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Avian influenza in migratory waterfowl and synanthropic birds in a wetland-agroecosystem interface in northwest Mexico

Influenza aviar en aves acuáticas migratorias y sinantrópicas en la interfaz de un humedal-agroecosistema en el Noroeste de México

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ABSTRACT

Avian influenza is a highly contagious viral disease caused by the avian Influenza virus (AIV). Waterfowl are considered their natural reservoirs, but highly pathogenic strains can cause mass mortality in both wild birds and poultry. Agroecosystems and coastal wetlands near migratory routes of wild birds are also habitats for many synanthropic animals, which may serve as a "bridge host" between poultry and other species. We aimed to analyze the molecular prevalence of AIV in terrestrial synanthropic birds and aquatic migratory wild birds within the context of the wetland-agroecosystem interface in Sonora, Mexico. A one-year survey was conducted. RT-qPCR was carried out to detect the molecular genome of AIV in ARN from cloacal samples. Our results show that avian influenza virus type A has been identified in 13 birds, from two estuarine species (*Anas crecca* and *Mareca americana*) and one ubiquitous bird (*Aythya marila*) inhabiting coastal and agricultural habitats. No evidence of positive doves was found. AIV-positive birds were more frequently found in adult female wild aquatic birds in the Yaqui Valley and southern Sonora's coastal wetlands, demonstrating the importance of these birds in introducing AIV to these sites, contributing to the ecoepidemiology of synanthropic birds, and highlighting the relevance of active surveillance in preventing outbreaks in this important Pacific migratory flyway.

Keywords: avian influenza, waterfowl, synanthropic birds, wetlands, agroecosystem, Sonora.

RESUMEN

La influenza aviar es una enfermedad viral altamente contagiosa ocasionada por el virus de la influenza aviar (VIA). Las aves acuáticas son el reservorio natural de estos virus; sin embargo, las cepas de influenza aviar de alta patogenicidad pueden ocasionar mortalidades masivas tanto en aves silvestres como en pollos domésticos. Los agroecosistemas y los humedales costeros que forman parte de las rutas migratorias de las aves albergan otras especies sinantrópicas que pueden constituir hospederos puente entre las aves domésticas, otras especies silvestres y mamíferos. El objetivo fue analizar la prevalencia del VIA en aves terrestres sinantrópicas y en aves acuáticas migratorias en la interfaz de un humedal-agroecosistema en Sonora, México. Se realizó un monitoreo de un año y la detección molecular del genoma viral de VIA en muestras de cloaca por RT-qPCR. Las aves positivas a VIA fueron 13 individuos de tres especies: dos acuáticas estuarinas (*Anas crecca* y *Mareca americana*) y una especie ubicua (*Aythya marila*) que puede usar tanto el hábitat del humedal como la zona agrícola del Valle del Yaqui, principalmente hembras adultas. No se encontraron palomas positivas. Esto demuestra la importancia de las aves acuáticas en estos sitios en la introducción de virus influenza aviar y aporta información sobre el papel de aves sinantrópicas en la ecoepidemiología de los VIA, así como la relevancia de la vigilancia activa para prevenir brotes en la ruta migratoria del Pacífico.

Palabras clave: influenza aviar, aves acuáticas, aves sinantrópicas, humedal, agroecosistema, Sonora.

INTRODUCTION

Avian influenza is a highly contagious viral disease that poses a significant challenge to global veterinary and public health. It is caused by the Influenza A virus, a segmented, single-stranded, negative-sense (ssRNA-) virus belonging to the genus Alphainfluenzavirus and the family Orthomyxoviridae (Walker *et al*., 2022). Waterfowl are considered its natural reservoirs, including members of the orders Anseriformes and Charadriiformes, such as gulls, swallows, shorebirds, ducks, geese, and swans (Blagodatski *et al*., 2021). Still, other orders of domestic and wild birds, as well as mammal species, including humans, can be infected (WHO, 2023). Avian influenza virus Hemagglutinin (H1-H16) and neuraminidase (N1-N9) subtypes are distributed worldwide. They are found in more than 100 species of birds (Simancas-Racines *et al*., 2023), whereas influenza-like viruses H17-H18 and N10-N11 are found in bats (CDC, 2024).

Most of the AIV strains cause middle to subclinical disease in infected birds. However, highly pathogenic avian influenza A viruses (HPAIV subtypes H5 and H7) can breach the intestinal and respiratory epithelial barriers, leading to systemic damage and death (Simancas-Racines *et al*., 2023). For instance, exposure to clade 2.3.4.4a H5N8 in 2014 and clade 2.3.4.4b H5N8 in 2016, as well as H5N6 in 2017, caused 60% and 100% mortality in Pekin ducks (*Anas platyrhynchos*), respectively (Verhagen *et al*., 2021).

Some AIV traits, such as genomic diversity, plasticity in hosts and environments, and bird behavior linked to feeding and roosting, might foster contacts between reservoirs and increase the risk of potential transmission or spillovers among susceptible species. Synanthropic animals found in anthropogenically modified landscapes (i.e., agricultural fields) may constitute "bridge host" species in the transmission of AIV between wild birds and domestic poultry farms or human settlements (Ringenberg *et al*., 2024). These animals could coexist with migratory wild birds in agroecosystems near their migratory routes, where the bodies of water facilitate the fecal-oral transmission of AIV. Once infected, synanthropic species, such as pigeons and doves, or other invasive species, can introduce the virus into poultry farms and domestic birds in rural areas (Shriner & Root, 2020).

This wildlife-agricultural interface scenario is common in the state of Sonora, located in northwest Mexico. Sonora has 1,209 km of coastline (INEGI, 2016) and 1,144,269 ha of agricultural land (INEGI, 2023). The coastal wetlands of northwest Mexico serve as the primary wintering grounds for waterfowl and shorebirds migrating through the Pacific Flyway (Palacios, 2019), one of the four main routes connecting the northern and southern hemispheres. Therefore, considering the notable fluxes of bird population migration through our wintering grounds, the continental and regional circulation of both low and highly pathogenic avian influenza strains, the possibility of zoonosis and/or bird infection transmission mediated by synanthropic species, we aimed to analyze the molecular prevalence of AIV in terrestrial synanthropic birds and aquatic migratory wild birds within the context of wetland-agroecosystem interface in Sonora, Mexico.

MATERIALS AND METHODS

Study design and sample collection

This observational epidemiological study was conducted in two Units for Wildlife Conservation Management (UMA, SEMARNAT), located near the Etchohuaquila (N 3,021,903, E 622,660 UTM) community and the other within the Tobari estuary (N 2,988249, E 608,768 UTM). Etchohuaquila is a small town surrounded by agricultural fields located in the south of Sonora state, within the Yaqui Valley, where agriculture and livestock are the main economic activities (INEGI, 2023). The Tobari estuary is part of the Gulf of California and includes 16,700 ha of waterfowl and shorebird habitat. It was declared a priority wetland for migratory shorebirds in Mexico by the Secretariat of Environment and Natural Resources (SEMARNAT) in 2008. In addition, the Tobari estuary was included in the Western Hemisphere Shorebird Reserve Network in 2014 due to its international importance for shorebirds and waterfowl along the Pacific Flyway migration route (Figure 1) (WHSRN, 2019).

Opportunistic sampling was conducted from hunted aquatic and terrestrial game wild birds from the two Units for Wildlife Conservation Management previously described. This sampling method was selected because it is convenient for rapidly and efficiently sampling many samples in a short period. The study was conducted from October 2018 to February 2019. The sample size was calculated, considering the 95% confidence interval and an expected prevalence of 0.25% and 3.60% of positivity for AIV in terrestrial and aquatic birds, respectively (Montalvo-Corral *et al*., 2011); this resulted in n=1,201 for terrestrial birds and n=103 for aquatic birds.

Cloacal samples were collected using Dacron sterile swabs. Bird species, age, and sex were recorded, along with the location and date of collection. Samples were placed in cryovials containing transport medium (Hank´s solution with 10% glycerol, supplemented with 100 µg/mL of penicillin-streptomycin and 0.02 mg/mL of amphotericin B) and stored in a double-layer container. They were refrigerated, transported, and pooled before storage at -80°C until analysis. The UMA administrators of hunting clubs granted permission for sampling as part of their monitoring and hunting bird activities. No intervention or experimental procedures were conducted with organisms or samples. All personnel handling the birds and collecting samples employed standard biosafety and biosecurity procedures and equipment during fieldwork tasks.

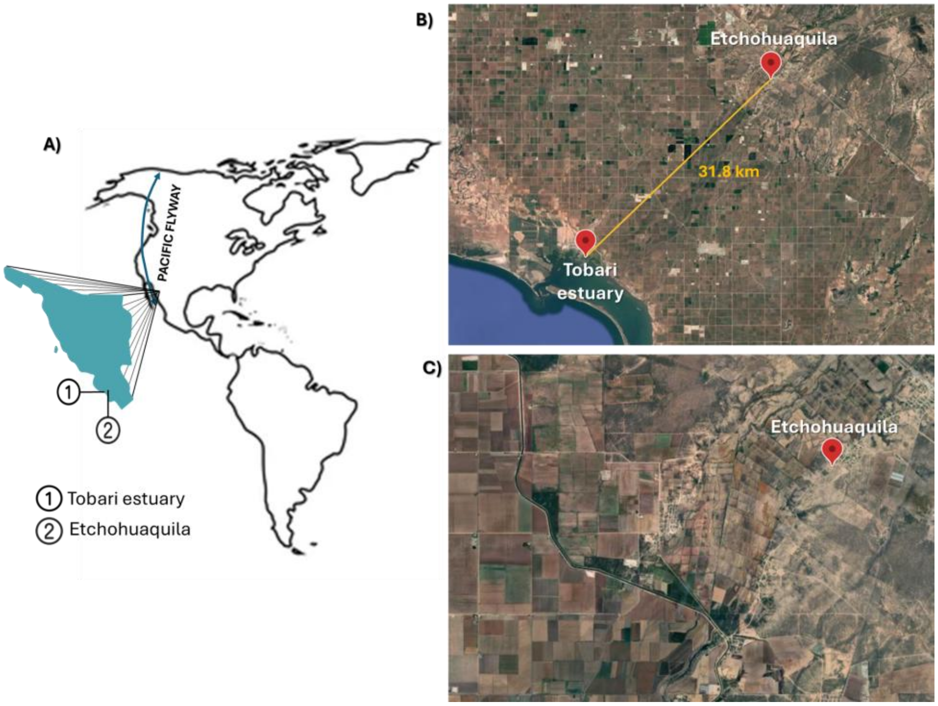

Figure 1. Sampling sites' geographical location. A) Location of the sampling sites respecting the Pacific flyway; B) Satellite image showing location and distance between the Tobary estuary and Etchohuaquila community; C) Satellite image showing the agroecosystem surrounding Etchohuaquila community.

Real-time RT-PCR (RT-qPCR)

RNA was extracted from pooled samples using the RNeasy Mini Kit (QIAGEN, Inc., California, USA) according to the manufacturer's instructions. Pools consisted of five samples collected from the same bird species on the same date. Once a pool was detected as positive, all the samples were reanalyzed individually to calculate prevalence. All these procedures were performed in a BSL-2 laboratory equipped with a Class II biosafety cabinet (LABCONCO, Missouri, USA) while wearing personal protective equipment and utilizing barrier containment measures.

The AIV virus matrix M and hemagglutinin 5 genes (H5 subtype-specific for the North American and Eurasian lineage) were analyzed in the samples by RT-qPCR using the One Step Ahead RT-PCR Kit (QIAGEN, Inc., Hilden, Germany), and a set of oligonucleotides previously described and tested for specificity including both avian North American and Eurasian lineage viruses and isolates of human, equine, and swine origin to demonstrate specificity for type A influenza virus by (Spackman *et al*., 2002). The molecular test had a specificity of 89%. The matrix, H5, and H7 RT-qPCR assay had a detection limit of 10^2 gene copies/µl (Montalvo-Corral *et al*., 2009). The PCR reaction mix consisted of 5 µl of RNA (50 ng), 10 µl of One Step Ahead RT-PCR MM 2.5x, 1 µl of One Step Ahead RT-Mix 25x, forward and reverse oligonucleotides (500 nM), TaqMan probes (300 nM), and RNAse-free molecular-grade water to complete a volume of 25 µl. Cycling conditions began with one cycle at 50°C for 10 min and one cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 10 s and 60°C for 20 s, with a final extension at 72°C for 2 min (Montalvo-Corral & Hernandez, 2010). Non-template and positive controls were used. This amplification was performed in the thermocycler (StepOne^{TM}, Applied Biosystems, California, USA).

Statistical analysis

The prevalence of AIV was calculated in the software STATA version 12 (Corporation, Texas, USA). The dependence between the variables (bird’s sex, age, date of sampling) and the presence of AIV was evaluated using Pearson's chi-squared test. Associations between sex and total viral load were analyzed using Analysis of Variance (ANOVA) with a significance level of $p < 0.05$. The analysis was conducted with the RStudio (IDE) 219 program.

RESULTS

The sample distribution consisted of 1201 terrestrial bird species from the agricultural valley of Etchohuaquila and nearby fields, as well as 102 dabbling duck and one diving duck species from the Tobari wetlands (Figure 2). Three species of synanthropic terrestrial birds were included in the study sample: 597 White-winged Doves, *Zenaida asiatica* (49.7%); 592 Mourning Doves, *Zenaida macroura* (49.3%); and 12 Eurasian Collared-Doves, *Streptopelia decaocto* (1.0%). Considering the three species, it was found that 50.8% were males, and 49.2% were females; 11.3% were juveniles, and 88.7% were adults. None of the terrestrial bird samples analyzed were positive for the matrix M and hemagglutinin 5 genes, indicating the absence of the AIV genome detection (Table 1). The Columbiformes, which are widely distributed in this agroecosystem, utilize a variety of habitats near poultry facilities, fields, and human settlements.

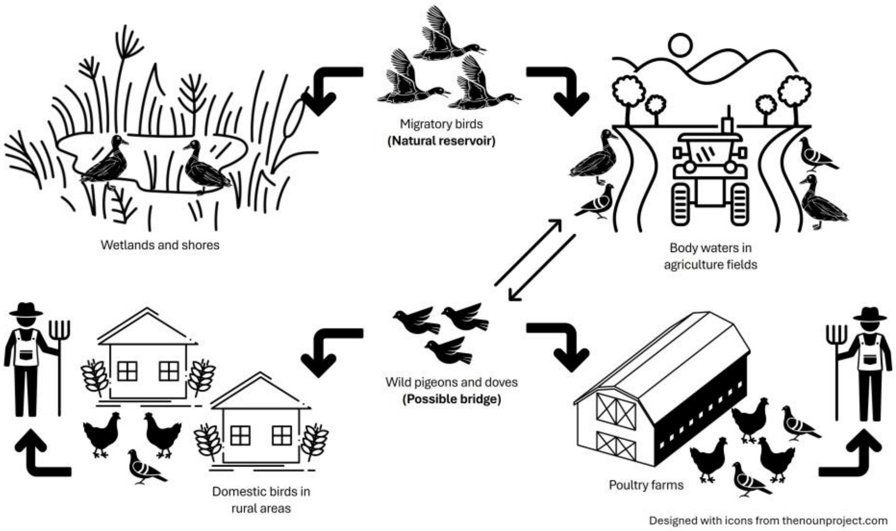

Figure 2. Schematization of the interaction of aquatic migratory and terrestrial synanthropic birds as possible “bridge hosts” for avian influenza virus

The eight waterfowl species sampled (n = 103) are described in Table 1. Thirteen positive cases for the matrix M gene were identified from three of the eight species of migratory aquatic birds sampled. Considering the total samples, the prevalence of AIV in aquatic birds during the 2018-2019 migration season in the Tobari estuary was 12.6%. The analysis by species shows a differential contribution to this prevalence. The highest positive samples were obtained from the dabbling ducks, specifically the Green-winged Teal, Anas crecca, followed by the American Wigeon, Mareca americana, and the diving duck, the Great Scaup or bluebill, *Aythya marila* (Table 1).

After identifying the AIV matrix M gene, the positive samples were analyzed for molecular subtype determination with H5-specific primers for the Eurasian and North American lineages. Neither sample was positive for the Eurasian H5 subtype; however, two individuals were positive for the North American lineage H5 subtype, each found in *Anas crecca* and *Aythya marila,* respectively (Table 1).

According to the positive cases in waterfowl by sex, we found that 9 of 13 cases were in female specimens. This represents a ratio of 2.25 positive female samples to male samples. Regarding age, 9 of 13 cases were adult birds, one was classified as juvenile, and three remained unclassified by age. Additionally, nine cases were identified in autumn, while the remaining four were discovered in winter, at the end of January. There were no statistically significant differences in age, sex, or collection month, as determined by the Chi-squared test (Table 2).

Table 1. Avian influenza virus A was detected by Real-time RT-PCR in wild birds sampled during the 2018-2019 season at the Tobari agroecosystem interface in Sonora, Mexico (n = 1,304)

Common name	Specie	n	Positive M[1] n (%)	Positive H5NA[2] n (%)
Terrestrial birds				
White-winged Dove	*Zenaida asiatica*	597	0	0
Mourning Dove	*Zenaida macroura*	592	0	0
Eurasian Collared-Dove	*Streptopelia decaocto*	12	0	0
Total terrestrial birds		1,201	0	0
Aquatic birds				
Dabbling ducks				
Green-winged Teal	*Anas crecca*	54	10 (18.5)	1 (1.9)
Blue-winged Teal	*Spatula discors*	8	0	0
Cinnamon Teal	*Spatula cyanoptera*	1	0	0
Redhead	*Aythya americana*	2	0	0
Gadwall	*Mareca strepera*	20	0	0
American Wigeon	*Mareca americana*	14	2 (14.3)	0
Northern Pintail	*Anas acuta*	2	0	0
Diving ducks				
Great Scaup	*Aythya marila*	2	1 (50)	1 (50)
Total aquatic birds		103	13 (12.6)	2 (1.9)

[1] M: avian influenza virus matrix gene (M) detection. [2] H5NA: North American H5 lineage subtype.

Upon thorough examination, we analyzed the potential differences by sex in the ten positive samples found in Green-winged Teal (*Anas crecca*). However, the ANOVA analysis revealed no statistically significant differences ($p > 0.05$).

Table 2. The proportion of samples tested for avian influenza virus and dependency analysis by sex, age, and month

Variable	Classification	Positive M[1] n (%)	Negative M[1] n (%)	p-value
Sex[3]	Male	4 (3.9)	44 (42.7)	0.354
	Female	9 (8.7)	46 (44.7)	
Age[3]	Adult	9 (8.7)	73 (70.9)	0.300
	Juvenile	1 (1.0)	9 (8.7)	
	Unidentified[2]	3 (2.9)	8 (7.8)	
Month[2,3]	December	9 (8.7)	55 (53.4)	0.796
	January	4 (3.9)	35 (34.0)	

[1] M: avian influenza virus matrix gene (M) detection. [2] The months in question are January and December, including the sampling on December 14, 2018, and January 24, 2019, respectively. [3] Chi-squared test in n= 103 samples

DISCUSSION

None of the terrestrial birds analyzed were positive for AIV; however, the overall prevalence found in the duck sample was 12.6%, higher than the results previously found in surveys carried out in the same wetland belonging to the Pacific Flyway migratory route region in Sonora, México, in aquatic birds with a prevalence of 3.6% (Montalvo-Corral *et al*., 2011). Similar results were found by Mateus-Anzola *et al*. (2021), with a prevalence of 3.6% of AIV-positive dabbling ducks in mid-winter in the Lerma river of Central Mexico. In addition, a study developed in the east of Mexico studied wild birds at a point of convergence of different migratory flyways (Pacific, Central and Atlantic), including samples of Anatidae and Columbidae families, and found an exceptionally high prevalence in terrestrial birds (49%) compared to aquatic birds (26%), without differences in the prevalence of resident (39%) and migratory (39%) birds (Cerda-Armijo *et al*., 2020). Although species that use the Pacific Flyway were included in the mentioned report, we agree with these authors that the differences in prevalence between the present and previous studies could be caused by the season of the year, species sampled, geographic variation, and also by the number of each represented species in the monitoring that increased the proportion of positive samples and the overall prevalence.

In the case of the three synanthropic dove species included in our research, they behave as either resident or long-distance migrants, capable of mobilizing up to 40 km from their perch to feeding sites within the agroecosystem. Their role as bridge species is controversial, as only a few studies included in AIV monitoring have found both positive and negative results. We did not detect any positive birds from doves included in our study. It has been argued that the very low AIV prevalence in the Columbine group is due to innate infection resistance mechanisms that they possess (Shao *et al*., 2023). Some epidemiological surveillance studies have evaluated the presence of LPAI AIV in *Streptopelia decaocto* and *Zenaida macroura*, without finding any positive samples (Kulak *et al*., 2010; Lefrançois *et al*., 2010; Cerda-Armijo *et al*., 2020). A study limitation is that the AIV genome, as determined by molecular diagnosis, doesn't reflect serological status, since we did not test for anti-influenza antibodies to evaluate the synanthropic birds' AIV exposure. Additionally, opportunistic sampling for terrestrial hunted game birds restricts the species included to cinegetic birds.

Nevertheless, the interaction between migratory birds and synanthropic birds and the conditions of coastal wetlands, where the bodies of water facilitate contact with fecal material, would lead to the spread of avian influenza viruses indirectly to poultry through the contaminated body that deposits the virus in water or feed and not necessarily by direct secretions, as well occurs with fomites, feathers or by mechanical spread (Abolnik, 2014; Shriner & Root, 2020). Previous research that simulated these natural conditions reported that starlings were susceptible to infection by exposure to AIV-contaminated water (Ellis *et al*., 2021), reinforcing the idea of the potential of some terrestrial birds to act as AIV bridge hosts and maintaining the importance of surveillance and the need for further analysis to test this possibility (Martelli *et al*., 2023). The three dove species included in our study are among the most abundant species in the Yaqui Valley, along with other pigeon species, which utilize different landscapes within the agroecosystem (Leyva-García *et al*., 2025).

It has been demonstrated that some terrestrial bird species play a role in the transmission of the influenza virus, as they can acquire the virus and exhibit symptoms (Liu *et al*., 2020; Sultankulova *et al*., 2022; Garcês & Pires, 2024). The possibility of exposure to the virus in other species of pigeons not included in the study is not ruled out because wild birds of the order Columbiformes have tested positive for antibodies and identification to AIV (Abolnik, 2014). Furthermore, birds of the *Streptopelia decaocto* are positive for HPAIV in territories affected by outbreaks in poultry (Verhagen *et al*., 2021). A seasonal, spatial, and demographic variation in avian influenza prevalence has been documented, with a peak in late summer, autumn, and winter in different regions of the Northern Hemisphere (Verhagen *et al*., 2021; Kent *et al*., 2022). Additionally, some authors have reported differences based on sex, finding a higher prevalence in female waterfowl (Nallar *et al*., 2015). Although we found a higher number of positive AIVs in females, this difference was not statistically significant due to the limited sample size.

Birds that migrate to Sonoran wetlands originate from regions in North America where the presence of the North American lineage H5 subtype has been documented, and, before 2006, was believed to be of low prevalence. Iša *et al*. (2022), in a genomic complementary study to Montalvo *et al*. (2011)'s waterfowl Sonora survey, found diverse subtypes of influenza virus, including the Low Pathogenic (LPAI) North American lineages H5N2 and H5N3 subtypes. Confirming the circulation and diversity of subtypes that waterfowl carry to the Pacific Mexican wetlands. After an introduction to new areas, AIV can circulate through the year and even persist until the following season. This phenomenon has been observed in the wetlands included in our study. Montalvo-Corral & Hernández (2010) identified two subtype H5 viruses in aquatic birds of Sonora and found a genetic identity of 94% between the two samples collected in different seasons. Furthermore, these viruses were highly related (99% and 98% identity) to viruses isolated in California from different consecutive years. However, more details of the genetic identity of the viruses found in the 2018-2019 season remain unknown.

Regarding the waterfowl species with positive samples, besides the Green-winged Teal (*Anas crecca)*, which had the highest influenza prevalence, the virus found in a Great Scaup (*Aythya marila*) stands out. Previously, Spackman *et al*. (2017) reported its susceptibility to infection with H5 HPAIV, and Huang *et al*. (2024) found a high AIV seroprevalence in this species. Conventionally, diving ducks are underrepresented in monitoring efforts. Diving ducks in the Yaqui Valley agroecosystem are found in canals and drains as part of the water irrigation system (Leyva-García *et al*., 2025). This interior area can blend with other terrestrial and aquatic species that can be bridge species in the transmission of viruses. This species is less represented in influenza monitoring studies and uses sites in the Valley, such as agricultural canals, which are not accessed by other species of ducks. As the Yaqui Valley region is one of the wintering sites for migratory species from North America, there is a possibility of introducing and mobilizing viruses. The circulation of newly arrived viruses can be maintained and spread among migratory populations of different species, potentially also to resident populations, promoting the transmission of VIA in wetlands and irrigation canals visited by birds. However, the latter could not be verified in the terrestrial birds studied.

Avian influenza viruses pose a persistent threat to animal and human health, as well as to wildlife biodiversity. Recently, significant mortalities have been caused by a highly pathogenic strain (HPAIV) H5N1 clade 2.3.3.4b in birds and mammals (Webby *et al*., 2024; Ospina-Jimenez *et al*., 2024). Thus, monitoring AIV in non-typical birds or reservoirs significantly contributes to understanding the ecology of the disease. The present research was conducted before the discovery and introduction of the highly pathogenic strain (HPAIV) H5N1 clade 2.3.3.4b in birds and mammals in North America and Mexico, as well as before poultry outbreaks in our region during the winters of 2022 and 2023 in farms from the Yaqui Valley (SENASICA, 2024). The results reported here are relevant to understanding the circulation, potential spread, and dynamics of viruses. Including those reasons reported by Ramey *et al*. (2018) regarding the importance of permanent surveillance of AIV in birds, such as the dispersion of wild bird viruses, the introduction of HPAIV strains, viral transmission between wild birds and poultry, the low probability of encountering and detecting a specific virus, and birds' immunity as outbreak triggers.

CONCLUSION

Avian influenza virus was identified in wild aquatic birds from the Tobari wetland habitats, demonstrating the importance of these sites in introducing this type of virus. However, the role of terrestrial species in transmission routes of the AIV to the domestic environment of this arid region has yet to be determined. A better understanding of the characteristics of individuals and their environment would help to explain the eco-epidemiology of avian influenza virus and other zoonotic diseases present in wild birds, which will allow the generation of base information for modeling the risk of introducing diseases to this region for possible prevention and control of outbreaks, and the importance of active surveillance.

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Errata Erratum

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