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Determining sex ratios and mitochondrial haplotypes of Hawai'i's endemic and introduced ducks

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Abstract

Human-facilitated introduction of invasive species is increasing rates of anthropogenic hybridization, further threatening endemic species. In fact, the endangered Hawaiian Duck (*Anas wyvilliana*; 'Koloa Maoli'), which is the remaining endemic duck species on the main Hawaiian Islands, is now threatened by genetic extinction through ongoing hybridization with feral Mallards (*Anas platyrhynchos*). In addition to hybridization, the Koloa adult populations are known to be heavily male-biased which can further reduce population growth rates in threatened species. However, it is unknown if the sex-bias occurs at hatch, or is from a female bias in post-hatch mortality that could be managed. Towards this, we tested for bias in offspring sex ratios among Koloa, feral Mallards, and their hybrids and investigate their respective mitochondrial DNA (mtDNA) diversity. Despite the reported male-bias among adult Koloa populations, we did not recover a significant deviation in clutch sex ratios from 50:50. Although we also recovered no sex-ratio bias among feral Mallard × Koloa hybrid populations, feral Mallard eggs did show a significant male bias. Next, we recovered previously reported mtDNA haplotypes across the Islands and discovered areas outside of Kaua 'i were dominated by Game-Farm Mallard haplotypes found around the world, which confirms these to be the source of current feral and hybrid populations. Moreover, we did not find significant change over time in the ratio of Game-Farm Mallards verses Koloa found at each location. Finally, we found that both species do perform inter and intra-specific brood parasitism, which could provide for further Mallard introgression. We conclude the male-biased sex ratio among adult Koloa are therefore driven by increased post-hatch female mortality, and that re-establishing Koloa outside of Kaua 'i likely hinges on the ability to manage these feral Mallard and hybrid populations.

Keywords Hawaiian Duck · Hybridization · Sex ratios · Feral mallards · Mitochondrial DNA · Nest parasitism

Zusammenfassung

Bestimmung des Geschlechterverhältnisses und der mitochondrialen Haplotypen einheimischer und eingeschleppter Enten auf Hawaii

Die vom Menschen begünstigte Einschleppung invasiver Arten führt zu einem Anstieg der anthropogenen Hybridisierungsrate, die die einheimischen Arten zusätzlich bedroht. Die vom Aussterben bedrohte Hawaiiente (*Anas wyvilliana*; „Koloa Maoli "), die letzte endemische Entenart auf den größeren der Hawai'i-Inseln, ist durch die ständige Vermischung mit wilden Stockenten (*Anas platyrhynchos*) vom genetischen Aussterben bedroht. Zusätzlich zu dieser Hybridisierung weiß man, dass die erwachsenen Koloa-Populationen einen sehr hohen Anteil an Männchen haben, was die Wachstumsraten der

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Populationen bedrohter Arten weiter verringern kann. Allerdings ist nicht bekannt, ob dieser Überanteil an Männchen schon beim Schlüpfen besteht, oder ob es sich um eine höhere Sterblichkeit weiblicher Tiere nach dem Schlüpfen handelt, die eventuell beeinflusst werden könnte. Um das herauszufinden, untersuchten wir das Geschlechterverhältnis der Jungen von Koloas, von wilden Stockenten und von ihren Hybriden und deren jeweilige mitochondriale DNA-Diversität (mtDNA). Trotz des hier beschriebenen Überanteils an Männchen in den adulten Koloa-Populationen konnten wir in den Gelegen keine signifikante Abweichung des Geschlechterverhältnisses von 50:50 feststellen. Obwohl wir auch bei den Populationen von Stockenten × Koloa-Hybriden keine Abweichung des Geschlechterverhältnisses feststellen konnten, zeigten die Gelege von Stockenten einen signifikanten Überanteil an Männchen. Als nächstes haben wir auf den Inseln schon früher berichtete mtDNA-Haplotypen aufgespürt und festgestellt, dass in bestimmten Gebieten außerhalb von Kaua'i die weltweit auftretenden Haplotypen der Wildenten dominieren, was bestätigt, dass diese der Ursprung der heutzutage auftretenden wilden und hybriden Populationen sind. Darüber hinaus konnten wir an den einzelnen Standorten über einige Zeit hinweg keine signifikante Veränderung des Verhältnisses von Wildenten zu Koloas feststellen. Schließlich haben wir herausgefunden, dass beide Arten inter- und intraspezifischen Brutparasitismus betreiben, was zu einer weiteren Ausbreitung der Stockenten führen könnte. Wir schließen aus alledem, dass das ungleiche Geschlechterverhältnis bei den erwachsenen Koloas auf eine erhöhte Sterblichkeit der Weibchen nach dem Schlüpfen zurückzuführen ist und dass die Wiederansiedlung von Koloas außerhalb von Kaua'i wohl von den Möglichkeiten abhängt, diese verwilderten Stockenten- und Hybridpopulationen zu steuern.

Introduction

The introduction of exotic species has led to increased frequencies of anthropogenic hybridization that has accelerated the genetic loss of native species (Rhymer and Simberloff 1996; Allendorf et al. 2001; Adavoudi and Pilot 2021; Moran et al. 2021). While in some cases hybridization has been used as a conservation tool (e.g., Asiatic Wild Ass [*Equus hemionus* ssp.], Zecherle et al. 2021; Florida Panther [*Puma concolor coryi*], Johnson et al. 2010; Taita White-Eye [*Zosterops silvanus*], Vedder et al. 2022; multiple listed under Chan et al. 2019), in other cases, it creates additional conservation risk (Rhymer and Simberloff 1996). The capacity for fertile hybrids creates a particular problem for waterfowl (Order Anseriformes), where interspecific hybridization is disproportionately common and often leads to viable and fertile hybrids that outcompete native populations (Ottenburghs et al. 2015). Consequently, predicting the impact of anthropogenic hybridization requires an understanding of the frequency of interbreeding and the viability of hybrid offspring (Lavretsky et al. 2019; McFarlane and Pemberton 2019; Wells et al. 2019).

A biodiversity hotspot, Hawai‘i is now also a global extinction epicenter and known as the bird extinction capitol of the world (Banko et al. 2001). Following early human settlement 1600 years ago, four species of geese and ducks became extinct, leaving only Laysan Ducks (*Anas laysanensis*), Hawaiian Ducks (*Anas wyvilliana;* ‘Koloa Maoli’, Koloa for short*)*, and Nenes (*Branta sandvicensis;* ‘Nēnē’) as the remaining waterfowl (Sorenson et al. 1999; Walther 2016). All of these are now listed as threatened or endangered species due to numerous anthropogenic factors (USFWS 2004, 2011). Successful translocation efforts were made for both Laysan Ducks and Nēnē to expand their ranges (Black 1995; Reynolds et al. 2015; USFWS 2020). In contrast, translocation efforts in the 1980 s to establish viable Koloa populations outside of Kaua‘i failed due to interbreeding with already established feral Mallard (*Anas platyrhynchos*) populations in those locations; thus, hybridization continues to be the proximate conservation concern to the future of Koloa (Wells et al. 2019).

Molecular data suggests that Koloa are a hybrid species that originated from the natural hybridization of wild Mallards and Laysan Ducks over the last 5000 years (Lavretsky et al. 2015). Koloa historically occurred across Kaua‘i, Ni‘ihau, O‘ahu, Maui, Moloka‘i, and Hawai’i, but by 1962 fewer than 500 Koloa existed on Kaua‘i and Ni‘ihau (Engilis et al. 2002) largely due to habitat degradation, hunting, and predation from introduced mammals, resulting in their federal listing under the Endangered Species Act in 1967 (Griffin et al. 1989). As part of recovery actions, Koloa were captive-reared and reintroduced onto O‘ahu, Hawai’i, and Maui until 1989 (Griffin et al. 1989; USFWS 2011; Wells et al. 2019). However, hybridization between reintroduced Koloa and the burgeoning feral Mallard populations on those islands produced fertile offspring (Browne et al. 1993; Fowler et al. 2009; Wells et al. 2019). Recent population genetic studies confirmed that pure Koloa persist mainly on Kaua‘i, with populations on other islands existing only as Koloa × feral Mallard hybrid swarms (Wells et al. 2019). Without immediate efforts, Koloa will continue to persist on only Kaua‘i and nearby Ni‘ihau with continued risk of extinction due to its existence in a single population and the potential for hybridization with feral Mallards (Mālama Hawai‘i and USFWS 2009).

Recent efforts were undertaken to develop analytical models that incorporate starting genetic ancestry and various life-history traits (e.g., clutch size, age to maturity) to optimize management strategies as a decision-making tool towards reversing consequences of hybridization across the

Hawaiian Islands (Hernandez et al. 2023). However, incomplete information on life-history traits including clutch sex ratios of Koloa, Mallards, and hybrids potentially may bias models and lead to erroneous predictions. Sex ratios, particularly in small populations, can have strong effects on birth rate (Johnson 1994; Rosche et al. 2018). While Koloa adults are known to have a 3:1 (male:female) sex ratio (Malachowski 2020), it remains unknown whether this ratio is present at hatch or is due to differences in post-hatch survival of female versus male Koloa. Haldane's rule is an evolutionary prediction that states that when two divergent lineages interbreed, the heterogametic sex—in this case females (ZW)—will be less viable due to chromosomal incompatibilities (Haldane 1922). Following this rule, the hybrid origin of Koloa (Lavretsky et al. 2015) may drive a disproportionate male to female ratio starting at hatch. If this is the case, any management efforts taken with Koloa will need to account for a naturally male-biased sex ratio. Alternatively, a balanced offspring sex ratio at hatch is also possible because any chromosomal incompatibilities may have been removed from the population over the thousands of years Koloa have existed (Lavretsky et al. 2014). Moreover, sex ratios of feral Mallard and Koloa × feral Mallard hybrid clutches are entirely unknown. It is possible that sex ratios among contemporary hybrids may be impacted by Haldane's Rule, perhaps even more so than Koloa, because they consist of newly formed hybrid swarms (Wells et al. 2019).

Low genetic variation, particularly with small populations, has been a cause for concern for decades within population genetics as it causes lower individual fitness and lower population adaptability (Lande 1988). Examining mitochondrial DNA (mtDNA) is one way of looking at the genetic diversity and can give us insight into whether small populations have just always been with low diversity due to their natural small population size or due to external pressures that need to be addressed. For example, the Nēnē once harbored greater genetic variability then today, but declined during human settlement (Paxino et al. 2002). So, alongside the Nēnē, we aim to further understand Koloa mtDNA ancestry dynamics across Islands. While previous studies have examined adult populations, it may not accurately reflect the subset of the breeding population, therefore, we made it our goal to examine this portion of the population exclusively. We compare mtDNA haplotype diversity to previously published data (Fowler et al. 2009; Wells et al. 2019) to look for proportional stability across time and space and determine whether eggs collected on Kaua'i remain at least maternally Koloa. In previous studies using mtDNA, all Koloa had one of five different haplotypes all falling within the New World (NW) B haplogroup, while all Mallards and most hybrids sampled in Hawai'i belonged to the Old World (OW) A haplogroup (Fowler et al. 2009; Wells et al. 2019), which is linked to Game-Farm Mallard domestic ancestry (Lavretsky et al. 2019, 2020, 2023b). Therefore, the identification of OW A haplotypes on Hawai'i in previous studies suggests mating to have proceeded between feral domestic female Mallards and Koloa males. This is a cause for concern as domesticated Mallard haplotypes may start to replace Koloa haplotypes, diminishing native genetic diversity. Given that these studies were done with samples from nearly a decade ago, or up to ten generations, this study provides a more contemporary update to earlier work by Fowler et al. (2009) and Wells et al. (2019).

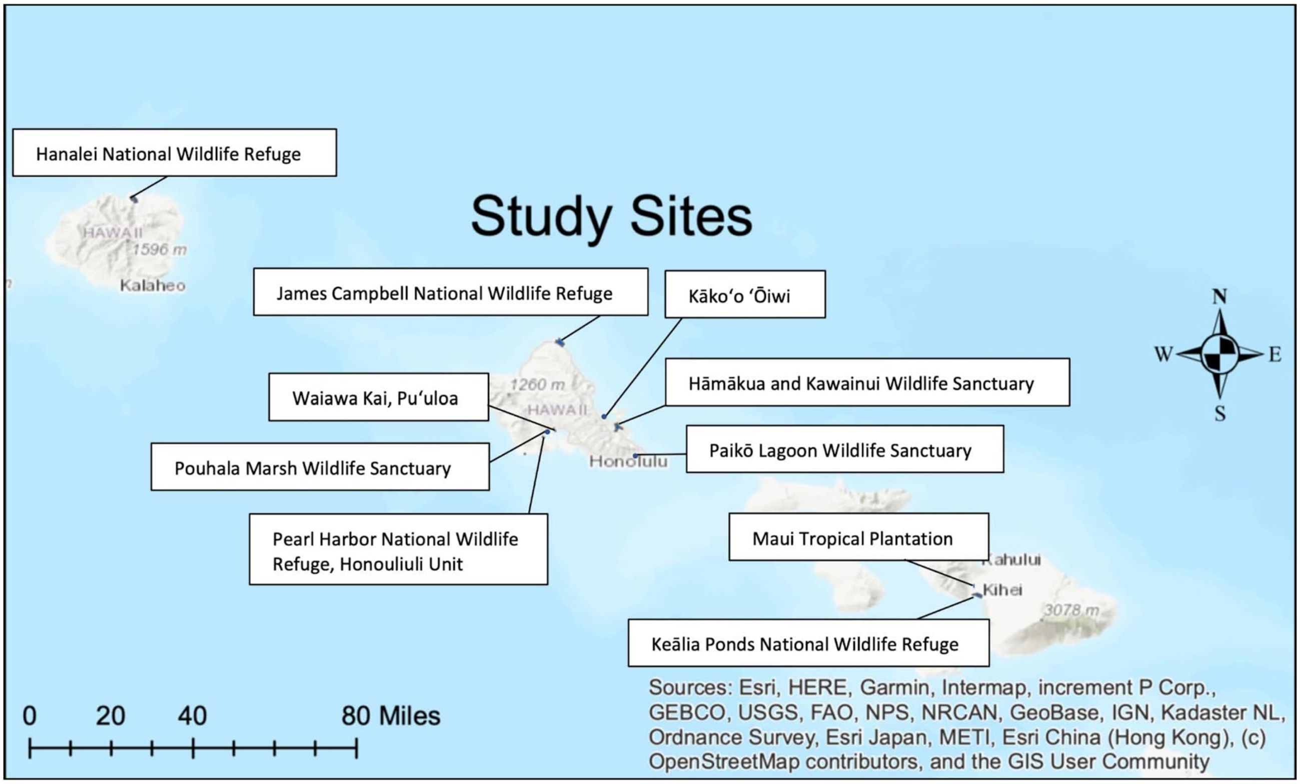
Finally, in addition to describing mtDNA diversity, clutches with complete mtDNA ancestry will be used to determine whether Koloa, Mallards, and/or hybrid females engage in nest parasitism. Nest parasitism (also known as brood parasitism, in which females lay eggs in the nests of other females of the same or different species) is extremely common in waterfowl and may result from limited nesting opportunities (Yom-Tov 2001) or maternal resource limitation (Sorenson 1991; Lyon and Eadie 2008). Additionally, sexual misimprinting has been considered as a possible mechanism underlying hybridization in brood parasitic species, but evidence is sparse (Rohwer et al. 2022). Mallards have been documented to parasitize a variety of other duck species (Weller 1959), and may also target Koloa nests. In Koloa and hybrid nests, eggs with differing maternal lineages (based on mtDNA) would indicate parasitism from an unrelated female, either from the same or other species. If both Koloa and Mallards have the potential to parasitize other nests, this may at least partially explain local levels of interspecific hybridization rates and hint at nesting and maternal resource limitations Koloa may be experiencing (Ottenburghs et al. 2016).

Methods

Nest collection

As a multiagency effort, eggshells, viable eggs, and/or nonviable eggs were collected from spring 2021 to spring 2023 from a total of eleven study sites across the Hawaiian Islands of Maui ($n = 2$), O'ahu ($n = 8$), and Kaua'i ($n = 1$) (Fig. 1). In general, populations on Kaua'i represent pure Koloa, Maui Tropical Plantation represent feral Mallards, and all remaining locations assumed to represent various hybrid forms (Wells et al. 2019). The study sites were chosen based on ease of collection, visitor frequency, and whether conservation efforts were already taking place.

Our goal was to collect 194 eggs or eggshells/site [~ 25 nests, with average of 10 eggs/nest for mallards (Batt and Prince 1979) and 8 eggs/nest for Koloa (Malachowski et al. 2018)], which would allow us to detect a 60:40 or more extreme deviation from parity (binomial power analyses:



Sources: Esri, HERE, Garmin, Intermap, increment P Corp., GEBCO, USGS, FAO, NPS, NRCAN, GeoBase, IGN, Kadaster NL, Ordnance Survey, Esri Japan, METI, Esri China (Hong Kong), (c) OpenStreetMap contributors, and the GIS User Community

Fig. 1 Study site locations moving east to west on Kaua'i, O'ahu, and Maui

$p_0 = 0.5$, $p_1 = 0.6$, $\alpha = 0.05$, $\beta = 0.2$, power $= 0.8$). However, egg availability fell short of this goal at some sites. Because Koloa are an endangered protected species, only post-hatch shells or inviable eggs were collected on Kaua'i, whereas viable and inviable eggs and eggshells were taken from Maui and O'ahu nests. The collected eggs and eggshells were shipped to the University of Texas at El Paso and held at − 80 °C. All collection efforts were done under Federal permit number MB11579C, State permit number WL21-02, and under Institutional Animal Care and Use Committee Protocol No. 24-11-056.

DNA extraction and molecular analyses

Membrane tissue was pulled from all eggs, as well as yolk or tissues and organs from the developing fetus if available. Genomic DNA was extracted for each sample using a DNeasy Blood & Tissue kit following the manufacturer's protocols (Qiagen, Valencia, CA, USA). The samples were run through gel electrophoresis with a 1% agarose gel to visualize amplification success and DNA quality based on a presence of a high molecular-weight band. DNA extractions were attempted up to three-times.

First, we molecularly assessed sex following optimized PCR-based methods targeting a CHD gene found on both sex chromosomes of birds (Çakmak et al. 2017). Primers CHD1F and CHD1R from Çakmak et al. (2017) were used, but optimized PCR reactions and thermocycler conditions were used as described in Lavretsky et al. (2023a). The PCR products were loaded on a 4% agarose gel, with successful amplifications expected to show one band for the homogametic sex (i.e., male) and two for the heterogametic sex (i.e., female). Each sample was run through PCR and gel electrophoresis three times to ensure accuracy. To detect a significant deviation from a 50:50 male:female ratio for each species, we used exact binomial tests.

Next, around 650 base pairs of the mtDNA control region were amplified using an optimized touch-down PCR protocol using L78 and H774 primers, and following optimized protocols outlined in Lavretsky et al. (2014). Amplification was verified using gel electrophoresis with a 1% agarose gel and was sent for Sanger sequencing on a 3130XL Genetic Analyzer at the University of Texas El Paso Border Biomedical Research Center's Genomic Analysis Core Facility. The raw Sanger sequences were aligned and edited using *Sequencher v4.8* (Gene Codes, Inc., Ann Arbor, MI, USA). 619 comparative mtDNA sequences for wild North American Mallards, known Game-Farm Mallards, Koloa, Laysan Ducks, and Khaki Campbells were also aligned and served as references (Fowler et al. 2009; Wells et al. 2019; Lavretsky et al. 2023b). We then reconstructed a haplotype network using PopArt (Leigh and Bryant 2015), as well as used *ArcGIS Pro* v3.2 (Environmental Systems Research Institute, Redlands, CA, USA) to map mtDNA diversity across sites. All sequences have been deposited in GenBank (accession numbers TBD). To find if there has been a significant change in Koloa to Mallard mtDNA haplogroups across locations and Islands, X^2 tests were done with our haplogroup numbers and those taken from Wells et al. (2019). Last, mtDNA haplotypes were compared within each clutch to see if there was more than one type, which would indicate brood parasitism. Note this is a conservative look at brood parasitism as it does not detect parasitism from females with the same haplotype, which may be important for areas such as Kauaʻi where one haplotype tends to dominant most of the population.

Results

Sampling effort and DNA extraction success

A total of 1270 eggs were collected across 168 nests on Oʻahu ($n = 114$), Maui ($n = 30$), and Kauaʻi ($n = 24$). Of the 1270 samples collected, 824 samples yielded high molecular weight DNA which was indicated by a strong distinct band on a gel.

Sex ratios

Of the 824 samples of successfully extracted DNA, 431 (~ 52%) were successfully sexed from Maui ($n = 51$), Oʻahu ($n = 330$), and Kauaʻi ($n = 50$; Table 1). Given the incomplete sampling of most nests, we compiled all eggs per site to understand sex ratios at the population level. First, we found no sex bias among Kauaʻi nests (exact binomial test: males = 20, total = 50, p-value = 0.20), and no bias for locations representing hybrids on Oʻahu and Maui (exact binomial test: males = 170, total = 338, p-value = 0.95). However, we did recover a high and significant (exact binomial test: males = 37, total = 43, p-value < 0.01) 6:1 male bias within Maui tropical plantation that represents feral Mallards (Table 1).

Table 1 Sex ratio results

Sex ratio per species	Male	Female	Exact binomial test *p*-value
Koloa overall ratio (2:3)	20 (40%)	30 (60%)	0.2026
Hybrid overall ratio (1:1)	170 (50%)	168 (50%)	0.9566
Mallard overall ratio (6:1)	37 (86%)	6 (14%)	0.000001

mtDNA diversity

For mtDNA, we successfully amplified and sequenced the mtDNA control region for 670 samples (of 824; 81%). As expected, all Laysan Duck reference sequences comprised a single and divergent haplogroup, which is now absent on the main Hawaiian Islands (Lavretsky et al. 2014, 2015; Fig. 2). Next, OW A mtDNA haplotypes dominated most Oʻahu ($n = 66$) and all Maui ($n = 11$) nests, and these grouped with reference Game-Farm Mallards and associated haplotypes that are now found world-wide due to their release and subsequent hybridization with endemic populations (Lavretsky et al. 2023b). In fact, the most common OW A haplotype found in this study was haplotype 7, which is the most dominant Game-Farm Mallard haplotype in other parts of the world (Wells et al. 2019; Lavretsky et al. 2023b; Fig. 3). Other nests in Northern parts of Oʻahu possessed another OW A haplotype (haplotype 14) that did not group with our reference samples (Fig. 3), but was found to have a 100% sequence identity with other published Game-Farm Mallards, Koloa × Mallard hybrids, and Pacific Black Ducks (*Anas superciliosa superciliosa*) from Hawai'i, China, and New Zealand that are present on the National Center for Biotechnology Information (NCBI) BLAST database.

Next, nests sampled on Kauaʻi possessed NW B mtDNA haplotypes with only one (of 118) egg being identified as OW A. Interestingly, whereas the dominate haplotype on Kauaʻi was 1, only one and two nests at Hāmākua and James Campbell National Wildlife Refuge on Oʻahu, respectively, carried haplotype 1 (Fig. 3). Instead, we recovered haplotype 2 as dominant NW B form among 51 individuals representing eight nests on Oʻahu (Fig. 3). Interestingly, a single NW B haplotype (i.e., haplotype 15) recovered in only Honouliuli and Pouhala was found to not overlap with any reference samples, and did not have any perfect matches on the NCBI database (Figs. 2, 3), suggesting that this is a previously undescribed NW B haplotype.

We found no significant differences (all X^2 p-value > 0.10) when comparing our OW A versus NW B mtDNA haplogroup ratios to those collected a decade ago by Wells et al. (2019); however, different frequency of haplotypes within each haplogroup were identified (Tables 2, 3). First, we recovered a shift towards OW A haplotype 7 today consistent with Wells et al. (2019), and that is shift away from a more even frequency of three major OW A haplotypes described in Fowler et al. (2009). Next, whereas we recovered the same NW B haplotype 1 as the predominant form found among Koloa, we only recovered four NW B haplotypes as compared to the five described in Fowler et al. (2009) and Wells et al. (2019). However, the two unrecovered NW B haplotypes were found in low frequency in both Fowler et al. (2009) and Wells et al. (2019), suggesting that we did not identify these due to a lack of sampling or perhaps these

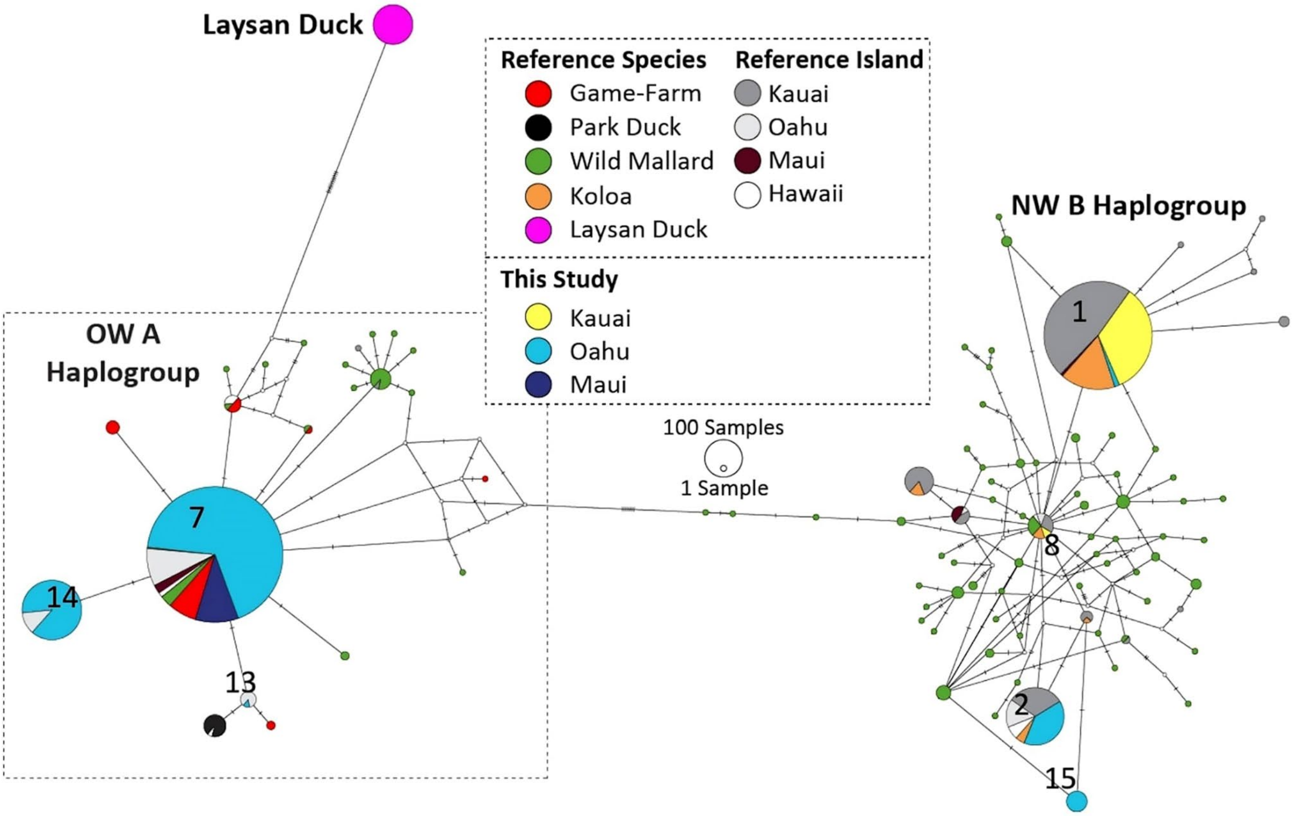


Fig. 2 Mitochondrial haplotype network reconstructed in PopArt (Leigh and Bryant 2015). The two major haplogroups (Old World A on the left, New World B on the right) and samples color coded by reference sequences of different species, sequences from a previous study distinguished by island, and samples from this study distinguished by island. Reference sequences include those for game-farm (domesticated origins) Mallards, Khaki Campbells, wild Mallards, Koloa, and Laysan Ducks. Included is also sequences from a previous study that examined birds from O‘ahu, Maui, Kaua‘i, and Hawai‘i Island, which are labeled as such for a side comparison with samples taken more recently in this study. Numbers on certain haplotypes are the haplotype number that was assigned to each one found in this study

have been lost since due to genetic drift. Moreover, the single novel NW B haplotype found here suggests that not all standing mtDNA diversity had previously been described either.

Finally, due to the low number of individuals sampled between overlapping sites sampled in this study and Wells et al. (2019), the only sites worth comparing haplotypes were Kaua‘i National Wildlife Refuge (KNWR) and Hāmākua. In Wells et al. (2019), they found nine different haplotypes on KNWR, we only found three, but with the dominant haplotype still being 1 (Table 4). Haplotype 2 and 3 commonly found in Wells et al. (2019), were also no longer detected (Table 4). For Hāmākua, whereas Wells et al. (2019) recovered four OW A haplotypes with haplotype 7 being the dominant haplotype, we recovered the same dominant haplotype but only two OW A haplotypes (Table 5). Together, whereas the overall ratios of OW and NW haplogroups have not changed when looking at all possible sites, there are evident frequency changes within sites that we posit is likely a result of genetic drift that explains the overall trend towards diminishing mtDNA diversity. Generally, we continue to confirm that OW A mtDNA diversity remains overwhelmingly represented on all Islands outside of Kaua‘i, supporting how translocation efforts of the 1980 s failed to account for the feral Mallard populations that resulted in eventual genetic swamping of those establishing populations (Wells et al. 2019).

Nest parasitism

A total of 39 out of 168 nests had complete clutch mtDNA sequences that included nine from Kaua‘i, 29 from O‘ahu, and one nest from Maui. Examining evidence for nest parasitism among these clutches, there was a single egg on Kaua‘i carrying the dominant OW A mtDNA haplotype within a nest that otherwise had NW B haplotype 1 (Table 6), suggesting that the one egg was a result of nest parasitism by a female with Game-Farm Mallard mtDNA ancestry. In addition to this nest, another nest on Kaua‘i had eggs carrying two different NW B mtDNA haplotypes

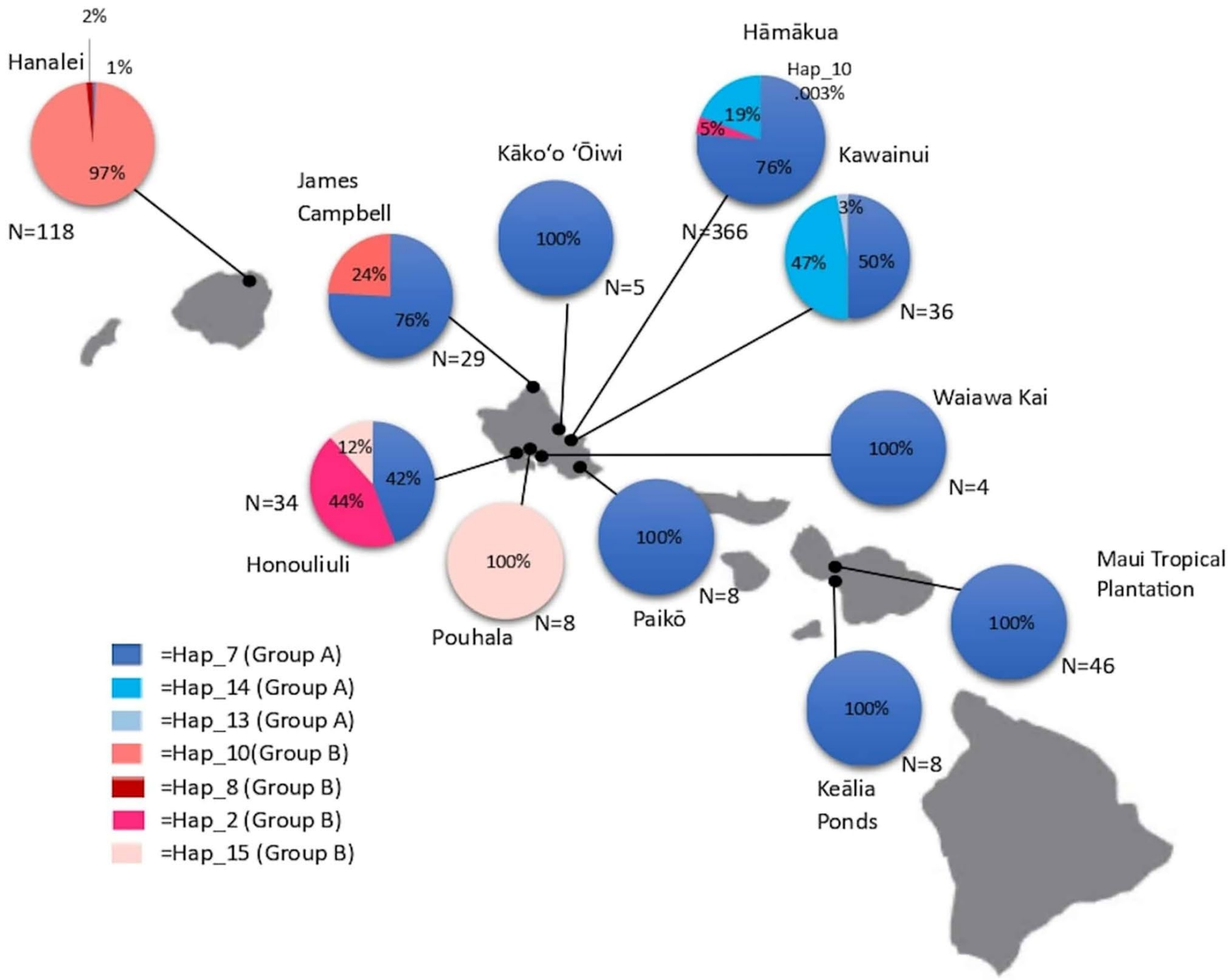


Fig. 3 Mitochondrial (mtDNA) haplotype ratios found per location, displayed geographically on a map. Haplotypes are denoted by number and haplogroup denoted by color

Table 2 Per site comparison of mitochondrial haplogroups OW A and NW B ratios between overlapping sites recovered in the 2010 s via Wells et al. (2019) and in this study, along with the resulting significance

Region	A:B ratios (current)	A:B ratios (2010s)	Chi-square *p*-value
KNWR	0:24	3:143	0.48
KPNWR	1:0	2:2	0.36
Hāmākua	66:4	57:1	0.25
JCNWR	2:1	4:9	0.25
Kawainui	7:0	1:0	N/A

Table 3 Per island comparison of mitochondrial haplogroups OW A and NW B ratios between overlapping sites recovered in the via Wells et al. (2019) and in this study, along with the resulting significance

Island	A:B ratios (current)	A:B ratios (2010)	Chi-square *p*-value
Maui	1:0	2:2	0.36
O‘ahu	75:5	62:10	0.11
Kaua‘i	0:24	3:143	0.48

(1 and 8), suggesting Koloa intraspecific nest parasitism (Table 6). Additional instances of nest parasitism at O‘ahu sites occurred at Hāmākua ($n = 6/87$ nests $= 6.8\%$) and Kawainui ($n = 2/13$ nests $= 15\%$). In all eight cases, there was a mix of different haplotypes from within the OW A mtDNA group, indicating parasitism within hybrid populations (Table 6).

Table 4 Comparison of individual haplotypes found at Kaua‘i National Wildlife Refuge in Wells et al. (2019) versus the number of nest haplotypes found in this study

Haplotype	Number of individuals	Number of nests
1	126	24
17	1	0
2	16	0
23	1	0
3	18	0
4	1	0
5	2	0
6	2	0
7	0	1 (parasitic egg)
8	3	1 (parasitic egg)

Table 5 Comparison of individual haplotypes found at Hamakua in Wells et al. (2019) versus the number of nest haplotypes found in this study

Haplotype	Number of individuals	Number of nests
13	6	0
14	12	13
2	2	3
7	30	56
9	11	0
1	0	1

Discussion

This is the first analysis of sex ratios and updated mtDNA ancestry of nests representing Koloa, Koloa × feral Mallard hybrids, and feral Mallards across Hawaiian Islands. Despite the adult population of Koloa on Kaua‘i exhibiting a 3:1 male:female ratio (Malachowski 2020), there was no significant difference from 1:1 in the offspring sex ratio in this population (Table 1). Hence, we conclude the bias in adult populations is likely due to increased female mortality post-hatch. A potential reason for a higher female mortality could be due to increased vulnerability to predation during nesting and incubation, which is known to generally cause a male-bias in waterfowl populations (Eygenraam 1957; Ferguson et al. 1981; Ohde et al. 1983). Evidence of higher female predation from non-native mammals has been documented specifically at Hanalei as well (Malachowski et al. 2022). Similarly, an even sex-ratio was found for populations of Koloa × feral Mallard hybrid populations of Maui and O‘ahu (Table 1). Together, this did not support that evolutionary (i.e., Koloa) or contemporary (i.e., Koloa × feral Mallard hybrids) hybrids suffer from Haldane‘s rule. Conversely, we recovered a significant male bias in clutches from feral Mallards on Maui (Table 1). It is possible that this male bias among the feral Mallard population may be due to allelic dropout during molecular sexing; however, tissue material and DNA quality did not differ from other populations, making allelic dropout an unlikely a source of bias in this case. Moreover, all samples were PCR amplified and gel checked three times to ensure accurate sexing. Doing so, recovered only six samples (~ 1% of all samples) that varied in band numbers between runs, indicating that allelic dropout was not a strong bias more generally. We posit potential causes for the male-bias among these feral populations may be their Game-Farm Mallard origins (Lavretsky et al. 2023b) where their domestic history has resulted in changes in

Table 6 Cases of nest parasitism, with island, site, dominant haplotype and haplogroup, as well as the number and ID of the alternative mtDNA haplotype recovered in the same nest

Island	Site	Dominant haplotype (haplogroup)	Number of eggs of other haplotype/haplogroup found in same nest
Kaua‘i	KNWR	Hap_1 (B)	1 Hap_7 (A)
Kaua‘i	KNWR	Hap_1 (B)	2 Hap_8 (B)
O‘ahu	Hāmākua	Hap_7 (A)	1 Hap_14 (A)
O‘ahu	Hāmākua	Hap_14 (A)	1 Hap_7 (A)
O‘ahu	Hāmākua	Hap_2 (A)	2 Hap_7 (A)
O‘ahu	Hāmākua	Hap_7 (A)	1 Hap_14 (A)
O‘ahu	Hāmākua	Hap_14 (A)	1 Hap_7 (A)
O‘ahu	Hāmākua	Hap_1 (B)	1 Hap_7 (A)
O‘ahu	Kawainui	Hap_14 (A)	1 Hap_7 (A)
O‘ahu	Kawainui	Hap_7 (A)	1 Hap_13 (A)

life-history traits, including clutch sex ratios (Solberg et al. 2013; Ahmad et al. 2020). Alternatively, the Trivers–Willard hypothesis predicts that offspring sex ratios will be biased towards the sex with higher variation in reproductive success (i.e., males for polygynous waterfowl) when mothers are in good condition (Trivers and Willard 1973). Feral Mallards are in tourist areas where they are consistently fed high caloric diets, presumably enhancing maternal condition, and thus, sex ratios could be shifted toward sons. Support for the Trivers-Willard hypothesis has been found in wild Mallards (Denk 2005), and may also occur in feral mallards. Similarly, local resource competition theory predicts that at high densities, offspring sex ratios will be biased toward the dispersing sex [i.e., males for female-philopatric waterfowl, Wells et al. (2024)], as they will not stay to intensify local competition with the mother and other relatives (Clark 1978; Gowaty 1993). Feral Mallards at the Maui tropical plantation are at high density, which could shift sex ratios toward sons. Local resource competition has rarely been tested in waterfowl (e.g., Jaatinen et al. 2013), but in some cases has had more support than Trivers–Willard (Wells et al. 2024). Future work would require additional, and more complete clutch sampling of these and other feral Mallard populations to determine whether the sex bias detected was a result of the small sample size in this study or truly due selection for male biased clutches.

Next, whereas Lavretsky et al. (2023b) was able to determine that Hawai'i's feral Mallards share the same Game-Farm Mallard ancestry as in New Zealand, mainland North America, and Eurasia based on nuclear DNA, they lacked mtDNA to confirm whether the same lineages found in the other locations existed in Hawai'i as well. Here, we can confirm that the OW A mtDNA haplotypes recovered in Hawai'i are indeed the same as those derived from Game-Farm Mallards released in New Zealand, mainland North American, and Eurasia (Lavretsky et al. 2023b). Together, Lavretsky et al. (2023b) nuclear and these mtDNA results confirm that Hawai 'i's feral Mallard populations originated from the release of the Game-Farm Mallard breed like elsewhere. What is more concerning is that since Wells et al. (2019), there has been one Game-Farm Mallard lineage in particular that has become more dominant, whereas Fowler et al. (2009) found there to be more even diversity of multiple lineages. This reinforces a major concern for wild waterfowl that just one domestic Mallard lineage may be globally replacing native haplotype diversity, diminishing the overall genetic degradation of related species (Champagnon et al. 2023).

Although Kaua'i is still dominated by Koloa haplotypes, feral Mallard mtDNA haplotypes have outnumbered Koloa haplotypes on O'ahu and Maui (Fig. 3). Despite the NW B mtDNA haplotype 1 being the dominant haplotype recovered across sampled nests on Kaua'i, it was only recovered across two and one nest on O'ahu's James Campbell NWR and Hāmākua, respectively (Figs. 2, 3). This lack of presence of haplotype was also something found in Wells et al. (2019). During the translocation efforts from the 1960 s to 1980 s, a variety of Koloa from Kaua'i, presumably including haplotype 1, were put on O'ahu. The lack of haplotype 1 tells us that the loss of this haplotypes in other locations is likely due to a combination of genetic swamping by OW A haplotypes and genetic drift since the translocation, or to the possibility that the nests sampled are just not fully representative of the adults present. Interestingly, a novel NW B mtDNA haplotype was recovered (i.e., haplotype 15, bottom solid blue haplotype in the NW group in Fig. 2, listed in Fig. 3). This haplotype was only found in one nest at Honouliuli and one nest at Pouhala. Nuclear ancestry analyses as part of another project confirmed these individuals with the novel haplotype 15 were hybrids, with an average ancestry of 62% Koloa and 38% feral Mallard, hinting that this may in fact be a new Koloa haplotype. The lack of sampling in these areas or low frequency of this haplotype may be why it was not detected in past studies. Similarly, the OW A haplotype 14 was only found on the North Shore of O'ahu, which was consistent with the findings of Wells et al. (2019), and this study confirmed to have perfect sequence similarity to previously published Game-Farm Mallards. Finally, mtDNA haplotype 2 was also found in low frequencies on Kaua'i but was the main NW B haplotype found on O'ahu in both this study and Wells et al. (2019), which may be due to stochastic events during the 1980 s translocation events simply capturing the haplotype in higher frequencies by chance. Nevertheless, compared to the 2010 s, proportions of NW B versus OW A mtDNA haplogroup ratios were similar (Tables 2, 3). Likely, the differentiation between the haplotypes represented between this study and Wells et al. (2019) is likely due to sampling chance because the different haplotypes that were found were represented in few low numbers. Therefore, we conclude there has been a general stabilization in regards to mtDNA diversity since initially being swamped by OW A mtDNA haplotypes occurring post-release, and as early as the late 1990 s (Wells et al. 2019).

Finally, this study provides the first empirical evidence of inter- and intra-specific nest parasitism among Koloa, feral Mallards, and their hybrids (Table 6), which is especially concerning if sexual imprinting in Koloa or hybrid nests could provide a mechanism for further Mallard introgression on Kaua'i (Sorenson et al. 2010; Rohwer et al. 2022). Although interspecific nest parasitism is infrequent in *Anas* ducks, recent molecular work with American Black Ducks (*Anas rubripes*) did find a proportion of females engaged in nest parasitism of related and unrelated females (Lavretsky et al. 2023a). In fact, previous work found at least one hybrid per generation in Kaua'i (Fowler et al. 2009; Wells et al. 2019). Here, a single case of interspecific parasitism on Kauaʻi was found, but no Mallards were observed as permanent residents of Hanalei National Wildlife Refuge by staff members during the time of this study. Thus, it is likely that the case of recovered interspecific parasitism is a result of a rare female with Game-Farm Mallard mtDNA ancestry. Nevertheless, continued monitoring is critical to ensure the genetic integrity of remaining Koloa. Similarly, parasitism was found among known Koloa × feral Mallard hybrid populations on Oʻahu (Table 6). These results suggest a potential conservation concern in future translocation efforts where hybrids and Koloa once again co-exist spatially, if sexual imprinting by interspecific offspring increases likelihood of hybridization. Interestingly, this study reports the first evidence of Koloa conducting intraspecific parasitism, with a single nest possessing a mixture of two different NW B mtDNA haplotypes (Table 6). We acknowledge that a lack of sampling may impact our true rate. The drivers of Koloa brood parasitism are unknown, but its occurrence could indicate that the parasitizing female was in either poor condition (parasitizing instead of incubating her own nest) or excellent condition (parasitizing in addition to incubating her own nest; Sorenson 1991). It is possible that some individuals are having clutch size constraints due to external pressures and limitations, causing them to parasitize nests in order for both the remainder of their nest to survive and for the best survival chance of that singular dumped egg (Lyon and Eadie 2017). Alternatively, parasitism may be a result of increased resource competition, where the parasitizing female may not be able to secure a nest or support the energetic investment in incubation (Sorenson 1991; Lyon and Eadie 2008; Harvey et al. 2021).

Together, our study fills in critical knowledge gaps regarding Koloa and non-Koloa life-history traits that will be integrated to re-optimize management models (Hernandez et al. 2023), which are critical for future Koloa translocation efforts. Nevertheless, updating ancestry assignments based on nuclear data is critical. Moreover, given that a unique Koloa mtDNA haplotype was recovered at Pouhala Marsh Wildlife Sanctuary and Honouliuli National Wildlife Refuge, expanding genetic surveying of other locations is also important when attempting to determine what Koloa ancestry remains from original captive breeding and supplementation efforts. Particularly, whereas sampling efforts did not include mountain ranges and streams, Koloa historically used these regions (Schwartz and Schwarts 1953; Swedberg 1967) and some do so today (Malachowski 2020); thus, upland areas should be considered for sampling in future studies. Most importantly, any future translocation efforts will require active control of feral Mallards, predators, invasive species, as well as continued habitat restorative efforts to increase habitat availability.

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Data availability The datasets generated and analyzed during the current study are available in the GenBank repository, NCBI accession numbers PV551321–PV551991.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

Ethical statement All collection efforts were done under Federal permit number MB11579C, State permit number WL21-02, and under Institutional Animal Care and Use Committee Protocol No. 24-11-056.

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