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A TEST OF SEX SPECIFIC GENETIC MARKERS IN THE HAWAIIAN HOARY BAT AND RELEVANCE TO POPULATION STUDIES

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ABSTRACT

We tested the utility of a protocol using genetic markers that previously proved successful to identify the sex of Vespertilionid bats on tissues collected from live bats and carcasses of varying age from the Hawaiian hoary bat (*Lasiurus cinereus semotus*). This molecular method is based on genes unique to X and Y chromosomes in mammals and previously was used successfully on North American hoary bats (*L.* c. cinereus). We amplified two markers within intron regions of the zinc-finger-X (Z*fx*) and zinc-finger-Y (Z*fy*) genes using a multiplexed polymerase chain reaction technique and obtained product bands that were easily visualized using gel electrophoresis. Genotyping determined the sex of 36 individual Hawaiian hoary bat carcasses previously assigned sex only by external genitalia and identified sex for 29 "unknown" bat carcasses that could not be classified by external genitalia. Employing this method for sexing Hawaiian hoary bats will permit more reliable evaluation of the ratio of males to females in subpopulations affected by fatalities from emerging threats. This is critical to the conservation and management of this endangered bat.

INTRODUCTION

The Hawaiian hoary bat was listed as a federally endangered species in 1970 under the Endangered Species Conservation Act of 1969 that protects native fish and wildlife found to be threatened with extinction under 16 USC 668aa(c). A Recovery Plan for the Hawaiian hoary bat was published in 1998 and lists research as the overall recovery strategy because of insufficient information on abundance, distribution, critical habitat needs, and population status of the Hawaiian hoary bat (USFWS 1998). In Hawai'i's modern environment, potential threats to population recovery for this species include timber harvest practices (especially during the pupping season), entanglement on barbed-wire fencing, exposure to pesticides, and fatal collisions with wind turbines (USFWS 2011).

In the last decade, turbines have been deployed to harvest wind power in many areas throughout the world, including Hawai'i. Hawaiian hoary bats are experiencing collisions with wind turbines on the islands of Hawai'i, Maui, and O'ahu. Information on sex ratios of downed bats may provide more accurate fatality estimates and help understand the potential impact of wind energy-associated fatalities to Hawaiian hoary bats (Arnett *et al.* 2013, Cryan *et al.* 2012, Frick *et al.* 2017, Hein & Schirmacher 2016).

The goal of our study was to apply a method of sex determination based on molecular genetics previously proven effective on Vespertilionid bats to test the probability of correct sex identification from Hawaiian hoary bat carcasses and live individuals. Carcasses in our study were estimated to range from less than one to seven plus days post mortem. We also discuss some benefits this research may provide toward improving the accuracy of modeling population take and impacts of wind energy on Hawaiian hoary bats.

METHODS

Sample Collection

The U.S. Geological Survey holds collections of biological samples from Hawaiian hoary bats dating from 2007. This tissue collection includes wing membrane and muscle tissue samples representing approximately 200 male, female, and unknown sex individuals from four Hawaiian

Islands (Hawai'i, Kaua'i, O'ahu and Maui). These tissue samples were obtained from live capture and release or from carcasses provided by wind energy facilities, Hawai'i State wildlife offices, and the U.S. Geological Survey's National Wildlife Health Center Honolulu Field Station. While new samples continue to be acquired periodically; our present study is limited to an analysis of tissues from 73 Hawaiian hoary bats obtained between 2009 and 2016 from the islands of Hawai'i, Maui and O'ahu (Figure 1 & Table 1).

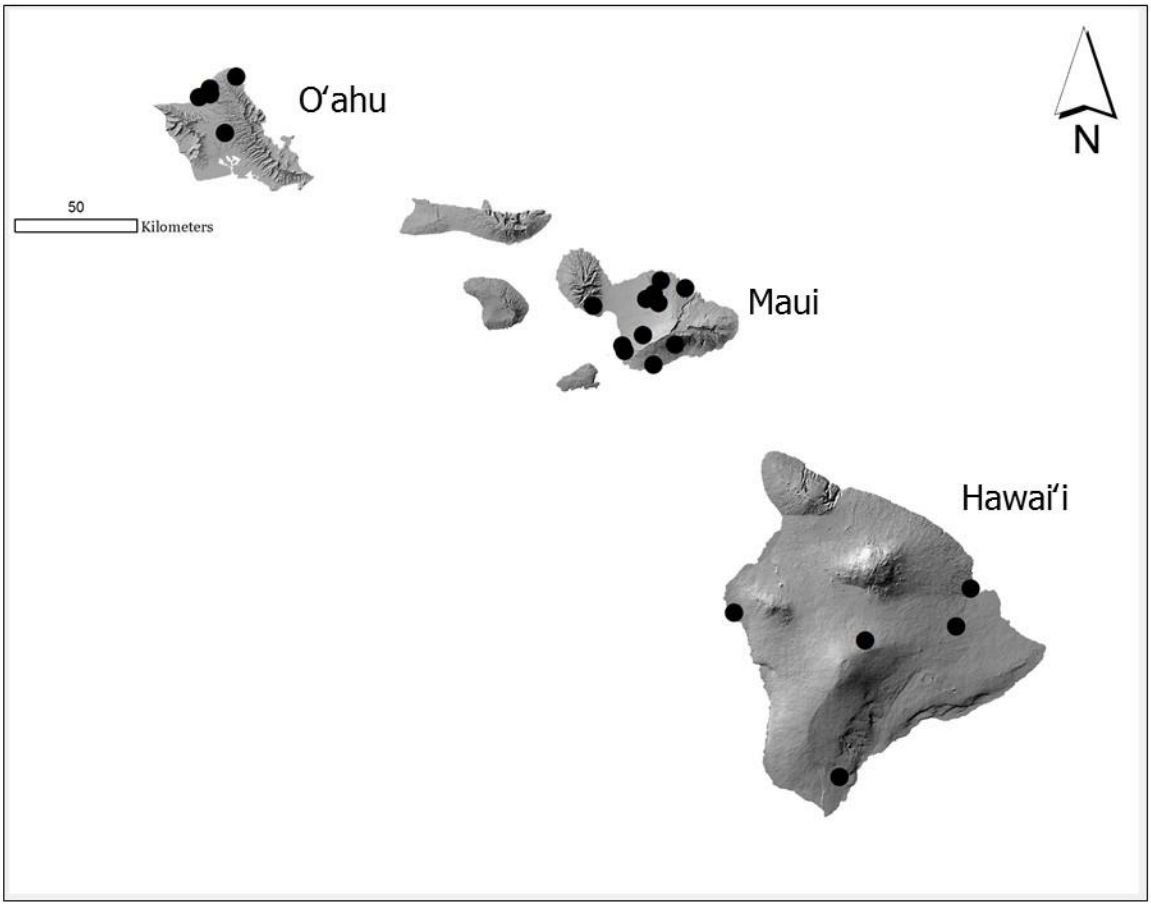

Figure 1. Map of the sampling locations for bats used in genetic sex testing.

Both samples from carcasses (n=67) and samples from captures (n=6) were used to test the genotyping method. For bat carcasses with associated downed wildlife reports and necropsies, we used the sex and the estimated time since death written on the report from the field observer. Field observers included both biologists and wind facility staff. In cases of wind fatality reports, estimated time of death was based on the frequency at which observers performed carcass searches and the condition of the carcass at the time of discovery. We used a χ^2 test of independence to assess the influence of carcass age (estimated time since death) on the number of bats with sex assigned as unknown. Only 55 case reports associated with carcasses provided time estimated since death, and we limited our χ^2 analysis of sex

determination on reports by external genitalia alone to this dataset. The sex of captured bats (n=6) was recorded during mist netting by wildlife biologists experienced in working with bats, and these samples were used to confirm the accuracy of the genotyping technique. We calculated sex ratios based both on identifications from external genitalia and genetic DNA testing. Sex ratio calculations were restricted to 65 bat carcasses previously categorized from examination of external genitalia as male, female, and unknown that had DNA successfully amplified during PCR. We used Fischer's exact test to assess if sex ratio was influenced by method of identification (observation of external genitalia or DNA genotyping) used to determine sex of bat carcasses.

Table 1. Numbers and types of bat specimens used for sex genotyping of Hawaiian hoary bats.

Island	Specimen Type	n	Recorded Sex[1]	n
Hawai'i	Capture	2	Male	1
			Female	1
	Carcass	3	Male	2
			Unknown	1
Maui	Capture	2	Male	2
	Carcass	28	Male	10
			Female	5
			Unknown	13
O'ahu	Capture	2	Male	1
			Female	1
	Carcass	36	Male	14
			Female	5
			Unknown	17
Total				73

[1]Sex that was recorded during collection of the bat and observed from external genitalia.

Live bats were captured using four-shelf, nylon mist nets (Kunz & Parsons 2009). Nets were opened at sunset across roads, trails, gulches, and ponds where bats had been observed flying. On some occasions, we used playback of Hawaiian hoary bat social calls to lure bats into mist nets. Nets were checked every 15 minutes and bats were placed individually in cloth holding bags until processing. Biological data collection included: sex and age class, reproductive condition, forearm length, and body mass (Kunz & Parsons 2009). We used a sterile 3 mm biopsy punch to obtain tissue samples from each wing. Bats were released at the site of capture within 40 minutes of netting.

Our methods follow the guidelines for capture, handling, and care of mammals recommended by the Institutional Animal Care and Use Committee (IACUC #04-039-12) of the University of Hawai'i at Hilo and the American Society of Mammologists (Sikes and Gannon 2011). We collected biological samples from bats as specified by US Fish and Wildlife Service permit TE003483-31 and Hawai'i Department of Land and Natural Resources permit WL 16-04.

Carcasses were refrigerated or frozen upon discovery, and tissue samples from necropsies were stored at -20 °C. Carcasses were assessed for external sexual morphology. Tissue samples were taken from soft wing membrane (Figure 2) with a sterile 3 mm circular biopsy tool. In some

cases, muscle tissue was cut away from the breast area of a fresh carcass with a sterile scalpel. All tissue samples were stored in 1.5 ml tubes containing NaCl-saturated 20% DMSO or on silica gel desiccant beads at ambient temperature in the field and later frozen at -20 °C until DNA extraction.

Figure 2. Examples of Hawaiian hoary bats used to test genetic sex determination, a live individual captured by mist net (left), and a desiccated carcass of unknown sex (right).

Genotyping Technique

DNA was isolated from bat tissues using a Qiagen DNeasy Blood and Tissue kit following the manufacturer's protocol for purification of total DNA from animal tissues (Qiagen, Valencia, CA, USA). After extraction, DNA quantity was estimated, and quality assessed with two different methods. First, 2 µL of the DNA sample was electrophoresed through a 1.5% agarose gel and visualized under UV illumination against a 1 kb bp ladder after staining with SYBR Safe (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Second, UV absorbance was measured using spectrometry in 3µL of each DNA sample on a NanoDrop 1000 machine. Isolated DNA was used in polymerase chain reactions (PCR) to amplify two gene sections on the X and Y sex chromosomes of Hawaiian hoary bats.

Sex determination PCR primers were multiplexed and consisted of two groups of fragment sizes (Korstian *et al.* 2013): the first primer set for the region within the *Zfx* intron yielded fragments of ~245 base pairs in length (F-ZFXBat: AGTCAAGGGRTGTCCATCR, R-ZFXBat: GTTTGYASACCAGGTTCCTC) and the second primer set for the region within the *Zfy* intron yielded fragments of ~80 base pairs in length (F-ZFYBat: GGTRAGDGCACAYRAGTTCCACA, R-ZFYBat: TGCYATTACAAAACCTTTRTAGATAC). DNA fragments were amplified using Qiagen's Multiplex Reaction Kit master mix following standard protocol. PCRs were 10 µL volume reactions each containing 20 ng template DNA, 0.5 µM of each X-primer, 0.35 µM of each Y-primer, 1X Multiplex Master Mix with HotStarTaq, Multiplex PCR Buffer containing 3mM MgCl2

pH 8.7, and dNTPs Mix. Cycling parameters were 1 cycle at 95 °C for 15 minutes, then 30 cycles of 30 seconds at 94 °C, 15 seconds at 57 °C, 30 seconds at 72 °C, and were carried out on an Eppendorf Pro S Thermal Cycler.

PCR products were checked for desired fragment size using gel electrophoresis and UV light visualization; the entire 10 µL volume reaction for each product was loaded into separate wells on a 1% agarose gel at 110 volts for 30 minutes and stained using SYBRSafe gel dye, with a 100 bp ladder for reference. The sex of each bat was determined based on the number of bands present for the individual in the gel lane (Figure 3). Males produced two bands, one resulting from the X chromosome intron, and another from the Y chromosome intron. Females produced only one large, very bright band, from the X-chromosome intron. The X-chromosome bands appeared at ~245 bp product size, and Y-chromosome bands at ~80 bp.

For a subset of 14 samples; 4 known males, 3 known females, 4 unknown males and 3 unknown females, we repeated both the PCR and gel electrophoresis two additional times to confirm repeatable banding patterns and correct genotype identification.

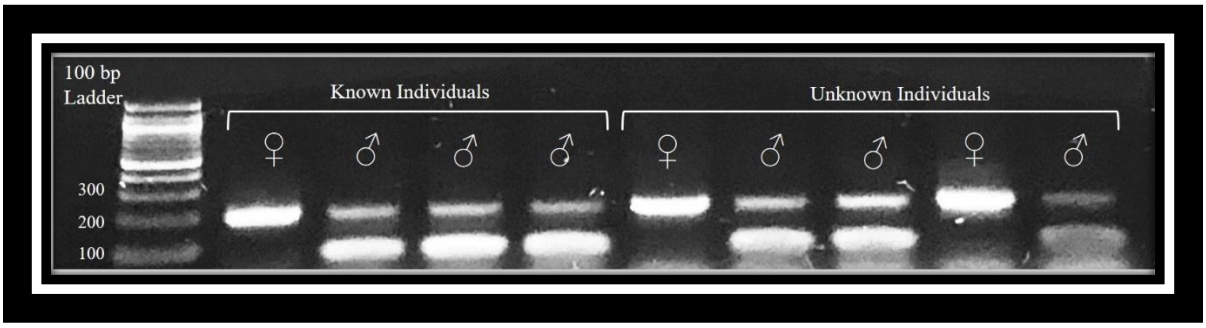

Figure 3. Bat genotype banding patterns on an agarose gel after successful amplification of zinc-finger X and Y specific PCR products and electrophoresis.

RESULTS

We determined sex by genotyping the X and Y chromosome introns (*Zfx* and *Zfy*) for 71 of 73 (97%) Hawaiian hoary bats sampled, including both capture and carcass samples. Extremely low DNA quantity and quality attributed to advanced decomposition of carcasses >7 days post-mortem likely were responsible for two samples that did not amplify during PCR. Samples from males (n=41) produced two bands, one resulting from the X chromosome intron, and another from the Y chromosome intron. Females (n=30) produced a single large, very bright band from the X-chromosome intron. X-chromosome bands appeared at ~245 bp product size, and Y-chromosome bands at ~80 bp (Figure 3). All 14 individuals of both sexes whose genotyping was replicated three times had a 100% confirmation among the three resulting outcomes.

PCR product bands from electrophoresis provided an 88.8% (32 of 36) match with the sex observed from external genitalia of bat carcasses. For all carcasses (Figure 4) that had sex recorded by a field observer (n=36), we calculated an 11.2% error for female bat carcasses

originally recorded as males; there were no male bat carcasses that had been recorded as females using external genitalia alone. Additionally, this genetic method provided identification of the sex of 29 individuals whose carcasses were categorized as unknown by field observation.

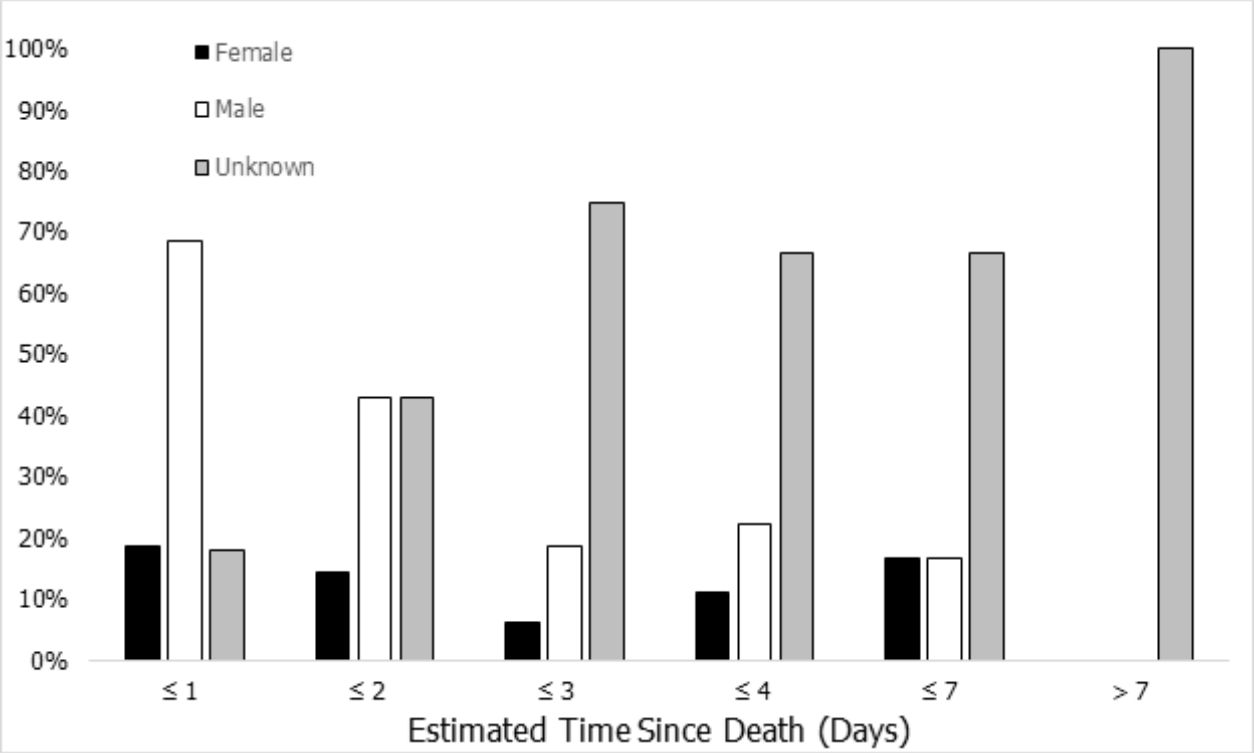

Figure 4. Sex identifications of carcasses (n=55) based on external genitalia recorded in wildlife reports as a function of the estimated time since death in days.

The accuracy of sex information generated by observers from carcasses in the field changed with time after death. Observers noted similar if not higher percentages of males to females from carcasses ≤4 days old. Carcasses of unknown sex dominated each category, except for carcasses ≤1 day, and often accounted for more than 50% of all carcasses. Due to extremely rapid rates of decomposition in the tropical climate of Hawai'i, the ability to assign sex to bat carcasses using external genitalia decreased significantly with estimated time since death (chi square χ^2=15.53, P=0.04). The sex ratio of Hawaiian hoary bat carcasses collected was significantly different using the two methodologies (Fischer's exact test, P=0.04; Table 2).

Table 2. Sex ratios based on external genitalia versus DNA genotyping for Hawaiian hoary bat carcasses.

Method	No. ♀	No. ♂	No. Unknown	Total	Sex Ratio/100 (♀:♂)
External Genitalia	10	26	29	65	28:72
DNA Genotyping	28	37	0	65	43:57

DISCUSSION

Sex genotyping was informative for 100% of the individuals analyzed in this study for which quality DNA was available, while only 88.8% of individual carcasses were correctly assigned sex using external morphology alone. For a subset of bats of known sex (3 ♀, 4 ♂) previously identified by external genitalia during capture by bat biologists, sex genotyping was 100% accurate, thus validating the utility of this method for Hawaiian hoary bats. We demonstrated that two published primer sets developed and validated for North American hoary bats, amplified regions on the *Zfx* and *Zfy* introns correctly and produced sex-specific variants in Hawaiian hoary bats. This method has shown itself to be useful in the identification of sex from bat carcasses found at wind facilities and submitted by citizens to wildlife agencies. Unless a bat carcass was quite fresh (≤2 days old), the sex of decayed or partially scavenged carcasses were difficult or nearly impossible to identify from external observation, leading to a male biased dataset in which females often were identified as unknown (Korstian *et al.* 2013, Nelson *et al.* 2018, this study). Observers noted males more often than females, but overwhelmingly they noted unknown sex. Sex determination of bat carcasses based on external genitalia often is unreliable unless limited to fresh carcasses and identifications are performed by trained personnel (Nelson *et al.* 2018). When valid sex identification is important, it can be confirmed by genetic analysis. The genotyping method that we describe also provides the opportunity to gain sex information even if a body fragment is all that was available. For example, we were able to genotype the sex of a bat from a single wing fragment.

Sex ratios of Hawaiian hoary bats may differ based on location, cause of mortality, and annual variation. In our study we grouped all carcasses together to provide a statewide overview, 43♀:57♂. Two previous studies reported sex ratios for the North American hoary bat (*Lasiurus cinereus*) using this sex genotyping technique and ratios varied by geographic location. Korstian *et al.* (2013) report hoary bats collected from northern Texas wind facilities at a ratio of 48♀:52♂, while Nelson *et al.* (2018) report hoary bats collected from wind facilities in Indiana were 61♀:39♂ (n=117). Although sex ratios from carcasses identified by the two different methods were statistically different in our study, our sample size of 65 carcasses was relatively small, and the ratio may change as more samples are analyzed. For example, Korstian *et al*. (2013) sampled 500 carcasses before a significant difference in the sex ratio of red bats (*Lasiurus borealis*) was detected.

Identification of sex from carcasses can be of great value in evaluating the impact of wind energy on local bat populations because the sex and age of bat carcasses often cannot be determined morphometrically. Hoary bats are sexually dimorphic, females are slightly larger than males, thus sex and age classification of a Hawaiian hoary bat cannot be identified reliably based on size alone (Jacobs 1996). For example, adult male forearms overlap with the range of

measurements for juvenile females. Genetic analysis allows accurate determination of sex from both adult and juvenile hoary bat (*Lasiurus cinereus*) carcasses (Nelson *et al.* 2018).

For an endangered bat species with limited live field collection opportunities, the importance of carcasses should be noted. A fresh carcass can contribute much more than hereditary genetic information, it also can provide details on bat foraging activity and prey types (Valdez & Cryan 2009). For example, Foo *et al*. (2017) was able to gain dietary information from genetic analysis of stomach content from hoary bat fatalities through the use of carcasses. It may soon be possible to genetically determine age from freshly collected bat wing tissues, researchers in Europe have developed and tested a molecular DNA methylation assay to establish age structure in the endangered Bechstein's bat (*Myotis bechsteinii,* Wright *et al.* 2018).

Sex information from genotyping is being employed in the study of genetic diversity, population structure, and historic population size in North American hoary bats (Korstian *et al.* 2015). Future studies of the evolutionary and phylogenetic relationships could compare Hawaiian hoary bat zinc-finger gene DNA sequences easily obtained from PCR products with this assay to that of the North American hoary bat. We suggest that this methodology has many potential applications for research across mammalian population genetics. Many mammal specimens throughout museum collections, especially those from 19th and early 20th Century collections lack records of specimen sex. Although this method of sex identification has not been tested yet on tissues from preserved museum specimens, we believe it could provide local collections with increased demographic information that was not captured by the original collectors.

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