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Xenosurveillance of Wild Pigs Using Mosquito Bloodmeals

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ABSTRACT: Feral swine are an abundant invasive species that are heavily managed in the United States. We used DNA extracted from the bloodmeals of mosquitos to detect free ranging feral swine in south central Florida. DNA was of a sufficient quantity and quality that downstream applications such as genotyping or Sangar sequencing were feasible. Preliminary analyses were able to detect feral swine in blood-fed mosquitos.

INTRODUCTION

Feral swine (*Sus scrofa*) are invasive, large-bodied ungulates that cause >$800 M in economic damages in the U.S. each year (Pimentel et al. 2005) and would cost billions of dollars in damage should they become a reservoir for a transboundary animal disease (Shwiff et al. 2020). In 2013, via congressional mandate, the USDA APHIS National Feral Swine Damage Management Program was established to mitigate feral swine damage (Miller 2020). Owing to the growing geographic distribution and economic damage caused by feral swine, financial and operational resources were allocated to develop nationwide disease surveillance strategies, removal efforts, and management tools. Efforts by USDA and cooperating agencies are ongoing to establish best practices for all aspects of feral swine detection, management, and control of feral swine in the U.S. and its territories.

The early detection of feral swine and a robust surveillance program of the pathogens that they carry are two important endeavors of the national program (Brown et al. 2020). Innovative tools such as the use of environmental DNA (eDNA) to detect and monitor populations are being developed and refined for feral swine detection (Williams et al. 2018, Piaggio 2021). One such application of eDNA is the use of blood sucking arthropods to sample and identify the blood of the target species using molecular barcodes. Termed xenosurveillance, this technique holds promise as a supplemental tool for surveillance and monitoring of feral swine (Atsma 2023).

Florida has been identified as having one of the highest feral swine population densities in the United States and is at high risk for the introduction of transboundary animal diseases (USDA 2018). Developing robust methods for the early detection of both feral swine populations and transboundary diseases in Florida and elsewhere will be necessary to mitigate damages. Here we characterize the ability of xenosurveillance to detect feral swine across a diversity of habitats and across seasons at the University of Florida Deluca Preserve in Osceola County, Florida. Our objectives were to 1) describe the quality of DNA extracted from those bloodmeals for downstream molecular analyses, 2) determine how rapidly we could detect pig bloodmeals in mosquitoes, and 3) quantify the number of pig bloodmeals we could detect.

METHODS

We conducted this study (Atsma 2023) at the University of Florida Deluca Preserve, a 27,000- acre working cattle ranch in the flatwood pine ecosystem of Osceola County, Florida (Figure 1). The property has diverse habitats within the ecosystem including upland pine flatwoods, cypress ponds, marshes, Florida scrub, improved and native pasture, and citrus groves. Located in the subtropical environment of south-central Florida, the climate has a wet season from June to October and a dry season from November to May (Austin et al. 1991).

From January to July 2022, we sampled eight sites representing four habitats (two apiece of scrub, upland pine forest, marsh, and citrus grove) for nine sampling bouts that lasted five days each (Atsma 2023). During this time, there was scant rainfall and no standing water. To include a wet season sample, we added an additional 5 sites in August that represented both marsh and pine forest. Those sites were sampled for five days.

Mosquitoes were collected using battery powered aspirators and pop-up resting shelters (Atsma 2023). Once collected, mosquitoes were killed by placing them into a cooler with dry ice, transferred to collection tubes, and frozen at -20˚C. Once in the lab, mosquitoes were sorted based on whether or not they were blood-fed. Blood-fed mosquitoes were identified to species using dissecting microscopes and taxonomic keys (Darsie and Ward 2005), then, blood from the abdomen was transferred to a QIAcard FTA Classic (Catalog Number ID: WB120205)
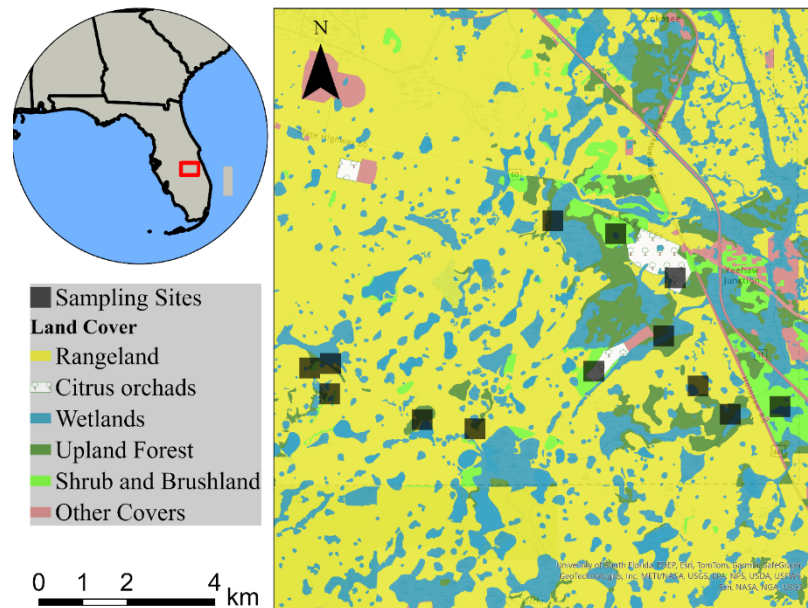
Figure 1. Location of the University of Florida Deluca Preserve within Florida, and of the 13 total sampling sites within the area's diverse habitats.

(Qiagen, Hilden, Germany), and scored on a 1 - 3 scale based on the level of digestion of the bloodmeal in the abdomen (Reeves and Burkett-Cadena 2023). DNA was extracted and bloodmeals were identified using established molecular barcoding methods (Reeves et al. 2018). We report the number of pig detections and time to first detection in each habitat type.

For a subset (n=110) of the feral swine bloodmeals, DNA was re-extracted from the FTA card using a Qiagen Puregene Blood and Tissue Kit following the manufacturer's instructions (Qiagen, Hilden, Germany). DNA was eluted in 50µL of DNA hydration solution and stored at 4°C for short term storage and at -20°C for long term storage. We quantified the DNA concentration and quality (spectrophotometric ratio of 260/280 and 260/230) of extracted DNA using a NanoDrop 2000 (Thermo Fisher Scientific, Walthan, MA).

To assess differences in DNA extraction yields among different levels of bloodmeal digestion, we performed a one-way ANOVA. Bloodmeal digestion was estimated by scoring each mosquito as a 1, 2, or 3 based on least to most digested (Reeves et al. 2018). The statistical test was performed using the command "aov" in the software R (R software version 4.2.0). We performed a Tukey's HSD Post Hoc analysis using the command "TukeyHSD" to determine pairwise differences among digestion categories. All analyses were considered significant if it had a reported p-value <0.05. The 95% confidence intervals for each category were calculated using the "confint" command on the software R.

RESULTS

Over the 50 days of mosquito collection, we collected 54,637 mosquitoes at 13 locations. The number of blood-fed mosquitoes (n=4,557) was a fraction of the total collection. During the 45 days of mosquito sampling during the dry season, we sampled eight sites each day and collected 2,071 blood-fed mosquitoes, and during the five days of wet season sampling at five sites we collected 2,482 blood-fed mosquitoes.

We identified 314 of the 4,557 blood-fed mosquitoes to be from feral swine (6.9%) which were collected from 10 species of mosquitoes (5 genera) (Atsma 2023). We detected 272 feral swine bloodmeals in the wet season and 42 wild pig bloodmeals in the dry season. Besides seasonal variation in the number of feral swine bloodmeals collected, detectability varied by habitat (Figure 2). Feral swine were detected on the first day of collection in upland pine forest (19 mosquitoes), after five days in Florida scrub (two mosquitoes), after six days in wetland habitat (19 mosquitoes), and after 30 days of mosquito surveys in citrus groves (two mosquitoes).

The results of the one-way ANOVA indicated a statistically significant difference ($F_{(2,107)} = 8.666$, $p = 0.00033$) in DNA concentration among the bloodmeal digestion categories (Figure 2, Table 1). Tukey's Post Hoc analysis indicated that the DNA concentration from bloodmeal score 1 (least digested) was significantly less than category 2 (p=0.007) and category 3 (p=0.005). The DNA concentrations obtained from bloodmeal score 2 and 3 were not statistically different (p=0.726) from one another (Figure 3). Reported means for DNA concentration, 260/280 ratio, and 260/230 ratio, and their associated 95% CI were similar for each bloodmeal category (Table 1).

DISCUSSION

We collected a total of 314 bloodmeals of feral swine from a diversity of mosquito species (Atsma 2023). While feral swine bloodmeals were collected throughout the year, 87% of them (272/314 bloodmeals) were detected in the
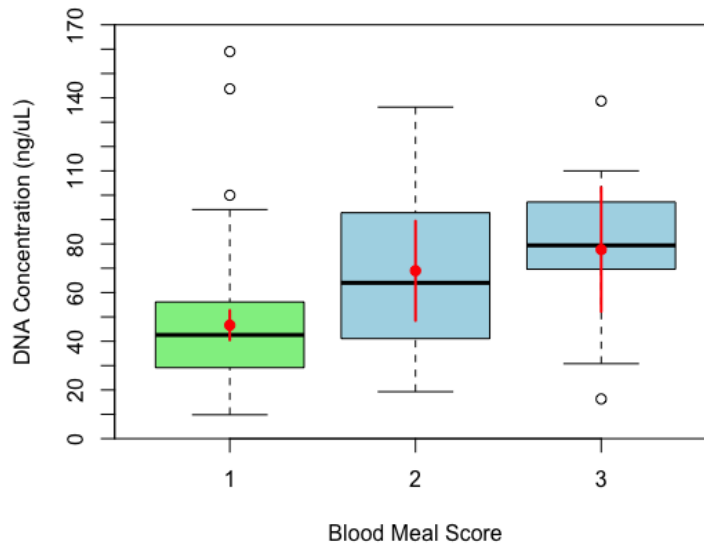
Figure 2. Boxplot of the median, upper/lower quartile, and the maximum and minimum of the DNA concentration for each of the three bloodmeal scores. Red points and brackets represent the mean DNA concentration and 95% CI for each of the three bloodmeal scores. Bloodmeal score 1 (green) was found to be statistically different from score 2 and 3 (blue). Bloodmeal score 1 represents the least digested bloodmeals and bloodmeal score 3 represents the most digested bloodmeal.

Table 1. DNA concentration, 260/280 ratio, and 260/230 ratio collected by the Thermo Fisher NanoDrop 2000 for each of the mosquito bloodmeal scores categories.

Bloodmeal Score	Mean DNA Concentration (95% CI)	Mean 260/280 ratio (95% CI)	Mean 260/230 ratio (95% CI)
1 (n = 83)	46.60 (40.53 – 52.67)	1.93 (1.90 – 1.96)	1.92 (1.84 – 2.00)
2 (n = 18)	68.97 (48.55 – 89.40)	1.98 (1.89 – 2.07)	2.15 (1.88 – 2.43)
3 (n = 9)	77.67 (52.21 – 103.12)	1.97 (1.86 – 2.09)	2.04 (1.70 – 2.38)

wet season during the August 2022 five-day sampling event. Feral swine bloodmeals were collected at each of the nine sites and in all four habitat types at the University of Florida Deluca Preserve (Figure 3). In three of the four habitats, detection of feral swine occurred in <6 days of sampling and during the wet season after only one day of sampling (Figure 3). We conclude that at this south-central Florida location, using mosquitoes to collect blood samples from feral swine was an effective and efficient method of collection, particularly during the rainy season.

Each bloodmeal represented a biological sample of a feral swine that had the potential to be used in downstream molecular applications. The combination of preserving blood on FTA cards and extracting DNA from the FTA cards using a Qiagen PureGene kit yielded extracted DNA that was high in quality and quantity (Table 1). Surprisingly, more digested bloodmeals (categories 2 and 3) yielded higher concentrations of DNA than the least digested bloodmeals (category 1) (Table 1). The reasons for this result are not known but could be the result of differential digestion of inhibiting molecules in the blood such as heme or autochthonous chitin. Despite the differences in DNA quantity, overall DNA yields from blood-

meals were high enough (47-78 ng/ul) to conduct downstream molecular analyses, such as genotyping with SNP chips or rtPCR, or sequencing using either Sanger methods or pyrosequencing.

All DNA extracted, regardless of digestion score, was of high quality, as represented by the calculated 260/280 and 260/230 absorbance ratios (Table 1). The 260/280 ratio represents the DNA to protein photospectrum absorbance peaks; a ratio between 1.8 and 2.0 suggests minimal contamination, a ratio <1.8 suggests contamination with protein and >2.0 suggests the overabundance of RNA. Across each of the bloodmeal scores in this study the group means were within the range of a pure sample. The 260/230 ratio represents the DNA to organic compound or salt absorbance; a ratio between 1.8 and 2.2 represents samples that have acceptable levels of organic compounds or salts that can otherwise inhibit a PCR reaction. Based on the estimated 260/230 ratio for DNA extracted from bloodmeals, downstream PCR reactions should not be inhibited by residues (Desjardins and Conklin 2010).

Our results suggest that bloodmeal analysis has the potential to be used for the detection and surveillance of feral swine populations. This method could be used for the
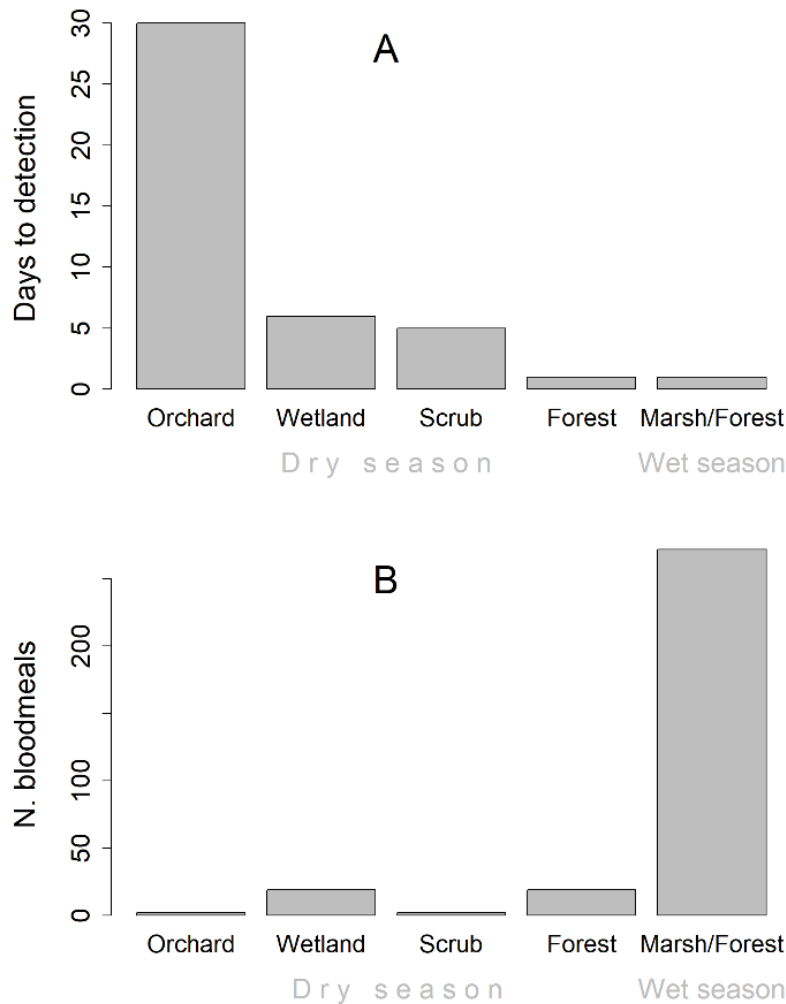
Figure 3. Detection of feral swine from blood-fed mosquitoes. A) Days of mosquito sampling at each habitat and season before the first detection of feral swine. B) number of mosquitoes fed on feral swine collected at each habitat and season.

detection of individuals in cases where the detection probability was low such as in isolated or low-density populations. Furthermore, this method could be used after depopulation events to identify undetected individuals or to verify the continued absence of feral swine on the landscape. Mosquitoes may more efficiently survey the landscape than more conventional detection methods such as camera traps or other wildlife survey techniques, making this tool a valuable addition to our current detection methods.

Given the high DNA quality and quantity that we observed in our study, a diversity of molecular techniques using DNA extracted from bloodmeals would likely be successful. For example, molecular markers of feral swine could be employed to identify individuals. This technique has previously been used to identify individual crow nestlings that were fed on by mosquitoes (Wheeler et al. 2021) and to identify humans that were fed on by malarial mosquito vectors (Mbewe et al. 2023).

Multiple genotyping assays have been developed to individually identify swine (Ramos et al. 2011, Beugin et al. 2017) and this information could be used to estimate population level parameters. Because xenosurveillance results in a blood sample without having to handle the target animals, it has the potential to efficiently sample a large proportion of individuals in a population. For example, a mark-recapture model of abundance estimation could be made by repeatedly sampling mosquitoes at the site of a targeted population. By comparing the proportion of marked (i.e., genotyped) individual pigs to unmarked (i.e., ungenotyped) pigs in serially collected bloodmeal samples, a population abundance could be estimated (Davis et al. 2020). Because of the importance of domestic swine in agriculture, the genome is very well characterized, and could also be exploited to better understand the population level variation in genome function in feral swine. For example, genes for pathogen susceptibility and immune function (Pierce et al. 2020, Bowden et al. 2023) in free-ranging feral swine could be assessed via bloodmeal analysis.

Feral swine bloodmeals could also be used to detect and survey for blood-borne pathogens in the hosts on which they feed. These pathogens do not have to be mosquito-borne; their nucleic acid simply needs to be circulating in the blood of the vertebrate host. Porcine blood-borne pathogens such as Circoviruses, Pseudorabies virus, African

Swine Fever Virus, and Classical Swine Fever Virus have the potential to be detected using this method. Bloodmeal analysis has not only been used to detect the nucleic acid of pathogens circulating in the blood (Mwakasungula et al. 2022), but bloodmeals have also been used to detect host antibodies to pathogens (Gyawali et al. 2020, Štefanić et al. 2022).

While our study conclusively demonstrated that bloodmeals from free-ranging feral swine can be effectively and efficiently used to detect this species on the landscape, there are multiple unknown factors that remain to be elucidated. Most importantly is understanding how many individual pigs are represented in the 314 bloodmeals. Still undetermined is the number of bloodmeals that contained more than one pig's blood, or how many mosquitoes fed on the same pig. Once optimized and validated, this technique will allow for demographic and epidemiological studies to be conducted.

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