial cytochrome oxidase I (COI) was amplified. PCR was carried out in 10 µL reactions consisting of 5 µL 2× Qiagen Multiplex PCR Master Mix (Multiplex PCR Kit, Qiagen), 5 µg BSA (Bovine Serum Albumin), 0.4 µM of each primer and 2 µL template DNA. Amplifications were performed using an Eppendorf Mastercycler™ pro S PCR System (Fisher Scientific) following the protocol of Boreau de Roincé et al. (2012). Each PCR set included a negative control to monitor contamination and a positive control containing pure *C. pomonella* DNA. Fragment analysis of PCR products was conducted using a Genetic Analyzer 3500/3500×L (Life Technologies) and was visualized using GeneMapper® Software 5 (Life Technologies).

Fecal samples contain low quality, low quantity DNA and PCR inhibitors. Finding the optimal extraction method for successful isolation and purification of target DNA was accomplished by comparing serial dilutions of target-free avian fecal samples spiked with known amounts of *C. pomonella* DNA (Table 1). Target-free fecal sample extractions were spiked with 0.075 ng of extracted *C. pomonella* DNA and subsequently diluted using diethylpyrocarbonate (DEPC) treated water (Thermo Fisher Scientific). Sensitivity was assessed through three PCR replicates of each dilution.

Each field sample was also amplified in triplicate to account for stochasticity and sources of error (Taberlet et al., 1999). We identified field samples as putative positives if the amplified fragments' fluorescence intensities in the electropherogram were ≥1000 and included a peak topology similar to that of the positive control in any one of the three replicates. Although there was low potential for other Tortricidae species in our study area, primers were not species-specific. Therefore, to verify the species identity of successfully-amplified PCR products, we Sanger sequenced putative positives (n = 2), a subset of negative field samples (n = 30), positive controls of *C. pomonella* DNA (n = 3) and negative controls (n = 7). Amplification products were purified using ExoSAP-IT (USB Corporation, OH) then cycle sequencing reactions were performed in 10 µL reactions with 3 µL of purified PCR product, 1 µM primer, 0.25 µL BigDye v3.1 and 2.275 µL sequencing buffer. Cycle sequencing cleanup was performed with Sephadex G-50 and PrepEase columns (USB, USA). Sequences were visualized on a Genetic Analyzer 3500/3500×l (Life Technologies), and aligned and edited using SEQUENCHER v5.9 (Gene Codes, USA). Final species identification from sequences was verified using a Basic Local Alignment Search Tool (BLAST) with the National Center for Biotechnology Information website (Altschul et al., 1990).

Table 1
Sensitivity of extraction kits for *C. pomonella* from avian fecal samples. Serial dilutions and amplification results of target-free avian fecal samples extracted using QIAamp® Stool Mini Kits and DNeasy® *mericon* Food Kits and spiked with *C. pomonella* DNA. Successfully amplified products had fluorescence intensities ≥1000 and are denoted with (+). Each symbol (+ or −) represents one replicate, and amplification in two out of three PCR replicates (multiple-tubes approach Taberlet et al., 1999) was considered positive (in bold).

DNA quantity	QIAamp® DNA Stool Mini Kit	DNeasy® *mericon* Food Kit
0.075 ng	+ + +	+ + +
0.0075 ng	+ + +	+ + +
0.00075 ng	− − +	+ + +
0.000075 ng	− − −	+ + −
0.0000075 ng	− − −	− − −
0.00000075 ng	− − −	− + −
0.000000075 ng	− − +	− − −

3. Results

Across the two sampling seasons we collected a total of 211 fecal samples from 31 bird species (Appendix Tables A.1 and A.2). Fecal samples were collected from 60% (211/352) of birds captured. The DNeasy® *mericon* Food Kit had superior DNA amplification of the spiked samples (Table 1). We therefore used the DNeasy® *mericon* Food Kit to extract DNA from field samples. Two samples (< 1%) had peaks in the fragment analysis (florescence intensity > 3300) that corresponded to expected size and peak morphology as assessed through positive controls of *C. pomonella*. Positive detection of *C. pomonella* DNA in one of these samples was further confirmed by sequencing and a BLAST search (0.5% of samples). We conservatively report one positive sample and one putative positive. Aligned sequences from *C. pomonella* positive control samples had 124 bases and 100% sequence identity match to reference accession numbers in GenBank (Appendix Table A.3). The aligned sequence from the one field sample resulted in 113 bases and 99% sequence identity with 112/113 base matches (one ambiguous base; Appendix Table A.3) to the same reference accession numbers in GenBank. Besides *C. pomonella*, no other species appear in any BLAST search. Although we were unable to successfully sequence the second amplified PCR product, both samples were collected from a single species, the brown-headed cowbird *Molothrus ater* (Boddaert). Extraction blanks and PCR negative controls were negative and none of the negative controls or negative field samples were successfully sequenced.

4. Discussion

We demonstrated the success of a DNA-based method to identify avian predators of an economically-important insect pest. Molecular analyses can be used to enhance our understanding of predator-prey interactions and help farmers evaluate and utilize the ecosystem services provided by birds. We found superior target DNA isolation and purification through higher amplification success using DNeasy® *mericon* Food Kits, similar to other low-quality, low-quantity DNA studies (Williams et al., 2017). Our study is the first to use this approach to identify the brown-headed cowbird as an avian predator of *C. pomonella*.

Analyzing bird diets can be challenging, especially for prey species such as *C. pomonella* which have life history phases that are difficult to observe (Symondson, 2002). Furthermore, studies in which *C. pomonella* are experimentally removed or where nest boxes are experimentally installed (e.g. Mols and Visser, 2002), are limited in their ability to definitively identify avian species providing the pest removal service. In light of these challenges, molecular scatology is a powerful, non-invasive indicator of biological pest control (Jedlicka et al., 2013). A variety of DNA-based methods are available for analyzing bird droppings, with advantages and disadvantages that must be considered in the context of the research questions. For example, metabarcoding could be used to identify many target pest species that may be consumed by avian predators (e.g., Cristol-Martínez et al., 2016) and metagenomics shotgun sequencing allows for an even broader study of all genomic material without the need to select amplification targets a-priori (e.g., Srivathsan et al., 2016). However, these approaches can be more expensive and necessitate a more comprehensive reference

database to appropriately interpret the data (Cristol-Martínez et al., 2016; Srivathsan et al., 2016). Selecting an optimal molecular method is therefore a matter of research needs, budget, and sequence database availability.

We hypothesized that several species of insectivorous birds would consume *C. pomonella* (McAtee, 1912), thus the limited number of positive detections that were restricted to a single species was surprising. While this may be due to limited predation, there are biological reasons that may have hampered the detection of *C. pomonella* DNA in our samples. Birds have relatively short gut retention of insects, ranging from ~1 h (Levey and Karasov, 1994) to ~4 h (Oehm et al., 2011). Birds in our study may have excreted the remains of their prey prior to sample collection. However, while mist netting in the mornings may have missed opportunities to detect birds consuming adult *C. pomonella* during periods of evening activity (Jedlicka et al., 2013), other life stages of *C. pomonella* were available to birds at all times of day. For instance, eggs are laid directly on apples and leaves, larvae are found on apple leaves and on the surface or the interior of the fruit, and pupae are typically in the tree bark or soil before reemerging as adults (Solomon et al., 1976). Consequently the probability of DNA detection may be influenced by avian foraging behavior (e.g., gleaning vs. fly-catching). Our sampling occurred throughout the entire apple growing season (Appendix Table A.1) and encompassed the overlapping life stages of the three *C. pomonella* generations that occur in our study region each year (Cranshaw and Hammon, 2013) which should have enabled us to examine predation of any *C. pomonella* life stage.

Applying molecular methods in an ecological context can help examine the tradeoffs associated with wildlife in agroecosystems, and inform management and conservation strategies (Karp et al., 2014; Mangan et al., 2017). Brown-headed cowbirds, which we identified as a consumer of *C. pomonella*, are brood parasites; they lay their eggs in the nests of other bird species and this host provides care to the eggs and young (Lowther, 1993). Therefore, even though we demonstrated that cowbirds contribute to this ecosystem service, the ecological costs of managing orchards to increase populations of this nest-parasitic species potentially outweigh the benefits to wildlife-friendly agriculture. While our results do not provide evidence that birds play a strong role in the control of *C. pomonella* in western Colorado organic apple orchards, the approach we present is one of many possible tools for understanding bird-mediated ecosystem services and avian feeding ecology, and supporting management decisions for sustainable agricultural practices and farmland biodiversity.

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