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Risk Factors for Exposure to Influenza A Viruses, Including Subtype H5 Viruses, in Thai Free-Grazing Ducks

A. L. Beaudoin[1], P. Kitikoon[2], P. J. Schreiner[1], R. S. Singer[1], J. Sasipreeyajan[3], A. Amonsin[3], M. R. Gramer[1], S. Pakinsee[3] and J. B. Bender[1]

[1] University of Minnesota Twin Cities, Saint Paul, MN, USA
[2] United States Department of Agriculture, Ames, IA, USA
[3] Chulalongkorn University, Bangkok, Thailand

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Correspondence:
J. B. Bender. 136F ABLMS, 1354 Eckles Ave, St. Paul, MN 55108, USA.
Tel.: +1 612-625-6203;
Fax: +1 612-624-4906;
E-mail: bende002@umn.edu

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Summary

Free-grazing ducks (FGD) have been associated with highly pathogenic avian influenza (HPAI) H5N1 outbreaks and may be a viral reservoir. In July–August 2010, we assessed influenza exposure of Thai FGD and risk factors thereof. Serum from 6254 ducks was analysed with enzyme-linked immunosorbent assay (ELISA) to detect antibodies to influenza A nucleoprotein (NP), and haemagglutinin H5 protein. Eighty-five per cent (5305 ducks) were seropositive for influenza A. Of the NP-seropositive sera tested with H5 assays (n = 1423), 553 (39%) were H5 ELISA positive and 57 (4%) suspect. Twelve per cent (74 of 610) of H5 ELISA-positive/suspect ducks had H5 titres $\geq$ 1 : 20 by haemagglutination inhibition. Risk factors for influenza A seropositivity include older age, poultry contact, flock visitors and older purchase age. Study flocks had H5 virus exposure as recently as March 2010, but no HPAI H5N1 outbreaks have been identified in Thailand since 2008, highlighting a need for rigorous FGD surveillance.

Introduction

Highly pathogenic avian influenza (HPAI) H5N1 has caused high mortality of wild birds and domestic poultry throughout Asia and in some parts of Africa, Europe and the Middle East (Webster et al., 2006; Emerging Centre for Transboundary Animal Diseases, 2011). Since HPAI H5N1 was first reported in Thailand in January 2004, there have been seven outbreaks, most recently in October and November of 2008 (Amonsin et al., 2006; Tiensin et al., 2007; Chaichoune et al., 2009; World Organization for Animal Health, 2011b). Recent outbreaks occurred largely in Central and Northern Thailand (World Organization for Animal Health, 2011b). Response measures have included stamping out, poultry movement control, biosecurity improvements and door-to-door surveillance (Buranathai et al., 2007; World Organization for Animal Health, 2011b). Influenza vaccination of poultry is prohibited in Thailand (Buranathai et al., 2007).

Thai HPAI H5N1 outbreaks have been intermittent, starting in the rainy season (July–September) or winter (October–January) (Chaichoune et al., 2009; Tiensin et al., 2009). No outbreaks began in or continued through the hot summer season (April–June). Despite this intermittent pattern, 2008 Central Thailand HPAI H5N1 isolates share the same clade 1 lineage as earlier Thai isolates, with genetic drift rates typically associated with sustained virus circulation (Amonsin et al., 2006, 2010; Buranathai et al., 2007; Chaichoune et al., 2009). This suggests an animal or environmental reservoir maintaining H5N1 viruses during summer prior to rainy season re-emergence. Because ducks can shed influenza viruses asymptomatically, domestic populations may sustain low-level H5N1 virus transmission during summer (Chen et al., 2004; Sturm-Ramirez et al., 2005; Songserm et al., 2006; Chaichoune et al., 2009). Free-grazing duck (FGD) flocks in Central Thailand were identified as a risk factor for HPAI H5N1 outbreaks through spatial analyses (Tiensin et al., 2005, 2009; Gilbert et al., 2006, 2007, 2008). In this management system, FGD farmers graze their flocks on post-harvest rice fields, where they eat residual rice, insects and snails, frequently sharing fields with resident and migratory wild waterfowl (Gilbert et al.,

2006). Owners identify grazing fields through social networks, built over years of experience, and if no nearby fields are available, flocks are moved out of the area, sometimes to other provinces (Beaudoin, unpublished data).

Free-grazing duck influenza research in Thailand has been largely conducted in Suphanburi Province, where irrigation facilitates two to three rice crops yearly with year-round duck grazing, and where almost 50% of all Thailand's duck outbreaks occurred during the second wave of HPAI H5N1 outbreaks in 2004 (Gilbert et al., 2006; Songserm et al., 2006). Despite the identification of FGD as a risk factor for HPAI H5N1 outbreaks, little is known about transmission within and among FGD flocks (FGDF) or the prevalence, incidence and viral subtypes of infection (Emerging Centre for Transboundary Animal Diseases, 2011). Our study goals were to improve the understanding of FGDF management and movement in Suphanburi Province and estimate the seroprevalence of influenza A antibodies, including those to H5 subtype viruses, in this population.

Materials and Methods

Study design

We conducted a cross-sectional study of FGDF within Suphanburi Province in July and August of 2010. Suphanburi last experienced HPAI H5N1 poultry outbreaks in November of 2005. In June 2010, the Thai Department of Livestock Development (DLD) completed a province-wide census and registration of FGDF. Flocks were identified through existing registration lists, communication with local livestock officers and registration announcements. The DLD considers flocks 'free-grazing' if they move away from home to graze. The census identified 340 flocks, registered based on owner residence in 9 of the 10 municipal districts of Suphanburi Province (Don Chang District had no FGDF). All were invited by local livestock officers to participate. Flocks located outside the province at the time of sample collection were excluded due to collection constraints. There were 139 flocks outside the province or unable to be contacted. All other owners agreed to participate, yielding a 201-flock study cohort (59% of registered flocks).

A total of 6254 samples were taken from ducks in the 201 flocks. All samples were tested with a screening test (NP ELISA) for influenza A antibodies. Due to resource constraints, only a subset of sera (1423 samples) was tested for H5 antibodies. For statistical analyses, flocks not currently grazing or with unknown grazing history ($n = 41$) or with incomplete survey responses ($n = 32$) were excluded. For the analysis of influenza A seropositivity, the final dataset consisted of 3978 ducks in 128 flocks, and for the H5 seropositivity analysis, the final dataset consisted of 962 ducks in 122 flocks.

Serum collection

Blood was collected at grazing locations during July and August 2010. With a test sensitivity of 96%, a sample size of 30 ducks per flock provided > 95% chance of detecting a single seropositive duck if the true flock seroprevalence was $\geq$ 10%. With this sample size, assuming a conservative flock-level seroprevalence of 50%, we have 95% confidence that the precision of the within-flock seroprevalence estimates is $\leq$ 18%. Convenience samples of ducks were selected from each flock for venipuncture.

NP ELISA

Sera were analysed by commercial enzyme-linked immunosorbent assay (ELISA) according to manufacturer instructions (FlockCheck Avian Influenza MultiS-Screen Ab Test kit; IDEXX Laboratories, Westbrook, ME, USA; referred to as NP ELISA). This screening test identifies avian antibodies to the highly conserved influenza A virus nucleoprotein (NP), allowing non-subtype-specific detection of antibodies against all subtypes. To obtain our positive cut-off value, we referred to one published study (Brown et al., 2009) and used unpublished duck-specific experimental data from that work to determine the optimal cut-off for duck serum. This cut-off has an expected 96% test sensitivity (Se) and 88% specificity (Sp). Sera were initially positive with a sample/negative (S/N) value of < 0.6, and suspect positive with $0.6 < S/N < 0.7$. Suspect samples were assayed in triplicate, and the mean S/N value was determined. To obtain a dichotomous outcome of duck-level seropositivity, samples with S/N (or mean S/N) of < 0.7 were considered positive. True seroprevalence (TP) estimates were calculated from the apparent prevalence estimates (AP) to adjust for imperfect Se and Sp using $TP = (AP + Sp\text{-}1)/(Se + Sp\text{-}1)$.

H5 ELISA

A subset of NP ELISA-positive samples was assessed using an ELISA that identifies avian serum antibodies to H5 subtype influenza A viruses (ID Screen Influenza H5 Antibody Competition, ID Vet, Montpellier, France; referred to as H5 ELISA). The kit was manufactured using a LPAI H5N2 virus isolated in Italy between 2005 and 2006. Samples were processed using manufacturer instructions and cut-offs (positive if S/N $\leq$ 0.35, suspect if > 0.35 and $\leq$ 0.39). Serum from ducks vaccinated with inactivated H5N1 vaccine was included as an additional positive control. Suspect-positive samples were considered positive for analysis. No sensitivity or specificity information could be obtained from the manufacturer.

H5 ELISA was used on a 1423-serum subset. Samples were selected from every flock with positive ducks. If there were < 10 positive samples in one flock, all samples were tested (45 samples from 13 flocks). All samples from 18 flocks with 100% seroprevalence (562 samples), including the youngest and oldest flocks from the 9 districts, were tested to estimate flock H5 seroprevalence. Five positive samples were randomly selected from all other flocks with positive ducks (1005 samples from 163 flocks). Thirty NP ELISA-negative samples were randomly chosen for assay.

Haemagglutination inhibition

H5-positive samples were further assessed using haemagglutination inhibition (HI) as published previously (World Organization of Animal Health, 2011). Pre-treatment of sera to reduce the effect of non-specific inhibitors was conducted by incubation of 100 μl serum with 400 μl of 20% kaolin, centrifugation and adsorption of the supernatant with 10 μl of 50% chicken RBC. The resulting supernatant yielded a starting dilution of 1 : 5 for the HI assay. HI was conducted with inactivated clade 1 influenza virus A/chicken/Thailand/CU-K2/2004/H5N1, and samples were considered positive with a titre $\geq$ 1 : 20.

Flock owner interviews

A 53-question survey regarding flock characteristics, management, grazing practices, movement history and experiences with HPAI H5N1 in 2004–2005 was developed. Questions were written in English, translated into Thai and back-translated into English to reduce loss of meaning through translation. Phone surveys were conducted with verbal consent after the blood collection. Seventeen flock owners could not be contacted, and data for those flocks were limited to age and seropositivity status. Interviews were conducted in Thai by native speakers, trained on the background and intentions of the study. Responses were recorded in Thai and translated into English. FGD owners with whom researchers had prior contact piloted the survey.

Analysis, NP ELISA

Descriptive statistics calculated with the full dataset (n = 201 flocks, 6254 ducks) characterized seroprevalence and age of flocks. Statistical analyses were conducted using the dataset with complete survey information and known grazing history (n = 128 flocks, 3978 ducks). For all analyses, duck-level serostatus was included as the dichotomous-dependent variable (seropositive or seronegative). Univariate analyses of duck and management parameters included t-tests, chi-square, Fisher's exact test and Wilcoxon rank-sum test. Multivariable analysis was conducted using generalized estimating equation (GEE) methods, with flock as a repeated measures variable. GEE methods account for similarities within clusters and may better model clustered discrete data than generalized mixed models (Dohoo, 2009). We assumed an exchangeable working correlation matrix, where every duck within a flock is equally correlated with every other duck in the flock. The full multivariable model included independent parameters with P-values of $\leq$ 0.25 on univariate analysis. Before inclusion in the model, independent variables were assessed for collinearity. Manual model backward and forward building techniques were used, and independent variables (or groups of indicator variables) with P-values of > 0.05 were removed. Model fit was guided by the quasi-likelihood under the independence model criterion (QIC), where QIC value decreases with improved model fit (Pan, 2001). As a measure of the precision of the parameter estimates, ratios of the lower and upper confidence limits were calculated and are presented with the multivariable model results (Poole, 2001). Statistics were conducted with SAS 9.2 software (SAS Institute, Inc., Cary, NC, USA).

Analysis, H5 diagnostics

Descriptive statistics were calculated for all samples tested for H5 seropositivity (n = 1423 ducks in 194 flocks). Statistical analyses were conducted using the dataset with complete survey information and known grazing history (n = 122 flocks, 962 ducks). Univariate and multivariable analyses were conducted as described above for NP ELISA data.

Ethical considerations

This work was approved by the University of Minnesota Institutional Review Board (no. 0701M01041) and Institutional Animal Care and Use Committee (no. 1003A78795).

Results

Suphanburi flock influenza A seroprevalence

The proportion of FGDF in each district that were included in this study can be seen in Fig. 1. Positive flocks were located in all nine districts with FGDF (Fig. 1). Of the 201 flocks sampled, 194 (97, 95% CI 95–98%) had at least one duck with influenza A antibodies identified by NP ELISA. All specimens were positive from 56 flocks (28%). The within-flock seropositivity distribution (Fig. 2) is left-skewed and, when adjusted for imperfect test sensitivity and specificity, has a median value of 100% (inter-quartile range (IQR) 93–100%). Among all ducks sampled, (n = 6254 ducks in 201 flocks), 85% (5305 ducks) were

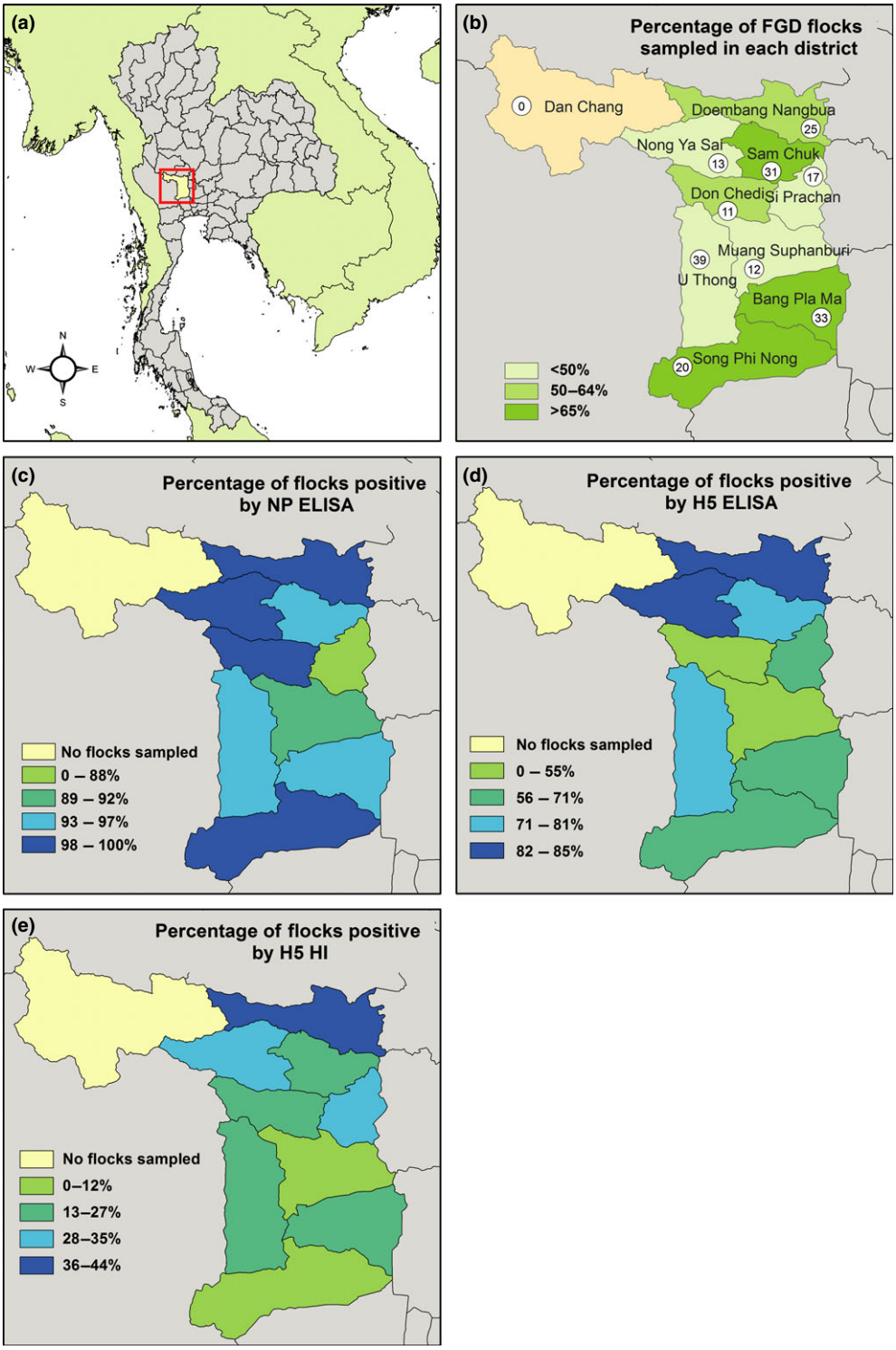

Fig. 1. (a) Sampled flocks and seropositivity in Suphanburi Province. Location of Suphanburi Province within Thailand. (b) Percentage of registered flocks in each district of Suphanburi that were included in the study, with number of flocks included in the circle. (c) Percentage of NP ELISA-positive flocks (with at least one NP-seropositive duck) in each district, $n = 201$. All districts had $\geq$ 88% of flocks positive for influenza A by NP ELISA. (d) Percentage of H5 ELISA-positive flocks (with at least one H5-seropositive duck) in each district, $n = 194$. The northern districts had the highest proportion of seropositive flocks. (e) Percentage of H5 HI-positive flocks (with at least one HI-seropositive duck) in each district, $n = 194$. Doembang Nangbua, the farthest north of the districts, had the highest proportion of seropositive flocks. Jenks natural breaks classification was used to generate the choropleth map data classes.

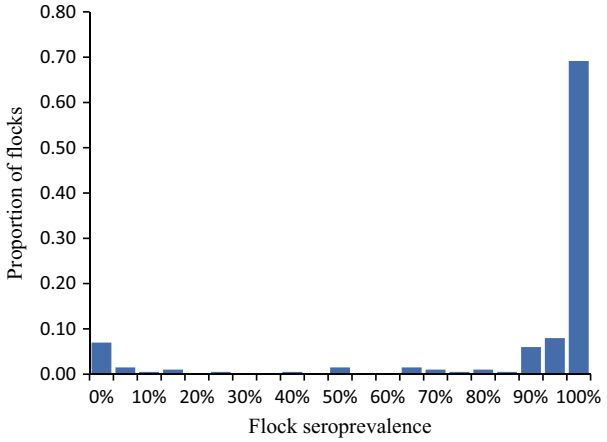

Fig. 2. Distribution of flock seroprevalence for avian influenza antibodies by NP ELISA, $n = 201$. The flock seroprevalence estimates included in the histogram are true prevalence estimates (apparent seroprevalence adjusted for imperfect test sensitivity and specificity).

seropositive on NP ELISA. Seropositive ducks were older than seronegative ducks, with mean (± standard deviation) ages of 9.1 (± 3.8) and 5.1 (± 3.2) months ($P < 0.01$), and median of 8 and 4 months, respectively.

Flock characteristics and management

Survey results can be seen in Table 1. In the 128-flock dataset used for analysis, flock size ranged from 300 to 11000 ducks (mean 2296, median 2000). Most flocks (99.2%) were of homogenous age, ranging from 1.5 to 24 months. One flock (0.8%) was of multiple ages. The most common breed was the egg-producing Khaki Campbell or a mix thereof (97.6%), although some owners raise Beijing (0.8%) or Cherry Valley (1.6%) meat ducks. Marketing of eggs occurs on average 2.4 days per week (median 2 days). In most cases, the eggs are picked up for sale (79.8%), rather than delivered to market by the owner (18.5%) or a combination of the two methods (1.7%).

After grazing on fields near home, FGDF are usually brought home to a barn or partially covered pen at night. Ducks are transported by truck (93.3%), by foot (5.6%) or by a combination of the two (1.1%). The average daily maximum distance to the grazing location is 5 km, although, rarely, flocks undergo daily movement up to 30 km. Ducks are penned together overnight for 12 h on average (range 10–13) and are kept in an open-sided roofed barn (79.5%) or an open uncovered area (20.5%). Flocks not brought back to a barn are penned in a dry location near the rice field.

Most owners (65%) move to the same locations for grazing each year, while others (35%) use variable grazing locations. Flock movement across administrative boundaries decreases with increased distance. Thirteen per cent of FGDF leave Suphanburi Province during a typical year. While in Suphanburi, 20% move outside their district, and while in the home district, 54% will move outside their subdistrict. Eighty-four per cent of FGDF move to other villages in their subdistrict to graze.

Nearly all flock owners reported purchasing their flocks as a group, with most ducks purchased at < 4 months old (86%). Median purchase age was 1 day. Ducks bought at < 4 months were from duck farms (95%) or from markets, dealers or neighbours (5%). Older ducks were obtained from farms (67%), or from dealers or markets (33%). The mean and median sale age of egg ducks is 24 months, the age at which production declines. Meat breeds are sold to slaughter at 3–5 months old. Ducks are sold to traders (95%), neighbours (1%) and slaughterhouses (4%). Flocks are usually sold as one group (80%), although small group sales (20%) may occur if purchased by another duck farmer or a small-scale trader. Market sales of live poultry are uncommon in Central Thailand, and 95% of owners reported never visiting live bird markets. Additionally, 95% of respondents never go to slaughterhouses, relying on traders as middlemen.

Nine per cent of respondents that owned ducks at that time ($n = 103$) reported having an infected flock during the 2004–2005 HPAI H5N1 outbreaks, and 19% had a flock culled. During the outbreaks, 65% raised ducks but did not graze, 28% stopped raising ducks and 7% continued grazing. When asked about the current flock, 23% of owners reported that the flock had at some time experienced a drop in egg production and 2.3% reported that at some time there had been sudden death of ducks. No flock owners reported seeing ducks with nasal discharge or neurologic abnormalities.

Univariate analysis, NP ELISA

Seropositive ducks had spent more time grazing (Table 1). Only some of the effect of grazing time was explained by duck age (data not shown), and both of these parameters were included in the multivariable analysis. Ducks from flocks having contact with other poultry had a higher odds of seropositivity, as did ducks with flock visitors and egg pickup, and ducks that had left the province. Other significant parameters include movement out of the village when within the home subdistrict, duck purchase age, night pen location and transport modality. Seventeen significant parameters were included in the multivariable analysis. Ducks that were seropositive were more likely to be in a flock that had ever exhibited a drop in egg production (OR = 5.1, $P < 0.001$) and less likely to be in a flock that had ever experienced sudden deaths (OR = 0.40, $P < 0.001$) (data not shown). These health history

Table 1. Results of univariate analyses of flock and duck factors for association with duck seropositivity on NP ELISA (n = 3978)

Parameter	Median for positive ducks (IQR)	Median for negative ducks (IQR)	*P*-value*
Flock size	2000 (1200, 2800)	2000 (1500, 3000)	< 0.01
Age (months)	8.0 (7.0, 10)	4.0 (3.0, 5.0)	< 0.01
Age when first grazed (days)	30.0 (25, 60)	30.0 (20, 30)	< 0.01
Time spent grazing over lifetime (months)	4.0 (2.5, 7.0)	3.0 (1.5, 3.5)	< 0.01

Parameter	Category	Number of positive ducks (n = 3268)	Number of negative ducks (n = 710)	OR	CI	*P*-value †
Duck use	Meat	82	13	Ref		0.28
	Eggs	3186	697	0.72	0.40, 1.3	
Grazed on rice fields for entire life to date	No	1988	336	Ref		< 0.01
	Yes	1280	374	0.58	0.49, 0.68	
Egg collection modality	No eggs	126	158	Ref		
	Bring to market	588	99	7.4	5.4, 10.2	< 0.01
	Picked up	2496	449	7.0	5.4, 9.0	< 0.01
	Combination	58	4	18.1	6.4, 51.4	< 0.01
Egg collections per week	$\leq$ 1	494	254	Ref		
	2–4	2381	415	3.0	2.5, 3.5	< 0.01
	$\geq$ 5	393	41	5.0	3.5, 7.0	< 0.01
Owner has other poultry	No	1709	415	Ref		
	Yes	1559	295	1.3	1.1, 1.5	< 0.01
Duck contact with other poultry	No	2389	648	Ref		
	Yes	879	62	3.8	2.9, 5.0	< 0.01
Visitors come to flock	No	1486	563	Ref		
	Yes	1782	147	4.6	3.8, 5.6	< 0.01
Ducks graze year-round	No	893	94	Ref		
	Yes	2375	616	0.41	0.32, 0.51	< 0.01
Ducks have moved out of province	No	3031	697	Ref		
	Yes	237	13	4.2	2.4, 7.4	< 0.01
Ducks have moved out of district	No	2760	589	Ref		
	Yes	508	121	0.9	0.72, 1.1	0.32
Ducks have moved out of village	No	1353	193	Ref		
	Yes	1915	517	0.53	0.44, 0.63	< 0.01
Duck purchase age	< 4 months old	2684	666	Ref		
	$\geq$ 4 months old	516	17	7.5	4.6, 12.3	< 0.01
	Eggs	39	24	0.40	0.24, 0.68	< 0.01
	Varying age	29	3	2.4	0.73, 7.9	0.15
Nightly pen location	Back to barn	2520	521	Ref		
	Near field	672	173	0.80	0.67, 0.97	0.03
	Either	76	16	0.98	0.57, 1.7	0.95
Transport modality	Truck	2788	592	Ref		
	Walk	130	59	0.47	0.34, 0.64	< 0.01
	Walk and truck	350	59	1.3	0.94, 1.7	0.12

*Non-parametric Wilcoxon test.
†Chi-square analysis for equality of proportions.

parameters were not included in the multivariable analysis, as they may be on the causal pathway between exposure and the outcome of influenza seropositivity.

Multivariable analysis, NP ELISA

The final GEE multivariable model included duck age at sampling, contact with other poultry, visitors to the flock and duck purchase age (Table 2), all which were positively associated with seropositivity. The final model's estimated working correlation was 0.004, indicating that, after controlling for independent parameters, 0.4% of the outcome variance of influenza A seropositivity occurs at the flock level. In other words, belonging to a certain flock has little to do with exposure to influenza A viruses, because seropositivity is ubiquitous.

Table 2. Results of multivariable analysis of flock and duck factors for association with duck seropositivity on NP ELISA (n = 3978)[a]

Independent variable	Category	OR[b]	95% CI	*P*-value	Confidence limit ratio
Age	2-month difference	2.3	1.7, 3.2	< 0.001	1.9
Contact with other poultry	No	Ref			
	Yes	3.9	2.1, 7.1	< 0.001	3.4
Visitors come to flock	No	Ref			
	Yes	4.5	2.1, 9.3	< 0.001	4.4
Duck purchase age	< 4 months old	Ref			
	≥ 4 months old	5.7	2.3, 13.8	< 0.001	6.0
	Eggs	1.2	0.75, 1.9	0.44	
	Varying age	0.50	0.19, 1.3	0.15	

[a]The working correlation estimate for this model was 0.004.
[b]Measures of association are adjusted for flock membership by inclusion of flock as a cluster variable.

H5 seropositivity

H5-seropositive ducks were located in all nine districts with registered flocks (Fig. 1). Of the 1423 NP ELISA-positive specimens assayed with H5 ELISA, 553 (39%) were positive for H5 antibodies, 57 (4%) were suspect and 813 (57%) were seronegative. Two (6.7%) of 30 NP ELISA-negative samples were positive on H5 ELISA. The manufacturer-provided negative and positive controls and the H5-positive serum controls consistently returned the expected result. The 1423 samples were from 194 different flocks. Of these, 151 flocks (78%) had at least one H5 ELISA-positive or suspect specimen, with the youngest flock being 2 months old.

The 610 positive and suspect samples were assayed using HI; 74 (12%) had a positive titre ($\geq$ 1 : 20; range 1 : 20–1 : 160), 90 (15%) had a negative but detectable titre (< 1 : 20) and 446 (73%) had no detectable titre. The 74 specimens with titres $\geq$ 1 : 20 belonged to 49 flocks, located all 9 study districts (Fig. 1).

All samples from 18 flocks with 100% seropositivity on NP ELISA (n = 564) were tested using the H5 ELISA to estimate flock-level H5 seroprevalence. Two of 18 flocks were seronegative for H5. The median within-flock seroprevalence was 44% (inter-quartile range 16–71), and maximum seroprevalence was 87%. Flock seroprevalence was not correlated with age (data not shown).

Analysis, H5 ELISA

Duck characteristics with regard to H5 seropositivity by H5 ELISA can be seen in Table 3. There was no difference in health history between H5-positive and H5-negative ducks (data not shown). Because H5 analysis includes only ducks positive for influenza A, there was selection bias in generating the dataset. Exposures that were evaluated in the NP ELISA analysis, as well as unmeasured exposures, influenced the ducks' selection for inclusion in the H5 analysis. Thus, the univariate results table (Table 3) is useful to appreciate the distribution of seropositive and seronegative ducks in each flock management category, but the statistical associations may be biased. The multivariable analysis is a more reliable estimate of the association between duck characteristics and seropositivity, as both measured and unmeasured exposures are controlled for by inclusion of flock as a cluster variable. After multivariable analysis (GEE modelling) controlled for flock, duck H5 seropositivity by ELISA is explained by duck age alone (Table 4). The estimated working correlation for the final model was 0.37, indicating that 37% of the outcome variance of duck H5 seropositivity is at the flock level.

Discussion

Influenza viruses

Our results indicate that FGDF in Suphanburi are widely exposed to influenza A viruses, including subtype H5, and these viruses have circulated in flocks as recently as May of 2010, as the youngest seropositive flock was 2 months old. While influenza A seropositivity in waterfowl is a normal finding (World Organization for Animal Health, 2011a), of the influenza-seropositive ducks tested, 39% were also positive on H5 ELISA, and 27% of these were positive by H5 HI, the international serological standard (World Organization of Animal Health, 2011). Because FGDF are moved around Suphanburi Province and blood was collected at only one time point, we are unable to determine where in Thailand the ducks were exposed to influenza viruses.

For even the oldest ducks, those with positive H5 titres were exposed when there were no known H5N1 outbreaks in Suphanburi Province (after November 2005). The assays used in this study do not distinguish between exposure to HPAI H5N1 and other H5 viruses (LPAI or HPAI), nor do they allow us to determine if the FGDF are maintaining virus circulation or were seropositive due to spillover virus from free-living birds. Nonetheless, FGD are a potential reservoir for influenza viruses, including subtype H5N1 between identifiable outbreaks, and these findings underscore the importance of year-round surveillance, including during summer months.

Recently, a small number of H4, H6 and H10 viruses were isolated from healthy Muscovy ducks at a central Bangkok live bird market (Wisedchanwet et al., 2011). Additionally, H12N1 viruses were isolated from wild ducks and a watercock in Central Thailand, both species which may frequent flooded rice fields (Wongphatcharachai et al., 2012). Other avian species known to feed at Thai rice fields, such as the little egret, Eurasian tree sparrow, scaly-breasted munia, common myna, white-vented myna, Asian open-bill

Table 3. Results of univariate analyses of flock and duck factors for association with duck seropositivity on H5 ELISA (n = 962)

Parameter	Median for positive ducks (IQR)	Median for negative ducks (IQR)	*P*-value*
Flock size	2000 (1200, 3000)	1550 (1300, 2800)	0.89
Age (months)	9.0 (8.0, 14)	7.0 (4.5, 10)	< 0.01
Age when first grazed (days)	30 (30, 60)	30 (25, 60)	0.22
Time spent grazing over lifetime (months)	4.0 (2.0, 7.0)	3.0 (2.0, 5.0)	< 0.01

Parameter	Category	Number of positive ducks (n = 405)	Number of negative ducks (n = 557)	OR	CI	*P*-value†
Duck use	Meat	0	15			< 0.01
	Eggs	405	542	–		
Grazed on rice fields for entire life to date	No	252	314	Ref		
	Yes	153	243	0.78	0.60, 1.0	0.07
Egg collection modality	Both/no eggs	14	55	Ref		
	Picked up	324	416	3.1	1.7, 5.6	< 0.01
	Bring to market	67	86	3.1	1.6, 6.0	0.01
Egg collections per week	≤ 1	38	148	Ref		
	2–4	276	331	3.2	2.2, 4.8	< 0.01
	≥ 5	91	78	4.5	2.8, 7.3	< 0.01
Owner has other poultry	No	220	305	Ref		
	Yes	185	252	1.0	0.79, 1.3	0.89
Duck contact with other poultry	No	347	437	Ref		
	Yes	58	120	0.61	0.43, 0.86	< 0.01
Visitors come to flock	No	206	211	Ref		
	Yes	199	346	0.59	0.45, 0.76	< 0.01
Ducks graze year-round	No	66	170	Ref		
	Yes	339	387	2.3	1.6, 3.1	< 0.01
Ducks have moved out of province	No	368	529	Ref		
	Yes	37	28	1.9	1.4, 3.2	0.01
Ducks have moved out of district	No	342	479	Ref		
	Yes	63	78	1.1	0.79, 1.6	0.50
Ducks have moved out of village	No	139	285	Ref		
	Yes	266	272	2.0	1.5, 2.6	< 0.01
Duck purchase age	Eggs	2	8	Ref		
	< 4 months old	331	481	Ref		
	Varying age	5	0	Ref		
	≥ 4 months old	67	68	1.4	0.99, 2.1	0.06
Nightly pen location	Back to barn	321	482	Ref		
	Near field	77	67	1.7	1.2, 2.5	< 0.01
	Either	7	8	1.3	0.47, 3.7	0.60
Transport modality	Truck	369	482	Ref		
	Walk	10	41	0.32	0.16, 0.64	< 0.01
	Walk and truck	26	34		0.60, 1.7	0.99

*Non-parametric Wilcoxon test.
†Chi-square analysis for equality of proportions.

Table 4. Results of multivariable analysis of flock and duck factors for association with duck seropositivity on H5 ELISA (n = 962)[a]

Independent variable	Category	OR[b]	95% CI	*P*-value	Confidence limit ratio
Age	2-month difference	1.3	1.1, 1.5	< 0.01	1.3

[a]The working correlation estimate for this model was 0.37.
[b]Odds ratio is adjusted for flock membership by inclusion of flock as a cluster variable.

stork, heron and dove, have been identified with confirmed HPAI H5N1 infection (Dierauf et al., 2006; Siengsanan et al., 2009). Of these infected species, the scaly-breasted munia, Asian open-bill stork and dove have tested positive in Thailand. Surveillance elsewhere in Asia indicates that a variety of LPAI subtypes has been present in wild or domestic avian populations since 2000, including H5N2 viruses in poultry of Japan and Taiwan, and H5N3 in domestic and wild species of Southern China (Alexander, 2007; Duan et al., 2007). Also identified in ducks of Southern China are

LPAI H3, H4, H6, H8, H10 and H11 viruses (Duan et al., 2007), and H9N2 subtype influenza has been endemic in Asian poultry since the 1990s (Swayne and Halvorson, 2008). These and other unclassified LPAI virus subtypes may have contributed to the nearly 100% flock influenza A seropositivity found here.

The large proportion of H5 ELISA-positive, H5 HI-negative samples (88%) may indicate exposure to a LPAI H5 virus, particularly as a whole-virus LPAI H5N2 was used in the ELISA. Additionally, while recent molecular studies show minimal virus evolution of the predominant clade 1 viruses beyond genetic drift in Central Thailand, there is some evidence of reassortment (Amonsin et al., 2010), and incursions of non-clade 1 HPAI H5N1 cannot be ruled out. Studies have shown limited cross-reactivity and low neutralizing titres between clade 1 antigens and clade 2.3.4 anti-serum (Chen et al., 2004, 2006; Balish et al., 2010). Low titres could also result from waning antibody titres or poor seroconversion. Experimental studies report low influenza HI titres in ducks after experimental infection (Saito et al., 2009). Regardless of aetiology, low titres could allow persistence of virus in ducks during the hot summer months as suggested elsewhere (Chaichoune et al., 2009; Amonsin et al., 2010; Magor, 2011). Additionally, minimal duck seroconversion may help limit virus evolution in Central Thailand, particularly in an unvaccinated population, as host immunity plays a role in influenza virus evolution (Webster et al., 1992).

Risk factors for influenza seropositivity

It is not surprising that age was associated with seropositivity, as older ducks accrued more time for exposure. Ducks that are grazed year-round and have grazed for their entire lifetime were less likely to be NP ELISA positive on univariate analysis, perhaps because they were kept at a lower flock density for longer periods than ducks that were penned or kept in a barn. When grazing, ducks move around the field in a loose group, resulting in a lower contact rate than would be experienced in a closed location. Ducks in confined pens or barns consume water from buckets or small ponds, rather than from water-covered rice fields, facilitating waterborne transmission. Duck distancing as a protective factor is further supported by increased odds of seropositivity for ducks undergoing vehicular transport and inter-provincial movement (Table 1). Transport is a high-density, stressful experience, often with two thousand ducks confined in one multilevel truck. High road density and short distance to highway junctions have been identified as spatial risk factors for poultry H5N1 outbreaks, supporting the association between transport and influenza infection (Ward et al., 2008; Paul et al., 2009; Rivas et al., 2010; Loth et al., 2010; Yupiana et al., 2010). One longitudinal study in Indonesia found that confining home-based scavenging ducks overnight decreased the risk of developing H5 antibodies (Henning et al., 2012), indicating that exposure risks may differ by country and duck management system. More research is needed to tease out the differences between FGDF that are moved from place to place and FGDF that graze only nearby the home.

In Thai smallholder flocks, biosecurity is often not practiced, and farm visitors are often other poultry owners or poultry-related traders. Such visitors have been identified as a risk factor for H5N1 outbreaks in Vietnamese smallholder poultry farms and in other countries (Henning et al., 2009; Fasina et al., 2011). Facilitated by people moving among flocks, movement of faecal material and virus-containing debris is a known risk factor for influenza transmission (Webster et al., 2006; Halvorson, 2008). In our univariate analysis, flocks that sold eggs had higher odds of seropositivity for influenza A (Table 1). Poultry traders onsite and the exchange of egg trays have been identified as risk factors for HPAI H5N1 infection (Biswas et al., 2009a; Desvaux et al., 2011). Contact between ducks and other poultry, which was associated on univariate analysis with influenza A seropositivity, has been previously identified as a risk factor for backyard chicken HPAI H5N1 outbreaks (Biswas et al., 2009b), and asymptomatic ducks and chickens living together have been found to be positive simultaneously for H5 virus RNA (Henning et al., 2010).

The final H5 multivariable GEE model indicates that 37% of the variance in duck H5 seropositivity is at the flock level (i.e. flocks must be in the right place at the right time for exposure). This differs from the flock-level variance identified in the NP ELISA analysis (0.4%). This finding may be due to a limited geographic distribution of H5 subtype viruses as compared to influenza A viruses generally; however, we cannot ignore the potential role of transmission variation. HPAI H5N1 viruses are preferentially shed via the respiratory tract in ducks, while other influenza A viruses replicate primarily in gastrointestinal mucosa (Perkins and Swayne, 2002; Sturm-Ramirez et al., 2004).

Study limitations

It is possible that there were unregistered FGDF in Suphanburi (and therefore not included in the study) who intentionally or unintentionally missed the registration call. In addition, flocks from other provinces could have been grazing in Suphanburi Province at the time of the study. These flocks would not have been registered in the province and thus not included in our study. We do not know management and movement details for the 139 flocks that were out of the province or could not be contacted, which may have led to systematic differences in the flocks included in our study. This may be especially true with regard to a difference

in movement practices for the flocks outside the province. We were able, however, to enrol 60% of the flocks in the province, yielding a large sample size for data analysis. In addition, due to careful questionnaire translation and interviewing, the data collected from the interviewed flocks are reliable and informative.

The sensitivity and specificity of the H5 ELISA could not be obtained from the manufacturer, and it is unknown how these attributes change with virus pathogenicity, subtype and time after infection. Two samples negative on the NP ELISA were positive on H5 ELISA, a finding perhaps due to imperfect sensitivity and specificity of the NP and H5 tests, respectively, or a longer persistence of antibodies to H5 antigens than NP antigens. As with any study that uses a diagnostic test with imperfect sensitivity and specificity, it is possible that some ducks were misclassified as to their seropositivity status. While misclassification with regard to flock management parameters in this analysis is likely non-differential, we cannot be certain that the sensitivity and specificity of the diagnostic tests used are not affected by a duck characteristic such as age. This study is limited by the inability to conduct the H5 subtype assays on all serum samples. In addition, the subset of samples for H5 analysis was not selected randomly (see Materials and Methods), because the authors were interested in identifying the flock seroprevalence of H5 where possible.

FGD surveillance

Free-grazing duck owners must register in the owner's home province and, after testing negative for HPAI H5N1 by cloacal swabbing, are given a flock passport and can graze within the province (Personal communication, Department of Livestock Development 2011). Pre-movement swabbing is required before leaving the province, but as flock movement often depends on rapidly fluctuating factors, including field availability, feed supply and flooding, FGD owners often leave their province on short notice and without testing (Beaudoin, unpublished data).

One approach for improved surveillance of these flocks could be cloacal and oropharyngeal sampling of FGDF *after* transport out of the province. If transport creates opportunities for within-flock influenza transmission and viral shedding, sample collection after 3 days (the approximate latent period) may provide a means to detect virus (Sturm-Ramirez et al., 2004; van der Goot et al., 2005; Tian et al., 2005; Bouma et al., 2009). Such a strategy may be accepted by flock owners for whom changing location when feed has been exhausted is imperative. This would also improve the knowledge of how many visiting FGDF are in a province at any time. Interviews with livestock officers indicate that because movements are rarely reported, the number of flocks in an area is often unknown (Beaudoin, unpublished data). While not ideal, identification of an HPAI H5N1-positive flock after transport is preferable to no diagnosis. Another method that would provide an opportunity to monitor flocks for HPAI H5N1, as well as generate data regarding LPAI viruses, would be sampling water from troughs that are used by potentially thousands of penned ducks each night. These containers present an environment for virus collection and persistence (Sturm-Ramirez et al., 2004; VanDalen et al., 2010). Additionally, as co-penning of ducks and chickens has been found to be a risk factor for backyard chicken outbreaks (Biswas et al., 2009b), one or more caged sentinel chickens in the night pen of FGDF may present a non-invasive and inexpensive way to monitor for subclinical virus shedding.

We have shown that some FGD in Central Thailand were exposed to H5 influenza viruses as recently as May 2010. This supports the hypothesis that H5 influenza viruses may circulate at low levels within FGD populations when there are no overt poultry outbreaks. Ongoing surveillance of FGDF, a population representing the intersection of wild birds and domestic poultry, is crucial to improving the knowledge of influenza viruses circulating in Thailand. Intensive antigen-based surveillance will also facilitate the generation of comprehensive panels of viruses for use in diagnostic tests and improving laboratory capabilities.

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Conflicts of interest

The authors have no conflicts of interest to report.

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