



5-2010

Molecular analysis of guano from bats in bat houses on organic pecan orchards

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Recommended Citation

Brown, Veronica Angelelli, "Molecular analysis of guano from bats in bat houses on organic pecan orchards." Master's Thesis, University of Tennessee, 2010.
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To the Graduate Council:

I am submitting herewith a thesis written by Veronica Angelelli Brown entitled "Molecular analysis of guano from bats in bat houses on organic pecan orchards." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Ecology and Evolutionary Biology.

Gary F. McCracken, Major Professor

We have read this thesis and recommend its acceptance:

James Fordyce, Alison Buchan

Accepted for the Council:

Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Accepted for the Council:

Carolyn R. Hodges
Vice Provost and Dean of the Graduate School

Molecular analysis of guano from bats in bat houses on organic pecan orchards

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Veronica Angelelli Brown
May 2010

This thesis is dedicated to my husband, Chris, and my daughters, Caroline and Maddie, whose support and encouragement keep me motivated. I also dedicate this thesis to my Granny, Peg Jamison, for being the most proud grandmother, even though she did not get to see its completion.

ACKNOWLEDGEMENTS

I would like to thank all those who provided support and encouragement during my degree program. I would like to thank my advisor, Gary McCracken, both for his support of my research and for encouraging me to push myself further. I also thank my committee members, Jim Fordyce and Alison Buchan, for valuable insight and suggestions through the course of this project. This project would not have been possible without John and Jimma Byrd along with the entire Bibin family: pecan growers committed to organic farming practices. I also thank Elizabeth Braun de Torrez for many nocturnal hours collecting guano samples, as well as for valuable discussion of results. My undergraduate research assistants, Ann Herron and Alicia White, deserve recognition for spending many hours looking through microscopes for stink bug fragments. Arijana Barun, Ben Fitzpatrick, Tom Hallam, Martin Nuñez, Amy Russell, Lara Souza, and Megan Todd-Thompson all provided support and advice on this research. Jim Dutcher, John Westbrook, and Jesus Esquivel provided information on insects. Maarten Vonhof and Amy Turmelle provided sequences to help me get started in primer design. The Knoxville Zoo and Barbara French provided assistance with fecal samples.

Funding was generously provided by Bat Conservation International, Graduate Women in Science, and a summer research award from the department of Ecology and Evolutionary Biology at the University of Tennessee.

ABSTRACT

Bats are generalist predators of night flying insects, including many crop pests. Pecan nut casebearer (*Acrobasis nuxvorella*), hickory shuckworm (*Cydia caryana*), and several stink bug species are some of the most damaging crop pests in pecan orchards. Attracting bats to agricultural areas using bat houses may reduce the numbers of these pests and, consequently, their economic impact. This study uses quantitative polymerase chain reaction (QPCR) of mitochondrial DNA found in the guano of bats living in bat houses on organic pecan orchards to document the consumption of pecan nut casebearer, hickory shuckworm, and corn earworm (*Helicoverpa zea*), which is one of the most destructive pests of many crops throughout the world. This study also uses direct sequencing of insect remains in bat fecal pellets to identify species of stink bugs consumed by bats in bat houses. Evidence that bats prey upon crop pests supports the hypothesis that bats are both economically and ecologically beneficial to pecan farmers and provides incentives for bat conservation.

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This Thesis is prepared in manuscript format for future submission to the journal *Acta Chiroptologica*, with coauthors Elizabeth Braun de Torrez and Gary McCracken. I completed all laboratory work. Elizabeth Braun de Torrez was responsible for the fieldwork in Texas. All lab work was done in Gary McCracken's lab, and he supervised the project.

INTRODUCTION

Predator-prey relationships can be challenging to document in bats, since direct observation of predation is difficult in fast-flying, nocturnal animals. As native, generalist predators of night-flying insects, bats have been shown to serve as significant agents for suppression of insect pests in agriculturally intensive areas (Federico et al. 2008; Cleveland et al. 2006). In particular, Brazilian free-tailed bats, *Tadarida brasiliensis*, have an estimated annual value of \$741,000 as pest control agents on cotton fields in south-central Texas (Cleveland et al. 2006). The diets of *T. brasiliensis* have been linked to the demographics and migratory movements of crop pests (Order Lepidoptera) in Central Texas, with approximately 31% of the bats' diet composed of moths (Lee and McCracken 2005, 2002). However, the moths could not be identified to species by visually examining fecal pellets because the soft-bodied insects break down thoroughly during mastication and digestion.

Broad-spectrum insecticides are commonly used to control insects, but these insecticides often have serious environmental and economic consequences. Pesticides have both direct cost to farmers (purchase and application the chemicals) and indirect costs to ecosystems and the environment (loss of non-target species, pesticide resistance, and public health costs) (Ellington et al. 2003, Cleveland et al. 2006). Recently, strategies for control of crop pests has moved more toward integrated pest management (IPM), which includes a combination of monitoring, chemical control, and biological control (Dutcher et al. 2003). Organic farming, which is a production system in which synthetic pesticides are avoided (Wickramasinghe et al. 2004), relies

entirely on non-chemical methods, such as biological agents, for pest control. To encourage pest suppression by bats in lieu of pesticide use, some organic farmers utilize bat houses to attract bats to their farms and orchards. Bat activity was found to be 61% higher and foraging activity 84% higher on organic farms versus conventional farms in Britain (Wickramasinghe et al. 2003), suggesting that organic farming practices may provide more suitable habitat for bats.

Crop pest species of the size consumed by bats, specifically Lepidoptera and Hemiptera (Whitaker 1996), are common pests of pecan orchards. Pecan is a highly nutritious food product that is widely cultivated in the southern United States and is expensive to produce relative to other nut species (Mizell 2003). The diversity of pests, the variation among orchards, and the masting trait of pecans (periods of large crop yields interspersed with relatively barren periods) make it difficult to attribute crop damage and impact on yield to a particular insect pest (Dutcher et al. 2003). However, several studies have found that the most notable pests of pecans include two moth species, pecan nut casebearer (*Acrobasis nuxvorella*) and hickory shuckworm (*Cydia caryana*), and several stink bug species (Order Hemiptera, Family Pentatomidae), specifically green stink bug (*Acrosternum hilare*), brown stink bug (*Euschistus servus*), dusky stink bug (*E. tristigma*), and southern green stink bug (*Nezara viridula*) (Wood 2003, Dutcher 2002, Cottrell 2001, Harris et al. 1998). In 2006 in Georgia alone, an estimated \$520,000 was spent to control pecan nut casebearer, and \$650,000 to control hickory shuckworm (Hudson & Dutcher 2006). Pecan nut casebearers damage pecans early in the growing season and feed only on pecan trees (Stevenson et al. 2003, Dutcher 2002). Hickory shuckworms are destructive to both hickory and pecan trees and are most damaging to pecans in the fall, although they are present in the orchards throughout the growing season (Dutcher 2002). Stink bugs are destructive to many crop species and are known to damage pecan trees in the fall (Dutcher et al. 2003).

Corn earworms (*Helicoverpa zea*; also known as cotton bollworm) are not known to damage pecans, but they are one of the most destructive agricultural pests in the Americas, affecting many crops surrounding pecan orchards, including corn and cotton (Jackson et al. 2008). By applying molecular techniques to DNA extracted from guano samples, *T. brasiliensis* have been shown to consume large quantities of corn earworms (McCracken et al. in prep).

Molecular scatology applies genetic techniques to the analysis of fecal material and can be applied to predator-prey relationships, allowing prey species to be identified to taxonomic levels that often are not feasible through traditional fecal analysis. Collecting and analyzing feces can provide a noninvasive and inexpensive method for obtaining genetic material for dietary analysis (Whittier et al. 1999). These techniques have been applied in several mammalian species, including seals (Casper et al. 2007), sea lions (Deagle and Tollit 2007, Deagle et al. 2005), and bats (McCracken et al. in prep, Clare et al. 2009). Given the difficulty in capturing and monitoring bats in the wild, molecular fecal analysis is particularly promising for studying predator-prey interactions in this group of volant mammals. The high consumption rate of corn earworm moths by *T. brasiliensis* was documented using quantitative polymerase chain reaction (QPCR) (McCracken et al. in prep), a highly sensitive method of simultaneously amplifying and quantifying a specific DNA sequence in a relatively short period of time (Troedson et al. 2007; Van den hurk 2007). When applied to fecal samples, QPCR allows many samples to be quickly screened for a specific prey item by using species-specific markers. Another molecular method recently applied to identifying prey DNA in fecal samples involves using non-specific primers to directly sequence DNA amplified from insect fragments found in guano pellets (Clare et al. 2009).

The objective of this study is to use molecular techniques to examine the consumption of crop pests by bats inhabiting bat houses on organic pecan orchards in Texas and Georgia. Here I analyze feces collected beneath bat houses for the presence of DNA from pecan nut casebearer, hickory shuckworm, corn earworm, and stink bugs using both QPCR and direct sequencing of amplified DNA products from insect fragments. Given that both orchards report reduced insect damage once bats began inhabiting the houses, we expect consumption of crop pests by bats to reflect pest insect abundances. This is the first study to document the diet of bats in pecan orchards.

METHODS

Study sites

This study was conducted on two organic pecan orchards, Pebble Hill Grove, a 27-acre orchard in Quitman, Georgia, and King's Crossing, a 100-acre orchard in San Saba, Texas (Figure 1; all Figures and Tables in Appendix I). Both orchards are certified organic, meaning that they use no insecticidal sprays. Bat houses were first installed at Pebble Hill Grove in 1996 and are currently inhabited by an estimated 3500 bats, predominantly Brazilian free-tailed bats (*T. brasiliensis*) and evening bats (*Nycticeius humeralis*). Bat houses were first installed at King's Crossing in 2003. An estimated 1500 bats live in the houses, mostly *T. brasiliensis*, with lesser numbers of *N. humeralis*, and cave myotis (*Myotis velifer*).

Fecal sample collection

Guano was collected by securing 4 oz collection cups (Fisher Scientific) to wooden boards beneath bat houses to passively collect feces. The cups were distributed under the houses to collect feces from different locations within the houses. Bats were not present in every bat house at every collection date, and collections were not made in rainy conditions. Feces were

put in 2 ml screw cap microtubes (Sarstedt) containing silica gel desiccant (4-10 mesh, Fisher Scientific) (Wasser et al. 1997) and stored at -20°C following collections. From the Georgia orchard in 2008 and 2009 and the Texas orchard in 2008, all of the feces from one collection cup were put into one tube. On nights of heavy bat activity, only the feces that would fit into one 2 ml tube were collected. From the Texas orchard in 2009, two fecal pellets from each collection cup were selected and placed into tubes individually. The largest pellets in the collection cups were preferentially selected.

Sampling in 2008 took place throughout the summer and sampling in 2009 was only during the time period of peak pecan nut casebearer activity, around May. On the Georgia orchard, 10 collection cups were placed under the bat houses at ca. 0700 and feces collected approximately 2 hours later. In 2008, samples were collected weekly under three bat houses; in 2009, samples were collected twice a week under two houses. Poor weather conditions ended sample collections prematurely in 2009. On the Texas orchard, 12 collection cups were placed under one bat house at ca. 2100 and feces were collected approximately 12 hours later. Collections were informed by insect activity in 2008; collections were made every 3-5 days in 2009. Collection dates for both locations are summarized in Table 1.

Fecal samples were collected from known species of bats captured in mist nets in Texas to serve as positive controls. Bats were placed individually in cloth bags for up to three hours immediately after capture, and feces produced were collected and placed in individual 2 ml tubes as described above. Wing tissue biopsies (2mm) of 7 *N. humeralis* and 12 *M. velifer* were placed in tubes containing 10% DMSO saturated with NaCl. Tissue samples were also collected from tree roosting bats found in the orchards but not the houses (2 *Lasionycteris noctivagans*, 3

Lasiurus borealis, and 1 *Perimyotis subflavus*) to aid in development of species-specific markers for bats.

To confirm insect markers, positive fecal controls known to contain pecan nut casebearer DNA were obtained by feeding adult moths to one adult male *N. humeralis* and one adult male *M. velifer*. The bats were fed mealworms and as many moths as they would eat. Feces were collected and stored as described above.

Insect monitoring

To document patterns of insect abundances and to provide insect tissue for genetic analysis, insects were collected using multiple pheromone lures with sticky and black light traps. Collections using pheromone lures were checked every 2-3 days during May on the Georgia orchard targeting pecan nut casebearers and the insect number was averaged across those days. Insect traps monitoring pecan nut casebearers in Texas were checked daily throughout the pecan growing season. Pheromone lures for hickory shuckworm moths were used in Texas only, but lures have limited efficacy in attracting hickory shuckworm moths (Bill Ree pers. com.). Corn earworms and stinkbugs were collected in black light traps in Texas, confirming their presence but not abundance. Monitoring for corn earworm in Georgia took place at the University of Georgia Tifton Station, located less than 100 kilometers north of the orchard (data accessed through the PestWatch database <http://www.pestwatch.psu.edu/sweetcorn/tool/tool.html>). Corn and cotton are common crops surrounding the Georgia orchard, and corn earworms are known to be prevalent in the area.

DNA extraction and amplification

DNA was extracted from 26 pecan nut casebearers, 12 hickory shuckworms, several stink bug species (*Acrosternum hilare*, *Euschistus servus*, *E. variolarius*, and *Thyanta custator*) and 45

other insects from the study areas using DNeasy Tissue DNA Extraction kits (Qiagen), with minor modifications. Incubations on excised insect abdomens from larger insects or the entire body of smaller insects took place overnight in buffer ATL with proteinaseK, followed by centrifugation and collection of supernatant to a new tube, then continuation of the manufacturer's protocol. DNA was also extracted from the bat tissue collected using DNeasy kits, following the manufacturer's protocol with an overnight incubation in buffer ATL with proteinaseK.

The high copy number of mitochondrial DNA compared to nuclear DNA makes mitochondrial DNA preferable for samples with degraded DNA (King et al. 2008; Farrell et al. 2000). Subsequently, approximately 650bp of the mitochondrial cytochrome oxidase I (COI) locus was amplified from both insects and bats for species-specific primer development using conserved primers. The COI PCR protocol using the primers LCO1490 and HCO2198 (Herbert et al. 2003; Table 2) is listed in Appendix II. The PCR products were purified using ExoSAP-IT (USB Corporation) and sequenced using the BigDye v3.1 Terminator Cycle Sequencing Kit (Applied Biosystems) on an ABI 3100 automated sequencer housed at the University of Tennessee Molecular Biology Resource Facility (Applied Biosystems). Insect and bat COI sequences were aligned using Sequencher v4.5 (Gene Codes Corporation) and analyzed for intra- and inter-specific variation. Sequences of COI from *T. brasiliensis* were obtained from Amy Turmelle (unpublished) and were included in this alignment.

Prey DNA isolated from predator feces is highly degraded and only relatively short fragments (<200-300 base pairs) amplify reliably (King et al. 2008; Taberlet et al. 1999). Species-specific COI primers and probes were designed using Primer3 software (Rozen 2000) to

amplify a <200 base pair fragment for both pecan nut casebearer and hickory shuckworm (Table 2). Each species-specific primer set was tested empirically for specificity.

Compounds in fecal material can inhibit the PCR process and may result in false negatives (Taberlet et al. 1999). As a positive control for the quality of the fecal extractions, species-specific primers for *M. velifer*, *N. humeralis*, and *T. brasiliensis* (Table 2) were designed. To confirm positive amplification of the fecal extractions, all samples were first screened using primers specific to *T. brasiliensis*, because this is the most common bat species in the roosts. Samples that did not amplify or amplified weakly with the primers for *T. brasiliensis* were then screened with primers specific to *M. velifer* and *N. humeralis*. Each sample that produced positive QPCR results for any of the insects was also screened with the three species-specific primer sets for bats. The PCR protocol for bat species-specific amplification is listed in Appendix II. PCR product was visualized on a 2% agarose gel (Sigma). Samples were confidently identified to bat species if they amplified with only one set of species-specific primers.

DNA was extracted from a single pellet from each guano collection cup using UltraClean DNA Kits (MoBio Laboratories), with incubation at 4°C for at least one hour, rather than the recommended 5 minutes. Since the bat houses hold a large number of bats, individual guano pellets selected from different locations under the houses are likely from different bats. The largest pellets were preferentially selected for extractions to assure the greatest quantity of insect DNA in the pellet. Because contamination is a concern for molecular analysis of samples with very low DNA content (Kohn & Wayne 1997), a negative control of feces collected from a captive colony of big brown bats (*Eptesicus fuscus*) fed a non-moth diet was included in each batch of extractions. For the Georgia orchard, DNA was extracted from 15 individual fecal

pellets per date in 2008 and from one pellet per collection cup per date in 2009. For the Texas orchard, DNA was extracted from 10 individual fecal pellets per mass-collection date in 2008 and 12 per date in 2009. DNA was extracted from the entire amount of feces collected (1-4 pellets) from the bats that were fed pecan nut casebearer.

Species-specific QPCR

The insect species-specific QPCR profile is listed in Appendix II. Size standards for QPCR were generated using PCR amplicons of genomic DNA of each insect species cloned into a standard vector (TOPO TA cloning kit; Invitrogen). Each QPCR run included a ten-fold dilution series of known gene copy number ranging from 10 copies to 1,000,000 copies, calculated using the methods of Rinta-Kanto et al. (2005). This dilution series served as a positive control of reaction conditions and allowed the results to be analyzed using Opticon Monitor 3 software (Biorad Laboratories). Negative controls (distilled water in place of template DNA) also were included in every QPCR to test for cross-contamination. Aerosol resistant tips were used for preparation of all reactions, and QPCRs were prepared under a laminar flow hood.

Species-specific QPCR of samples collected in 2008 was performed in single reactions during the time periods of expected peak pecan nut casebearer and hickory shuckworm activity in pecan orchards. Table 1 summarizes the dates and numbers of reactions performed. Because false negatives are often associated with PCR of fecal samples (King et al. 2008; Taberlet et al. 1996), samples from 2009 were run in duplicate to improve detection of low quality DNA and were only screened for pecan nut casebearer.

A subset of samples for corn earworm cytochrome oxidase II (COII) were screened using the methods of McCracken et al. (in prep), running all reactions in duplicate. Samples were

screened approximately weekly throughout the summer of 2008 at both locations. Samples were considered positive if at least one of the duplicate reactions amplified.

The QPCR results for corn earworm from Georgia in 2008 were analyzed using the logistic procedure of SAS 9.13 (SAS 2003) to determine whether a correlation with insect abundance was evident. In these models, the response variable was the presence or absence of corn earworm DNA in any of the fecal samples analyzed for each date. The independent variables analyzed were (1) the number of corn earworms caught in pheromone traps each night, (2) the average number of corn earworms caught on the sample night and the previous night, and (3) the maximum number of corn earworms caught on the sample night and the previous night.

To confirm that the reactions amplified the correct DNA sequence, all samples that showed positive QPCR results for pecan nut casebearer and hickory shuckworm and two samples positive for corn earworm were cloned using a TOPO TA cloning kit (Invitrogen). Cloned plasmids were harvested from *E. coli* cultures using FastPlasmid Mini Purification System (5 Prime) and sequenced as described above using the M13R primer (5'-CAGGAAACAGCTATGAC-3') that recognizes the plasmid.

Direct sequencing of insect fragments

Using a needle and fine forceps to visually identify stink bug fragments under a microscope, fourteen fecal pellets were examined per date from the Georgia orchard from April through October 2008, for a total of 336 guano pellets. This method is ideal for identifying stink bug fragments in guano because they tend to be very distinctive and easily identifiable under a dissecting microscope. The stink bug fragments were washed several times in ethanol prior to DNA extraction with a DNeasy kit as described above. The PCR for stink bug amplification using insect-specific COI primers (Table 2) is listed in Appendix II. The PCR product was

purified and sequenced as described above. The resulting 221 bp sequences were compared to stink bug sequences obtained in the lab and to published sequences on GenBank using the blast(n) function to identify the sequences to species level.

RESULTS

Through the use of QPCR, mitochondrial DNA sequences of pecan nut casebearer and corn earworm were identified from guano samples collected beneath bat houses in organic pecan orchards in Georgia and Texas. Through the use of QPCR and direct sequencing of insect remains from fecal pellets, mitochondrial DNA sequences of hickory shuckworm and stink bugs were identified from guano samples collected in Georgia.

Taxonomic identification of guano

One hundred and seventy four fecal samples were tested with three species-specific primers for bats. The remaining 672 fecal samples amplified strongly with primers specific to *T. brasiliensis* and, thus, were not tested with additional reactions. Ninety (52%) of the samples tested with the three bat species-specific primers yielded an amplicon with only one primer set and were, therefore, identified to bat source (78 *T. brasiliensis*, 12 *N. humeralis*, 0 *M. velifer*). All of the fecal samples confirmed from *N. humeralis* were from the Texas orchard, and *M. velifer* was not confirmed in either bat house. Three samples did not amplify in any reaction; this lack of detection could be due to PCR inhibitors not being fully removed in the DNA extraction process or the fecal pellets could have been from a species of bat not detected by these primers. The remaining 81 samples gave ambiguous results and could not be identified to bat species.

Controlled feedings

Pecan nut casebearer DNA was recovered through QPCR from fecal material collected from bats fed a diet of pecan nut casebearer. The QPCR products, measured as copy number

standardized by fecal sample weight, ranged from 3.3×10^4 to 7.5×10^8 copies per gram. The lowest copy number came from a bat that consumed only half a moth, and the highest copy number came from a bat that consumed seven moths.

Identification of pecan nut casebearer DNA in bat guano

Eight samples (1.4%) out of the 576 fecal samples screened for pecan nut casebearer produced positive QPCR results. All eight positive samples yielded only pecan nut casebearer gene sequences upon sequencing of cloned product. Figure 2 displays the dates of insect detection plotted against insect abundance data. Insect trap data indicate that pecan nut casebearers had a short, sharp peak of abundance in early May that increased dramatically over one or two nights. Seven of the eight positive samples coincided with periods when pecan nut casebearers were caught in pheromone traps. Positive samples were detected in Georgia in both years, but no samples from Texas in 2008 were positive. Five of the positive samples amplified with only one set of bat species-specific primers and were, thus, confidently identified as from feces of *T. brasiliensis*; one was identified as *N. humeralis*. Five of the eight samples were run in duplicate, but only one amplified in both reactions, indicating a low amount of insect DNA. The QPCR products ranged from 1.1×10^3 to 5.5×10^4 copies per gram of fecal material.

Identification of hickory shuckworm DNA in bat guano

Eight samples out of the 210 fecal samples screened for hickory shuckworm produced positive QPCR results. Only three of these eight positive samples (1.4% of total samples) yielded gene sequences consistent with hickory shuckworm upon sequencing of cloned QPCR products. The three dates with positive results were August 20, September 26, and October 2, 2008 and all were from the Georgia orchard. One of the three positive samples was confidently identified as feces from *T. brasiliensis*. The QPCR products ranged from 3.9×10^3 to 6.4×10^4

copies per gram of fecal material. The cloned sequences that did not correspond with hickory shuckworm matched through the primer regions but differed from the hickory shuckworm sequence at 3 to 16 bases. None of these gene sequences matched with any identified sequences using the BLAST function of Genbank, although they are consistent with Lepidoptera COI sequences. Given the non-specificity of the primers and probe and the low detection rate, no further analysis was done on hickory shuckworm.

Identification of corn earworm DNA in bat guano

Thirty-six (7.13%) of the 505 fecal samples screened for corn earworm produced positive QPCR results. Twenty-six of the positive samples were confidently identified as feces from *T. brasiliensis* and one from *N. humeralis*. The single positive sample from *N. humeralis* was from Texas on May 3, 2008, a time when corn earworms are in low abundance. The QPCR products ranged from 7.6×10^1 to 8.0×10^5 copies per gram of fecal material. Figure 3 shows the dates that positive samples were obtained for both locations in 2008, along with corn earworm abundance for Georgia. No data are available on the numbers of corn earworm in Texas near the pecan orchard. However, 13 of the 14 samples positive for corn earworm in Texas were from July 5 through August 11, a time period when corn earworm moths are known to be in high abundance, as they emerge from fruiting corn in southern Texas (Westbrook et al. 1995). Samples positive for corn earworm in Georgia occurred throughout the sampling period. Insect abundance data from pheromone traps also indicate that corn earworm moths were present throughout the sampling period.

The logistic regression analyses showed no statistically significant relations between QPCR positive fecal samples and corn earworm abundance in pheromone traps in Georgia ($\chi^2 = 0.17$, DF = 1, P>0.67) (Figure 3). The average and maximum numbers of corn earworms over

two nights were also not significant ($\chi^2 = 0.001$, DF = 1, $P > 0.97$; and $\chi^2 = 0.02$, DF = 1, $P > 0.88$ respectively).

Identification of stink bugs in bat guano

From five dates in Georgia, 22 insect fragments in guano pellets were identified visually as potentially from stink bugs. DNA from 7 of these fragments was successfully sequenced and identified as southern green stink bug (*Nezara viridula*), with 100 percent identity over 189 bp to Genbank Accession number AY839161. These fragments were from guano collected over 4 nights in August and September 2008. The remaining fragments failed to amplify.

DISCUSSION

Molecular methods of analyzing predator diets are the most practical means for evaluating consumption of soft-bodied prey (McCracken et al. in prep; Clare et al. 2009). This is the first study to document the consumption of pecan nut casebearer and hickory shuckworm moths by bats. Previous studies have shown that bats consume insects of the family Pentatomidae (Lee and McCracken 2005; Whitaker and Clem 1992), but this is the first study to document stink bug consumption to the species level.

QPCR detected pecan nut casebearer both during peak insect emergences and during periods of low insect abundance. The insect collections document the brief local periods of pecan nut casebearer emergence in each orchard. Pheromone traps only attract male moths during reproductive periods, and there is no information on females in these orchards (Marvin Harris, pers. com.). Positive samples from dates when pecan nut casebearers appear to be absent from the orchards could be reflective of female movement or of local emergences in nearby orchards.

While this study verifies that bats are consuming pecan nut casebearers, the low numbers of fecal samples that were positive for the DNA of this pest suggests that the bats are not specializing on this species of moth as a major food source. As highly mobile animals that often forage over great distances (Lee and McCracken 2005), bats can take advantage of variation in prey abundance, and the local activity of bats is expected to respond quickly to local changes in prey abundance (Pocock and Jennings 2008). Pecan nut casebearers are small moths (approximately 8.47 mm long; Ree and Knutson 1997) that tend to remain close to pecan orchards (Harris et al. 1997) and exhibit sharp peaks of abundance. The bat houses on both orchards are dominated by *T. brasiliensis*, which are known to forage in open, uncluttered areas and at high altitudes, owing to their wing morphology of high wing loading and high aspect ratio (Gillam et al. 2009; McCracken et al. 2008). This type of flight may decrease this species ability to detect local peaks of insect abundance within orchards. Vertical separation of the bats and insects may prevent high rates of interaction within this predator-prey system. In contrast to *T. brasiliensis*, *N. humeralis* tend to forage in clearings within forests (Carter et al. 2004) and may, therefore, more frequently encounter insects that remain below canopy within orchards.

Although the markers developed for hickory shuckworm produced several false positives, subsequent cloning positively identified three samples as containing hickory shuckworm DNA. These markers have the potential to detect hickory shuckworm DNA in guano samples but would require further development to be reliable. The three positive samples were collected in the fall, when hickory shuckworms are known to cause the most damage to pecan orchards. Thus, bats are consuming this crop pest at a time when consumption of this pest may mitigate crop damage.

Tadarida brasiliensis have been shown to exploit large emergences of corn earworms from corn and cotton fields in southern Texas (McCracken et al. in prep), and the present study

indicates that bats residing on pecan orchards are large consumers of corn earworms as well. This study also documents the consumption of corn earworms by *N. humeralis*, a species not previously shown to consume this crop pest. Because corn earworms are not known to damage pecan trees, this documentation of consumption suggests that the bat houses are benefiting other parts of the agroecosystem, in addition to the orchards containing the bat houses. The lack of a correlation between corn earworm abundance and the number of QPCR positives in Georgia suggests that the bats are able to capitalize on these moths, even when the moths are in low abundance. Given the distances that bats can travel to forage, the bats may also be capitalizing on emergences of corn earworms from locations that are not detected with the pheromone trap data.

Because stink bugs have hard exoskeletons that resist mastication and digestion, sequencing remains found in guano samples is a fairly straightforward and inexpensive means of identifying the insects to species. In this study, southern green stink bug remains were positively identified from fecal samples collected in the fall, when stink bugs are most detrimental to pecan trees. Because stink bugs damage a wide variety of crops, their consumption by the bats living in bat house benefits other components of the agroecosystem, in addition to the pecan orchard.

This study positively identifies several crop pests in the diets of bats in bat houses; however, the frequency of positive samples in feces was low compared to previous findings of high occurrence of corn earworm moths in the diets of *T. brasiliensis* in corn and cotton agroecosystems. The low percentage of positive samples could result, in part, from the sampling regime. Fecal pellets were analyzed individually in order to document the number of bats consuming the pests. However, Whitaker et al. (1996) suggest that five fecal pellets are necessary to accurately assess the diet of a single Brazilian free-tailed bat. Similarly, a

molecular study of sea lion diets showed that prey DNA from different meals consumed on the same day was not well mixed among scats, implying that the DNA in each scat represented only some of the prey consumed by an individual (Deagle et al. 2005). The method of extracting DNA from single pellets used here may represent only a portion of the dietary content of a meal and/or may not provide sufficient template DNA for reliable amplification. Extracting DNA from pooled fecal samples could allow a broader description of a colony's activity, while still collecting samples in a non-invasive manner.

Another possible explanation for the reduced insect crop damage reported in the orchards studied here is that the presence of the bats in such high numbers acts as a deterrent to moth activity. Behavioral changes in bat prey have been previously noted (Fenton and Fullard 1979, Fullard 2001, Svensson et al. 2002), although a recent study on the response of ovipositing mosquitoes to bat activity failed to show any behavioral changes (Reiskind and Wund 2009). Bats use echolocation to locate aerial insects, and tympanate insects can detect and evade bat activity (Fenton and Fullard 1979). Predators can affect prey population densities through such trait-mediated interactions, when prey alter their phenotypic traits (here behaviorally) to reduce their risk of mortality in the presence of predators (Preisser et al. 2005). Trait-mediated interactions may, therefore, indirectly benefit plants by suppressing herbivores even when the predators consume few prey items (Preisser et al. 2005).

By monitoring for insects in the orchards, direct comparisons can be made between numbers of insects and the occurrence of specific insects DNA in fecal samples. More frequent fecal collections in 2009 yielded a higher level of detection of pecan nut casebearers during the brief periods of peak abundance. This highlights the need to consider the biology of the insect being studied when selecting a sampling regime. For an insect, such as pecan nut casebearer,

with brief, episodic activity, daily collections of insects and guano may be necessary to detect brief shifts in consumption. In contrast to the sharp peak of pecan nut casebearer abundance, corn earworm moths are present in the environment throughout the growing season and periods of peak abundance tend to extend over several weeks (Westbrook et al. 1995), potentially explaining the different consumption patterns of the two insects. Bat activity has been shown to increase several days after the initial increase in corn earworm numbers over corn and cotton fields in Texas, suggesting that bats return to forage in areas of high insect activity (Kennard 2008). Bats may be better able to exploit insects, such as corn earworm, with longer periods of abundance, rather than insects, such as pecan nut casebearer, with sharp, episodic peaks in abundance.

Documentation that bats roosting in bat houses on pecan orchards are consuming insect pests that attack pecans and surrounding crops indicates that growers can encourage the natural pest control services of insectivorous bats through the use of bat houses. Although the levels of detection were low, this study successfully detected multiple species of crop pests being consumed by multiple bat species roosting in bat houses. Given the reports from growers that the orchards providing the houses no longer require insecticides, the presence of the bats appears to benefit the orchards. This benefit could be from both direct consumption of the insects and from predator avoidance by the insects. By encouraging the presence of a native, generalist predator, growers may be able to reduce insect damage to their crops without the costs and hazards associated with insecticides, while also providing supplementary roosting habitat for a variety of insectivorous bat species.

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APPENDIX I



Figure 1. Map of sampling locations in the southeastern United States.

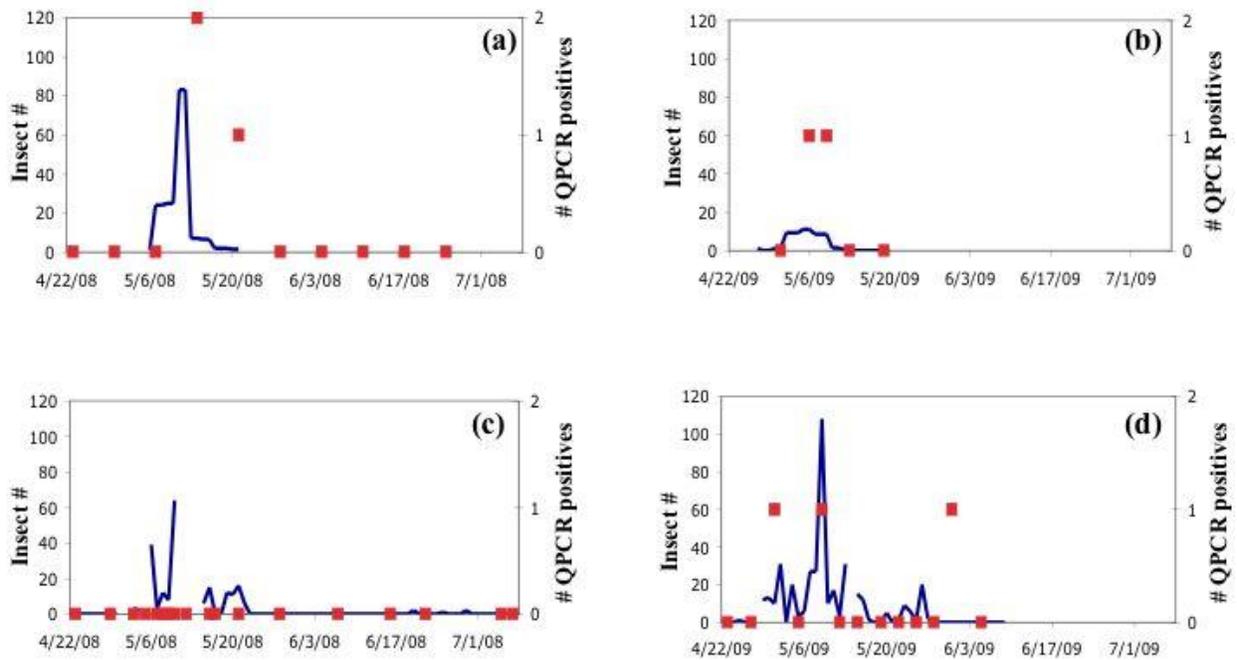


Figure 2. Plots of pecan nut casebearer (*Acrobasis nuxvorella*) abundance versus number of QPCR positive fecal samples. Lines indicate numbers of insects captured in pheromone traps. Dots represent the number of fecal samples positive for pecan nut casebearer DNA using QPCR. (a) Georgia 2008. (b) Georgia 2009. (c) Texas 2008. (d) Texas 2009. Fifteen samples were analyzed on each date in Georgia in both 2008 and 2009. In Texas, 10 samples were analyzed per date in 2008 and 12 samples per date in 2009.

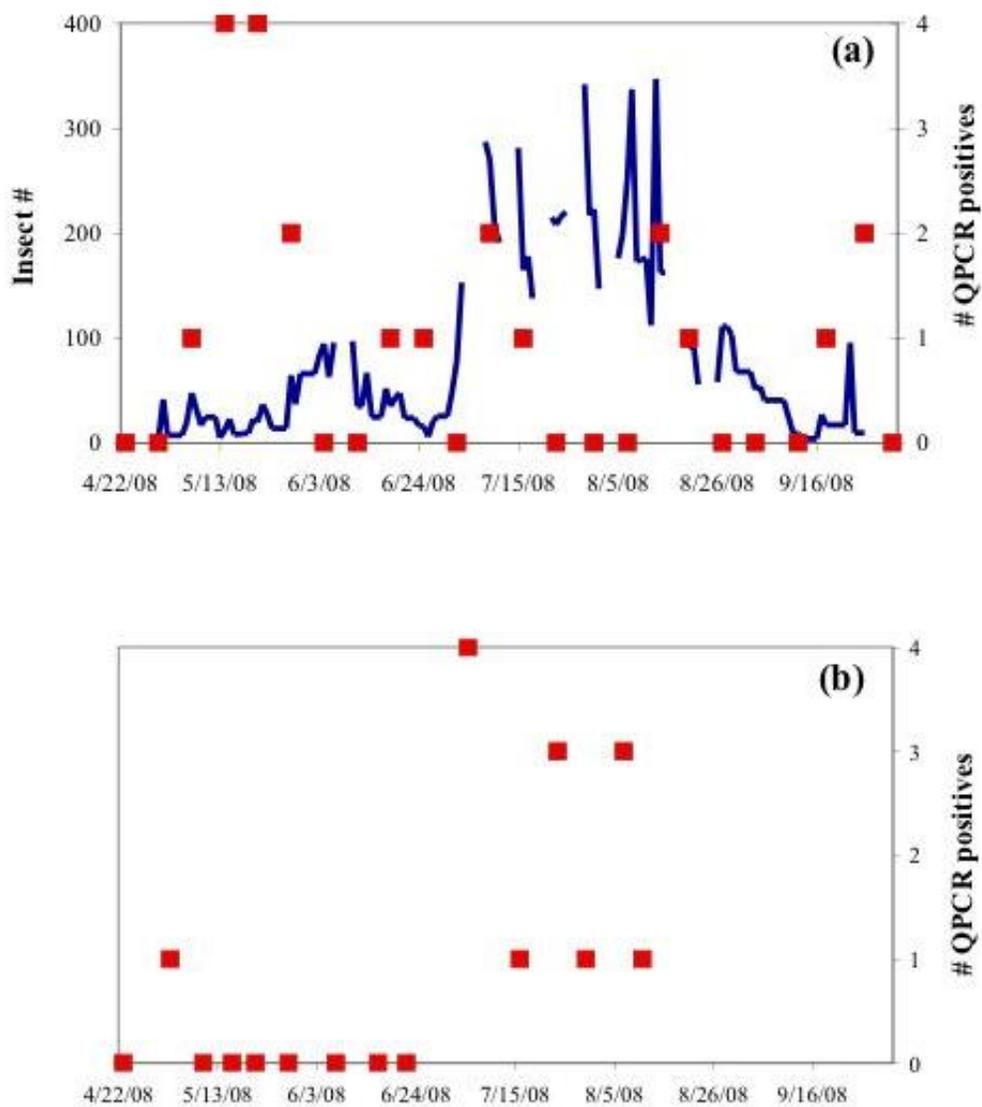


Figure 3. Plots of corn earworm (*Helicoverpa zea*) abundance versus number of QPCR positive fecal samples. Lines indicate numbers of insects captured in pheromone traps. Dots represent the number of fecal samples positive for corn earworm DNA using QPCR. (a) Georgia 2008. Insect trap collection data obtained from the PestWatch database. (b) Texas 2008. No data on insect abundance are available for Texas. Fifteen samples were analyzed on each date in Georgia, and 10 samples were analyzed per date in Texas.

Table 1. Summary of fecal collection and molecular analysis by location and year.

		Georgia 2008	Georgia 2009	Texas 2008	Texas 2009
fecal collections	dates frequency # collection dates # DNA extractions	4/23/08-10/2/08 weekly 24 360	5/1/09-5/19/09 3-6 days 5 90	4/23/08-8/11/08 1-13 days 24 240	4/23/08-6/5/08 3-5 days 13 156
pecan nut casebearer QPCR	dates # reactions single/double reaction	4/23/08-6/5/08 150 single	5/1/09-5/19/09 90 double	4/23/08-7/7/08 180 single	4/23/09-6/5/09 156 double
hickory shuckworm QPCR	dates # reactions single/double reaction	7/16/08-10/2/08 180 single	- - -	7/30/08-8/11/08 30 single	- - -
corn earworm QPCR	dates # reactions single/double reaction	4/23/08-10/2/08 355 double	5/1/09-5/19/09 90 double	4/23/08-8/11/08 150 double	4/23/08-6/5/08 84 double
stinkbug sequencing	dates # pellets analyzed	4/23/08-10/2/08 336	- -	- -	- -

Table 2. Primer and probe sequences.

Species	Primer name	Sequence	Reference
	LCO1490	5'-GGTCAACAAATCATAAAGATATTGG-3'	Herbert et al. 2003
	HCO2198	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	Herbert et al. 2003
hickory shuckworm (<i>Cydia caryana</i>)	Cyca COI F	5'- TTC ATC TAA TAT TGC TCA TAG AGG TA -3'	
	Cyca COI R	5'- GAG CTG TAA TAC CTA CTG CTC ATA C -3'	
	Cyca COI probe	5'- d FAM-TTC TCT TCA TTT AGC TGG AAT TTC TTC TA-BHQ-1 3'	
pecan nut casebearer (<i>Acrobasis nuxvorella</i>)	Acnu COI F	5'- CCA TGG TGG AAG ATC AGT TG -3'	
	Acnu COI R	5'- TTT GAT CGA AAG ATA ATC CAT TTA -3'	
	Acnu COI probe	5'-d FAM-TTC CTT ACA TTT AGC TGG AAT CTC CTC AA-BHQ-1 3'	
Mexican free-tail bat (<i>Tadarida brasiliensis</i>)	Tabr COI F	5'- TGC TGC AAG CAC TGG TAG AG -3'	
	Tabr COI R	5'- TCC CTT AGC CGG AAA TTT AG -3'	
cave velifer (<i>Myotis velifer</i>)	Myve COI F	5'- GAG AAA GTG CGG GAG GTT T -3'	
	Myve COI R	5'- CCT TTC TAC TAC TGC TTG CCT CA -3'	
evening bat (<i>Nycticeius humeralis</i>)	Nyhu COI F	5'- GGC CTC CGT AGA TCT GAC CA -3'	
	Nyhu COI R	5'- AAA GGA GCA CGG CTG TAA T -3'	
stink bugs (family Pentatomidae)	insect alt COI F	5'-TCATTCTTGAYCCTTCAGGA-3'	Kavar et al. 2006
	insect COI R	5'-CTGTAAATATGTGATGTGCTC-3 '	Muraji et al. 2000

APPENDIX II

Protocol for COI PCR

The PCR for mitochondrial COI amplification consisted of 12.5 μ L volumes, each containing 1 μ L DNA, 1X PCR Gold buffer (Applied Biosystems), 2.5 mM MgCl₂, 0.8 mM dNTP Blend (Applied Biosystems), 25 ng each primer (Integrated DNA Technologies; LCO1490 and HCO2198 in Table 2), and 0.0625 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). The PCR amplification profile included an initial hot start denaturation for 10 min at 95°C, followed by 35 cycles of 95°C for 45 sec, 60°C (for insects) or 46°C (for bats) for 45 sec, and 72°C for 45 sec, with a final extension at 72°C for 10 min.

Protocol for bat species-specific PCR

The PCR for bat species-specific amplification consisted of 12.5 μ L volumes, each containing 1 μ L DNA, 1X PCR Gold buffer, 2.5 mM MgCl₂, 0.8 mM dNTP Blend, 25 ng each primer (Table 2), 0.0625 units of AmpliTaq Gold DNA polymerase, and 2.5 μ g BSA (Sigma). The PCR amplification profile consisted of 10 min at 95°C, followed by 35 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 10 min.

Protocol for insect species-specific QPCR

The insect species-specific QPCR profile, each consisted of 25 μ L volumes, containing 4 μ L DNA, 1X PCR Gold buffer, 3 mM MgCl₂, 0.8 mM dNTP Blend, 50 ng each of species-specific primers (Table 2) and probe (BioSearch Technologies; Table 2), 5 μ g BSA, 0.125 units of AmpliTaq Gold DNA polymerase. The PCR amplification profile consisted of 10 min at 95°C, followed by 42 cycles of at 95°C for 30 sec and combined annealing and elongation at 58°C for 45 sec. Optical data were acquired following each 58°C step.

Protocol for PCR of stink bug fragments

The PCR for stink bug amplification consisted of 25 μ L volumes, each containing 6 μ L DNA, 1X PCR Gold buffer, 2.5 mM MgCl₂, 0.8 mM dNTP Blend, 50 ng each insect COI primer (Table 2), 5 μ g BSA, 0.125 units of AmpliTaq Gold DNA polymerase. The PCR amplification profile consisted of 10 min at 95°C, followed by 40 cycles of 95°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 10 min.

VITA

Veronica Angelelli Brown was born in 1979 in Knoxville, Tennessee. She graduated with honors from Farragut High School in Knoxville, Tennessee in 1997. She graduated magna cum laude from the University of Tennessee, Knoxville, in 2000 with a Bachelors of Science degree in Ecology and Evolutionary Biology. After receiving her undergraduate degree, she worked as a mammal keeper at the Knoxville Zoo. She then worked as a laboratory technician at the University of Tennessee where she developed her research skills in molecular work with both animals and plants. Veronica received her Masters of Science degree in Ecology and Evolutionary Biology from the University of Tennessee in May 2010.