Spring 4-2006

Beet Armyworm(*Spodoptera exigua*)DNA Detection in the Diet of Mexican Free-Tailed Bats(*Tadarida brasiliensis*)

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Senior Honors Project 2006

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Abstract

Mexican free-tailed bats (*Tadarida brasiliensis)* are thought to be large consumers of crop pests in Mexico and the American Southwest. The economic benefit of their predation could be enormous due to reduced crop loss and the reduced need for pesticide applications. To document the agro-ecosystem services of the bats and assist conservation action for the preservation of this bat species, our goal is to establish on a molecular level that bats do indeed consume specific crop pests by identifying prey DNA in bat feces.

This senior thesis involves developing a gene probe to detect the DNA of the beet armyworm (*Spodoptera exigua)* in the fecal material of bats that forage over crops in Texas and Mexico. Beet armyworms are causing extensive damage to crops. The goal of the project is to identify and amplify a species-specific region of the mitochondrial cytochrome oxidase II gene from moth DNA in the bat feces. Some of the techniques to be used include DNA extraction from Mexican free-tailed bat feces, quantitative PCR primer and probe design, polymerase chain reactions (PCR), quantitative PCR, and cloning.

After this technology is developed, it can be applied to the fecal material of wild bats from Southern Texas to document whether Mexican free-tailed bats are in fact large consumers of the beet armyworm by detecting the actual amount of beet armyworm DNA in the bat feces.

Introduction

Mexican free-tailed bats (*Tadarida brasiliensis*) are small bats that inhabit vast regions of North, Central, and South America. They feed twice nightly and find their prey in the dark by echolocation. Foraging heights vary dramatically for these bats, ranging from 30-60 meters above the ground (Caire et al. 1984), 200-300 meters above the ground (Griffin and Thompson 1982), and as high as 1200 meters above the ground (McCracken et al. 1997). In one night, a

single colony may even forage over an area of 400 km^2 (Williams et al. 1973). Over 100 million bats migrate from their wintering grounds in Mexico into Texas each year where they form maternal colonies in which most of the bats are female (Lee and McCracken 2005). Their great numbers and ability to hunt at varying altitudes and over great areas make them natural insect predators.

Moths, which serve as a major food source for these bats and are devastating agricultural pests, migrate nightly in massive numbers from the Rio Grande River Valley in Mexico and arrive in the early morning hours at crop fields some 400 km north in Texas (Wolf et al. 1990). One study found that Lepidopterans, which includes moths, were consumed at 1.9-10.3% of the bats' diet by volume in the evening feeding and at 40.9-89.8% by volume at the pre-dawn feeding, which supports the theory that the diet of *T. brasiliensis* varies according to the availability of migratory moths (Whitaker et al. 1996). Lee and McCracken (2005) did a much more comprehensive study and found that Mexican free-tailed bats do in fact exploit migratory moths, as is evident from the increased percentage of the bats' diet that consists of moths during the peak periods of moth emergence. The same paper showed Mexican free-tailed bats consume at least 12 orders and 35 different families of insects as part of their diet, with moths being one of the most frequently occurring prey items. Also emphasizing the diverse diet of these bats and the relative importance of moths in their diet, McWilliams (2005) found 11 orders and 38 families of insects in the feces of a *T. brasiliensis* colony at Carlsbad Cavern in New Mexico, with Lepidoptera comprising up to 79% of the diet at certain times of the year. All of these studies relied on physical examination of feces content, which made species-specific insect determination difficult or impossible in the case of moths.

Another characteristic of these bats that makes them potentially valuable as predators of crop pests is their high energetic requirements. Reproductive females spend several hours foraging and emerge earlier in the evening to feed and return later in the morning, due to the increased energetic demands associated with reproduction and lactation (Lee and McCracken 2001), which can double or triple the feeding rate for these reproductive females (Kunz et al. 1995). These energetic demands are estimated to require food intake ranging from 39.4-73.4% of the reproductive female body mass (Kunz et al. 1995). By contrast, non-reproductive females and males emerge later to feed and return earlier because of their less extensive energetic demands (Lee and McCracken 2001). However, these colonies are comprised of mostly females, so the energetic demands of most of the bats are quite high.

The Bracken Cave colony near San Antonio, Texas, is the largest colony, with 20 million bats. This colony is so large that the National Weather Service Doppler radar station near the cave picks up the massive bat colony at feeding times and registers the activity the same as it would a gigantic storm in the area (McCracken and Westbrook 2002). The Winter Garden region southwest of San Antonio is a major agricultural region where bats go to feed twice a day, consuming as much as 1,000 tons of insects each night (McCracken and Westbrook 2002). The moths that hatch from eggs laid in Texas can migrate north and damage crops, indicating these bats might even protect farmland further north and act as an initial defense against crop pests, which cost farmers over $1 billion per year (McCracken and Westbrook 2002).

The economic benefit of these bats is thought to be quite large due to decreased crop losses and the minimized need for pesticide applications, which are expensive and threaten biodiversity and sustainability in agricultural regions. The dangers of pesticide use to society at large are also important to consider. In the United States alone, some 300,000 illnesses stem

from the massive application of pesticides each year (Harper and Le Beau 2003). Clearly conserving these bats is important, but first documentation of what they are eating and how much of it they are consuming is needed.

The beet armyworm, (*Spodoptera exigua*), is a species of migrating moth that causes quite extensive crop damage across the southern United States. It was introduced into Oregon from Southeast Asia in 1876, where it quickly became established across North America (Greenberg et al. 2001). Several billion *S. exigua* migrate to Texas in June of each year from the Lower Rio Grande Valley in Mexico (Lee and McCracken 2005), causing massive crop damage, most notably to peppers, tomatoes, cabbage, peanuts, soybeans, cotton, and corn (Ruberson et al. 1994). These moths have established themselves as a major crop pest, and in 1995 an outbreak devastated crops in the Winter Garden agricultural region in Texas (McCracken and Westbrook 2002). In 1998 alone, the beet armyworm caused cotton losses of approximately $19.2 million (Williams 1999).

The beet armyworm is difficult to combat, most notably because of its tendency to develop resistance to pesticides, weakening the effectiveness of agrochemicals against this pest (Brewer et al. 1990). Its damage is offset by natural predators such as arthropods and pathogens, but liberal applications of pesticides, such as organophosphates, may in fact cause increases in the numbers of *S. exigua* due to decreases in the numbers of these natural predators (Ruberson et al. 1994). Also, the generalist predators of *S. exigua* that are harmed by pesticide applications consume crop pests other than *S. exigua* (Ruberson et al. 1994). This means that harming these natural predators can also cause an increase in the numbers of the other crop pests preyed upon by the natural predators. To further complicate matters, *S. exigua* is tolerant to many pesticides at normal application levels, and the eggs are deposited on the undersides of leaves where

pesticides are less effective (Ruberson et al. 1994). Pesticides are clearly not an effective long-term strategy for controlling beet armyworm infestations.

Fortunately, Mexican free-tailed bats could possibly serve to limit crop damage from the beet armyworm. Migrating species of the order Lepidoptera, which includes *S. exigua*, have higher lipid contents that enable them to travel long distances (Angelo and Slansky 1984). The prevalence and nutritional quality of *S. exigua* could make it an ideal food source for Mexican free-tailed bats, especially reproductive females with high energetic demands. Due to the fact that soft-bodied moths are digested far beyond the point where species-specific determination of what the bats are eating is possible through manual examination of the feces, DNA analysis of the feces is required. The DNA evidence proving on a molecular basis that *Tadarida brasiliensis* eats specific moths is becoming available through the work of our lab, and quantitative results of how much of certain species of moths the bats are eating is currently in development. My research represents part of the next step in this larger body of research, seeking to provide the technology to answer both of these questions for *Spodoptera exigua*.

Materials and Methods

The first step of this research, which was conducted prior to the start of this senior project, involved sequencing the mitochondrial cytochrome oxidase II (COII) gene for *Spodoptera exigua*, *Spodoptera frugiperda*, *Helicoverpa zea*, and *Heliothis virescens*, all related moth pests collected from the same region in Texas. This was also performed on other insects from the study area. This provided the exact sequence of this region for each moth species [see Fig. 1]. The sequence was amplified using the nonspecific primers A-tLEU (5'-ATGGCAGATTAGTGCAATGG-3') and B-tLYS (5'-GTTTAAGAGACCAGTACTTG-3') published by Liu and Beckenbach (1992) in a basic polymerase chain reaction (PCR).

Polymerase chain reactions enable scientists to synthetically replicate DNA in a lab setting. A mitochondrial gene is good to use in research that seeks to eventually detect and quantify DNA from bat feces because it has a much higher copy number per cell (each cell contains several mitochondria) than genomic DNA, so it is more likely to survive the harsh chemical process of digestion and still be detectable in bat feces. It also helps to detect shorts regions of DNA, which are more likely to exist intact after DNA is sheared during digestion.

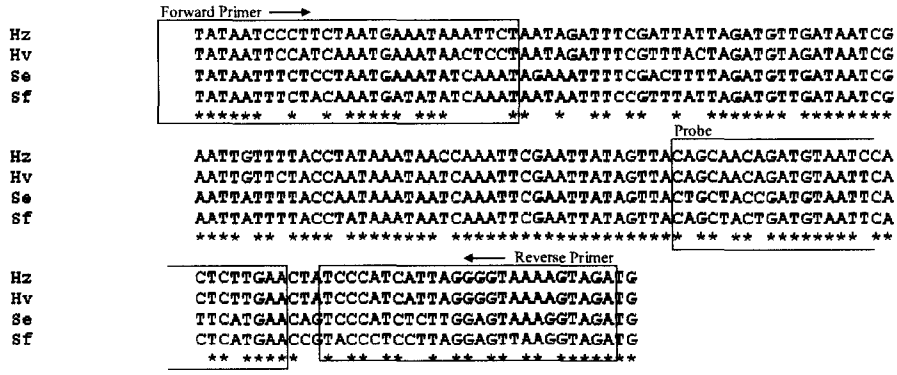

Figure 1. Cytochrome Oxidase II (mitochondrial) 158 bp DNA fragment that is species-specific. Primers and probes can be designed for the above regions of high variability.

Liu & Beckenbach 1992
Mol Phylogen Evol
1: 41-52

Identifying regions of variability between these four moth species allowed for the design of more specific primers that can amplify smaller regions of DNA within the original COII region in a species-specific fashion. The main goal of the research was to develop a quantitative PCR (qPCR) primers/probe set capable of not only detecting *S. exigua* but also quantifying the amount of DNA present in a given reaction volume or fecal sample. Quantitative PCR primers and a probe were designed to amplify what was named the I5 region of the COII gene for *S. exigua*. The reverse primer sequence is 5'-ATTTGCATCTACCTTTACTCCAAGAGAT-3', the

forward primer sequence is 5'-TTGAATTTGACTCTTATATAATTTCTCC-3', and the probe sequence is 5'-FAM d(TGAAATATCAAATAGAAATTTTCGACTTT) BHQ-1-3'.

Quantitative PCR works by having a probe anneal to a specific region of the DNA strand near the primer. This probe has a fluorescent reporter molecule that will fluoresce when a new molecule of DNA is produced through the PCR reaction. It is prevented from fluorescing before a new molecule of DNA is produced by being bound in the probe to a quencher molecule. The 5'→3' exonuclease activity of *Taq* DNA polymerase will degrade the probe as a new strand of DNA is being produced in the 5'→3' direction, releasing the reporter from the quencher. Figure 2 illustrates this. The fluorescent reporter is what is detected by the qPCR machine.

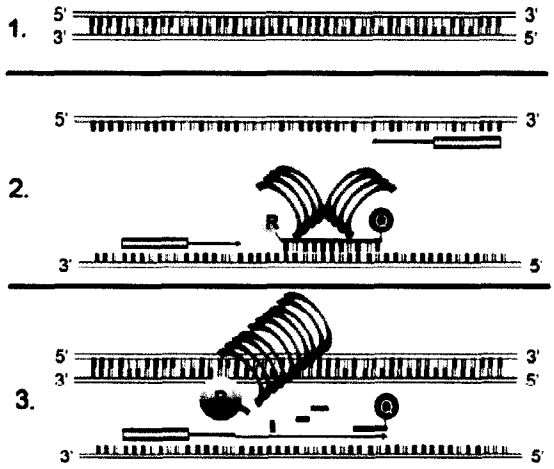

Figure 2. As the probe is degraded in the course of the polymerase chain reaction, the reporter is released and can fluoresce.
Diagram from Bextine (2005).

The qPCR machine records this fluorescence in the form of C(T), or the first cycle number at which fluorescence is detected. Standards are generated that contain known quantities of the DNA region of interest. The machine generates a standard curve from these standards and

uses the C(T) values generated from the standard curve to calculate the quantity of DNA in each sample based on the C(T) value for that sample.

A PCR temperature gradient using a sample of *S. exigua* genomic DNA and a fecal sample from a big brown bat (*Eptesicus fuscus*) fed *S. exigua* in the laboratory was used to determine at which temperature the COII gene for *S. exigua* would amplify most robustly and provide the greatest yield of DNA. This temperature, determined to be 56° C, was used for the elongation steps of the basic and qPCR reactions.

After the qPCR protocol was established, the product of a basic PCR using the qPCR primers was cloned to prove scientifically that what was being detecting through qPCR was in fact the I5 region of the *S. exigua* COII gene. A DNA sample from *S. exigua* tissue obtained in the lab was used for this. Without this verification, the qPCR primers could be detecting something other than the region of interest and result in false positive results once the primers were used on actual field samples. This was also performed on DNA from the feces of Mexican free-tailed bats collected during controlled feedings.

The qPCR standards were made by cloning DNA samples taken directly from *S. exigua* moths. The cloning reaction is required to make such high concentrations of very pure DNA. The DNA samples were quantified using a florometer, and dilutions were made to yield standards containing 10^6 copies/µl, 10^5 copies/µl, 10^4 copies/µl, 10^3 copies/µl, 10^2 copies/µl, and 10^1 copies/µl.

All basic PCR reactions were carried out in a Biometra T-Gradient Thermoblock machine using the following program parameters: 95° C for 10 minutes, followed by 37 cycles of 95° C for 45 seconds and 56° C for 1 minute. Basic PCR reactions were carried out with 12.5 µl reaction volumes consisting of 1.25 µl 10X PCR Gold Buffer (Applied Biosystems), 1.75 µl 25

mM $MgCl_2$ (Applied Biosystems), 1.0 µl 10 mM dNTP mix (Applied Biosystems), 0.25 µl Moth COII I5 Se F a primer (Integrated DNA Technologies), 0.25 µl Moth COII I5 Se R a primer (Integrated DNA Technologies), 0.25 µl 10mg/ml BSA (Sigma), 1.0 µl DNA sample, 0.0625 µl Amplitaq Gold (Applied Biosystems), and 6.6875 µl dH_2O. Basic PCR products were visualized by gel electrophoresis on a 2% agarose gel in 1X NaOH buffer. These were then stained in an ethidium bromide solution and visualized under ultraviolet light.

All qPCR reactions were carried out in a MJ Research PTC-200 Peltier Thermal Cycler with Chromo4 Continuous Fluorescence Detector using the following program parameters: 95° C for 10 minutes, followed by 46 cycles of 95° C for 45 seconds and 56° C for 1 minute. This differs from the basic PCR protocol by incorporating a "plate read" after each 56° C step to detect fluorescence. qPCR reactions were carried out with 25.0 µl reaction volumes consisting of 2.5 µl 10X PCR Gold Buffer (Applied Biosystems), 3.5 µl 25 mM $MgCl_2$ (Applied Biosystems), 2.0 µl 10 mM dNTP mix (Applied Biosystems), 0.5 µl Moth COII I5 Se F a primer (Integrated DNA Technologies), 0.5 µl Moth COII I5 Se R a primer (Integrated DNA Technologies), 0.5 µl 10mg/ml BSA (Sigma), 0.5 Moth COII Se Probe (Biosearch Technologies), 2.0 µl DNA sample, 0.125 µl Amplitaq Gold (Applied Biosystems), and 12.875 µl dH_2O.

A total of 119 DNA samples extracted from the feces of *Tadarida brasiliensis* bats from Texas colonies were analyzed with quantitative PCR. These fecal samples were collected in the Winter Garden region of Texas at midnight and 6:00 AM by placing captured bats in bags for six hours, collecting any feces, and releasing the bats. DNA was extracted from the samples using the MO BIO UltraClean Fecal DNA kit, with minor modifications. One sample indicating it contained a large amount of *S. exigua* DNA was cloned to prove the sample did in fact contain *S. exigua* DNA.

This field sample thought to contain *S. exigua* DNA because of positive identification through qPCR was amplified through the basic PCR protocol to make multiple copies of any DNA that was present. The 3' adenines needed for the cloning reaction were added by incubating 1 µl of a 1/5 dilution of Promega *Taq* DNA polymerase and 5 µl of the standard PCR product for 10 minutes at 72° C. This is done because the Amplitaq Gold used in the basic and qPCR reactions does not add these terminal adenines. This DNA was then cloned for further analysis with the Invitrogen TOPO TA Cloning Kit (pCR 2.1-TOPO Vector).

Twenty bacterial colonies were selected as a result of the cloning reaction and allowed to grow overnight in LB broth containing ampicillin at 37° C. These were then cleaned and the plasmids were extracted from the *E. coli* with the Eppendorf FastPlasmid Mini Kit. A small sample from each cleaned and extracted plasmid was digested with the enzyme EcoR1 at 37° C for two hours to cleave the DNA inserts. These were run in a 2% agarose gel by means of gel electrophoresis to determine if the inserts in the samples were the size expected for the I5 region of the COII gene. For those that were the right size, the cleaned and extracted plasmid for each sample was sequenced using the BigDye Cycle Sequencing Terminator Ready Reaction Kit (Applied Biosystems). The sequencing reaction products were cleaned using Centrisep Spin Columns (Princeton Separations) before being sent to the Molecular Biology Resource Facility (University of Tennessee). The sequence data was then compared with the previously sequenced *S. exigua* data for the COII I5 region to determine if the DNA amplified and cloned from the field sample was indeed from *S. exigua*.

Results

The Texas fecal sample (#644) shown through quantitative PCR to contain *S. exigua* DNA was collected at a bridge over Seco Creek in Uvalde County, Texas, at 6:00 AM on June

27, 2005, and had a fecal weight of 0.075 g. The C(T) value for first fluorescence detection in the field sample was 30.345 [see Fig. 3], and the qPCR indicated the sample contains 2518.35 copies of DNA per 2 µl of DNA sample. Figures 4-5 represent the standard curve generated through qPCR.

Of the 20 bacterial colonies selected and digested with EcoRI after the cloning reaction, 12 were determined to contain DNA inserts of the appropriate size and were sequenced. The sequences obtained were aligned with the consensus sequence for the I5 region of the COII gene for *S. exigua*. An example of one cloned sequence compared with the consensus sequence is shown in Figure 6.

When plotted on a graph on *S. exigua* numbers in the summer of 2005, the positive sample was shown to have been collected at the time of a moderate peak in insect numbers [Fig. 7].

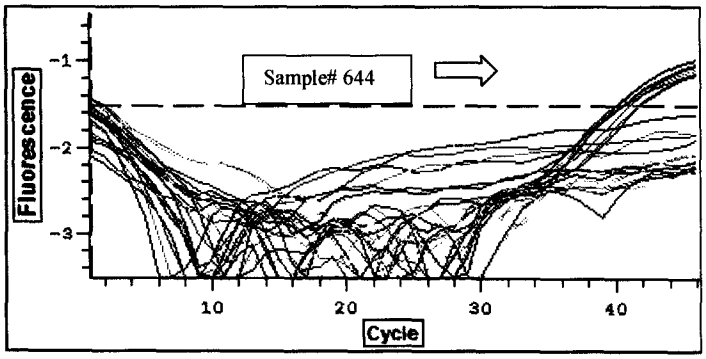

Figure 3. This graph shows the earlier and greater intensity fluorescence of sample 644 compared with the other samples shown to contain no relevant amounts of *S. exigua* DNA.

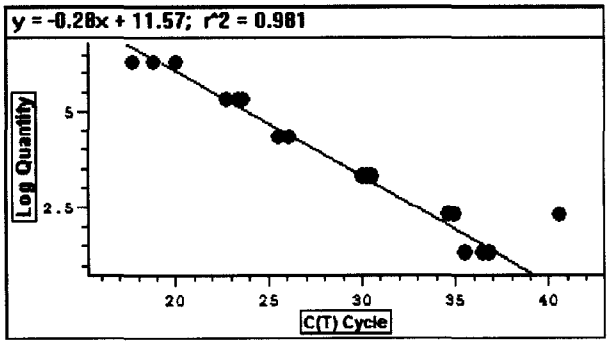

Figure 4. This graph shows the standard curve generated that allows for the calculation of the amount of DNA present in each sample.

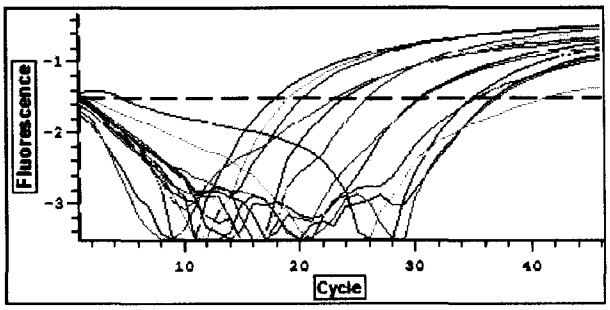

Figure 5. This shows the fluorescence patters of the standards used to generate the standard curve.

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CLUSTAL W (1.83) Multiple Sequence Alignment

Se              TTGACTCTTATATAATTTCTCCTAATGAAATATCAAATAGAAATTTTCGACTTTTAGATG
Clone           TTGACTCTTATATAATTTCTCCTAATGAAATATCAAATAGAAATTTTCGACTTTTAGATG
                ************************************************************

Se              TTGATAATCGAATTATTTTACCAATAAATAATCAAATTCGAATTATAGTTACTGCTACCG
Clone           TTGATAATCGAATTATTTTACCAATAAATAATCAAATTCGAATTATAGTTACTGCTACCG
                ************************************************************

Se              ATGTAATTCATTCATGAACAGTCCCATCTCTTGGAGTAAAGGTAGATGCAAAT
Clone           ATGTAATTCATTCATGAACAGTCCCATCTCTTGGAGTAAAGGTAGATGCAAAT
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Figure 6. These sequences represent the cloned sequence from the Texas# 644 sample in comparison to the consensus sequence for the I5 region of the *S. exigua* COII gene. The sequences are the same, indicating that this field sample in fact contains *S. exigua* DNA. All stars represent perfect matches between the two sequences.

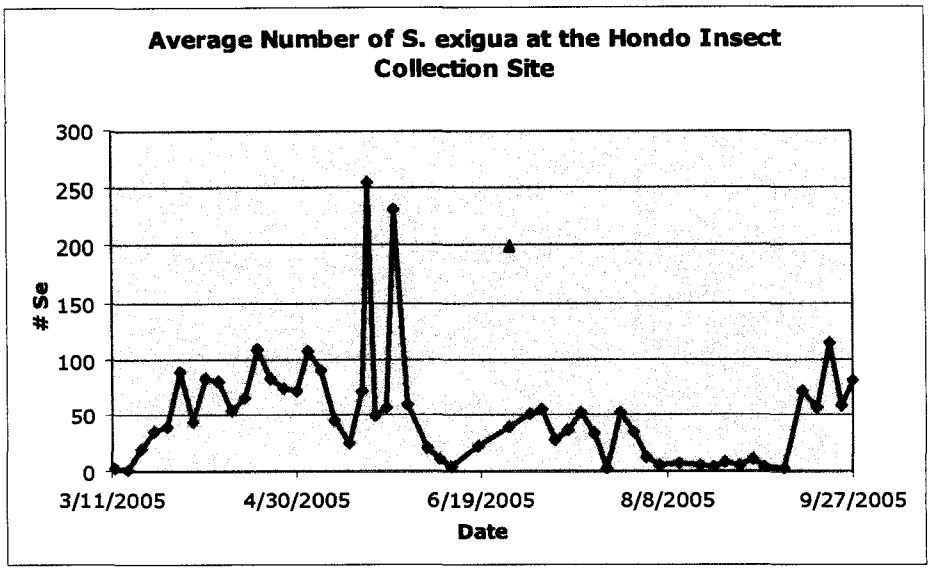

Figure 7. This graph shows the positive sample (in red) was collected during a moderate peak in the number of insects in the area. This insect data was collected by an insect scout very close to Seco Creek.

Discussion

This project was successful in identifying the DNA of *S. exigua* in bat feces collected from wild Mexican free-tailed bats. The one sample indicated through qPCR to contain *S. exigua* DNA was cloned, and the cloning reaction and subsequent DNA sequencing reactions proved that the qPCR reactions were accurate and that the sample did contain *S. exigua* DNA. Furthermore, because the cloning and sequencing reactions yielded no sequences that did not match the consensus sequence for *S. exigua*, the qPCR primers did not amplify any extraneous DNA sequences. This minimizes the concern of false positives in qPCR detection.

This senior thesis research represents only the first step in determining if Mexican free-tailed bats are large consumers of the beet armyworm. This research has been successful in developing the technology necessary to accurately and scientifically determine if fecal samples collected from wild bats contain beet armyworm DNA. The next step is to analyze far more field

samples to determine if several bats are in fact feeding on the massive numbers of beet armyworm moths that are present at certain times of the year. Although the moths are present in such large numbers, there is no guarantee that the bats are consuming this particular species. Also, *S. exigua* is smaller than other local moth species and might be harder to detect because of this. If it is determined in the future that beet armyworm moths are in fact being consumed by many Mexican free-tailed bats, it may be possible to lab feed bats beet armyworm moths and extract DNA from their feces to correlate the number of moths eaten with the quantity of DNA detected through qPCR of fecal samples. This would allow for the calculation of how many moths are being consumed and for the valuation of the potential agro-economic benefits of such consumption. Also, correlating the number of samples shown to contain *S. exigua* DNA with the number of the moths in the area over time could provide valuable information about insect emergence and movement and how these affect the feeding behaviors of Mexican free-tailed bats.

The overarching goal of this research and similar research on other moth species is the conservation of this species of bat. Mexican free-tailed bats live in aggregated colonies, indicating that any threat to their habitat could be quite destructive to the maintenance of the species. Endangering these bats could offset the natural predator-prey relationship and cause giant peaks in the numbers of crop pests and the amount of money needed to combat them. Therefore, determining what benefits Mexican free-tailed bats provide through natural predation of crop pests is of great importance. The cost of maintaining these naturally occurring colonies of Mexican free-tailed bats is likely very small, and the benefit to society could be enormous. This research represents the first but vital step in determining the role *Tadarida brasiliensis* plays in combating *Spodoptera exigua*.

Acknowledgements

I would like to thank Dr. Gary McCracken for allowing me the privilege of conducting my senior research in his lab and for funding this work. Also deserving of thanks are Kim Kennard, Annie Tibbels, and Dr. Mel Eldridge for teaching me and helping me along the way. My sincerest gratitude goes to Veronica Brown, whose help, guidance, and knowledge provided the foundation necessary for me to complete this project.

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