CONSERVATION AND IMMUNOGENOMICS OF THE ENDANGERED WHITE-WINGED DUCK

By

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ABSTRACT

The White-winged Wood Duck (WWD) is an endangered species native to southeast Asia. Efforts to conserve the species via captive breeding have been hindered because captive populations appear to be highly susceptible to avian tuberculosis (avian TB). This infection results in the premature mortality of over 80% of these birds. Therefore, despite the fact that birds can be readily bred in captivity, long-term maintenance is not yet possible. The underlying basis of this susceptibility is unknown. Thus, in my research I seek to understand the causes of this high disease susceptibility. Doing so will allow the development of strategies to reduce infection rates and facilitate conservation efforts aimed at maintaining this iconic species.

In Chapter 1, I provide a review of captive WWD biology, specifically the management of the North American captive population, to serve as an important resource for future husbandry protocols aimed at improving the lifespan and reproductive success of WWD. I also identify priorities for the future of WWD in captivity. Due to their precipitous decline in captivity from

avian TB, in Chapter 2 I aim to characterize the effects of inbreeding in captivity relative to a wild population, and in particular, describe variation in immune-relevant portions of the genome.

Finally, in Chapter 3, I characterize the microbiome of two pond types at Sylvan Heights Bird Park (SHBP). This comparison between natural bottom ponds and cement bottom ponds will help guide future management decisions of WWD in relation to mitigating exposure to the organism responsible for avian TB, *Mycobacterium avium* subsp. *avium.*

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LIST OF FIGURES

CHAPTER 1: Captive status and future of the White-winged Duck

INTRODUCTION

The White-winged Duck (WWD) (*Asarcornis scutulata*) is teetering on the edge of extinction and is one of the most endangered ducks in the world (BirdLife International, 2022). Conservationists, ornithologists, and aviculturists have been working to save this species for almost a century, using both *ex situ* and *in situ* approaches (Green, 1992; Mackenzie & Kear, 1976). While efforts to breed the WWD in captivity have helped the species to persist, there has not been a significant improvement in its population size in the wild (BirdLife International, 2022). There has been a drastic decline in the size and health of the North American captive WWD population over the last decade (Cook, 2016). However, the captive WWD would likely have disappeared entirely without collaborative effort across institutions and individuals.

This dissertation chapter summarizes over 40 years of captive breeding effort at Sylvan Heights Bird Park (SHBP). The status of the WWD in the United States is increasingly precarious, and SHBP has led the effort to maintain this species in captivity since the 1980s. Since the initial collection of individuals from the wild in 1970, *Mycobacterium avium* subsp. *avium*, the organism responsible for avian tuberculosis (hereafter, avian TB), has plagued the captive birds and ultimately prevented the establishment of a sustainable captive breeding population. WWDs have high susceptibility to avian TB compared to other waterfowl species (Cromie et al., 1992; Green, 1990), which results in their premature death (Tomlinson et al., 1991).

Ultimately, for continued persistence in captivity, this species needs a scientifically sound strategy of capturing a diverse set of founders using targeted wild collections. Due to their CITES I status, their status as Endangered on the IUCN endangered species list (BirdLife

International, 2022) and additional protections in the countries they inhabit (Ounsted, 1993), this collection will require coordination across numerous agencies at national, state, local, and NGO levels. As their history in captivity has been plagued by avian TB, potential captive-rearing facilities will need to demonstrate their ability to maintain populations with reduced bacterial exposure (Cook, 2017; Tomlinson et al., 1991). The story of WWD management is an informative lesson involving miscommunication between stakeholders, in both time and space. Numerous individuals and agencies have spent their valuable time and resources to arrive at similar outcomes when it comes to conserving this species in captivity, especially when it comes to their relationship with avian TB (Green, 1993; Ounsted, 1993; Tomlinson et al., 1991). As individuals and organizations continue to work on both *in situ* and *ex situ* WWD aims, communication between groups will need to be improved and resources pooled for a more concerted approach.

For the first time, in this review of captive WWD biology, I will focus on the management of the North American captive population through the lens of SHBP, which will serve as an important resource for the future of the worldwide WWD population. Moreover, I will relate lessons learned for *ex situ* conservation of other waterfowl species based on a recent, sharp decline in this captive population. This chapter has three aims:

1) Provide an overview of WWD husbandry in North America since 1969
2) Discuss current husbandry protocols and provide future directions for improving the lifespan and reproductive success of WWD.
3) Identify future priorities for captive WWD management.

The White-winged Duck

The WWD is one of the larger duck species and is named for its wing covert feathers that are white and unmistakable in flight (Figure 1.1A). The original scientific name for the WWD was *Cairina scutulata,* and it was placed in tribe Cairininia, or greater wood ducks based on morphological and behavioral similarities (Green et al., 2005; Muller, 1842). This tribe also included the Muscovy Duck (*Cairina moschata*) and the Hartlaub's Duck (*Pteronetta hartlaubi)* (Todd, 1979). All three species are large bodied tropical forest birds and thought to be related to the perching duck tribe. This group is poorly defined, and its members have been shown to be paraphyletic (Bulgarella et al., 2010; Zelenkov & Kurochkin, 2012), making it difficult to reconcile the taxonomy as the group contained multiple evolutionary lineages. However, recent research using mitochondrial genes has placed WWD as monotypic sister genus within the pochard tribe (Gonzalez et al., 2009; Johnson & Sorenson, 1999). The pochard tribe Aythini includes genera *Aythya*, *Netta*, and *Rhodonessa* (e.g. Canvasbacks, Redheads, and Red-crested Pochards) and are commonly known as diving ducks (Johnson & Sorenson, 1999).

Adult WWD males typically weigh between 2.5 and 3.8 kilograms, and adult females between 1.9 and 2.3 kilograms (Green et al., 2005; Todd, 1979). However, size should not be relied upon when differentiating sexes as there can be overlap. Their contrasting color pattern of white, black, and brown is cryptic when perched in the dense forest understory (Johnsgard, 2010; Kear & Hulme, 2005). There is natural variation in the amount of white on the head (Figure 1.1A), with some individuals having white extend past the mantle (chest) (Gill, 1994). The "high white" plumage phenotype is more common in the lower latitudes of its range, specifically Indonesia and Malaysia (Holmes, 1977; Mackenzie, 1990), suggesting possible genetic differentiation across the WWD range.

The WWD was originally found throughout South and Southeast Asia, but now small, fragmented populations persist only in India, Thailand, Bangladesh, Burma, Vietnam, and Sumatra (Figure 1.1B) (Choudhury, 2007; Green & Crosby, 1992; Marcell, 2016; Saikia & Saikia, 2011; Sharma et al., 2015). While seemingly versatile in captivity, wild WWD rely on flooded forest wetlands (Green & Crosby, 1992; Holmes, 1977; Mackenzie & Kear, 1976). Small family groups or single pairs are found in tropical forests around ponds and marshes, and they generally prefer secluded sections of forest (Choudhury, 2006; Green, 1993). They are omnivorous, regularly feeding on seeds, grains, rice, plants, snails, invertebrates, insects, and fish (Kear & Hulme, 2005; Todd, 1979). Their main food items are found in still or slow-moving water. They typically do not graze on land or dive for food items, but forage in a manner similar to dabbling ducks (e.g. *Anas*), feeding along shallow water edges by tipping forward. However, they are more crepuscular in nature than typical dabblers.

At the southern extent of their range, WWD appear more tolerant of human encroachment and can be seen foraging in rice fields (Holmes, 1977). However, they seem sensitive to disturbance of their diurnal roosts (Green, 1993; Sharma et al., 2015) and will spook when approached. Suitable habitat is rarely found above 4500 feet, with optimal habitat often below 750 feet (BirdLife International, 2022) making them sensitive to sea-level rise in their southern range. They depend on larger trees with hollows for nesting sites. As secondary cavity nesters, WWD are particularly sensitive to deforestation along lowland river systems (Green, 1992; Green & Crosby, 1992). While they appear catholic in their choice of nest tree, *Gluta renghas* trees appear commonly in WWD literature perhaps indicating a preference (Green, 1992; Holmes, 1977; Mackenzie & Kear, 1976). WWDs will use artificial nest boxes, but there has not been much success with their use in the wild as both breeding adults and eggs can be

targeted easily by human hunters (M. Lubbock, personal communication, 2022, Green 1990, 1996).

Like many rare species today, the WWD was considered common by prominent 19th century ornithologists (Green & Crosby, 1992; Sclater, 1880). However, ornithologists believe the species has been in decline for the past 100 years (Johnsgard, 2010; Mackenzie & Kear, 1976; Scott, 1954), with current estimates as low as five percent of its original population size (Green et al., 2005). Recent estimates put the population of mature adult WWDs between 250-1000, with the largest populations in India (BirdLife International, 2022; Choudhury, 2006). WWDs are difficult to accurately survey due to their cryptic, shy and non-migratory nature. Other non-migratory waterfowl species of conservation concern such as Brazilian Mergansers (*Mergus octosetaceus*), Torrent Ducks (*Merganetta armata*), and Salvadori's Teal (*Salvadorina waigiuensis*) also have uncertain population estimates with large confidence intervals (Gill, 1994; Green, 1996; Johnsgard, 2010). These waterfowl have short non-seasonal movements and spend their lives in small family groups which further makes accurate population counts labor intensive. Global population estimates for these types of waterfowl are therefore difficult to assess, relying heavily on model estimates rather than actual observations (Bird et al., 2021). Non-migratory waterfowl species are also more prone to being highly threatened than migratory species (Green, 1996), as non-migratory status correlates with reduced range size and vulnerability to stochastic events.

Early White-winged Duck conservation

WWD protection in India began as early as 1937, and in 1952 it was placed on the Indian Special Protected List (Marcell, 2016). Although hunting WWD is illegal throughout the world,

the ability to enforce restrictions varies across countries, as does the severity of penalties (Hilborn et al., 2006). Much of the wild WWD population is found in protected areas, wildlife sanctuaries, and national parks, though these areas also see more surveys and birders than private or unprotected areas (Suryawanshi et al., 2019). Rural education and ecotourism have proven to be important in combating illegal hunting (Sharma et al., 2015). The WWD also seems to benefit indirectly from protection of several umbrella species such as the Sumatran Rhino (*Dicerorhinus sumatrensis)* and Indian Elephant (*Elephas maximus indicus*) (Allen et al., 2020; DeNormandie, 2000). Conservation efforts for the Sumatran Rhino specifically continues to be an important part of WWD conservation as their habitat requirements and extant populations share a high degree of overlap (Choudhury, 2006; Lynam, 1999; Reilly & Spedding, 1997).

Sir Peter Scott established the Wildfowl and Wetlands Trust in 1946 and paved the way for many of the programs and initiatives that protect the WWD today (Scott, 1954). He was the founding chair of the World Wildlife Fund (Scott, 1989), that selected the WWD as the 406th focal species project in 1968. As a result, the International Waterfowl Research Bureau and the World Wildlife Fund reported that immediate action was needed to ensure the survival of WWD in Assam. Two years later they published a report with three aims: 1. to collect WWD to establish captive flocks in India, England, and the USA, 2. to study and breed WWD in captivity, and 3. to create wild preserves in India or suitable areas where future captive birds could be reintroduced (Green, 1990, 1992; Mackenzie & Kear, 1976). Though WWD were collected, the captive flocks established in England and USA are now in decline, and a captive flock was never established in India. Nor were wild preserves created specifically for WWD in India or elsewhere.

In 1975, the Endangered Waterfowl Group was created by the International Waterfowl Research Bureau, the Wildfowl Trust, the International Council for Bird Preservation (now BirdLife International), and the International Union for Conservation of Nature. The Endangered Waterfowl Group selected the WWD to be the first threatened waterfowl species to have its status reviewed (Green, 1992; Mackenzie & Kear, 1976). While these papers provide a comprehensive overview of wild WWD biology, there has not been an updated assessment in 20 years, during which time the captive population experienced a prolonged decline. Despite early recognition of the threats WWD faced in the wild and multiple efforts to maintain captive breeding populations, there has not been any significant improvement to the future of WWD populations in captivity or the wild.

General captive history of the White-winged Duck

WWD have never been held in large numbers in captivity anywhere in the world compared to most species of waterfowl. The first recorded pair was housed at London Zoo in 1851 (Mackenzie & Kear, 1976). One of the first captive breeding records was in 1936 at the collection of the Schuyl family in Rotterdam, Holland (Mackenzie & Kear, 1976; Scott, 1954). These birds were imported in 1934 from Alipore Zoological Garden, India. Alipore received birds from Edward Charles Stuart Baker who collected the juvenile birds from Assam while stationed in the India Police Service (Mackenzie & Kear, 1976). M. Schuyl described the WWD breeding aviary as small, approximately 2,500 ft^2, shaded by surrounding trees and grasses. A central pond was also described as small, with the female nesting in a box set three feet from the ground (Green et al., 1992). It took the female two years before breeding. Compared to other waterfowl species that have been collected as adults, it is unusual that Schuyl's WWD bred at all.

Often, wild adult waterfowl take many years to settle into captivity and many fail to breed at all (M. Lubbock, personal communication, 2022). This is one of the reasons that contemporary breeding programs for other species of waterfowl source wild stock from eggs rather than adults. None of Schuyl's birds have contributed to the current captive WWD population.

In the hopes of establishing a captive breeding population, the Wildfowl and Wetlands Trust at Slimbridge, England received 10 WWD collected as wild adults from Thailand in 1955 (Green, 1993). However, none of those birds reproduced, and the last individual died in 1961. The cause of death of six of these individuals was evaluated by necropsy. One died from aspergillosis shortly after being imported. The other five all died from avian TB (Cromie et al., 1992; Green et al., 1992). There are no detailed records on the locations of where the Thai WWD were collected. However, since adults were collected, there is a reduced chance they were all siblings or closely related. Regardless, the fact that these Thailand birds also appeared to be prone to avian TB suggests that WWD as a species is sensitive to this pathogen.

The current captive population in Europe and North America was established from birds collected in Assam in 1969 (five males and one female) and in 1970 (two males and two females) (Mackenzie & Kear, 1976). Both collections took place on the same tea plantation in upper Assam (Figure 1.1B); M. J. Mackenzie collected eggs and hand-reared ducklings (M. Lubbock, personal communication, 2022). In contrast to the 10 Thai WWDs, it was known that all 10 Assam founders were closely related as they were from the same nest site. Staff of the Wildfowl and Wetland Trust centers located at Slimbridge and Peakirk, U.K., were able to grow their captive WWD populations to a total of 86 individuals by 1976 (Richardson, 1996). During that time, necropsies of deceased adults revealed avian TB was responsible for the deaths of 25 out of 29 individuals (Cromie et al., 1992). Yet, husbandry records showed that birds lived

several years longer on average than today's expected lifespan (3-5 years) of captive WWD, allowing the population to grow during that time (Cook, 2016; Cromie et al., 2000).

History of captive White-winged Ducks in North America

In 1959, Philadelphia Zoo imported the first WWD into the United States, but this bird (studbook #001) did not contribute to the current captive population (Duckworth, 1996). Between 1974 and 1990, the Wildfowl and Wetlands Trust exported birds to the National Zoo (Washington, DC), Denver Zoo (CO), Minnesota Zoo (MN), Orlando Zoo (FL), Goodewood Game Bird Farm (AL), Ripley Waterfowl Conservancy (CT), and Sylvan Heights Bird Park (NC). However, all the individuals in North America are descended from only two wild founders (studbook numbers 002 and 003), a pair the Wildfowl and Wetlands Trust imported from Assam in 1969. Lineages from the other wild founders all terminated in three generations. The current North American population is estimated to be approximately 15 generations removed from the founding pair (Cook, 2016; Duckworth, 1996).

A 1976 review of captive European WWD biology mentioned little about the then novel population of North American captive birds (Mackenzie & Kear, 1976). While there is still some ambiguity in the avicultural records, there is a relatively complete narrative of captive WWD history in North America with known founders (studbook numbers 001 through 007) (Cook, 2016). This is rare for birds of conservation concern, where numerous wild collections from multiple stakeholders often contribute to the captive global population (Witzenberger & Hochkirch, 2011). Furthermore, when considering their generation length and average lifespan, the relative completeness of the North American WWD pedigree is even more noteworthy (Cook, 2016; Pemberton, 2008). Other avian species with long generation times and long

lifespans have captive lineages that are easier to trace back (e.g. raptors), but in general avian captive history is not nearly as well documented as that of mammalian species (Leus et al., 2011; Princee, 2016).

A comprehensive review published in 1992 showed that avian TB continued to be a significant contributor to WWD deaths in Europe (Green et al., 1992) and between 1976 and 1991, 102 of 121 (84%) WWD deaths were due to avian tuberculosis and there was no sex difference in mortality rate (Cromie et al., 1992). However avian TB began to significantly affect the North American population after 1999, primarily due to an increase in mortality in birds aged 2-6 years (Cook, 2016). Again, there was no significant difference in mortality between the sexes. Between 2010 and 2019, the International Wild Waterfowl Association, Akron Zoo, and SHBP performed biannual health screens on WWDs located at Akron Zoo, Hiram College (Hiram, OH), and SHBP. To reduce TB exposure in healthy WWD, birds with low body condition scores and elevated white blood cell counts were preemptively euthanized as experience showed they would not be alive by the following health assessment (K. Cook, personal communication, 2022). In late stage avian TB infection, birds shed higher levels of the mycobacterium (Cromie et al., 2000). This monitoring program was paused after 2019 due to the Covid-19 pandemic, and as of this writing, it has yet to resume.

The North American Regional White-winged Wood Duck studbook was first published in September 1996, but it did not include any management recommendations (Duckworth, 1996). There were 32 North American facilities that had participated in the studbook up to 1996, but only 19 were actively holding WWDs at that time. The WWD population could be considered robust during this time, with 187 individuals, including 94 males, 93 females, and no birds of unknown sex (94.93.0). The captive North American population size reached its height in 1999

(197 birds) and has been in decline ever since (Figure 1.2), with sharp declines in 2001 and 2019. Unfortunately, it has been my experience at SHBP that their association with avian TB has reduced the number of institutions interested in working with WWD, and this has accompanied their decline. *Mycobacterium* is difficult to eradicate in aviaries and it remains impractical to diagnose and treat avian TB once a bird is infected and/or displaying symptoms (Buur & Saggese, 2012; Riggs, 2005; Saggese et al., 2007; Tell et al., 2003). As of June 15th, 2022, 41 (21.18.2) individuals across 12 institutions remained in the captive North American population. Targeted and biologically relevant captive breeding policies could have helped maintain a more stable captive population. In order to achieve this, aviculturists must recognize the successes and failures throughout the history of captive WWD husbandry and incorporate adaptive management strategies for success (Blais et al., 2022).

Lessons learned from past husbandry of White-winged Ducks

In 1981, the Association of Zoos and Aquariums (AZA) began to manage species across facilities with Species Survival Programs (SSPs) (AZA, 2021). Public perception and species protection policies at the local, state, and national level no longer allow zoos to regularly replace captive animals with wild ones (Powell et al., 2019). The SSP program was initiated to move AZA towards sustainable captive populations (Powell, 2019). There are three levels within the SSP: Green, Yellow, and Red and the WWD is currently a yellow SSP program (Cook, 2016). Three quantitative criteria were established in order to designate which SSP level to manage a species: its population size, number of AZA member facilities where it is housed, and projected genetic diversity. Projected genetic diversity is defined as percent allelic diversity at 100 years or 10 generations, with some allowances for group mating systems (e.g. flocks/herds). While this

matrix can be important when managing a species in long term captivity, it tends to be most relevant for captive populations derived from species with relatively robust wild populations (Powell et al., 2019). This is due to higher numbers of founders for the captive population and a large current captive population able to maintain long term genetic diversity without the addition of new founders (Princee, 2016). This approach forces facilities to be extremely critical when selecting species to devote resources to (Powell et al., 2019). There are allowances for species listed as Extinct in the Wild, Critically Endangered, or Endangered by IUCN that allow them status as a Red SSP regardless of the criteria. Yet, many facilities misinterpret the original intent of the SSP and ultimately focus primarily on species that can achieve genetic sustainability. With the declining WWD population size, many facilities invested in other species of waterfowl and reduced their WWD breeding efforts. Recent work has shown that, declining species benefit from attempted sustainability programs compared with unmanaged programs (Putnam et al., 2021). Future WWD holders will need to balance the core principles of the SSP program with what is practical.

While SSPs can maintain species with adequate genetic diversity across facilities, there are species that can be hindered by this approach (Powell, 2019; Wildt et al., 2019). Often, SSPs benefit species with a long-life span and low reproductive rate (Leus et al., 2011; Powell et al., 2019). It can be difficult to coordinate recommendations in time across facilities for short-lived species that may reproduce prolifically for only a few years, as is the current case with the WWD. The Pink-headed Fruit Dove *(Ptilinopus porphyreus)* is an example of a species whose North American captive population was not benefiting traditional management by an SSP (Foote, 2017). While the program's calculated management recommendations would have maintained genetic diversity for 100 years, it was not possible to move birds among facilities

quickly enough to breed them consistently. Transfers between facilities take time, resources, coordination, health certificates, quarantine periods, and permits. Additionally, transfers can be stressful for the animals. In the case of the fruit doves, that are selective about their mates, these policies often resulted in paired birds failing to bond and not reproducing. It would take several years to re-analyze breeding recommendations, and longer still for facilities to coordinate movements across the country. Chuck Cerbini and Jeff Sailer gathered most of the Pink-headed Fruit Doves within the United States at Toledo Zoo. This change in management strategy had drastic effects on the North American captive population. With the ability to quickly pair and re-pair birds, Toledo was able to follow SSP recommendations at a pace suited to the reproductive lifespan of this dove. Assembling this concentration of birds involved cooperation and trust across facilities and between individual staff members. Whereas it was shown that this strategy can work, it can be difficult to convince facilities to de-diversify their collections and instead dedicate holding and breeding spaces to a reduced number of species, as this conflicts with zoos' and aquariums' interests in providing diversity to incentivize visitation. A future captive breeding program for WWD should mirror this focused effort because it allows for real time decision-making.

The Endangered Waterfowl Breeding Center (EWBC) was built in 2007 as a partnership between Akron Zoo and Hiram College. Located at the college's J.H. Barrow Field Station, the EWBC was designed to house WWD in an aseptic environment. This project, under the supervision of SSP manager, Dr. Kim Cook from Akron Zoo, was meant to help us understand how environmental exposure influenced WWD susceptibility to avian TB. While WWDs lived significantly longer in this environment compared to birds at SHBP, the only successful breeding produced a single duckling in 2010 (Cook, 2017). This suggests that the husbandry protocols

used in that facility to maintain a sterile environment are counterproductive to reproduction. It is unclear, however, which aspects of indoor husbandry (e.g., lighting, cleaning) negatively impacted breeding. At this facility, college students were responsible for the majority of daily care. Therefore, it is conceivable that WWDs under the care of professional aviculturists in indoor facilities could still be a viable option, though such a program may be cost-prohibitive (M. Lubbock, personal communication, 2022). In 2010, based on the work of the late Dr. Jody Modarelli, six WWD from this facility were relocated to outdoor facilities to determine if birds raised in a low avian TB environment could develop robust immune systems post adolescence. Sadly, all six birds were dead by 2014 due to avian TB (Modarelli et al., 2010, 2011) with no successful breeding (Cook, 2017).

WWD care at SHBP has been modified gradually since the late 1990s when avian TB first started noticeably influencing North American captive reproduction efforts. In general, waterfowl are hand reared in a traditional "dry to wet" brooder system, with brooders that are approximately 3ft by 2ft in size (Gereg, 2017). In this system, ducklings are given access to heat, food, and small bowls of running water for the first several days. Ducklings are kept in groups of 3 to 20, depending on species. Based on their clutch size, WWDs are generally kept in groups of 3 to 11 individuals. After the first week, ducklings are given access to larger bodies of water to begin the water proofing process of applying preen gland oil to their feathers. After three weeks, ducklings are moved to a larger wet brooder (30 square feet) and have access to natural lighting and reduced heat sources to improve their thermoregulation abilities. Once feather waterproofing is sufficient, birds are moved to larger outdoor areas. Throughout this process, groups of similar size and species type are slowly combined to form larger groups. After 6 weeks, adolescents are moved to large outdoor flight cages where they will remain until adulthood. WWDs have

gradually been removed from this general rearing protocol, and instead spend longer durations indoors while rarely being mixed with other species (N. Hill, personal communication, 2022). In addition to reducing interspecific competition, this also allowed SHBP to reduce exposure to avian TB by reducing the number of birds within the brooder.

Between 2013 and 2014, SHBP again modified several of its management protocols for WWD. Six inches of soil was removed from the primary WWD aviary and replaced with a layer of lime and gravel. Creating alkaline soil with lime has been shown to reduce avian TB loads, though the duration of effectiveness after such treatments is unknown (Riggs, 2005). Additional trees for perching above the water and substrate were added with the goal of reducing environmental exposure. This strategy was based on their tree-nesting ecology and may mitigate exposure in the wild as well, though mycobacterium levels and tolerance to avian TB in wild WWD are unknown. Anecdotally, these modifications appeared to be beneficial. The average lifespan increased from ~3 years to ~5-6 years. Nevertheless, in 2019, SHBP noticed a drastic increase of avian TB mortality. Reduced fertility and number of reproduction attempts coupled with this increased mortality have left only nine WWDs at SHBP, with an additional 35 birds at 11 other institutions in North America as of October 2022. Furthermore, at SHBP, 8 of 9 are less than 3 years old, and only one female remains. Peak breeding occurs after 3 years, but they tend to be more susceptible to avian TB after this age. Only two other facilities have successfully bred WWD between 2019 and 2022, resulting in only five new individuals.

Optimizing White-winged Duck husbandry protocols going forward

Based on the past 50 years of WWD captive husbandry, practical aviaries will need to be built outdoors on novel ground. While wild WWDs prefer forested areas, captive WWD breed

readily in open areas. Shade cloth and privacy screens can allow for seclusion and cover while still providing ample sunlight necessary to reduce mycobacterium (Riggs, 2005). Water features should be designed to reduce soil, fecal, and detritus particle loads, and come from either well, filtered, or other non-surface water sources. If possible, the use of ultraviolet filters would be beneficial in reducing bacteria, though their effectiveness at reducing *Mycobacterium avium* varies (Bohrerova & Linden, 2006; Peccia & Hernandez, 2004). Water should not be shared between aviaries. While there are merits to a natural bottom pond with a diverse microbial community, I do not foresee natural ponds being beneficial for WWD in North America. Natural ponds would have to be large, well planted, and have limited historical waterfowl exposure in order to be viable options for housing WWD. Aviaries should be heavily perched to encourage birds off the ground and reduce mycobacterium exposure. Accordingly, pinioning (surgical flight restriction) should continue to be avoided in North American captive WWD husbandry. For optimal breeding success, daily husbandry practices should minimize disturbance to the birds which would require aviary footprints above 450 ft^2. Rectangular aviaries (e.g. 15ft wide by 30ft long) have proven successful in other waterfowl breeding programs (M. Lubbock, personal communication, 2022). Water and food sources close to the front of the aviary allow husbandry staff to minimize time in the aviary while still allowing for adequate sanitization. Avicultural staff should be trained in waterfowl husbandry and propagation to optimize chances of successful breeding.

For a novel population of captive WWD, the species should be kept separate from other captive waterfowl. Pairs should be selected that maximize breeding success while balancing genetic diversity. If possible, housing young adults together initially and monitoring formation of pair bonds would lead to stronger pairs. For weakly bonded pairs, allowing parents to rear

offspring has worked well to reinforce the bond (N. Hill, personal communication, 2022). In waterfowl husbandry, ducklings are either raised by the parent or raised without the parent (i.e. hand reared) and both methods should be used with WWD. Subsequent breeding seasons could make use of double clutching (removing first clutch of eggs so the female produces a second clutch). The parents should be allowed to parent rear the 2nd clutch if possible. After two failed breeding seasons, valuable pairs should have their aviary environment changed and given a 3rd breeding season before re-pairing. Assisted hatching, hand rearing, and even use of commercial diets can protect individuals that would not have survived in the wild. Unfit offspring (i.e. small, weak, deformed) should not be allowed to contribute to the future population as captivity can favor deleterious alleles inadvertently (Christie et al., 2012). While this would cause a slower initial population growth, it will be critical to the future success of the captive population.

White-winged Duck future directions

Given the precipitous decline of the North American captive WWD population (Figure 1.2), SHBP began the process of importing captive WWD from Europe in 2019. However, both the covid-19 pandemic and increased regulatory requirements due to highly pathogenic avian influenza (HPAI) have so far prevented the importation. Importing birds from Europe is a practical first step to counter the decline of WWD in North America. Without the addition of captive European WWDs, I estimate the North American captive population will be extinct by 2027 (current population + average number of viable offspring – average number of deaths). Ultimately, importing captive WWD from current holdings in Europe will only delay decline within captivity as all are descendants of the same founders collected in 1969-1970 (Cook,

2016). New individuals with novel alleles will need to be collected from wild populations to increase the genetic diversity.

Wild WWD collection will require coordination across numerous agencies at the national, state, local, and NGO level. An example of a successful modern partnership for captive breeding is the Brazilian Merganser (*Mergus octosetaceus*), one of the world's most critically endangered waterfowl species. With less than 250 individuals known, this species is extinct throughout much of its historical range and now found only in Brazil. In 2006, the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) and the Chico Mendes Institute for Biodiversity Conservation (ICMbio) established an action plan for conservation of the Brazilian Merganser (Hughes et al., 2006). In 2011, after confirming the wild population was not increasing, ICMbio approved a captive propagation component to become a part of the conservation plan (M. Lubbock, personal communication, 2022). A thorough understanding of genetic diversity across the known wild population coupled with feasible collection locations allowed for eggs of diverse founders to be collected and raised in captivity at central propagation facility at Zooparque Itatiba (Maia et al., 2020; Vilaça et al., 2012).

Understanding how husbandry practices can influence the captive WWD population is only part of the solution for WWD. With current science advances making genetic sequencing quicker and more affordable, genetic data could be leveraged in creative ways to maximize the chances of success. Like in the example of the Brazilian Merganser, understanding the genetic diversity in the wild is crucial to the sampling design of a captive breeding program. Given the broad distribution of WWD in Southeast Asia, it is likely that there is genetic differentiation among populations, and this needs to be accounted for to optimize genetic diversity in future captive efforts. However, given how poorly captive and wild populations are doing, and how difficult

importation is, there may not be the flexibility to strategically choose which locations to collect birds from. Further, extensive sequencing could be used to gain an understanding of the genetic basis of mycobacterium susceptibility (see Chapter 2) which could aid selective breeding. Finally, the advent of gene editing tools could even make it possible to enhance immunity of WWD. Further, we can leverage molecular tools (such as metabarcoding or qPCR) to understand the ecology of *Mycobacterium* in aviaries (Chapter 3), which will help improve husbandry practices.

While collection of genetically diverse birds from the wild is the ideal scenario for future husbandry of WWD in captivity, for practical reasons it should not be considered exclusive of other approaches. As with other captive programs, non-releasable wild adults from rehabilitation centers should be considered valuable sources of new genes. As with the Brazilian Merganser, the global captive WWD population's best chance of success would most likely include coordination through a primary facility within the species' native range. This facility would ideally be situated in the native habitat of WWD to facilitate multiple collections across time and space, allowing researchers to target specific locations to maximize genetic diversity. Facilities in North America or Europe would most likely not be permitted to collect in this manner but would most likely be restricted to one collection event from preordained locations without regard to genetic diversity. Overall, a combination of improved husbandry protocols, careful genetic management, international conservation partnerships and habitat preservation efforts is needed.

CONCLUSION

The future of captive WWD in North America relies on the critical importation from Europe to allow us to continue working on improving husbandry protocols and maintain

relationships between stakeholders. Without establishing a lead facility in the native range of WWD, it will be difficult to target diverse founders for a sustainable captive breeding population. It will be important to foster connections with local communities and governments that are directly involved with WWD conservation as the facility develops its breeding program. WWD facilities in North America and Europe should continue to engage with each other as they work on improving husbandry protocols that mitigate exposure to avian TB.

Figure 1.1. Image of White-winged Ducks and geographical locations of wild sightings, captive founder collection site, and wild samples collection site.

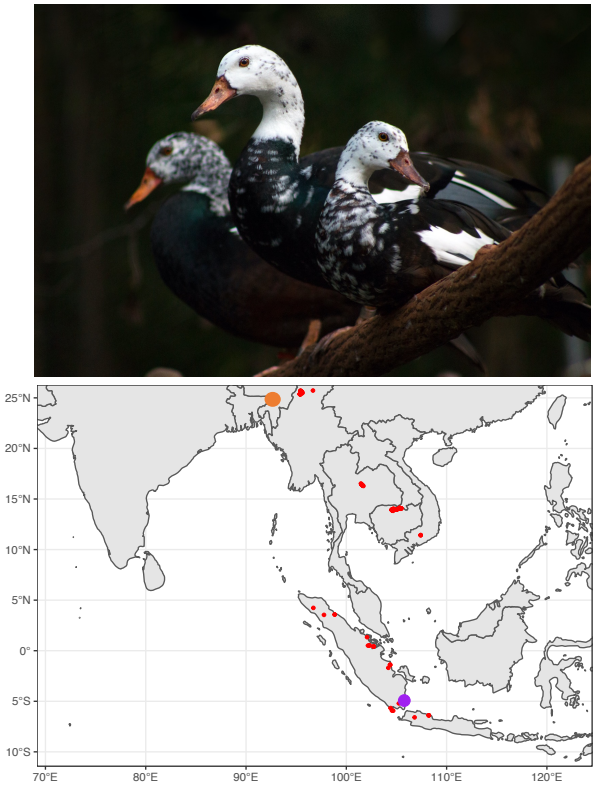
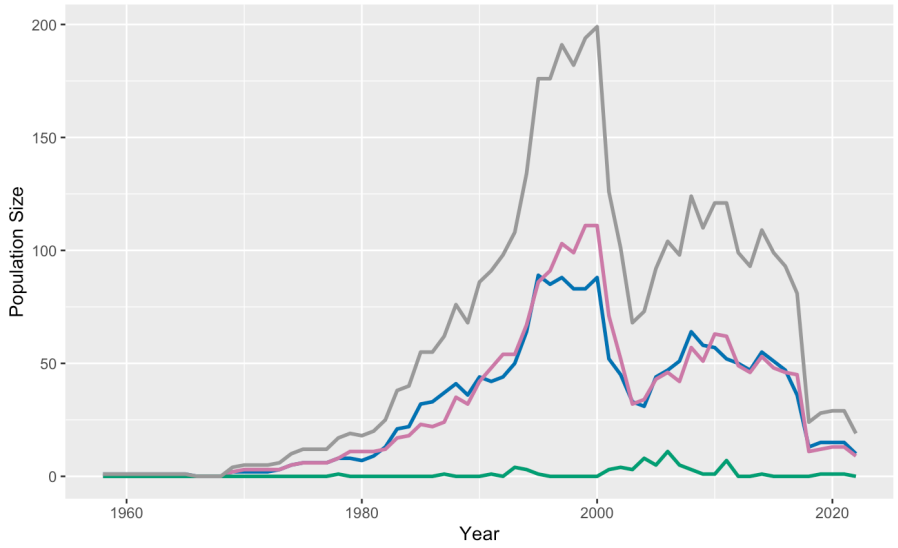


Figure 1: (a) White-winged Ducks (WWD) at Sylvan Heights Bird Park, photo credit Katie G. Lubbock. The diagnostic white wing covert feathers can be seen in the foremost individual. A slight gradient can be observed between the darker individual in the background and the lighter individual in the front. This can be much more extreme, with individuals showing complete white chests to almost no white on the head. (b) A current range map of WWD. Red dots are gbif observations between 1901 and 2020 that included coordinates. Purple dot is the collection location of the wild WWD in Way Kumbas National Park. Orange dot is the location in Assam where the captive birds were originally collected from.

Figure 1.2. Population size of captive North American White-winged Duck population between 1959 and 2022.



Population size of captive North American WWD between 1959 and 2022. Date current to June 15th, 2022. Total population (grey), male population (blue), female population (pink), unknown sex (green). Individuals who died under six months of age are not included.

CHAPTER 2: Conservation genomics of the White-winged Duck

INTRODUCTION

The White-winged Duck (WWD) (*Ascornis scutulata*) is one of the rarest species of waterfowl in the world. The WWD was originally found throughout South and Southeast Asia, but now small, fragmented populations persist only in India, Thailand, Bangladesh, Burma, Vietnam, and Sumatra (Figure 1B) (Choudhury, 2007; Green & Crosby, 1992; Marcell, 2016; Saikia & Saikia, 2011; Sharma et al., 2015). WWD rely on flooded forest wetlands (Green & Crosby, 1992; Holmes, 1977; Mackenzie & Kear, 1976). Small family groups or single pairs are found in tropical forests around ponds and marshes, and they generally prefer secluded sections of forest (Choudhury, 2006; Green, 1993). They are omnivorous, regularly feeding on seeds, grains, rice, plants, snails, invertebrates, insects, and fish (Kear & Hulme, 2005; Todd, 1979). Due to their shy nature and IUCN Endangered status, there is nothing known about the genetic differences across their range (BirdLife International, 2022). However, there is visible phenotypic variation in the amount of white plumage on the head (Chapter 1, Figure 1.1A), with some individuals having white extend past the mantle (Gill, 1994). The "high white" plumage phenotype is more common in the lower latitudes of its range, specifically Indonesia and Malaysia (Holmes, 1977; Mackenzie, 1990), suggesting possible genetic differentiation across the WWD range. However, nothing is known about population genetic structure in this species.

The WWD was considered common by prominent 19th century ornithologists (Green & Crosby, 1992; Sclater, 1880). However, ornithologists believe the species has been in decline for the past 100 years (Johnsgard, 2010; Mackenzie & Kear, 1976; Scott, 1954), with current estimates as low as five percent of its original population size (Green et al., 2005). Recent

estimates put the population of mature adult WWDs between 250-1000 individuals, with the largest populations in India (BirdLife International, 2022; Choudhury, 2006). As secondary cavity nesters, WWD are particularly sensitive to deforestation along lowland river systems (Green, 1992; Green & Crosby, 1992). In addition to habitat loss, hunting, hydro-power development, and pollution all continue to contribute to the decline of the WWD (Choudhury, 2000).

Due to their decline, 10 wild birds were collected in Assam between 1969 and 1970 to start a captive breeding program in the United Kingdom (Mackenzie & Kear, 1976). In 1974, North American facilities began importing WWD from this captive breeding program. Despite the initial collection of 10 individuals, the North American population is only descended from two individuals. The peak of the captive North American population was in 1999, when there were 197 birds spread across 19 facilities (Cook, 2016). However, as of June 15th, 2022, only 41 birds are left.

The status of the WWD in the North America is increasingly precarious, and Sylvan Heights Bird Park (SHBP), in North Carolina, has led the effort to maintain this species in captivity since the 1980s. Since the initial collection of individuals from the wild in 1970, *Mycobacterium avium* subsp. *avium*, the organism responsible for avian tuberculosis (hereafter, avian TB), has plagued the captive birds and ultimately prevented the establishment of a sustainable captive breeding population (Saggese et al., 2007). WWDs have high susceptibility to avian TB compared to other waterfowl species (Cromie et al., 1992; Green, 1990), which results in their premature death at around 3 years (Cook, 2016; Tomlinson et al., 1991). The decline of North American population has been exacerbated by a growing challenge of maintaining breeding age adults who do not succumb to avian TB prior to reproducing. As there

are individuals who survive or resist infections and live well into their teens, it is critical to understand patterns of genetic variation that may contribute to differences in susceptibility. With this in mind, I set out to characterize genome-scale patterns of variation from both the North American captive population and from wild WWD populations. I aimed to characterize the effects of extreme inbreeding in captivity relative to a wild population, and in particular, to describe variation in immune-relevant portions of the genome.

METHODS

Overview: whole genome sequencing of the White-winged Duck

I conducted the first genome-wide analyses of this endangered species, which included the first *de novo* whole genome sequence. I also sequenced 30 WWD individuals across three distinct groups: current captive, historical captive, and wild. My analyses included 14 males, 13 females, 3 currently unknown sex birds. Captive birds with a studbook number had known life history traits (i.e., studbook number, sex, hatch date, death date, health history, sample location, etc.). The use of whole genomes in conservation genetics has greatly increased in the last decade as the associated technologies become more affordable and practical (Luikart et al., 2018; Ouborg, 2010). However, this data set is one of the first to include whole genomes from *in situ* and *ex situ* individuals of an endangered avian species across a thirty-year period.

Current captive White-winged Duck samples

In order to chart changes in genetic diversity in the captive population at SHBP, I sampled birds from among the current population and from birds sampled at the same facility in the early 1990s. All animal procedures were reviewed and approved by East Carolina

University's IACUC under AUP D351 (PI: S. McRae). The current captive sample consisted of ten individuals (six males and four females) hatched between 2008 and 2014. In order to select samples that captured the diversity of the current captive population, I used the CRAN package kinship2 version 1.6.4 in R (Therneau et al., 2015) to produce a pedigree in 2018 of the 651 individuals that had existed in the North American population. As stated in the previous chapter, all captive North American WWD were a part of a studbook program, and each individual was assigned a unique studbook number that started with the original 7 founders (studbook #001 to #007) (Cook, 2016). I mapped known traits including sex, age, cause of death, and sample availability in order to objectively select a diverse representation of birds to sequence for the current North American population. A female WWD (studbook #510) was selected for the first *de novo* whole genome assembly of a WWD. (A female was selected due this being the heterogametic sex.)

Historical captive White-winged Duck samples

The historical captive sample consisted of nine birds hatched between 1985 and 1990 in North America at three locations; SHBP, NC; Goodewood Game Bird Farm, AL; and St. Louis Zoo, MO. Three of the individuals were unable to be associated with their corresponding studbook number and consequently have limited known historical information. However, these WWD were still alive at SHBP during sample collection by Dr. Michael Sorenson in 1992. Despite the birds being hatched at three different locations, they had all been transferred to SHBP prior to collecting blood. The six birds with known studbook numbers are #056, 092, 096, 097, 106, and 116.

Wild White-winged Duck Samples

Feather samples were collected from each of 11 wild WWD in the Lampung Province of Indonesia on the southern tip of the island of Sumatra between June 1999 and February 2001, primarily in Way Kambas National Park (WKNP) by Nancy Drilling (Marcell, 2016). Two males and eight females were captured with mist nets, and one male was captured by a local farmer and held in captivity for 11 days before being released. Males were marginally larger across all metrics; however, two males were reported as hatch year (HY) and were likely immature. Two females had records related to breeding (were accompanied by broods), and I would estimate breeding season between late February and May for this region. WKNP was recorded as one of the biggest remaining populations of WWD in the early 1990s (Marcell, 2016). The wild WWD samples in my dataset were a result of an extensive monitoring program to collect information on breeding biology, specifically population size, habitat use, and juvenile survivorship and dispersal within WKNP. That study estimated a population size of 40 adults producing 120 offspring a year. In 2011, the WKNP Bird Club, AleRT, the Oriental Bird Club, and the Save Indonesian Endangered Species (SIES) Fund, reported the population was down to 12 individuals, citing illegal fishing in WWD primary habitat as the major contributing factor (Saikia & Saikia, 2011).

DNA extraction and sequencing

The ten current captive samples included DNA extracted from blood, liver, lung, and spleen. Seven of the current WWD samples (studbook # 512, 458, 465, 498, 548, 592, and 619) were extracted on June 5th, 2017, with a Qiagen DNeasy Tissue and Blood kit. DNA libraries were prepped with an Illumina TruSeq PCR-free kit prior to sequencing. Samples were taken

from two current captive WWD (studbook # 370 and 343) on May 23rd, 2017, and stored in a -80 freezer until extraction on June 26th, 2019. Historical captive WWD DNA samples were all extracted from blood collected in 1992 at SHBP and extractions were performed by Dr. Mike Sorenson and stored in a -80ºC freezer at Boston University until being sent to East Carolina University in 2019.

All 11 wild WWD feather samples collected by Nancy Drilling were sent to Dr. Mike Sorenson where DNA was extracted and preserved in a -80ºC freezer until they were sent to East Carolina University in 2018 and sequenced.

Due to project funding and sample availability, I performed three rounds of sequencing:

1) A single bird for the *de novo* genome (current captive studbook #510). I used a MagAttract DNA extraction kit for Illumina HiSeq 4000 at Hudson Alpha (Huntsville, Alabama).
2) Seven birds for initial analyses (current captive studbook # 512, 458, 465, 498, 548, 592, and 619).
3) Twenty-two birds (current captive studbook # 343, 370; historical captive studbook # 56, 92, 97, 96, 106, 112, 116, and 3 WWD unknown studbook number birds; 11 wild WWD samples). These samples were all sequenced at the University of Illinois Roy J. Carter Biotechnology Center. The libraries were prepared with a Hyper Library construction kit from Kapa Biosystems. The wild samples, which were more degraded, were pooled and sequenced on two lanes while the other 11 samples (1 current, and 10 historical) were pooled and sequenced on 1 lane for 151 cycles on a NovaSeq 6000.

De novo genome assembly

For the *de novo* genome, I used the 10x Genomics Chromium platform because of the link-read sequencing approach, which provides better reconstruction (relative to other short read-based methods) for previously difficult regions of the genome (Mohr et al., 2017). I assembled the genome using the 10x Chromium Supernova platform (version 2.0) (Weisenfeld et al., 2017). Per the platform recommendations, I used Trim Galore! (version: 0.3.8) to trim the first 23bp of read 1 and first base pair of read 2 (command: trim_galore ${R1.fastq.gz} ${R2.fastq.gz} --clip_R1 ${23} --clip_R2 ${1} --paired). The supernova software package includes two processing pipelines and one post-processing pipeline. FASTQ files were generated with supernova mkfastq and then assembled (command: supernova run --id = ${sample_assembly} --fastqs = ${sample_fastq} –maxread = ${600000000} --localcores ${36} --localmem ${600}. The final FASTA assembly file was produced by the command: supernova mkoutput --style = pseudohap2 –asmdir = ${/path/assembly} --outprefex = ${output} --localcores ${32} --localmem ${600}. Following the manufacturer's guidelines, I attempted assemblies under varying number of reads surrounding a target depth of ~56x coverage.

Read mapping and variant calling

Although my original intent was to use the *de novo* genome as a reference for mapping, and I conducted preliminary analyses using this genome, in 2019 the Vertebrate Genomes Project published a platinum quality Tufted Duck (*Aythya fuligula*) genome (GenBank accession GCA_009819795.1) (Mueller et al., 2021). Due to the quality of the genome, its chromosome level annotation, and the Tufted Duck's close relatedness to the WWD (Gonzalez et al., 2009), I remapped my 30 WWD genomes to the Tufted Duck's genome instead of the *de novo* WWD

genome. Other studies have also used a hybrid approach between mapping reads to a *de novo* genome and a platinum genome, including a recent paper between White-fronted Goose (*Anser erythropus*) and the Mallard (*Anas platyrhynchos*) (Díez-del-Molino et al., 2020).

I used the Burrows-Wheeler Alignment package BWA-MEM (version: BWA-0.7.17(r1188)) to map my sequences to the reference genome (bwa mem ${genomic.fna} -t 6 read1 read2 > ${file.sam} | samtools view -bS -@ 6 ${file.sam} > ${file.bam}). The reference genome was indexed with bwa index prior to read mapped (samtools index ${file.bam} > ${file.bai}). I confirmed using samtools flagstat (samtools flagstat ${bam.file}) that mapping rates between the *de novo* WWD and Tufted Duck genome were similar for each individual. Initial analyses performed on the WWD reference mapped genomes were comparable to those done with the tufted reference mapped genomes.

I used bcftools (version 1.13) mpilup to generate a VCF file with the following options, bcftools mpileup -f | bcftools call -m -Oz -f GQ -o. Downstream analyses also included just variant sites (bcftools call -mv). The VCF files were filtered using vcftools (version 0.1.15, command: --max-missing 0.7 --max-meanDP 50 --minDP 5). I identified SNPs using both samtools and the analysis of next generation sequencing data (ANGSD) software (Durvasula et al., 2016). ANGSD is particularly useful in that it estimates genotype likelihoods and uses these to estimate downstream parameters taking genotype uncertainty into account.

Population genetics and demographic inference

I use both variants identified in samtools and genotype likelihoods from ANGSD to describe key aspects of genetic variation. I used the program PCAngsd (Durvasula et al., 2016) to produce a principal component analysis plotted in R. I estimated the inbreeding coefficient for

each individual using ANGSD version: 0.911-44-glc0ebb6 (angsd command: angsd -bam /${sample.txt} -doGLF ${3} -GL ${1} -out ${/destination} -ref ${/ref.fna} -anc ${/anc.fna} -doMaf ${1} -SNP_pval ${1e-6} -doMajorMinor ${1} -minMapQ ${30} -minQ ${20} -nThreads ${32}) and tested for associations between inbreeding coefficient and lifespan (Oh et al., 2019).

I estimated individual heterozygosity using vcftools (version: 0.1.15, command: vcftools –gzvcf ${sample.vcf.gz} –keep${samples.txt} –het –out ${sample_het}) and π for each of the three populations at the genome and chromosome level using Pixy (version: 1.0.0.beta) genome wide command: pixy --stats pi --vcf ${sample.vcf.gz} --populations ${samples.txt} --window_size ${10000} --n_cores ${8} --output_prefix ${name}, chromosome wide command: pixy --stats pi --vcf ${sample.vcf.gz} --populations ${samples.txt} --chromosomes '${chromosome_name}' --window_size ${10000} --n_cores ${8} --output_prefix ${name})(Korunes & Samuk, 2021). I also estimated π using pixy for chromosome 33 in 100bp sliding windows (command: pixy --stats pi --vcf ${sample.vcf.gz} --populations ${samples.txt} --chromosomes '${chromosome_name}' --window_size ${100} --n_cores ${8} –output_prefix ${name}). Additionally, genome and chromosome π was measured using vcftools (version: 0.1.15, command: vcftools --gzvcf ${vcf.gz} --window-pi ${10000/1000} --window-pi-step ${1000/100}). I also estimated relatedness among individuals using vcftools (version: 0.1.15, command: --gzvcf ${sample.vcf.gz} --relatedness2 --out ${name}) (Manichaikul et al., 2010).

Finally, I assessed long-term population demography using the pairwise sequentially Markovian coalescent (PSMC) method (H. Li & Durbin, 2011). The parameters for the PSMC analyses were –N30 –t5 –r5 –p4 + 30*2 + 4 + 6 + 10 with a mutation rate of 1e-9 and a generation time of two years (Nadachowska-Brzyska et al., 2015, 2016). I selected the mutation

rate based on rates in similar avian taxa (Brüniche-Olsen et al., 2021; S. Li et al., 2014; Martin et al., 2022), and a generation time of 2 years based on estimates of wild WWD (Green, 1993).

Runs of homozygosity

I then estimated runs of homozygosity at the population levels using bcftools roh (version: 1.13, command: bcftools roh -G30 –AF-tag AF –S ${samples.txt} -o ${samples.vcf.gz}), and the allele frequencies estimated from bcftools (command: bcftools +fill-tags ${sample.vcf.gz} -Oz -o ${sample_plugin.vcf.gz} -- -t AF) (Narasimhan et al., 2016). The genomic inbreeding coefficient (Froh) was calculated as the percentage of ∑Lroh/Lauto where ∑Lroh is the total length of all autosome ROHs and Lauto is the total length of the autosome genome (Ceballos et al., 2018; Fu et al., 2019; McQuillan et al., 2008).

Genetic differentiation between White-winged Duck populations

I used vcftools --weir-fst-pop to calculate Fst estimates between the four groups of WWD (wild/historical captive, wild/current captive, wild/captive (historical + current), and current captive/historical captive). For genome wide estimates I used a sliding window of 10kb in 1kb steps (command: --fst-window-size ${10000} --fst-window-step ${1000}). I also calculated Fst between the four groups for chromosome 33 (1kb windows with 100bp steps), as it contains the most major histocompatibility complex (MHC) Class I and II genes (Mueller et al., 2021).

Based on the immunological challenges faced by WWD, I was particularly interested in understanding diversity in immune-related parts of the genome. The major histocompatibility complex is well known for this role (Alcaide et al., 2009; Lan et al., 2019; Stervander et al., 2020) and may function in avian TB immunity (Buur & Saggese, 2012; Chen et al., 2015;

Dhama et al., 2011). At the same time, the MHC is a complex, multi-gene family and is difficult to assemble and annotate in even the highest quality genomes. As a proxy for MHC diversity, I characterized diversity patterns specifically on chromosome 33, a region I identified as containing many key MHC genes including MHC Class I and Class I regions.

Principle component analyses

The three populations of WWD were separated in both time and space. The wild WWD were collected between 1999-2001 in Indonesia from birds that most likely had minimal interaction with the main land population of wild WWD, which is where the captive birds were originally sourced from in ~1970 (Upper Assam, India). The closest historical and captive bird were separated by at least 2 generations in the pedigree. The average lifespan for the six historical birds (three of the nine were unknown) was 9.9 years, and all birds were hatched between 1985 and 1990. The average lifespan for the 10 current captive birds is 5.1 years and all birds were hatched between 2001 and 2014. If current captive WWD #343 and #370 are excluded from those statistics, the average lifespan for the remaining 8 current captive birds was 2 years and 8 months, with birds hatched between 2008 and 2014.

Sequencing depth variation

My initial SAMtools-based analyses described above did not account for a bias in sampling depth for the wild WWD, which was a result of sequencing them for a higher target of 20x coverage due to the age and value of the samples (captive samples were sequenced for a target 10x coverage). Initially, the average depths for both current and historical captive groups was 10.5x while the wild WWD group was 16.6x. I corrected for this using SAMtools view -s to

subsample the bam files so all individuals were approximately 10x and confirmed this did not affect the overall trends observed in initial analyses. ANGSD analyses were carried out specifically to accommodate genotype uncertainty caused by variation in depth.

RESULTS

De novo genome assembly

Using varying numbers of reads (403 million to 813 million, I generated five assembles and compared assembly statistics to select the final assembly which used 771 million reads, 53x coverage, edge N50 = 8.43Kb, contig N50 = 114.79Kb, phase block N50 = 508.88Kb, scaffold N50 = 5.22Mb, missing 10Kb 3.34%, assembly size = 1.07Gb. The primary statistic used for selection was the cumulative score of scaffold N50 and contig N50 (average contig N50 = 122.0Kb/standard deviation = 14.78kb, average scaffold N50 = 2.19Mb/standard deviation = 1.79Mb, average cumulative score = 124.20Kb/standard deviation = 13.82Kb). This WWD genome had better assembly statistics compared to other published waterfowl genomes (Mueller et al., 2021; Zhu et al., 2021). The 2013 the mallard genome (*Anas platyrhychos)* was assembled with short read data using the 60x SOAPdenovo v 1.03 (assembly size = 1.10 Gb, contig N50 = 26 kb, and scaffold N50 = 1.23 Mb) (Zhu et al., 2021). At the time of this assembly, one of the best non-model avian genomes was the 2014 Crested Ibis (*Nipponia nippon*) genome that was constructed *de novo* from high coverage sequence reads (156x) via SOAPdenovo (contig N50 = 67 kb, scaffold N50 = 10.7Mb, and an assembly size of 1.22Gb (Feng et al., 2019). I believe this assembly is an important example of low cost (<$3000 USD) *de novo* genome of sufficient quality for downstream analyses. The *de novo* WWD genome assembly was published as part of the Bird 10,000 Genomes (B10K) project (Feng et al., 2020).

Read mapping

Due to mapping rates for three of the wild WWD samples (average mapping WWD reference = 31.26%, tufted reference = 31.38%) I excluded three of samples that were too degraded for downstream analyses (WILDWD_2, WILDWD_4, WILDWD_16). GC content was highest for these samples (52%-60%), compared to the other wild samples (44%-48%), historical samples (41%-43%), and current captive samples (40%-44%). Disregarding these samples, my original WWD reference genome, an average of 97.60% of reads mapped for all individuals. Average mapping rates for each group for the WWD reference were historical WWD = 99.11%, current WWD = 98.78%, wild WWD = 94.57%. These percentages were marginally higher for reads mapped to the tufted duck reference genome (all WWD = 97.63%, historical WWD = 99.21%, current WWD = 98.96%, wild WWD = 94.72%). My mapping rates were comparable to other studies on avian *de novo* genomes (Feng et al., 2019; Robledo-Ruiz et al., 2022).

Genetic variation in White-winged Ducks

After filtering, using SAMtools I identified 30,034,593 SNPs in total. On average, each historical captive WWD contained 1,923,936 (±363,132) heterozygous sites, each current captive WWD contained 1,175,529 (±108,228) heterozygous sites, and each wild WWD contained 1,405,933 (±67,685) heterozygous sites. Another study on an endangered avian species that had gone through a severe bottleneck before being a captive breeding program also showed similar trends in a reduction of heterozygosity between historical and contemporary samples (Feng et al., 2019).

The average historical captive WWD had an inbreeding coefficient of 0.057 (±0.093), the average current captive WWD had an inbreeding coefficient of 0.254 (±0.108), and unexpectedly, the average wild WWD had an inbreeding coefficient of 0.381 (±0.060). For the 17 WWD with known ages (10 current captive and 7 historical captive), I observed a correlation between inbreeding coefficient and lifespan with more highly inbred individuals dying younger (Figure 2.1, $p = 0.0004$). I tested for relatedness among individuals and the closest related historical captive birds was 0.134 (2^{nd} degree relative), closest current captive birds were 0.170 (2^{nd} degree relative/full sibling), and the closest wild birds was 0.169 (2^{nd} degree relative/full sibling) (Manichaikul et al., 2010). However, the average relatedness for all the historical captive birds was 0.086 (±0.136, $n = 9$) (3^{rd} degree relatives), current captive birds was 0.093 (±0.137, $n = 10$) (3^{rd} degree relatives/2^{nd} degree relatives), and wild birds was 0.120 (±0.154, $n = 8$) (2^{nd} degree relatives).

Overall genetic diversity was low for all three populations. The average genome wide estimates of π for the historical captive birds was 0.0013, current captive birds were 0.0008, and wild birds were 0.0011. A broad comparison across 14 phyla which included 167 species showed π ranged from 0.0005–0.05, with a median of ≈ 0.0065 (Leffler et al., 2012). All three groups of WWD are on the low end of this range, and are considerably lower than comparable studies of birds (Dutoit et al., 2017).

The average number of ROHs across all individuals was 2,360 (±1,519) and the average number of ROHs for historical captive birds was 751 (±759), current captive was 2,321 (±491), and the wild birds was 4009 (±1086). The mean value of the total length of ROHs across all WWD was 95,188,707 (±66,722,613), and the total length of ROHs for historical captive was 26,656,796 (±47,174,420), current captive was 124,602,685 (±39,326,164), and wild WWD was

134,306,641(±56,461,821). Average ROHs length for all WWD was 36,895 (±17,157) and I saw the longest average ROH lengths in the current captive WWD (53,130bp ±10,712), followed by wild WWD (32,209bp ±6,076), and then historical captive WWD (23,543bp ±13,985). I plotted the number of ROHs verse the total length of ROHs in the autosome (Figure 2.2) and all three populations differentiated except one historic individual (studbook #106). Despite this individual being hatched in 1990, it was the first progeny produced from studbook pair #096 and #093 and was a part of the 7th generation, which had seen four sibling pairings. The other six historical birds with known pedigrees all averaged 4th generation (±1) with 2 sibling pairings. I also calculated that the level of genomic inbreeding (Froh = ∑Lroh/Lauto) was high overall (Díez-del-Molino et al., 2020), but highest in the wild (Froh = 0.12) and current captive (Froh = 0.11) birds, and lower in the historic captive (Froh = 0.023) birds.

Genetic differentiation between White-winged Duck populations

All three of my sampled populations are clearly differentiated in Principal Component space, with PC1 strongly differentiating captive birds (historical/current) with wild birds (Figure 2.3). This corresponds to relatively high Fst values for each pairwise comparison (historical versus wild = 0.0914, current versus wild = 0.1668, historical versus current = 0.01874, collective captive versus wild = 0.0848). The distinction is clearest between the wild birds and the current captive birds. The underlying priority of any captive conservation program is to preserve individuals for the eventual release into the wild. The longer a species is maintained in captivity, the greater the challenge to preserving robust and genetically viable individuals becomes. In the case of the highly endangered Brazilian Merganser, fragmented populations only exist in several locations, including the National Park Chapada dos Veadeiros (PNCV), Parque

Nacional Serra da Canastra (PNSC), and Alto Paranaiba region (APR) (Maia et al., 2020). Fst estimates across these three populations were similar to differentiation between wild WWD and captive WWD (PNCV/APR Fst 0.131, WWD wild/captive Fst 0.167).

Functional genetic diversity in wild White-winged Ducks

Despite overall low genetic diversity, as expected the MHC region (chr33 in the tufted duck) harbored the greatest genetic diversity for each population. The average π for chromosome 33 for the historical captive WWD was 0.0057, current captive was 0.0039, and wild was 0.0084. Interestingly, and in contrast to overall patterns of genetic diversity, wild birds have maintained higher levels of functional genetic diversity than captive birds, specifically in MHC class I exons.

Demographic history of wild White-winged Ducks

Consistent with low overall levels of genetic diversity, PSMC analyses revealed long-term population declines that started over the last million years and continued through the Pleistocene (Figure 2.4). The global WWD population is estimated to have experienced a drastic reduction in size, with a steady decline from 100,000 pairs at the beginning of the Pleistocene to 20,000 pairs at the start of the Holocene (11.7ka). The rate of decline increased during the last interglacial period around 120ka.

DISCUSSION

Low genetic diversity

Estimates of genome wide π diversity for the WWD were extremely low, similar to other endangered or threatened avian species, including the Crested Ibis (*Nipponia nippon*), Kea (*Nestor notabilis*), and the Kiwi (*Apteryx mantelli*) (Le Duc, 2015; S. Li et al., 2014; Robinson et al., 2016). Surprisingly, I found that wild-captured WWD from Indonesia harbored the lowest levels of genetic diversity ($\pi = 0.0010$). Although I have not sampled wild birds from northern India, this finding suggests important differences in the demography of these different populations. Populations in Indonesia, like the one sample here, may have suffered historical bottlenecks in the colonization of islands in the archipelago at the southern extreme of the WWD range.

Less surprisingly, I observed the expected decline in genetic diversity over time in captive birds. This corresponded with longer and more numerous runs of homozygosity. Critically, I observed a strong correlation between inbreeding levels and lifespan. This provides crucial evidence that inbreeding is contributing to poor outcomes in captivity and argues strongly for improving the genetic diversity of captive bred birds.

Among population genetic differentiation

I found strong evidence of genetic differentiation among captive and wild populations. Since the wild bird samples were from Indonesia, and the captive founders were sourced from India, this could suggest substantial genetic differentiation among populations in their wild range. Further sampling is needed from across the WWD range, including wild birds in India, to assess the extent of inter-population variation. Comparing samples specifically in the state of Assam would provide valuable insights to this dataset by providing a more direct comparison

between genomic changes between the contemporary founding wild population and the captive samples.

Other species of conservation concern with similar range sizes to the WWD show evidence of strong differentiation between populations. The Indian Rhinoceros (*Rhinoceros unicornis*) showed and Fst of 0.2 between populations in Assam and Nepal (Zschokke et al., 2011). Most studies looking at differentiation within species across south east Asia and Sundaland show genetic and phenotypic variation (Lim et al., 2014, 2015), though ecology (i.e. dispersal distance, reproductive rate, etc.) has been associated with the degree of differentiation (Burney & Brumfield, 2009). As a non-migratory, nonsocial species of waterfowl, the WWD most likely has strong differentiation between populations throughout its range.

Functional genetic differentiation

Due to the avian TB pathogen risk to WWD, I was particularly interested in characterizing diversity in parts of the genome involved in immunity. Based on experimental work, the differences in MHC diversity between the wild and captive WWD could be related to the captive populations' increased risk. Across all samples, MHC diversity remains higher than rest of the genome. However, unlike rest of genome, captive birds had less MHC diversity than wild, Indonesian birds. Therefore, it appears that long-term declines in the wild have proceeded without sapping critical functional diversity. Whereas extreme inbreeding in captivity has led to losses in these critical parts of the genome. This highlights the importance of incorporating novel, wild-derived lineages to the population.

Wild White-winged Duck population trends

The wild, Southeast Asian WWD population has shown a steady decline that started well before the last glacial maximum and subsequent sea-level rise. This result is in marked contrast to my initial expectation, which was that population declines for this species were purely anthropogenically driven. Instead, it appears that long-term environmental changes have acted in conjunction with anthropogenic effects, leading to the current conservation crisis for this species. A number of other species from these regions show similar long-term declines. The estimated effective population sizes for the mainland Clouded Leopard (*Neofelis nebulosa*) and the Sunda Clouded Leopard (*Neofelis diardi)* are distinctly different in their historical trajectories (Bursell et al., 2022). Similar to the WWD, the Sunda Clouded Leopard shows a steady decline in population size over the last million years. The same differences in predicted historical population sizes has been also been observed for the Sumatran Rhino (*Dicerorhinus sumatrensis*) and Indian Rhino (*Rhinoceros unicornis*) (Kutschera et al., 2018).

The Pink-headed Duck (*Rhodonessa caryophyacea*) has not officially been seen in the wild since 1949. This species shares several similarities with the WWD, including overlap in their geographic range. Of greater import is the similar feeding style and relationship of WWD to Aythini (pochards). The PSMC does not provide reliable estimates, but the recent population size of Pink-headed Duck seems to have remained consistent for much of the last 100,000 years before its decline during the Holocene. During most of the Pleistocene, the Pink-headed Duck population was estimated to be between 15,000 and 25,000 birds. By contrast, the WWD has seen a drastic reduction in population throughout the Pleistocene, with a steady decline from 100,000 pairs to 20,000 (Ericson et al., 2017). This is cause for concern as recent anthropogenic factors such as hunting and habitat loss could be behind the Pink-headed Ducks extinction, but

the decline in global WWD populations over the last million years suggests it may be more difficult to reverse.

CONCLUSION

As evidence of the 6th mass extinction accumulates, the WWD is a species at the forefront despite numerous individuals, facilities, and organizations working to conserve them. My study paints a complex picture in which long-term environmental changes have been compounded by anthropogenic effects. In species with broad geographic ranges, like WWD, these long-term and short-term changes vary in scope and in their genetic consequences, and so understanding patterns throughout the range is critical. Further samples are needed across the WWD's native range to understand the genetic connectivity of the populations and to assess overall diversity. Our current understanding is limited to wild birds from Indonesia, which show an inbred population with lower diversity than expected. Despite this, the population appears to have maintained higher functional diversity in MHC regions compared to captive WWD collected from Assam. Comparison between historical and current captive WWD suggests that the Assam population of WWD had higher diversity and were genetically distinct from the Indonesian population. Currently, most extant wild WWD are thought to occur in Assam, and introduction of some birds from this population into the captive breeding stock could provide a valuable source of genetic variation. Moving forward, to bolster the captive breeding program, understanding differences in avian TB susceptibility across WWD populations will be critical. Captive birds originating from Thailand that died from avian TB suggest that members of more than one population are at risk from this disease.

Figure 2.1. Higher levels of inbreeding associated with shorter lifespans in captive White-winged Ducks. Current captive individuals highlighted in purple and historical captive birds in orange. Age of bird at time of death. Inbreeding coefficients calculated with angsd.

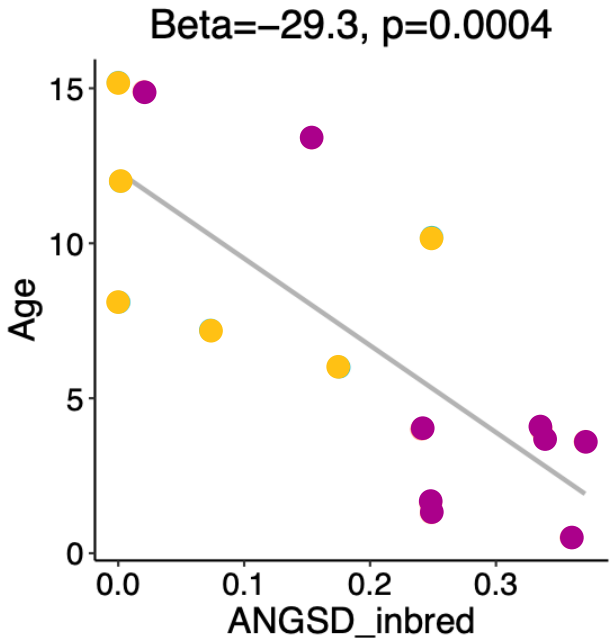


Figure 2.2. Distribution between the number and sum of runs of homozygosity. The total number of ROH segments in the autosomes plotted against the total combined length of all ROH segments in the autosomes.

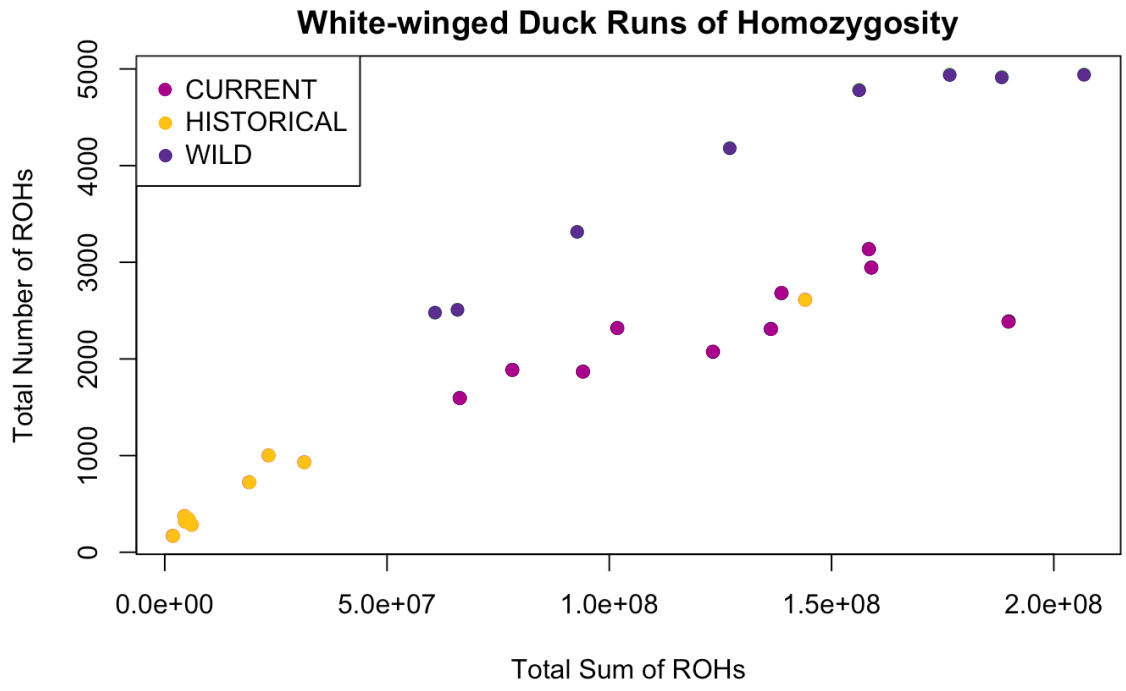


Figure 2.3. Principle component analyses of White-winged Duck samples and allele frequencies shows differentiation between populations. Wild and captive individuals separate distinctly on PC1, while PC2 shows a transition between all three populations.

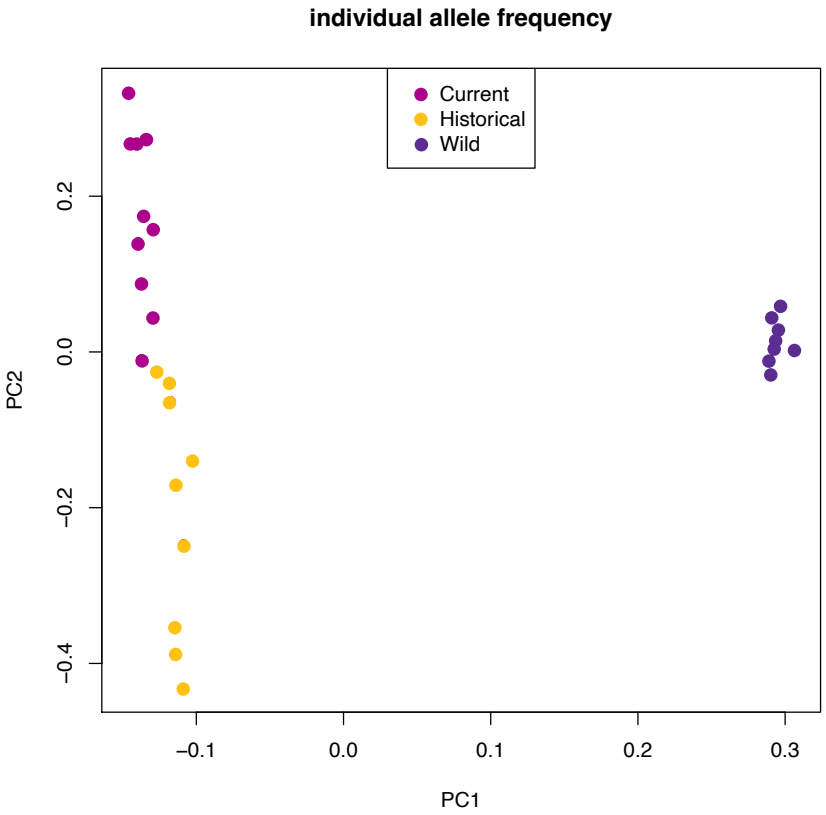
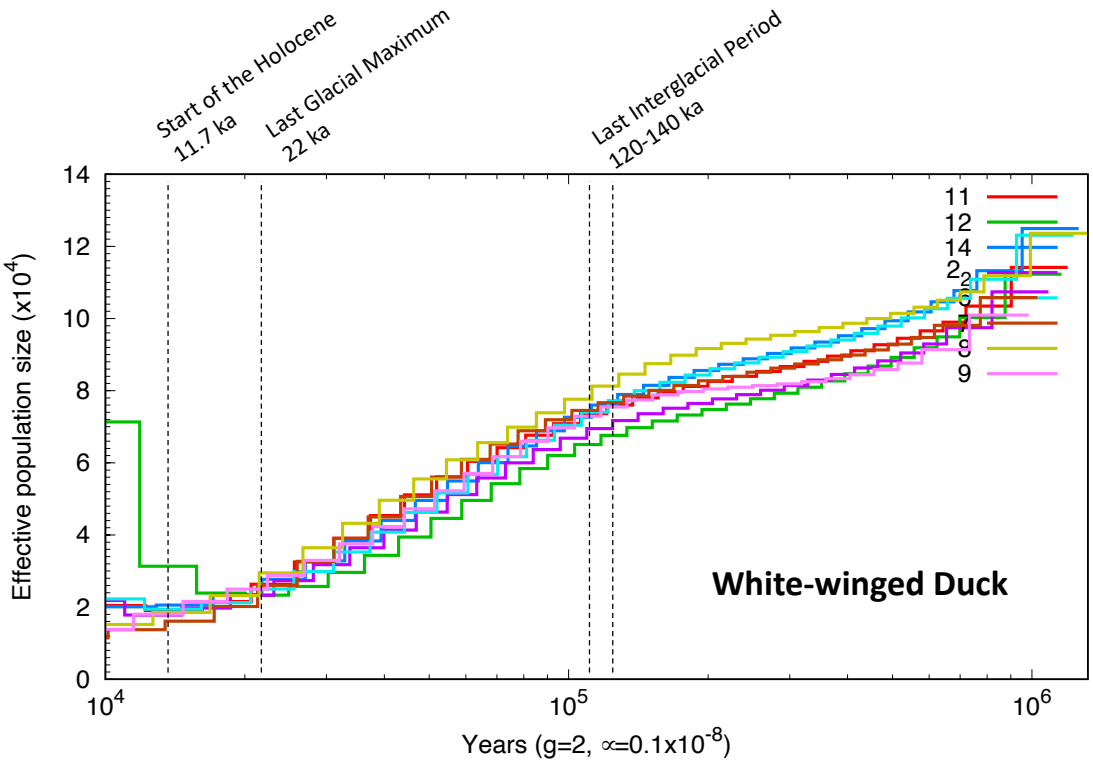


Figure 2.4. PSMC of the White-winged Duck. The PSMC shows that the wild White-winged Duck population I sampled has been in decline for the last million years, well before anthropomorphic affects. In addition, the decline appears to be steady and not subject to any large events that affected effective population.



CHAPTER 3: Characterization of environmental microbiomes within aviary ponds

INTRODUCTION

Ecological microbiome communities are shaped by their environment but also play a key role in shaping their own environment. Research on host-environment-microbiome interactions has exponentially increased over the last decade (Cullen et al., 2020). Protocols on how to define, survey, and analyze microbial communities has seen tremendous change as the field of microbiome research progresses (Berg et al., 2020). Zoos and aquariums have readily embraced this field as there is little question that host-microbiomes influence important factors such as disease susceptibility, nutrition, and growth (Fan & Pedersen, 2021). Due to the complexity of these interactions, most zoological studies focus on host-microbiomes rather than ecological microbiomes, as the later can be difficult to standardize (Clayton et al., 2016; Wills et al., 2022). However, as our ability to characterize microbial communities improve, researchers will have better tools to allow them to understand the complex communities that are a part of the ecological microbiome (Tsuji et al., 2019).

As one of the rarest ducks in the world, the White-winged Duck (WWD) (*Ascornis scutulata*), captive breeding is an import part of their future. A captive breeding project was initiated in 1970 from only several birds collected from Assam, India. Since the initial collection of these founders, *Mycobacterium avium* subsp. *avium*, the organism responsible for avian tuberculosis (hereafter, avian TB), has plagued the captive birds and ultimately prevented the establishment of a sustainable captive breeding population (Saggese et al., 2007). WWDs have high susceptibility to avian TB compared to other waterfowl species (Cromie et al., 1992; Green,

1990), which results in their premature death at around 3 years (Cook, 2016; Tomlinson et al., 1991).

As I look to the future of the WWD in captivity, improving our understanding of how management decisions impact ecological microbial communities (specifically in relation to *Mycobacterium* species) will be paramount for success. In Chapter 1, I outlined management protocols for WWD in captivity based on the collective knowledge of individuals who have worked with this species for over 50 years. These protocols all center on practical methods of reducing the exposure of *Mycobacterium* to WWD in an environment still conducive to WWD propagation (novel ground, ample sunlight, use of filters, use of non-surface water, cement ponds, etc.) (Buur & Saggese, 2012; Drewe et al., 2009; Tomlinson et al., 1991). To determine the efficacy of these decisions, and identify new possible solutions, I characterized the ecological microbiome of the source well water, and two ponds at Sylvan Heights Bird Park (SHBP). These two ponds differed in their construction, cement-bottomed versus natural bottomed, but also differed in their avian communities. I aimed to begin to understand the uniquity of *M. avium* throughout the part, and the relationship between pond construction and microbial communities. This initial characterization of ponds at Sylvan Heights will help guide future management decisions of WWD in relation to mitigating exposure to the organism responsible for avian TB, *Mycobacterium avium* subsp. *avium.*

METHODS

Location and pond types

SHBP is an avicultural facility located in Scotland Neck, NC (USA) that has been a part of the WWD captive breeding program since the beginning (Green, 1990). The facility is

approximately 28 acres and uses four wells to provide unfiltered ground water to ponds and aviaries. All four wells were drilled to an average depth of 75ft (±22ft) and were cased to 52ft (±12ft) with pumps suspended at 25ft (±8ft). Average water depth is 16ft (±3ft) below grade. Each well ties into a main water distribution system that runs throughout the park.

At SHBP, waterfowl ponds are all flow through systems, with a constant input of water from the distribution line that exits through an overflow. Aviaries have two connected ponds (water enters first pond from input of the distribution line, overflows into a second pond, and then exits via an overflow). Best practice is to have the input and output points as far apart as possible so that the entire pond is cycled with no stagnant spots.

I characterized the microbiome across two pond types SHBP, a cement bottomed pond and a natural bottomed pond. The cement bottom ponds selected were in SHBP's primary WWWD aviary (here after WWD will refer to the bird, and WWWD will refer to the aviary and pond samples). A cement bottomed pond is a practical method of reducing the amount of detritus that accumulates due to the ability to easily drain and clean the pond. (Though, WWD still succumbed to avian TB in this aviary.) The natural bottomed ponds selected were in SHBP's South American waterfowl aviary. This aviary has over 25 species of waterfowl along with a large colony of Chilean Flamingos (*Phoenicopterus chilensis*), resulting in one of the "dirtiest" ponds based on turbidity. This aviary rarely has waterfowl succumb to avian TB infections.

Sample collection

I collected samples at both aviary locations (WWWD and SA) and from a source location (SOURCE). Each location represents two ponds that are connected in a flow through system. I collected multiple samples along three transect points: water inflow at pond one (PRE: 3

samples), midway point between inflow and outflow of pond two (MID: 3 samples), and outflow (EXIT: 2 samples). I also collected samples at three locations where a well tied into the mainline (SOURCE: 3 samples).

Samples were collected in June 2017 from SHBP. Samples were collected in sterile 500 ml wide-mouth HDPE bottle. Prior to collection, each bottle was rinsed 3x with sample water before collecting the sample on the 4th fill. Sample collection took approximately two hours and samples were transported on ice to East Carolina University for filtering.

Filtration

To collect microbial samples for DNA extraction, the water samples were filtered through Pall Life Science Supor Membrane Disc filters (gridded S-pack, diameter: 47mm, pore size: 0.2µm, pin #66234, lot #T43125). Samples SH_1 through SH_8 were pushed on a 5-run system at 10PSI. Samples SH_9 through SH_19 were pushed on a double run at 13 PSI. Samples SH_1 through SH_16 each contained 300ml of water, while samples SH_17 through SH_19 contained 400ml. Filtration time varied from 10 minutes to 140 minutes based on the amount of particles in the sample. Filters were stored in a -80°C freezer at East Carolina University until DNA extraction.

DNA extraction, PCR, library preparation, and sequencing

I extracted DNA in September 2017 using a Qiagen PowerWater Kit (Carlsbad, CA, USA) (GIIN#04053228029151, lot#154046056). I followed manufacturer protocols (Quick-start Protocol version 1 DNeasy PowerWater Kit) except (1) I incubated samples in Powerbead tubes for five minutes at 65°C before vortexing, (2) incubated samples for five minutes following the

addition of elution buffer to the filter membrane, and (3) eluted purified DNA with 50µl of the elution buffer (Rubin et al., 2014).

I amplified the V4-V5 region of the bacterial 16 subunit of the ribosomal RNA gene (16s rRNA) with PCR following the Earth Microbiome Project protocol (Caporaso et al., 2012). Each PCR reaction contained 13.75µl H2O, 5µl 5x Colorless GoTaq Flexi Reaction Buffer (Promega), 2.5µl MgCl2 (25mM), 0.625µl dNTPs (40mM), 0.5µl barcoded 515f forward primer (10µM), 0.5µl barcoded 806r reverse primer (10µM), 0.125µl GoTaq DNA Polymerase (5U/µl, Promega), and 2µl DNA template. Each reaction had the same thermal cycler conditions of 94°C for 2 minutes, followed by 30 cycles of (35 seconds at 94°C, 45 seconds at 55°C, and 120 seconds at 72°C), then 72°C for 2 minutes and held indefinitely at 4°C.

PCR reactions underwent PCR cleanup using Agecourt AMPure XP magnetic beads (Pasadena, CA, USA). Amplicon DNA concentration was measured with an Invitrogen Qubit 2.0 (Carlsbad, CA, USA). I pooled 10ng DNA from samples SH_1 through SH_17, 9.2 ng from SH_18, and 1ng from SH_19 together with a mock sequence sample and sequenced at the Indiana University Center for Genomics and Bioinformatics on one lane of an Illumina MiSeq using 2x150bp reads.

Microbial community analyses

Sequences were analyzed using mothur v1.40.5 pipeline (Schloss et al., 2009). I merged read 1 and read 2, removed duplicates, and then removed chimeras with the VSEARCH algorithm (Rognes et al., 2016). To classify my remaining sequences into microbial taxa, I aligned them to the SILVA 128 database (Quast et al., 2012). Using the known mock sequence error rates, I classified sequences into operational taxonomic units (OTUs) based on 99%

similarity. I then performed a Microbial Community Analyses in Rv4.1.3 (package Vegan version 2.0-10) to classify microbial communities based on Fisher's alpha for richness, Shannon's diversity index, Simpson's evenness index, and Pielou's index (Fisher et al., 1943; Pielou, 1966; Shannon, 1948; Simpson, 1949). I rarefied abundances prior to a minimum of library size (Cameron et al., 2021). I tested for differences between groups (location, transect, and a factor of transect location) using an ANOVA and then performed a post hoc t-test to evaluate interactions between groups. I then tested for differences using a permutational multivariate analysis of variance (PERMANOVA) and visualized with a principal coordinates analysis (PCoA). I removed OTUs with less than 10 reads, then calculated relative abundance. I then removed small taxa (<.001), combined common taxa, and then visualized with a bacterial community composition plot (Lin & Peddada, 2020b). I plotted relative abundance for 4 OTUs (0113, 1461, 2496, and 3114) that were classified as *Mycobacterium*.

MicrobiomeAnalyst

In order to plot absolute microbial abundance of each sample, I also performed an analysis using an alternative pipeline, Marker Data Profiling in MicrobiomeAnalyst (Dhariwal et al., 2017). This pipeline allowed for single factor comparisons between my samples as the design matrix was unbalanced which made including SOURCE samples difficult in parts of the previous R pipeline. (SOURCE location had 3 samples, other locations had multiple samples as a factor of transect location, i.e., location SA in transect PRE had 3 samples.) Per pipeline recommendations, I removed OTUs with less than 2 reads per sample, OTUs with less than four counts in 20% of the samples, OTUs with low variance (10% inter-quartile range), and then rarefied samples to the lowest library size. I scaled the data using total-sum scaling (divide

number of reads for OTU in a sample by the total number of reads in each sample) to reduce the influence of variation between read counts across samples (Lin & Peddada, 2020a). At both OTU and phylum level I examined alpha diversity by calculating bacterial community richness (chao1), community diversity (Shannon), and community evenness (Simpson) (Chao, 1984; Shannon, 1948; Simpson, 1949). To assess beta diversity at OTU and phylum levels, I calculated Bray-Curtis Index distances between each sample (Bray & Curtis, 1957). I tested for differences using a PERMANOVA and visualized with a PCoA. Finally, I re-plotted absolute abundance for OTU0113.

RESULTS

I sequenced 19 samples and had an average of 18,875 reads per sample (minimum count per sample: 8,022, maximum count per sample: 37,436). The number of reads was as follows in descending order, WWWD_PRE (28,177 ±9,470), WWWD_MID (21,817 ±2,345), SA_PRE (20,739 ±7,397), SOURCE (20,419 ±5,691), SA_MID (15,548 ±3,204), WWWD_EXIT (9,995 ±1,760), and SA_EXIT (9,268 ±1,762).

Microbial community diversity

I plotted Shannon's diversity and Simpson's evenness indices in ggplot2 (Figure 3.1 and Figure 3.2, respectively). Broadly, in the natural bottomed SA ponds, diversity and evenness decreased as you moved between PRE, MID, and EXIT. However, in the cement bottomed WWWD ponds, diversity and evenness increased as you moved between PRE, MID, and EXIT. The ANOVAs for Shannon's diversity index showed no significant differences between locations and transects (Shannon's diversity by location, F-value = 0.0066, p-value = 0.9369, by

transect, F-value = 1.1686, p-value = 0.3499, by transect as a factor of location, F-value = 2.1517, p-value = 0.1670.) Simpson's evenness by location, F-value = 0.3717, p-value = 0.5557, by transect, F-value = 5.3490, p-value = 0.0263*, by transect as a factor of location, F-value = 0.2365, p-value = 0.7937 (this result showed significance in Simpson's evenness between transects, *post hoc t*-test then determined which transects). The result of the *post hoc t*-test showed this significant interaction in Simpson's evenness between PRE and MID samples (df = 10, p-value = 0.0269*). *Post hoc t*-test results for the other contrasts were PRE/EXIT: df = 10, p-value = 0.1056 and MID/EXIT: df = 10, p-value = 0.8681.

The PERMANOVA showed significant differences between microbiome composition based on samples grouped by transect (df = 3, F = 3.0966, p = 0.0019*), location (df = 1, F = 6.7249, p = 0.0009*), and transect as a factor of location (df = 2, F = 4.2556, p = 0.0009*). I visualized this in a PCoA (Figure 3.3), with PCoA 1 accounting for 30.2% of the variance, and PCoA 2 accounting for 21.6%. Samples did not group by transect. However, they were distantly grouped by location. SOURCE was most similar to WWWD_EXIT and WWWD_MID.

There were clear differences for location and transect based on comparisons of relative abundance of bacterial community composition at the genus level (Figure 3.4). For the SA samples, unclassified Gammaproteobacteria, *Flectobacillus*, and *Flavobacterium* all dominated the start of the transect, but disappeared in the midpoint and exit samples. For the WWWD samples, *Streptococcus*, unclassified Rhodobacteraceae, *Acinetobacter*, and *Paludibacter* were all identified at the start of the transect in low levels but disappeared in the midpoint and exit samples. Additionally, community composition changed considerably as samples moved along the transect. SA saw an overall reduction in abundance of consistent community members moving from pre to exit. WWWD saw a steep increase in overall bacterial abundance between

pre and midpoint samples, but there was substantial succession between these two transects. The bacterial community members that were consistent throughout SA yet not present initially in WWWD, became dominant in by the end of the WWWD transect (Comamonadaeae and Chitinophagaeae).

MicrobiomeAnalyst

Due to the recommended stringent filtering steps in the MicrobiomeAnalyst platform, my initial 7,992 OTUs were filtered down to 501 OTUs used for these analyses (filtering for ≥ 2 removed 4,212 OTUs, filtering for low count minimum of 4 reads in 20% of samples removed 3,223 OTUs, filtering for low variance in 10% of inter-quantile range removed 56 OTUs) (Cameron et al., 2021; Dhariwal et al., 2017). While I do see the same relative patterns of diversity and evenness in this pipeline between samples, I were only focused on reporting a single factor comparison in absolute abundance between samples for a specific OTUs.

Mycobacterium OTUs

I identified read counts for the four *Mycobacterium* OTUs. However, only OTU0113 had enough reads across all samples to remain in downstream filtering analysis steps (451 reads). The removed *Mycobacterium* OTUs were OTU1461 (total: 12 reads (SOURCE: 10 reads, WWWD_PRE: 2 reads)), OTU2496 (total: 6 reads (SOUCE: 2 reads, WWWD_PRE: 4 reads)), and OTU3114 (total: 4 reads in WWWD_MID). A single-factor comparison in MicrobiomeAnalyst between sample type showed OTU0113 was significant between locations (p-value = 1.198E-6, FDR = 8.578E-6). Absolute number of reads for OTU0113 was highest in SOURCE and there was a significant difference between abundance in between WWWD and

SA, with highest read counts in WWWD_MID (μ = 56, ±26.8), then WWWD_EXIT (μ = 12.5, ±2.1), and then WWWD_PRE (μ = 10.6, ±3.2).

Based on the relative abundance, *Mycobacterium* OTU0113 was more prevalent in the natural bottomed SA pond compared to the cement bottomed WWWD pond, however there is no significant difference between locations (Figure 3.5). Relative abundance is highest in SA_PRE > SA_EXIT > SA_MID. In general, the average number of reads in a sample per location corresponds with the average number of reads in OTU0113. Based on absolute counts of *Mycobacterium* OTU0113, the SOURCE samples had the highest abundance and there was a difference in abundance between WWWD and SA, with highest counts found in WWWD_MID > WWWD_EXIT > WWWD_PRE (Figure 3.6).

DISCUSSION

There are differences in the microbial composition of the ecological microbiome between two pond transects at SHBP despite both receiving water from the same source. Multiple factors likely contribute to these observed differences. For the two ponds in this study, the age of the water (time spent in distribution system) varies due to the distance from their respective source wells. The water in SHBP distribution system has been considered homogenous, however ponds may receive varying proportions of ground water from each well depending on their respective distance from where a well ties into the distribution system. The natural bottomed ponds (SA) are less than 32 meters from where a well ties into the water distribution system, whereas the cement bottomed ponds (WWWD) are over 250 meters from the closest well. Water should not be considered homogenous across the distribution system.

In addition to the physical location of the ponds, the bird communities and total bird biomass associated with each pond also differs. Based on WWD mortality from *M. avium*, we'd expect these birds to be amplifying and shedding abundant *M. avium* back into the environment. The high bird biomass in the SA, however, could interact in complex ways with the natural bottomed ponds to generate turbid water with complex microbial communities. This turbidity might itself impact our ability to characterize microbial communities in the SA transect as I tended to recover a smaller number of reads in these analyses.

Although this analysis cannot tease apart the complex contributions of birds and pond construction to microbial community structure, it does provide a clear description of *Mycobacterium* presence and absence throughout the park. Most strikingly, *Mycobacterium* was detected everywhere, including from the source well, revealing a major challenge for WWD husbandry.

Regardless of differences between absolute and relative counts of reads in the *Mycobacterium* OTUs, detection of *Mycobacterium* in the SOURCE samples supports the need for filtering water immediately prior to entering a pond with WWD. In urban water distribution systems, *Mycobacterium avium* readily survive treatment plants and will recolonize distribution and household plumbing (Falkinham III et al., 2001). Furthermore, in domestic water distribution systems, water age (time spent in distribution and home plumbing system) has been correlated with greater *Mycobacterium avium subsp. avium* abundance (Haig et al., 2018).

CONCLUSION

In relation to future management decisions for WWD in captivity, this descriptive analysis on aviary pond microbiomes will impact husbandry protocols. One of my primary

objectives was identifying where *Mycobacterium* could be detected. In aviculture, well water or ground water has always been considered a relatively clean source (M. Lubbock, personal communication, 2022), especially compared to surface water. Since *Mycobacterium* is present in SOURCE samples, regardless of species or relative abundance, filtration will be important to implement in future WWD husbandry at this site.

This descriptive analysis is a single time point of microbial community composition across two aviary pond locations. In addition to having different bottom substrates, there are other confounding variables (size of pond, amount of input water, amount of sunlight, stocking density of birds, etc.) that make real world ecological microbiome comparisons difficult regarding pinpointing primary factors of influence. Further research is needed to understand the factors that change community composition across ponds at SHBP. A major goal going forward is to characterize temporal patterns of change in *Mycobacterium,* specifically *M. avium* subsp. *avium.* abundance at the park. I have already collected water samples toward this effort. PCR-based characterization would provide a more cost-effective approach to determine when and where *M. avium* subsp. *avium* is most abundant and may provide additional insights into effective husbandry techniques.

Figure 3.1. Diversity indices along SA transect and WWWD transect.

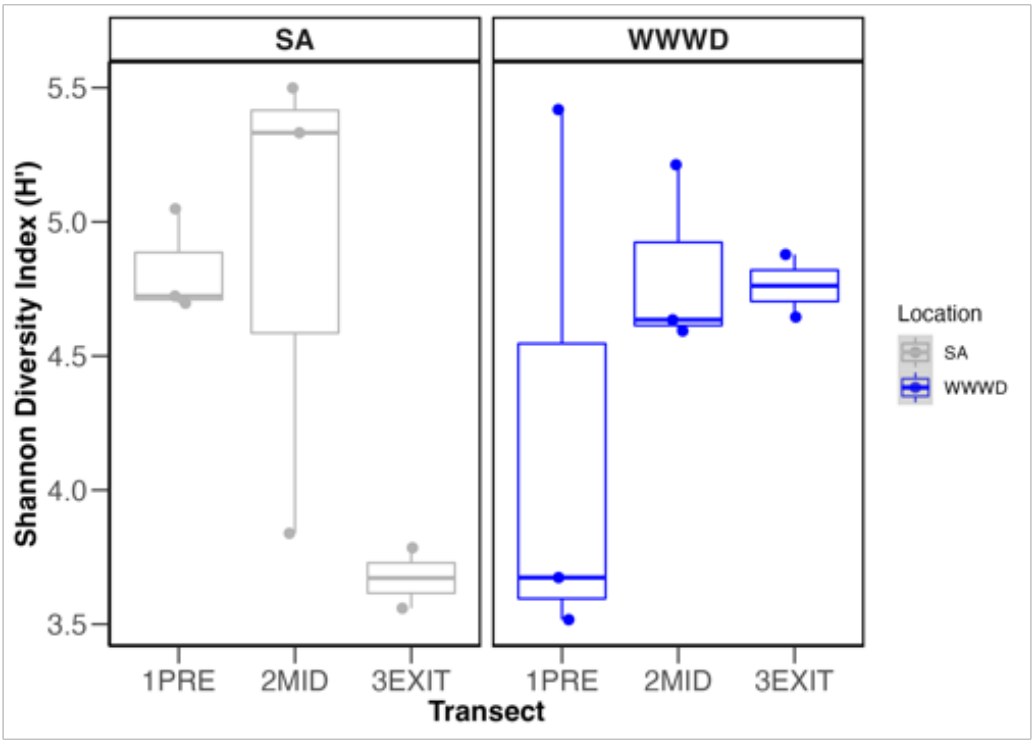


Figure 3.2. Evenness indices along SA transect and WWWD transect.

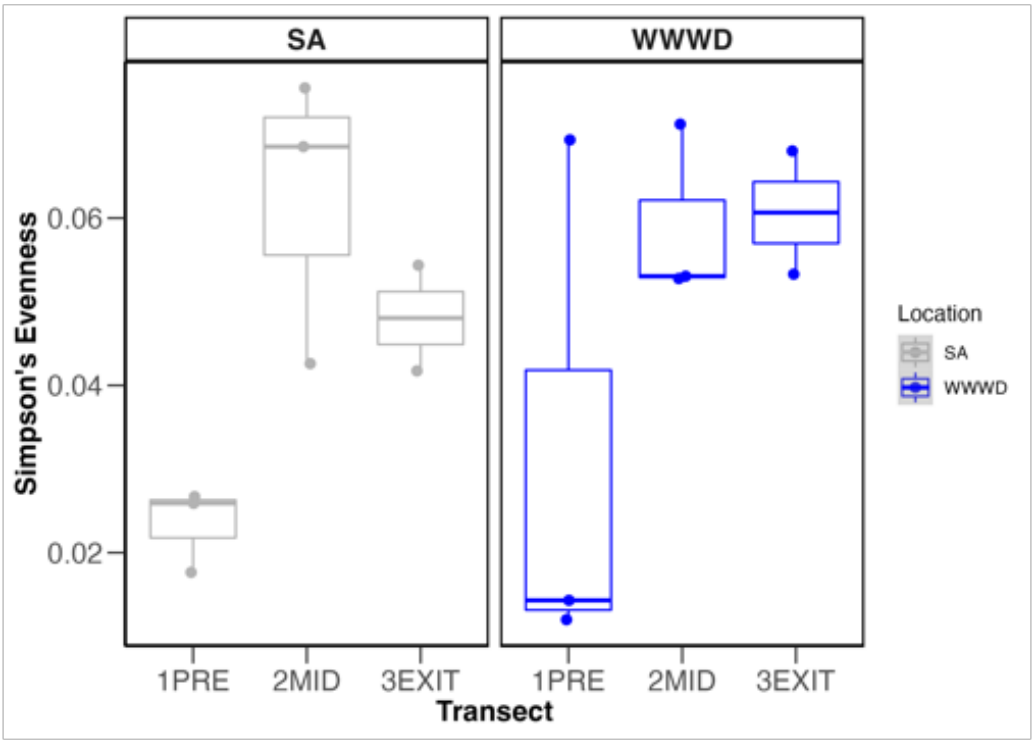


Figure 3.3. PCoA of all 3 locations (SA, WWWD, and SOURCE) along transect points.

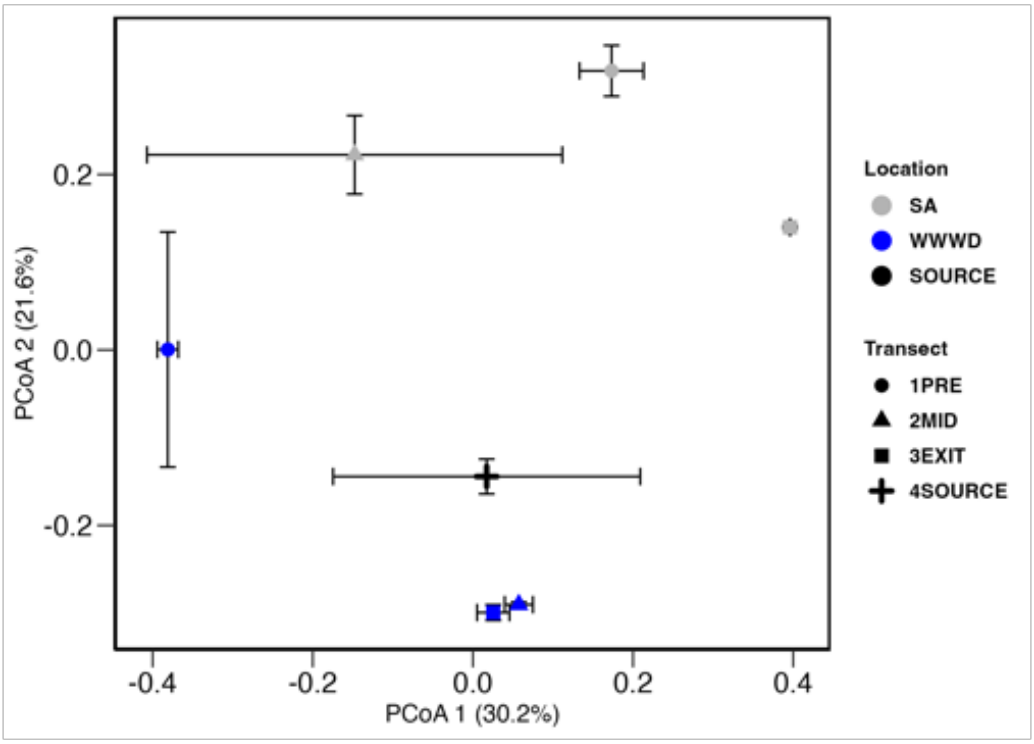


Figure 3.4. Community composition between SA transect and WWWD transect.

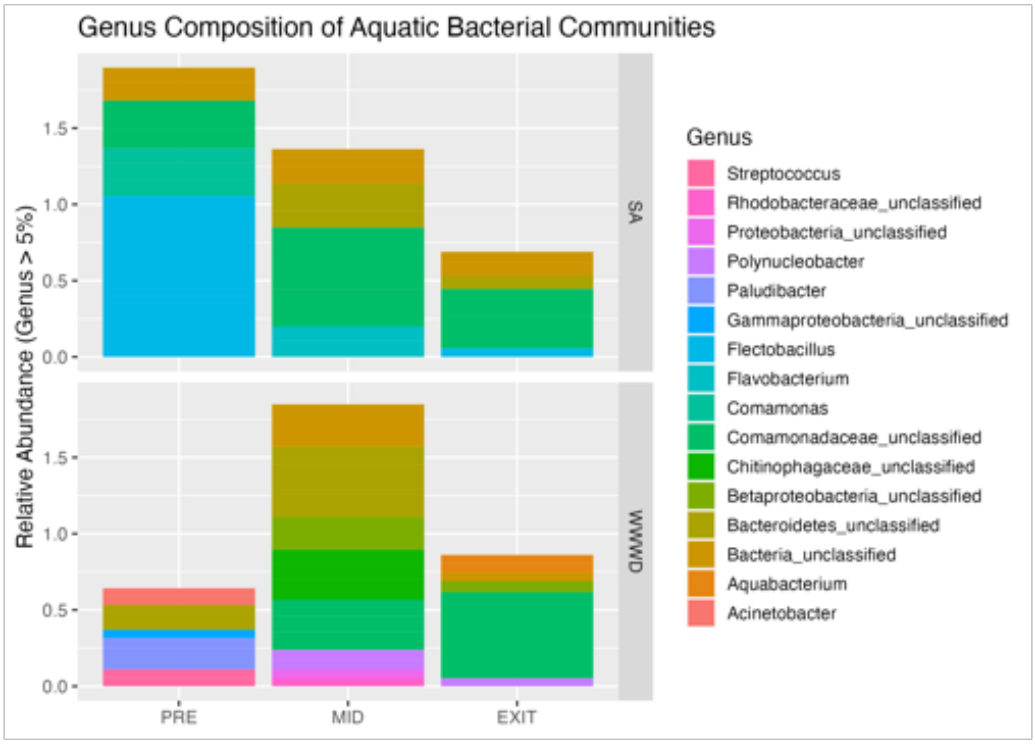
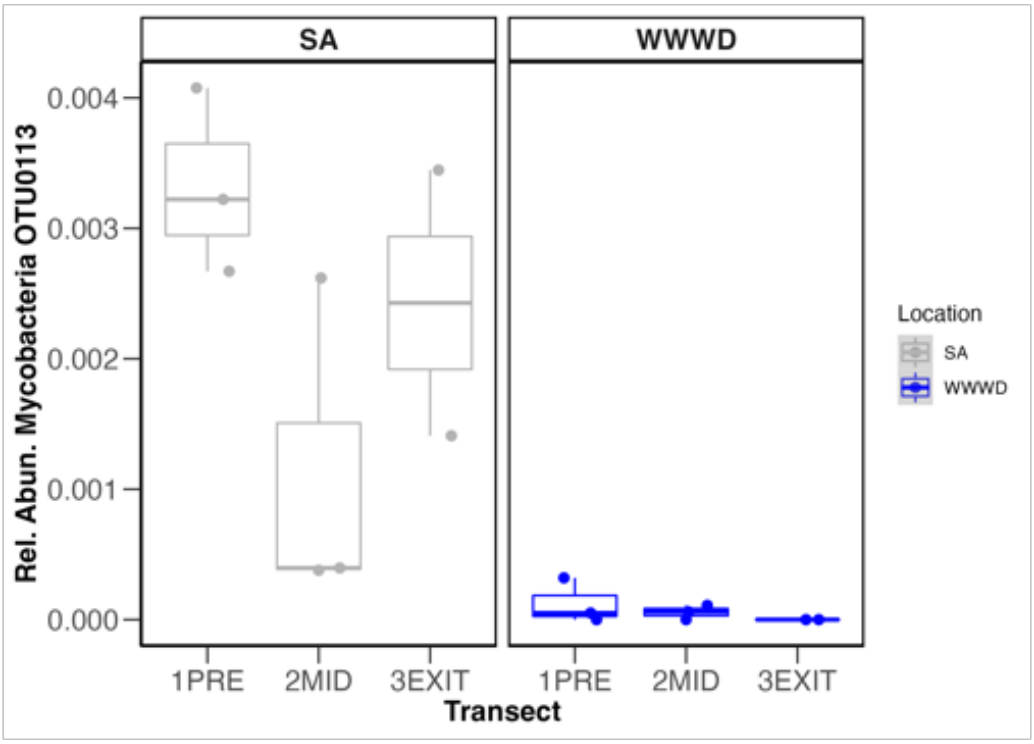


Figure 3.5. OTU0113 relative abundance between SA transect and WWWD transect.



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APPENDIX A: Animal Use Declaration

All animal procedures were reviewed and approved by East Carolina University Institutional Animal Care and Use Committee under AUP D351 (PI: Susan B. McRae).

APPENDIX B: IACUC Approval Letter

Animal Care and Use Committee
003 Ed Warren Life Sciences Building | East Carolina University | Greenville NC 27354 - 4354
252-744-2436 office | 252-744-2355 fax

August 4, 2020

Susan McRae, Ph.D.
Department of Biology, ECU

Dear Dr. McRae:

Your Animal Use Protocol entitled, "Molecular sex and fecal diagnostics of captive birds at Sylvan Heights Bird Park" (AUP #D351a) was reviewed by this institution's Animal Care and Use Committee on 08/04/2020. The following action was taken by the Committee:

"Approved as submitted"

****Please contact Aaron Hinkle prior to any hazard use****

A copy of the protocols is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

Sincerely yours,

Jamie DeWitt, Ph.D.
Vice-Chair, Animal Care and Use Committee

JD/GD

enclosure