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Serological Surveillance of Wild Waterfowl in Northern Australia for Avian Influenza Virus Shows Variations in Prevalence and a Cyclical Periodicity of Infection

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SUMMARY. The virological surveillance of 3582 wild waterfowl in northern Australia from 2004 to 2009 for avian influenza virus (AIV) found an apparent prevalence (AP) of 1% (31 of 2989 cloacal swabs; 95% CI: 0.71%–1.47%) using a Taqman Type A real-time reverse transcription polymerase chain reaction test and no viral isolations from 593 swabs tested by the embryonating chicken egg culture method. From serological testing using a nucleoprotein competitive enzyme-linked immunosorbent assay for AIV antibody, 1131 of 3645 sera had ≥40% inhibition, indicating an apparent seroprevalence of 31% (95% CI: 29.5%–32.6%). This value suggests that the low AP from virological testing does not reflect the dynamics of AIV infection in these populations. Spatiotemporal and species variations in seroprevalence were found at wetland sampling sites, with consistently higher values at Kununurra in Western Australia (AP = 39%, 95% CI: 36.9%–41.4%) compared to other locations. At Kununurra, seroprevalence values had a two-year cyclical periodicity and suggest this location is a hotspot of AIV activity. From hemagglutination inhibition (HI) testing using multiple subtype antigens, the highest AP of HI reactions were to H6 and H5 subtypes. The phenomenon of cyclic periodicity in NP seroprevalence at Kununurra is hypothesized as being related to the prevalent H6 subtype that may have either become predominant or cycled back into a mostly AIV naïve flock. The inclusion of serological testing provided insight into the dynamics of AIV infection in wild birds such as species risk profiles and spatiotemporal patterns, important epidemiological information for a risk-based approach to surveillance.

RESUMEN. La vigilancia serológica de aves acuáticas silvestres en el norte de Australia para el virus de la influenza aviar muestra variaciones en la prevalencia y en la periodicidad cíclica de la infección.

La vigilancia virológica de 3,582 aves acuáticas silvestres en el norte de Australia del año 2004 al 2009 para el virus de la influenza aviar (AIV) mostró una prevalencia aparente (AP) de 1% (31 de un total de 2,989 hisopos cloacales; con un intervalo de confianza 95%: 0.71% –1.47%) utilizando un método de transcripción reversa y reacción en cadena de la polimerasa con un Taqman tipo A sin aislamiento viral de 593 hisopos analizados por el método de cultivo en huevos embrionbados de pollo. A partir de las pruebas serológicas utilizando un ensayo inmunoenzimático competitivo para anticuerpos contra la nucleoproteína del virus de la influenza aviar, 1,131 de 3,645 sueros mostraron ≥40% de inhibición, lo que indica una aparente seroprevalencia del 31% (IC del 95%: 29.5% –32.6%). Este valor indica que la baja prevalencia aparente mostrada por las pruebas virológicas no refleja la dinámica de la infección por el virus de la influenza aviar en estas poblaciones. Variaciones espacio-temporales y por la especie en la seroprevalencia fueron encontradas en sitios de muestreo localizados en humedales, con valores consistentemente altos en Kununurra en Australia Occidental (Prevalencia aparente = 39%; IC del 95%: 36.9% –41.4%) en comparación con otros lugares. En Kununurra, los valores de seroprevalencia tenían una periodicidad cíclica de dos años y sugieren que esta ubicación es un punto de actividad importante del virus de influenza aviar. Mediante el análisis por inhibición de la hemaglutinación (HI) utilizando múltiples antígenos de subtipo, la prevalencia aparente mas alta por inhibición de la hemoaglutinación fue contra los subtipos H6 y H5 subtipos. Se estableció la hipótesis del fenómeno de la periodicidad cíclica en la seroprevalencia en Kununurra que está relacionada con el subtipo H6 prevalente que pudo convertirse en predominante o que ha estado reciclándose en la mayoría de las parvadas susceptibles a influenza aviar. La inclusión de las pruebas serológicas proporciona una idea de la dinámica de la infección por el virus de influenza aviar en aves silvestres, como los perfiles de riesgo de las especies y patrones espacio-temporales, que es información epidemiológica importante para un enfoque basado en el riesgo para la vigilancia.

Key words: avian influenza virus, exposure risk, northern Australia, prevalence, surveillance, waterfowl

Abbreviations: AAHL = Australian Animal Health Laboratory; AI(V) = avian influenza (virus); AP = apparent prevalence; c-ELISA = competitive enzyme-linked immunosorbent assay; CI = confidence interval; C_t = cycle threshold; DAFWA = Department of Food and Agriculture WA; ECE = embryonating chicken egg; GMT = geometric mean titer; HA/H = hemagglutinin; HI = hemagglutination inhibition; HPAI = highly pathogenic AI; LPAIV = low pathogenicity AIV; NI = neuraminidase inhibition; NP = nucleoprotein; OR = odds ratio; RRT-PCR = real-time reverse transcription polymerase chain reaction; WA = Western Australia

There is overwhelming scientific evidence that wild waterfowl are the natural reservoir hosts for avian influenza viruses (AIV) with the detection of numerous viruses across a broad range of subtypes. Many biological traits of waterfowl (particularly ducks) favor the epidemiology of AI viruses that have adapted to their aquatic hosts in a relationship often referred to as evolutionary stasis. Features of this ecological paradigm include a highly dispersive host, infection that is asymptomatic, and excretion of prodigious amounts of virus laden feces into relatively stable freshwater ecosystems that favor transmission to other sympatric hosts (21). However, the panzootic of highly pathogenic AI (HPAI) H5N1 virus in Eurasia from late 2003 that prompted heightened focus on the role of wild birds has provided greater insights into the epidemiology of AIV.

Population size, density, and age structure are important factors in the epidemiology of AIV in wild birds (17). In North America, where waterfowl population size is large and movement behavior predictable, long-term surveillance of waterfowl has consistently isolated AI viruses with prevalence ranging from 5.1% to 45.5%,

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and in juvenile ducks, very high viral isolation rates to 61% (11,14). In Australia with its harsher environment, waterfowl population size is much lower, and movement behavior more unpredictable and responsive to rainfall, viral detection rates are much lower (8,9). In North America, surveillance has revealed an annual periodicity in virus and subtype prevalence, both seasonally and cyclically, with peaks in prevalence followed one to two years later by lower prevalence. In northern Europe, waterfowl have similar AIV ecology to North America, with AIV prevalence mostly higher in the fall than in the spring and higher in juveniles than adults (16,20).

Biological factors such as behavior, morphology, and physiology are also important determinants of AIV ecology in waterfowl. Dabbling ducks, especially the mallard, are hosts with high exposure risks to AIV due to their morphology and aquatic filter feeding habits (6). The highly dispersive behavior of waterfowl, responsive to the critical survival demands of feeding and breeding, provides potential dissemination of viruses into new regions. Molecular and physiological features of the innate immune system of ducks may contribute to natural resistance to AI viruses. These could include unusual features of duck IgY serum antibody, evident as weak anti-hemagglutinin (HA) responses to low-pathogenicity AIV (LPAIV) infection (15) and the presence of RIG-I helicase in epithelial cells that stimulates early antiviral interferon responses (1).

Prior to 2004, there had been minimal surveillance of Anseriformes for AIV in northern Australia, and globally, few studies have included serological testing that can potentially provide greater insights into the dynamics of AIV infection in wild birds. This study outlines the findings from the surveillance of more than 3500 waterfowl in northern Australia.

MATERIALS AND METHODS

Surveillance. The locations and timing for wild bird surveillance activities across northern Australia were determined and limited by factors such as seasonality, accessibility, and host availability. Hence only a few locations across northern Australia were identified as suitable for sampling wild waterfowl. These sites were reliably frequented by waterfowl, congregating to diurnally roost in the late dry season months (August to November) as other wetlands receded. It was also vital that each site provided good access and terrain to allow deployment and positioning of bulky cannon netting and sampling equipment, and that each site was assessed as low risk for dangerous estuarine crocodiles (*Crocodylus porosus*). Surveillance at other times of the year was not possible due to the summer monsoon presenting access issues and waterfowl dispersed across vast floodplains. The cannon netting method of bird capture was used primarily; however, some waterfowl were also caught by mist netting and walk through traps, and low numbers of samples were provided by indigenous hunters. Flocks of waterfowl mainly consisted of magpie goose (*Anseranas semipalmata*) and plumed whistling duck (*Dendrocygna eytoni*). Magpie geese were morphologically aged; however, this was not possible for plumed whistling ducks that have only subtle plumage age differences. The main locations and numbers sampled annually between 2004 and 2009 were wetlands around Kununurra in Western Australia (WA; from 2004, $n = 1853$), Atherton in Queensland (from 2007, $n = 871$), and Kakadu National Park and around Darwin in the Northern Territory (from 2005, $n = 634$). The capture of wild birds and collection of samples for testing was primarily conducted at each of these key locations only once per annum due to limited resources, and timed for optimal abundance and density of birds for cannon netting. Limited sampling also occurred at other opportunistic sites across northern Australia.

Blood samples were taken mostly from the medial metatarsal vein to a maximum safety volume of 1% body weight and transferred into either a 5-ml plain clotted blood container (Techno Plas, St Marys, Australia), or a 3.5-ml serum separation vacutainer (Becton, Dickinson and Company, Plymouth, UK). Following clot retraction, samples were centrifuged for 5 min at 3000 $\times$ *g* in a Hettich EBA20 (Hettich Lab Technology, Tuttlingen, Germany), with harvested sera stored at −20 C until processing. Samples of the cloacal mucosa were taken using sterile cotton-tipped wooden swabs (Eurotubo, Deltalab, Rubi, Spain) and placed into individually labeled 1-ml vials of viral transport media (3) and chilled prior to storage at −170 C. The surveillance of wild birds was undertaken under license and animal ethics approval from the Department of Parks and Wildlife WA and Murdoch University, respectively.

Laboratory testing. Serum samples were screened using an Australian Animal Health Laboratory (AAHL) nucleoprotein competitive enzyme-linked immunosorbent assay (NP c-ELISA) for AIV antibody at either AAHL or at the Department of Food and Agriculture WA (DAFWA), based on standard methods (19). Most c-ELISA positive samples (>60% inhibition) and many in the weak positive range (40%–60% inhibition) were also tested by hemagglutination inhibition (HI) (22), using subtype H5 and H7 antigens, and where possible against a panel of H1–H16 antigens, subject to availability of sera and antigens. A HI titer ≥8 was considered a significant reaction when interpreting test results for wild waterfowl sera in this study, ensuring test internal quality control standards were followed and test results repeatable, based on the findings from an evaluation of this cutoff in another study (2). Subject to availability of sera, samples positive to subtypes H5, H7, and H9 were then tested at AAHL by neuraminidase inhibition (NI) based on standard methods (22), prioritizing N1–N3 subtypes, which were the predominant subtypes associated with H5 and H9 viruses circulating in Asia. Prior to 2005, virological samples ($n = 593$) were routinely tested for AIV by embryonating chicken egg (ECE) viral isolation (five swabs from the same species collected at the same time/pool) according to standard methods (22). Thereafter, all samples ($n = 2989$), were tested at AAHL or DAFWA by Taqman AIV Type A real-time reverse transcription polymerase chain reaction (RRT-PCR) in pools of three to five swabs according to the AAHL method of Heine and Trinidad (10). At DAFWA, minor variations included extraction of RNA from 50 μl samples using a Magmax 96 viral isolation kit (Ambion Inc., Austin, TX) on an Applied Biosystems Magmax Express magnetic particle processor (Life Technologies Corporation, Melbourne, Australia) according to the manufacturer's instructions. At both laboratories, cycle threshold (C_t) <37 is considered positive, with $C_t = 37$–40 an indeterminate and $C_t \geq 40$ negative.

Statistical analyses. The apparent prevalence (AP) of NP c-ELISA positives (seroprevalence) included results from both the positive and weak positive ranges and was compared between species and groups using the Pearson's chi-square test and odds ratio (OR) in Statistix 9.0 (Analytical Software, Tallahassee, FL). The means of c-ELISA percentage inhibition by species or groups were compared by the two-tailed *t*-test in SPSS (version 17 for Windows; SPSS Inc., Chicago, IL). For HI results, the geometric mean titer (GMT) and standard deviation were calculated using the method of $\log_2$ transformation of reciprocal titers followed by calculation of the antilogarithm (7). The 95% confidence interval (CI) for proportions was calculated using the exact binomial method, and the 95% CI for the OR was calculated using the normal approximation method in Statistix 9.0.

RESULTS

Virology. No AIV was detected from the 593 swabs tested by ECE viral isolation, and of the 2989 swabs tested by Taqman Type A RRT-PCR, 31 were positive (AP = 1%, 95% CI: 0.71%–1.47%; median $C_t = 31.5$). There were more Type A RRT-PCR positives at Kununurra ($n = 19$) compared to the other sampling locations, and significantly higher ($P < 0.01$, OR = 3.0, 95% CI: 1.3–6.5) positive prevalence in plumed whistling ducks (AP = 1.6%, 95% CI: 1%–2.4%) than magpie geese (AP = 0.55%, 95% CI: 0.2%–1.1%). All 31 positive Type A RRT-PCR samples were negative to H5 and H7 subtype-specific primers by Taqman RRT-PCR, with only one

Table 1. Virologic and serologic test numbers by family and by species from 2004 to 2009 with NP c-ELISA seroprevalence and mean inhibition values.

Family and common name	Scientific name (2)	No. tested by virology[BC]	No. c-ELISA ≥40%/no. tested[BC]	AP (95% CI) NP c-ELISA ≥40% inhibition	Mean c-ELISA % inhibition
Anseranatidae					
Magpie goose	*Anseranas semipalmata*	1700	340/1745	19.5[A] (17.6–21.4)	31[A]
Anatidae					
Grey teal	*Anas gracilis*	25	15/25	60[A] (38.7–78.9)	54[A]
Pacific black duck	*Anas superciliosa*	31	13/31	41.9 (24.5–60.9)	46
Plumed whistling duck	*Dendrocygna eytoni*	1786	757/1806	41.9[A] (39.6–44.2)	43[A]
Anseriformes total		3582	1131/3645	31.0 (29.5–32.6)	37

[A]Species value with significant differences compared to the group value (minus that individual species data).
[B]One virus isolated from a plumed whistling duck, RRT-PCR results not shown.
[C]Species data not shown where sample size <25, Anseriformes total includes these data.

positive by ECE virus isolation (H6N1 subtype). The virological test numbers for each species are summarized in Table 1.

Serology by NP c-ELISA. Of 3645 samples, 1131 sera had ≥40% inhibition (Table 1), indicating a seroprevalence of 31% (95% CI: 29.5%–32.6%). For each species, seroprevalence and mean inhibition value were compared to the group value (minus that individual species data). Those species with a significantly higher seroprevalence value were plumed whistling duck ($P < 0.0001$, OR = 2.83, 95% CI: 2.4–3.3) and grey teal ($P = 0.003$, OR = 3.4, 95% CI: 1.5–7.5), with the magpie goose having a significantly lower seroprevalence ($P < 0.0001$, OR = 0.34, 95% CI: 0.3–0.4).

Spatial analysis of NP c-ELISA data shows overall that seroprevalence was significantly higher ($P < 0.0001$) in the waterfowl at Kununurra (AP = 39%, 95% CI: 37%–41.4%), compared to Atherton (AP = 24%, 95% CI: 21%–26.7%) and the Northern Territory (AP = 25%, 95% CI: 21.7%–29%). Seroprevalence over the three years at Atherton ranged from 20.6% to 26.6%. Temporal analysis of Kununurra annual test data showed a cyclical seroprevalence at a two-year periodicity, with every peak year followed by a significantly lower seroprevalence year ($P < 0.0001$). The peak years at Kununurra were 2005 (AP = 68.3%), 2007 (AP = 61.1%), and 2009 (AP = 63.8%), and low seroprevalence years were 2004 (AP = 31.1%), 2006 (AP = 7.2%), and 2008 (AP = 28%) (Fig. 1). The highest OR was found when comparing the data between 2005 and 2006 ($P < 0.0001$, OR = 27.7, 95% CI: 18.5–41.5), and between 2007 and 2006 ($P < 0.0001$, OR = 20.2, 95% CI: 13.4–30.3). The seroprevalence was significantly higher ($P < 0.001$) in plumed whistling ducks compared to magpie geese every year from 2006 to 2009 (Table 2). Seroprevalence values were compared between juvenile ($n = 79$) and adult magpie geese ($n = 471$) at Kununurra from 2007 to 2009, and in all years seroprevalence values were higher in adults than juveniles.

HI and NI serology. From approximately 11,000 HI tests using multiple hemagglutinin antigens and antisera, HI reactions with titers ≥8 were detected in 152 of 1131 sera that had NP c-ELISA ≥40% inhibition test results, with 69% of all reactions from the Kununurra samples. Some sera were not tested against all subtypes due to either insufficient sera or a shortage of antigens. Overall, plumed whistling ducks ($n = 104/1806$) had more than twice the number of HI reactions ($P < 0.0001$, OR = 2.75, 95% CI: 1.9–4) compared to magpie geese ($n = 38/1745$). Overall the most prevalent HI reactions were against H6 (43% of reactions, GMT = 18; 50% of reactions at Kununurra) and H5 subtypes (27% of reactions overall and at Kununurra; GMT = 18.4), with fewer reactions against H9 (8.6% of reactions; GMT = 19) and H2 subtypes (6.5% of reactions; GMT = 17). Low numbers of sera were reactive to H3, H4, H7, H8, H11, H13, H15, and H16 subtypes (<2% of reactions for each subtype; GMT range = 8-20),

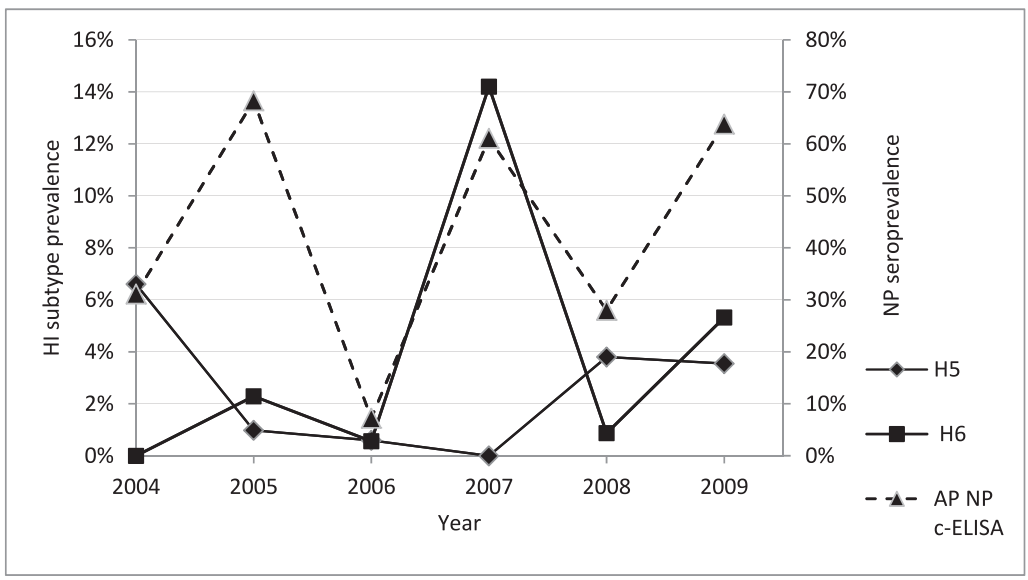

Fig. 1. Prevalence of H5 and H6 subtype HI reactions (read from *y*-axis on the left side) compared to the NP c-ELISA seroprevalence (read from *y*-axis on the right side) in the serum samples from Kununurra waterfowl from 2004 to 2009.

Table 2. Comparison of NP c-ELISA seroprevalence between magpie geese and plumed whistling ducks with odds ratio, and the proportion of birds from both species with ≥60% and ≥80% inhibition results at Kununurra from 2004 to 2009.

Year	Magpie goose Test+/no. tested (AP; 95% CI)	Plumed whistling duck Test+/no. tested (AP; 95% CI)	OR (95% CI)	Proportion of birds with ≥60%–100% inhibition	Proportion of birds with ≥80% inhibition
2004	21/79 (26.6; 17.3–37.7)	12/27 (44.4; 25.5–64.7)	2.2 (0.9–5.5)	18%	8%
2005	0[A]	209/306 (68.3; 62.8–73.5)		48%	24%
2006	3/226 (1.3; 0.3–3.8)	36/314 (11.5; 8.2–15.5)	9.6 (2.9–31.7)	5%	3%
2007	53/133 (40; 31.5–48.7)	115/142 (81; 73.6–87.1)	6.4 (3.7–11.1)	43%	25%
2008	19/190 (10; 6.1–15.2)	76/151 (50.3; 42.1–58.6)	9.1 (5.2–16.1)	22%	13%
2009	129/227 (56.8; 50.1–63.4)	51/55 (92.7; 82.4–98)	9.7 (3.4–27.7)	39%	28%

[A]Very low numbers of magpie geese were available for capture in 2005.

with no reactions against subtypes H1, H10, and H14. From the results for Kununurra, prevalence values for HI H5 and H6 and NP c-ELISA were compared (Fig. 1). For 2007, results suggest a correlation between high NP c-ELISA seroprevalence and a peak in HI H6 reactions. Of those sera (from all sample locations) tested against H5 subtypes, 43 of 996 sera had HI titers ≥8 to H5N1 Clade 1 (A/chicken/Vietnam/8/2004; GMT = 17.6, range of titers 8–128), nine of 872 sera (two cross-reactive with H5N1 Clade 1 antigen) had HI titers ≥8 to H5N3 (A/shearwater/Australia/1975; GMT = 23.6), with no reactions in 360 sera to a H5N1 Clade 2 antigen (A/chicken/Konawe selatan/BBVM204/2007). Only three of 982 sera were HI reactive against a H7N3 subtype (A/chicken/Victoria/224/1992; GMT = 20). Of the HI reactive sera available for NI testing (H5 = 26, H9 = 8, cross-reactive = 1), there were NI subtype reactions only in the HI H5 reactive sera (N1 = 3, N2 = 1, N3 = 3).

DISCUSSION

The virological surveillance of 3582 waterfowl across northern Australia from 2004 to 2009 found an apparent prevalence of 1% in swabs that were tested for AIV by Taqman Type A RRT-PCR. This value is significantly lower ($P < 0.0001$) than the 2.4% Type A RRT-PCR positive detection rate in waterfowl from southeast Australia ($n = 10{,}231$) (8) and markedly lower than the 21.7% mean virus prevalence over 32 years in North America (14) and global estimates of 7.7% virus prevalence (18). These spatial differences in AIV prevalence could be related to a range of host-virus factors such as variations in exposure risk, immune status, species susceptibility, population size and structure, feeding and movement behaviors, virus ecology, infection dynamics, and differences in sampling strategies. In this study, there was only a 3.2% virus isolation rate from RRT-PCR positive swabs, and it is unclear, given the moderate RRT-PCR reactions, why this was so low. It is possible that viral viability was compromised by the tropical field conditions with diurnal temperatures of 30–40 C.

From serological testing by NP c-ELISA, 1131 of 3645 samples from waterfowl had a ≥40% inhibition result, representing a 31% seroprevalence. These findings suggest that virological testing alone does not always reflect the dynamics of AIV infection, a finding consistent with other wild bird studies that have included serological testing (4,5,23). Moreover, low rates of viral detection are likely related to the timing of sampling and the narrow window of opportunity to detect virus that is normally excreted for a short duration, or possibly other factors such as preexisting immunity in the flock. There was significantly higher NP seroprevalence at Kununurra compared to Atherton and the Northern Territory, and at Kununurra, values suggest considerably higher likelihood of AIV infection in plumed whistling ducks compared to magpie geese (OR range = 6.4–9.7). The NP seroprevalence data from annual sampling over six years at Kununurra suggests that AIV infection was cyclical with a two-year periodicity (Fig. 1), and the flock profile of NP c-ELISA results provided further insight into the infection dynamics in peak seroprevalence years. For instance in the peak years, 39% to 48% (mean = 43.5%) of birds had ≥60%–100% inhibition values, and 24% to 28% of birds (mean = 25.6%) had ≥80% inhibition values (Table 2). Based on AIV postinoculation data from one study (3) where percentage inhibition waned below 80% from 42 days to 60% by about 8 months, this suggests that at the time of sampling there had been exposure to AIV infection in 25% of birds during the previous 6 weeks and in at least 40% of birds during the preceding eight months. Seroprevalence in magpie geese was higher in adults compared to juveniles, though limited numbers of juveniles ($n = 79$) were available for assessment.

From HI testing, more reactions were detected in plumed whistling ducks than any other species, and more reactions were in samples from Kununurra than any other location. The most prevalent HI reactions were to H6 and H5 serotype antigens. Samples tested against H5 serotypes reacted primarily against the Vietnamese H5N1 Clade 1 subtype rather than the Australian H5N3 subtype, with no sera reactive to the Indonesian H5N1 Clade 2 subtype. Moreover, the H5 serotype reactions were in twice as many samples from plumed whistling ducks than magpie geese and were detected in four sample years at Kununurra. Testing for the NA serotype in sera that were HI reactive to H5 and H9 serotypes detected NI reactions in seven H5 HI reactive sera to N1, N2, and N3 subtypes. These results suggest that an LPAI H5 subtype in various N1, N2, and N3 subtype combinations may be circulating with other subtypes in northern waterfowl populations. Interestingly, there were few HI reactions against the Australian H5N3 and H7N3 subtypes that could not be explained in this study. One hypothesis is that antigenic drift of the HA gene of the older H5N3 isolate has occurred, resulting in a circulating H5 virus that is temporally closer to the more recent H5N1 Clade 1 virus. The HI reactions to the Vietnamese H5 Clade 1 subtype antigen in this study are more likely related to this antigenically related subtype, rather than a HPAI H5N1 virus, given the absence of this virus in Australia (8).

From long-term studies in North America, cyclic AIV infection with a 2–3 yr periodicity was identified in waterfowl. This phenomenon was hypothesized as being related to waning humoral immunity accompanied by cell-mediated immunity that provides some cross-protection against heterologous subtypes, a high population mortality rate, and an influx of immunologically naïve juveniles (13). In this study, cyclic periodicity of NP seroprevalence in the waterfowl at Kununurra alternating each year from high to low values could not be confirmed by virological testing. However, from HI testing there was evidence that H6 and H5 were predominant subtypes, and that the circulation of these subtypes is

likely to be associated with the fluctuations in NP seroprevalence. In some years HI H6 and H5 reactions were almost nonexistent, further suggesting that these subtypes cycled in and out of the population. These events are likely to be dependent on the level of flock immunity and degree of maintenance of these subtypes in the population. Furthermore, these findings are consistent with hypotheses that predominant subtypes change and cycle over time, raising uncertainty about how viruses move in and out of wild bird populations and how viruses are maintained when not circulating in a population (14). A hypothesis for the phenomenon at Kununurra is that a wave of subtype virus infection, most likely a H6 subtype, moved rapidly through the flock, resulting in high levels of flock infection and immunity. This subtype immunity is sustained in the flock and the virus either cycles out only to reappear in the second year when flock immunity has waned sufficiently, or changed through attrition and recruitment of naïve juveniles, or is sustained at low levels of infection in the flock. The H6 subtype is the most likely candidate for these events at Kununurra and is a known duck adapted subtype (13,16). In the waterfowl of Kununurra, H6 was the predominant subtype by HI testing, with peaks in prevalence followed by troughs where it was almost nonexistent. The consistent trough in NP seroprevalence subsequent to a peak year at Kununurra may reflect an inability of other extant heterologous subtypes, in the absence of H6 subtype, to initiate significant infectivity and stimulate NP antibody production in these birds. To fully explain the phenomenon of cyclical AIV seroprevalence at Kununurra, where roost flock density is high and conditions are ideal for transmission of virus between hosts that were previously dispersed during the breeding season requires further long-term epidemiological studies.

There are marked differences in the ecology of Australian waterfowl when compared to the northern hemisphere that are likely to influence AIV epidemiology. In Australia, waterfowl ecology is inextricably driven by an unpredictable climate, reflected in highly dispersive behavior, and influencing population size and mortality rates. Variability in rainfall is evident as episodic flooding of wetlands with high numbers of waterbirds at low density and conversely, in drought with congregating flocks in high density (12). In northern Australia, plumed whistling ducks are primarily grazers that also feed on plants in shallow waters, and magpie geese feed on aquatic plants such as the bulbs of sedges. Although both show some dabbling behavior, they do not filter feed like dabbling ducks, and hence may be less exposed to AIV. The challenging conditions in Australia contrast markedly to the seasonal regularity of the northern hemisphere with its considerably larger populations of waterfowl, where wetland availability is less variable, and where strong philopatry drives regular migration (12).

Serological testing has revealed useful epidemiological information about the dynamics of AIV infection in the waterfowl of northern Australia, evident in cyclical patterns of infection, species differences in exposure risk profile, and circulation of HA specific serotypes. Serological testing for AIV antibodies should be considered in wild bird studies to develop a risk-based surveillance strategy, particularly in situations where new populations are to be sampled or when low rates of viral shedding are encountered, or where virological testing through the annual cycle is not possible. Inclusion of AIV serological testing for antibodies with different longevities can, however, present interpretative difficulties in wild birds that can have multiple infections, be in different stages of infection, have anamnestic responses or cross-reactive or weak production of HA antibodies, and have varying immune status and with subtype-specific antibody tests, if there is a mismatch of antigens. The findings in this study provide further insight into an AIV epidemiology in wild waterfowl that is inherently complex, intriguing, and challenging to understand and fully explain.

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