NO EVIDENCE FOR HIGH PATHOGENICITY AVIAN INFLUENZA IN WAVED ALBATROSS *PHOEBASTRIA IRRORATA*

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Received 23 March 2024, accepted 21 May 2024

ABSTRACT

JIMÉNEZ-UZCÁTEGUI, G., VÉLEZ, A., VEGA, P., BUENDIA, V., MONTENEGRO-BENALCÁZAR, V., SEVILLA, C. & CRUZ, M. 2024. No evidence for high pathogenicity avian influenza in Waved Albatross *Phoebastria irrorata*. *Marine Ornithology* 52: 349–353. http://doi.org/to_come

Highly pathogenic avian influenza (HPAI) is caused by the *Alphainfluenzavirus influenzae* species (type A, subtype H5N1), which has been detected in mammals (including humans) and marine birds in the Americas, including the Galápagos Islands. The Waved Albatross *Phoebastria irrorata* is a marine endemic species of Ecuador. Most of its breeding population nests on Española Island in the Galápagos Archipelago, and it forages at sea in the eastern South Pacific. This marine bird shares its feeding areas with the Peruvian Pelican *Pelecanus thagus*, Peruvian Booby *Sula variegata*, Guanay Cormorant *Leucocarbo bougainvillii*, Humboldt Penguin *Spheniscus humboldti*, Sanderling *Calidris alba*, Belcher's Gull *Larus belcheri*. These six species nest on the mainland of Ecuador and Peru; all have tested positive for HPAI H5N1, which has been particular concern to researchers. Therefore, we used a real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR), to test for HPAI H5N1 in choana-tracheal and cloacal samples from Waved Albatross at the Punta Suárez and Punta Cevallos colonies on Española Island, which have been under investigation for more than two decades. None of the birds tested positive for HPAI H5N1. Despite negative results, it is important to implement preventive and precautionary measures to avoid the spread of this viral disease to the Galápagos Archipelago. Avian influenza can have a high impact on the isolated Galápagos Islands and, therefore, we must deeply understand the possible means of entry of this virus.

Key words: avian flu, Galapagos Islands, marine birds, prevention, spread, surveillance

INTRODUCTION

Based on the hemagglutinin (protein viral genome) cleavage site and the infection's clinical severity (pathogenicity), avian influenza is classified into two categories: Highly Pathogenic Avian Influenza (HPAI) and Low Pathogenic Avian Influenza (LPAI; Hansen 1999, Alexander & Brown 2009, Castro-Sanguinetti *et al.* 2024). HPAI is caused by the Influenza A virus (*Alphainfluenzavirus influenzae)*, subtypes H5 and H7. This RNA virus may cause high mortality in birds, even without clinical signs (Gerlach 1999, Hansen 1999, Castro-Sanguinetti *et al.* 2024), and it affects domestic birds, wild birds, and mammals (including humans; Gerlach 1999, Hansen 1999, Aiello *et al*. 2000, CDC 2023). In wild birds, it is more common among waterfowl, shorebirds, and marine birds, with no clinical signs. The H5N1 subtype of HPAI was first detected in 1996, but it is constantly evolving, and the current outbreak involves clade 2.3.4.4b (Lee *et al.* 2017, Castro-Sanguinetti *et al.* 2024). The virus spreads along migration pathways (Hansen 1999, Aiello *et al*. 2000) both directly, through respiratory secretions, conjunctival discharge, and feces, and indirectly, through mechanical vectors such as contaminated water or fieldwork materials (Gerlach 1999).

According to the World Organization for Animal Health (WOAH), HPAI H5N1 has been reported by several countries in Asia, Europe, Africa, and the Americas (PAHO/WHO 2024). The country with the most severe outbreak in South America has been Peru, which has recorded positive testing on more than 100 000 birds involving 24 species, including the Peruvian Pelican *Pelecanus thagus*, Peruvian Booby *Sula variegata*, Guanay Cormorant *Leucocarbo bougainvillii*, Humboldt Penguin *Spheniscus humboldti*, Sanderling *Calidris alba*, Belcher's Gull *Larus belcheri* (Gamarra-Toledo *et al.* 2023, Leguia *et al.* 2023, Vernimmen 2023, Castro-Sanguinetti *et al.* 2024). A Waved Albatross *Phoebastria irrorata* (WAAL) was found dead on Las Gaviotas Beach, Peru, in May 2023, and it tested positive for HPAI H5N1 (MIDAGRI-SENASA-DSA 2023). Peru has implemented an avian influenza monitoring system since 2006 to improve its ability to detect and respond to potential outbreaks; therefore, new cases can be reported quickly (Castro-Sanguinetti *et al.* 2024).

WAAL is endemic to Ecuador and has been categorized as a Critically Endangered species (Harris 1973, Freile *et al.* 2019). Most (99.9%) WAAL breed on Española Island in the Galápagos Archipelago, with the remainder breeding on La Plata Island, which is on the continental coast (Harris 1973). WAAL mostly forage in Humboldt Current waters off Ecuador and Peru, from Manabi Province in Ecuador to Callao Province in Peru. However, they are known to occur from latitudes 5.5°N (Cocos, Costa Rica) to 38°S

(Harris 1973, Spear & Ainley 2008, Awkerman *et al.* 2014). Many of the other marine birds listed above also occur in these waters (Murphy 1936, Spear & Ainley 2008).

As part of an ongoing effort to monitor WAAL populations, we assessed the presence of HPAI H5N1 in the Galápagos by sampling breeding colonies at Española Island using a real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) protocol.

METHODS

Study area

The Galápagos Archipelago is ~960 km west of the Ecuador coast, and it is composed of 13 islands and ~100 islets and rocks (Snell *et al.* 1996). We collected samples on Española Island at Punta Suárez, where population studies (i.e., survival, reproduction, diseases analysis, toxicology, etc.) have been ongoing since 2002 (Tourist plot: 01°22′18″S, 089°44′24″W; Harris plot: 01°22′30″S, 089°44′6″W), and at Punta Cevallos, where the studies have been ongoing since the 1990s (01°23′36″S, 089°37′15″W; Fig. 1).

Samples

Both choanal-tracheal and cloacal samples were collected using sterile swabs from 51 WAAL adults. More specifically, 41 individuals were sampled at Punta Suárez (25 from the Tourism plot and 16 from the Harris plot; 30 in June 2023 and 11 in October 2023), and 10 were sampled at Punta Cevallos in October 2023 (Fig. 1). Swabs were placed in individual labeled cryotubes with 0.5 mL of DNA/RNA Shield 1x (Zymo Research; Orange, USA) in an ice-filled cooler on site then transferred to a freezer (–4 °C) on the boat. Analysis of HPAI H5N1 was done on the third day after collection in the Galápagos Agency for Regulation and Control of Biosafety and Quarantine Laboratory (GBA).

We also conducted physical examinations to document the heart rate, respiratory rate, weight, and body temperature of each bird as indicators of general health (Jiménez-Uzcátegui *et al.* 2021).

We used protocols approved by the Galápagos National Park Directorate. One of the authors (GJU) has a research permit from the park directorate (PC-48-23) to work on avian health. No animals were injured during the study, and the samples were collected using non-invasive sampling methods that meet international standards (Serafini *et al.* 2023).

Genetic analysis

RNA was extracted using the Purelink Viral RNA/DNA Mini Kit (Invitrogen; Waltham, USA) following the manufacturer's instructions. We used 200 µL of each sample for the extraction of viral genomic RNA. The RNA was diluted from the columns with

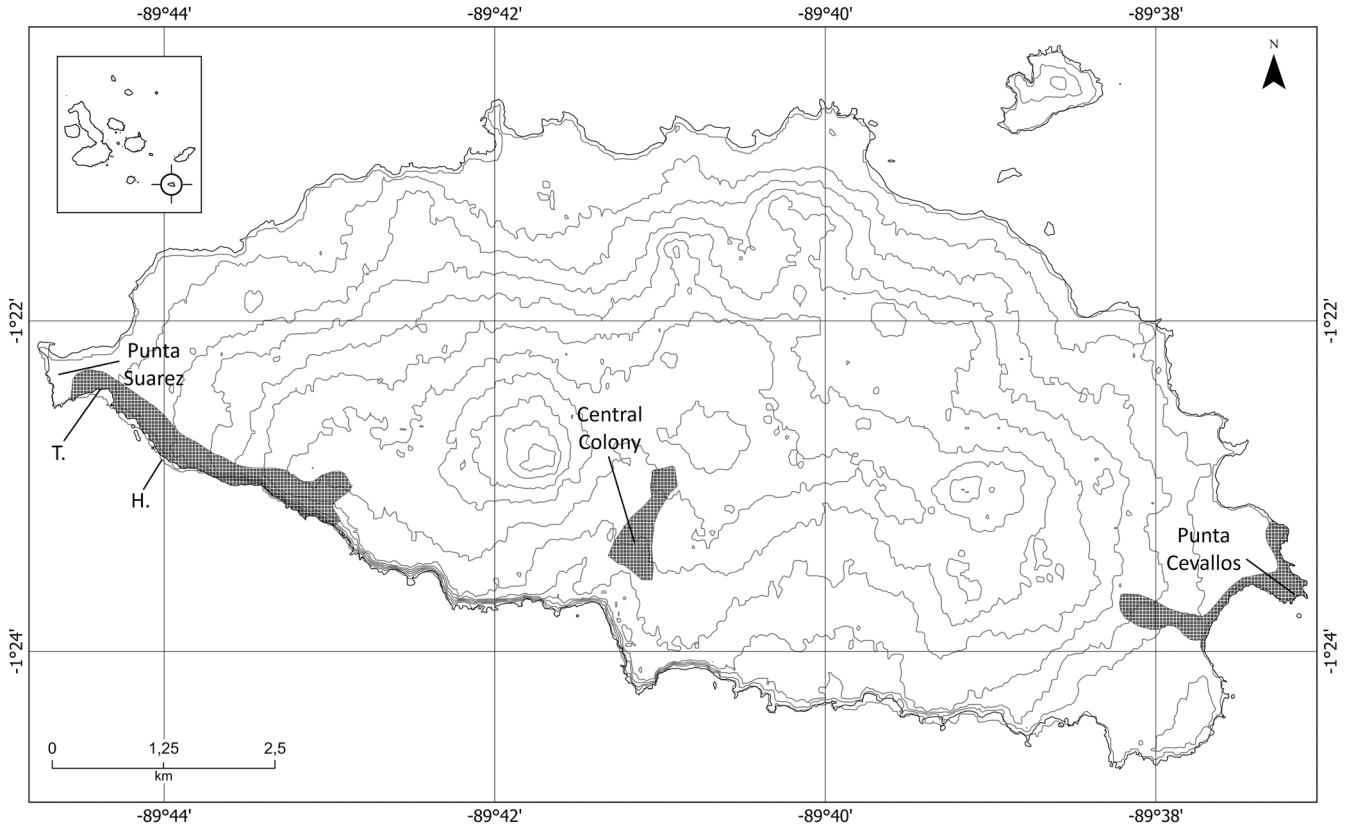

Fig. 1. Waved Albatross *Phoebastria irrorata* breeding areas on Española Island, Galápagos Archipelago, Ecuador, are indicated by the hatched areas. The geographical features for which the breeding areas are named (Punta Suárez and Punta Cevallos) are indicated with lines. Study plots where Waved Albatross samples were collected are indicated by T (Tourist plot) and H (Harris plot) in the Punta Suárez breeding area and by the line in the Punta Cevallos breeding area. Image modified from Harris 1973.

40 µL RNase-free water from the kit and either used immediately for further testing or stored at −80 °C.

We used diagnostic qRT-PCR protocols from a European Union Reference Laboratory (Istituto Zooprofilattico Sperimentale Delle Venezie in Legnaro, Italy) specializing in avian influenza and Newcastle disease. We used the TaqMan Fast Virus One-Step Master Mix for qPCR kit (Thermo Fisher Scientific; Waltham, USA) with a 25 µL reaction volume containing 20 pmols of the sense primer, 10 pmols of the antisense primers. The primer and probe sequences are shown in Table 1. The qRT-PCR step conditions for all primer sets for HPAI type A (Heine *et al.* 2015) were 50 °C for 10 min, 95 °C for 10 min, 95 °C for 15 sec, and 60 °C for 45 sec, followed by 45 cycles on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad; Hercules, USA; same machine used for all PCR). For detection of Eurasian H5 and H7 avian influenza virus, the PCR conditions were as follows, 50 °C for 30 min, 95 °C for 15 min, 95 °C for 10 sec, 54 °C for 30 sec, and 72 °C for 10 sec, followed by 40 cycles (Slomka *et al.* 2007, 2009). For interpretation of results, we considered samples producing a cycle threshold below 45 to be positive.

RESULTS

None of the 51 WAALs sampled at Punta Suárez and Punta Cevallos were positive for HPAI H5N1. The qRT-PCR analysis was conducted at the GBA laboratory, and testing was endorsed by the Instituto Nacional de Investigación en Salud Pública del Ecuador (INSPI; National Public Health Research Institute of Ecuador). The following averages (± standard deviation) were collected during the physical examinations: heart rate 101 ± 11 beats/minute, respiratory rate 20 ± 5 breaths/minute, cloacal temperature 39.7 °C ± 1.3 °C, and body weight 3.7 ± 0.4 kg (see Jiménez-Uzcátegui *et al.* 2021). We did not observe any individual with clinical signs of any respiratory diseases, and we found no dead individuals in either colony (Fig. 1).

DISCUSSION

The negative PCR results for HPAI H5N1, the normal metrics during physical examinations, and the absence of any clinical signs of respiratory infection indicated healthy WAAL populations at Española Island. The previous study of this disease in WAAL was in 2001 at Punta Cevallos, where all individuals tested negative (Padilla *et al.* 2003). WAAL does not appear to be a current reservoir of avian influenza (see Gerlach 1999, Hansen 1999). However, given that an infected WAAL individual was found dead on the beach in Peru (~1000 km away) raises our vigilance (see MIDAGRI-SENASA-DSA 2023). We also must be vigilant for the emergence of the disease in the Galápagos, where 28 other marine bird species and marine mammals occur, including endemic species and subspecies (Jiménez-Uzcátegui 2022).

Contagions may spread among Galápagos species in two ways. First, they may spread directly from infected individuals through contact with respiratory secretions, conjunctival discharge, and feces (Gerlach 1999). This could occur in marine feeding areas and at sea within multispecies foraging flocks. Second, contagions may spread indirectly via mechanical vectors such as shoes, tourist gear, fieldwork materials, and contaminated water (i.e., principally by humans; Gerlach 1999, Aiello *et al*. 2000). Therefore, management actions are critical to preventing the introduction and spread of H5N1. Similarly, following the precautionary principle (i.e., risk assessment, early action, transparency, public engagement, adaptive management) would also be critical to prevent the spread of HPAI H5N1 if it was introduced.

For many unique Galápagos species living in small populations, it is important to implement passive and active surveillance to monitor HPAI H5N1, especially in breeding areas. Consistent surveillance results in timely reporting of any infection, and this is how this virus has been detected early on the mainland (in Ecuador and neighboring countries) and coastal islands (see

TABLE 1
Primer and probe oligonucleotides (oligo) used in real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) assays on Waved Albatross *Phoebastria irrorata* swabs collected in 2023 at Española Island in the Galápagos Archipelago, Ecuador. Samples were initially processed using primers for the highly pathogenic avian influenza (HPAI) type A virus. Positive samples were processed further using the Subtype H5 or Subtype H7 primers.

Assay	Oligo name	Sequence (5′ to 3′)
Type A	Sense primer IVA D161M	5′-AGA TGA GYC TTC TAA CCG AGG TCG-3′
	Antisense primer IVA D162M1	5′-TGC AAA AAC ATC YTC AAG TCT CTG-3′
	Antisense primer IVA D162M2	5′-TGC AAA CAC ATC YTC AAG TCT CTG-3′
	Antisense primer IVA D162M3	5′-TGC AAA GAC ATC YTC AAG TCT CTG-3′
	Antisense primer IVA D162M4	5′-TGC AAA TAC ATC YTC AAG TCT CTG-3′
	Probe IVA MA	5′-FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA/BHQ1-3′
Subtype H5	Sense primer H5LH1	5′-ACA TAT GAC TAC CCA CAR TAT TCA G-3′
	Antisense primer H5RH1	5′-AGA CCA GCT AYC ATG ATT GC-3′
	Probe H5PRO	5′-FAM- TCW ACA GTG GCG AGT TCC CTA GCA-TAMRA-3′
Subtype H7	Sense primer LH6H7	5′-GGC CAG TAT TAG AAA CAA CAC CTA TGA-3′
	Antisense primer RH4H7	5′-GCC CCG AAG CTA AAC CAA AGT AT-3′
	Probe H7pro11	5′-FAM-CCG CTG CTT AGT TTG ACT GGG TCA ATC T-BHQ1-3′

Jiménez-Uzcátegui 2022, Castro-Sanguinetti *et al.* 2024). These actions need to be coordinated by a committee of scientists and managers, in collaboration with the community and tourism groups.

ACKNOWLEDGEMENTS

We wish to express our appreciation to our donors: Hurtigruten Foundation, Fondo para Control de Especies Invasoras de Galápagos (FEIG), Rapid Response Finance (RRF), Sophie Lenoir of Tunememi Conservancy, Inventagri, and anonymous sponsors for financial support of the Galapagos Penguin, Flightless Cormorant, and Waved Albatross Project at the Charles Darwin Foundation. We are grateful to our collaborators, laboratory technicians, and more than 95 assistants and volunteers who helped with this research starting in 2009, especially Johannes Ramírez, Jipson Valle, and Andrea Coloma. We thank Johny Mazón Redín for the map design. Thanks to the Galápagos National Park Directorate for granting research permit PC-48-23; the Agencia de Regulación y Control de la Bioseguridad y Cuarentena para Galápagos for use of the laboratory facilities; and the Charles Darwin Research Station for their unconditional support. Finally, we appreciate the suggested edits by all reviewers and referees who helped us to improve our paper. This publication is contribution number 2633 of the Charles Darwin Foundation for the Galapagos Islands.

AUTHOR CONTRIBUTIONS

The following contributions were made by authors: Conceptualization—GJU, CS, MC. Methodology—GJU. Software—GJU, AV, PV. Validation—GJU, VMB, CS, MC. Formal analysis—GJU, AV, PV, VB, VMB. Investigation—GJU, AV, PV, VB. Resources—GJU, CS, MC. Data curation—GJU, VB, VMB. Writing (original draft)—GJU, AV. Writing (review and editing)—GJU, AV, PV, VB, VMB, CS, MC. Project administration—GJU, CS, MC. Funding acquisition—GJU, CS, MC. All authors have read and agreed to the published version of the manuscript. The authors declare no conflict of interest.

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