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Influenza in Canada geese*

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The role of wild avian species in the natural history of influenza is unknown. A serological study was carried out to ascertain the prevalence, distribution, and types of influenza antibody in several wild Canada goose populations. Geese were trapped and blood samples were obtained in each of 4 consecutive years, 1966–69. Antibody to influenzavirus was found in 66 (4.7%) of the 1 401 Canada geese tested by the haemagglutination inhibition (HI) test. Antiribonucleoprotein antibody was found in 8 of 1 359 sera tested by the agar gel precipitation (AGP) test. An increase in the percentage of reactors was seen each year. This increase was greater in two refuges with nonmigratory flocks. HI antibody was found against the turkey/Wisconsin/66, turkey/Wisconsin/68, turkey/Canada/63, and turkey/Alberta/6962/66, or closely related viruses. No antibody was found against duck/Ukraine/1/63 or human A/Hong Kong/68 virus at a time when the latter was prevalent in human populations, suggesting that Canada geese played no direct role in spreading the virus.

Canada geese were experimentally exposed to turkey/Wisconsin/66 and turkey/Wisconsin/68 viruses; mallard ducks were exposed to turkey/Wisconsin/66 virus. HI antibody developed in 75% of the geese and 40% of the ducks but was generally short-lived. Anti-RNP antibody was detected in 15% of the exposed geese but in none of the ducks. Virus was recovered from 3 of 10 adult ducks but not from geese. None of the birds showed signs of disease.

The public health significance of type A influenza infection in avian species is not known. Indirect evidence suggests, however, that birds may play a role in the epidemiology of human influenza (Webster & Pereira, 1968; Tumová & Pereira, 1968).

Most studies of avian influenza have been concerned with the economic importance of the disease in domestic fowl. Type A influenzaviruses have caused severe economic losses in domestic poultry when the infecting agent was one of the highly virulent strains such as fowl plague, turkey/England/63, or chicken/Scotland/59 (Stubbs, 1965; Wells, 1963; Pereira et al., 1965). Economic losses caused by the less virulent strains are more difficult to measure but are probably just as important since these viruses affect egg production, hatchability, and the rate of food conversion (Bankowski & Mikami, 1964; Lang et al., 1965).

The importance of influenza in wild birds has been studied only superficially and it is not known whether they are a possible source of infection for domestic species, a cause of mortality in economically or aesthetically important wild species, or whether they are important in the epidemiology of human influenza.

In recent years, only one isolation from a wild bird species has been reported: tern/South Africa/61 virus was isolated from common terns, *Sterna hirundo*, during an epizootic on the coast of South Africa in 1961 (Becker, 1966). Easterday et al. (1968) tested 50 serum samples representing 9 species of North American wild birds for influenza antibody and found HI antibody in 11 of the sera tested (from snow geese, *Chen hyperborea*, and Canada geese, *Branta canadensis*. More recently, Dasen & Laver (1970) demonstrated A/Singapore/57 antineuraminidase antibody in the serum of shearwaters in Australia.

There have been few reports on the experimental

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infection of wild birds with influenza. Homme & Easterday (1970) described the experimental exposure of pen-reared pheasants (*Phasianus colchicus*), mallard ducks (*Anas platyrhynchos*), and trapped wild Canada geese to turkey/Wisconsin/66 virus. Virus was recovered from pheasant and mallard tracheal swabs for up to 10 days after exposure; no virus was recovered from the Canada geese.

The serological study of some North American Canada goose populations described in this report represents an extension of the work described by Easterday et al. (1968). The purpose of this study was to expand the information available regarding the prevalence of influenza antibody in Canada geese in North America and to look for differences in antibody prevalences in several populations of Canada geese that might help clarify the epidemiology of influenza in geese or establish a relationship between influenza in geese and other hosts.

The experimental infections described in this report were intended to provide information that would be useful in interpreting the results of serological surveys and in evaluating the role of these species as potential hosts of some influenzaviruses.

MATERIALS AND METHODS

Canada goose populations

Canada geese were collected at 8 refuges in the USA between 1966 and 1969. The birds use these refuges as follows:

Seney Wildlife Refuge, Michigan. This federal area in the upper Michigan peninsula is the nesting ground for geese from the Tennessee Valley population in the Mississippi flyway. Migrants from other populations in the same flyway also use it as a rest area during spring and autumn migrations.

Crex Meadow Refuge, Wisconsin. This federal refuge in north-west Wisconsin is the home of a captive breeding flock of geese derived from hunting season cripples collected at Horicon Marsh Refuge in southern Wisconsin and from birds acquired from a private aviculturist. Transient geese of the Eastern Prairie and Mississippi Valley populations mix with the resident population during migrations.

Green Bay, Wisconsin. Bay Beach Municipal Park in Green Bay is the home of a nonmigratory population of geese. Migrants in the Mississippi flyway, primarily the Mississippi Valley population, mix freely with these birds.

Horicon Refuge, Wisconsin. This federal–state refuge in south-east Wisconsin serves as a " staging area " or early migration collecting point for most of the birds of the Mississippi Valley population.

Squaw Creek Refuge, Missouri. This federal refuge in north-west Missouri is a resting area for migrant birds from the Western Prairie population of the central flyway and the Eastern Prairie population of the Mississippi flyway. It serves as the southern wintering ground for many birds in these populations.

Swan Lake Refuge, Missouri. This federal refuge in central Missouri is an important wintering ground for birds of the Eastern Prairie population of the Mississippi flyway. It is also a resting area for migrants flying south.

Horseshoe Lake Refuge, Illinois. The state refuge in the extreme south of Illinois has in recent years become an important overwintering ground for birds of the Mississippi Valley population. Many of the geese moving south from Horicon Refuge overwinter there.

Wilson Hill Refuge, New York. This state hunting preserve in the north-west of New York State is in the Atlantic flyway. Migrant birds from Canada use this as a resting area, and it is also a summer nesting area for both migrant and sedentary flocks.

Geese were trapped for sampling in winter or early summer. Summer collections were made from flightless moulting birds and winter collections from free-flying birds. The birds were classified as juvenile, adult, or unknown. The juveniles were the young of the year, and the adults were 1 year old or more. Many birds were sexed, but those whose sex was not established are classified as " unknown ".

Laboratory procedures

Viruses. Virus strains used to test all sera were : A/Hong Kong/1/68(H3N2), turkey/Wisconsin/66(Hav6N2), turkey/Wisconsin/68(Hav5N?), turkey/Canada/63(Hav6Neq2), turkey/Alberta/6962/66(Hav 4N?) and duck/Ukraine/1/63(Hav7Neq2). Viruses were cultivated in the allantoic cavity of 10-day-old embryonated eggs. Eggs were incubated for 30 hours and the allantoic fluids were harvested and stored at $-65°C$ until used. The turkey/Wisconsin/66 and turkey/Wisconsin/68 viruses were used as infective allantoic fluids in the experimental infections of Canada geese and mallard ducks.

Haemagglutination (HA) test. The haemagglutination test was used to determine the HA titre of virus used in haemagglutination inhibition (HI) tests and to confirm the presence of virus in virus neutralization tests. Chicken erythrocytes (RBC) at a concentration of 0.5% in 0.01 M phosphate buffered saline, pH 7.2, were used in the HA test.

Blood collection. Blood samples were collected by peripheral venepuncture or by cardiac puncture and the serum was stored at −20°C until tested.

Haemagglutination inhibition (HI) test. The HI test was conducted by the technique of Jensen (1956) except that macroplates[1] were used. The serum to be tested was treated by a modification of the technique described by the National Communicable Disease Center (1969). To 0.2 ml of test serum, 0.8 ml of receptor destroying enzyme (RDE) was added, and the mixture was incubated for 12–18 hours at 36°C. Then 0.6 ml of a 2.5% w/v sodium citrate solution was added; the mixture was held at 56°C for 30 minutes and was then cooled to 4°C. To the cooled mixture was added 0.4 ml of a 20% chicken RBC suspension; this mixture was held for 1 hour at 4°C and then centrifuged at low speed for 10 minutes; the serum was decanted and either tested immediately or held at −20°C for no more than 24 hours and tested.

Virus neutralization (VN) test. The virus neutralization test was carried out in 10-day-old embryonated eggs by the technique of Jensen (1956). Three eggs were inoculated in each 4-fold dilution with 0.2 ml of serum–virus mixture. Simultaneous titration of the test virus was carried out using three eggs per dilution. Eggs were allowed to incubate for 30 hours after inoculation; they were then chilled for 12 hours and 0.5 ml of allantoic fluid was harvested into plastic macroplates and tested for HA activity.

Agar gel precipitin (AGP) test. The AGP test for ribonucleoprotein (RNP) antibody was performed as described by Beard (1970).

Experimental infections

Experimental birds. The Canada geese used in these experiments were wild adults trapped at Horicon Refuge. Mallard ducks were reared in pens from hatchlings obtained from commercial sources. Three age classes of ducks were used: juveniles, 2 weeks old; subadults, 6 weeks old; and adults, 1 year old.

Exposure methods. Aerosol exposure of birds was carried out as described by Beard & Easterday (1965).

Contact exposure of birds was effected when the aerosol-exposed birds were returned immediately after exposure to the rooms in which they had been held with previously unexposed birds.

Virus recovery. Tracheal swabs for virus isolation attempts were collected on the same days on which blood samples were taken. Sterile cotton-wool-tipped applicators were inserted 8-15 cm into the trachea and immediately immersed in 1.0 ml of brain–heart infusion broth (Difco) containing 2 mg of dihydrostreptomycin and 100 000 IU of penicillin K per ml. These samples were tested within 2 hours or were stored at −65°C until tested. The broth in which the swabs had been immersed was tested by inoculating 0.1 ml of it into the allantoic cavity of 10-day-old embryonated eggs. After incubation for 30 hours the eggs were chilled and the allantoic fluid was harvested and tested for HA activity.

Experimental procedures

Experiment 1. Ten Canada geese were exposed to aerosols that contained $10^{2.8}$–$10^{3.1}$ 50% egg infective doses (EID_{50}) of turkey/Wisconsin/68 virus per litre of air. The birds were bled and swabbed on days 4, 8, 12, 16, 29, and 39 and were tested for antibody conversion by HI and AGP tests and for virus isolation by egg inoculation of tracheal swab washings.

Experiment 2. Five Canada geese were exposed to aerosols that contained $10^{3.7}$ EID_{50} of turkey/Wisconsin/66 virus per litre of air. Immediately after exposure these birds were placed in a room with five unexposed Canada geese, which then became the contact-exposed group. All geese were bled and swabbed on days 6, 10, 14, 18, 22, 26, 32, and 48 and were tested for antibody and virus isolation as in experiment 1.

Experiment 3. Five mallard ducks in each age group (juvenile, subadult, and adult) were exposed to aerosols containing $10^{3.4}$–$10^{3.7}$ EID_{50} of turkey/Wisconsin/66 virus per litre of air. Immediately after exposure the birds were placed with unexposed groups of the same age class. There were 5 adult, 5 subadult, and 10 juvenile birds in the contact-exposed group. The ducks were bled and tested

[1] Disposo-tray, 96-U-WS, supplied by Linbro Co., New Haven, Conn., USA.

Table 1. Influenza haemagglutination inhibition test results from Canada goose serological survey: distribution by refuge and year of collection [a]

Refuge	1966	1967	1968	1969	Total
Seney	0/45 (0.0)	NT	NT	5/86 (5.8)	5/131 (3.8)
Crex Meadow	1/25 (4.0)	NT	4/77 (5.2)	14/143 (9.8)	19/245 (7.8)
Green Bay	0/29 (0.0)	NT	1/47 (2.1)	14/134 (10.4)	15/210 (7.1)
Horicon	1/75 (1.3)	NT	6/108 (5.6)	16/334 (4.8)	23/517 (4.4)
Squaw Creek	1/39 (2.6)	NT	NT	NT	1/39 (2.6)
Swan Lake	0/104 (0.0)	NT	NT	NT	0/104 (0.0)
Horseshoe Lake	NT	1/55 (1.8)	NT	NT	1/55 (1.8)
Wilson Hill	NT	NT	NT	2/100 (2.0)	2/100 (2.0)
Total	3/317 (1.0)	1/55 (1.8)	11/232 (4.7)	51/797 (6.4)	66/1401 (4.7)

[a] The figures in each column give the number of positive reactors/the total number tested, and in parentheses the percentage that were positive. NT = not tested.

as in the first two experiments, but only the adult ducks were swabbed for virus isolation.

RESULTS

Serological survey

Of the 1 401 Canada geese tested for influenza HI antibody, 66 (4.7%) had HI titres of 1 : 20 or greater (Table 1). Birds with positive titres were found in 12 of the 15 collections. The three negative collections were made in 1966. The highest proportion of reactors was found in the 1969 collections made at Crex Meadow and Green Bay, 9.8 and 10.4%, respectively. The proportion of reactors at each of the refuges increased each year, from 1.0% in 1966 to 6.4% in 1969.

Positive reactions occurred against the turkey/Wisconsin/66, turkey/Wisconsin/68, turkey/Canada/63, or turkey/Alberta/6962/66 virus strains; there were none against the A/Hong Kong/68 or duck/Ukraine/1/63 strains. Forty serum samples had demonstrable antibody against a single test virus; 26 had demonstrable antibody against two or more test viruses. Three sera gave HI titres of 1 : 160 but in general the titres were between 1 : 20 and 1 : 80.

As far as possible the distribution of reactors by age and sex was determined. The highest proportion (7.6%) of reactors was in a group for which the age and sex were unknown. Among the identified birds the highest proportion (5.7%) was among the adult females. There were many more positive adults (4.8%) than juveniles (1.6%). There was little difference between the number of positive samples collected in winter (3.7%) and in summer (5.5%), and approximately equal numbers of samples were collected in the two seasons.

In addition, 1 359 of the 1 401 goose sera were tested for type-A RNP antibody by the AGP test: 8 (0.6%) of the 1 359 sera were found to be positive (Table 2). Three of the 8 positive sera were negative for HI antibody. All but 1 of the 8 positive sera were collected in 1969, and 5 were collected from Horicon Refuge in 1969.

Virus neutralization tests were carried out on 4 HI-positive and 2 HI-negative sera. The results of the neutralization tests correlated well the HI test results.

Experimental infections

Of the 10 geese exposed to aerosols of turkey/Wisconsin/68 in the first experiment 8 had HI antibody by day 12 after exposure; only 2 of the 8 had antibody 27 days later. Anti-RNP antibody was demonstrated in 1 goose on post-exposure days 8, 12, and 16, and in a second goose on days 8, 12, and 29. Virus was not recovered from the tracheal swabs from any of the 10 exposed geese.

HI antibody developed in all 5 geese exposed to aerosols of turkey/Wisconsin/66 virus. The antibody was of short duration, being demonstrated at

Table 2. Haemagglutination inhibition test results of all Canada goose sera found to be positive by agar gel precipitin test [a]

Bird no.	Refuge	Date	Age/Sex	turkey/ Wisconsin/ 66	turkey/ Wisconsin/ 68	turkey/ Canada/ 63
23	Green Bay	summer 1969	U/U	160	80	80
48	Horicon	winter 1969	adult/U	20	0	20
49	Horicon	winter 1969	adult/U	160	160	0
54	Horicon	winter 1969	adult/U	80	20	0
62	Horicon	winter 1969	adult/U	20	40	0
70	Wilson Hill	summer 1969	U/U	0	0	0
71	Crex Meadow	summer 1968	adult/male	0	0	0
72	Horicon	winter 1969	adult/U	0	0	0

[a] The figures indicate the reciprocals of end dilution. U = sex or age unknown.

most 32 days after exposure. Only 2 of the contact-exposed birds developed antibody. One of the aerosol-exposed geese had anti-RNP antibody on post-exposure days 10 and 14. Virus was not recovered from tracheal swabs collected from any of these birds.

In the third experiment, 8 (4 subadults and 4 adults) of the 15 ducks exposed to aerosols of turkey/Wisconsin/66 virus developed HI antibody. Only 1 had antibody 67 days after exposure. Of the 20 ducks exposed by contact to the aerosol-exposed ducks, 2 of 5 adults, 1 of 5 subadults, and 3 of 10 juveniles had HI antibody of low titre (they all had titres of 1 : 20 except one adult which had a titre of 1 : 80). No anti-RNP antibody could be demonstrated in any of the ducks.

Sera collected on day 37 after exposure from 3 aerosol-exposed birds (no. 466, 475, and 481) were tested for virus neutralizing antibody. All 3 birds had virus neutralizing antibody titres of 1 : 128 when tested against 250 EID_{50} of turkey/Wisconsin/66 virus. All 3 had shown HI antibody also, although 1 bird (no. 466) no longer had a detectable HI titre by day 36. Virus was isolated from tracheal swabs taken from 5 adult ducks (3 aerosol-exposed, and 2 contact-exposed birds).

DISCUSSION

The widespread geographical and temporal distribution of Canada geese with influenza HI antibody suggests that influenza infections have occurred in many of the goose populations of eastern and central North America for at least 4 years. Reactors were found in 12 of the 15 collections and in each of the 4 years when samples were taken. Reactors were found in several populations within the Mississippi flyway, in the only population sampled from the Atlantic flyway, and perhaps in one population from the central flyway. The positive serum from the Squaw Creek Refuge could have been either a Mississippi or a central flyway bird, since birds from both flyways use this refuge. No significant difference ($P > 0.05$) could be demonstrated in the proportions of reactors from the different refuges. No significant difference was found in the proportion of reactors by year, but an increase in the prevalence of reactors was seen between 1966 and 1969. When considered by individual refuges, a significant difference ($P < 0.05$) was found between the proportion of reactors at Crex Meadow and Green Bay refuges. These two refuges had a significantly higher proportion of reactors in 1969 (9.8% and 10.4%, respectively). Both have sedentary flocks of geese, captive and nonflying at Crex Meadow and flying but nonmigratory at Green Bay. The majority of the birds sampled at these two locations were from these nonmigratory populations, which suggests that sedentary flocks had a higher reactor rate than migrant birds, particularly in 1969. There is also some suggestion of an increase in reactor rates between 1966 and 1969 in the migratory flocks sampled at Horicon and Seney refuges, where samples of birds of different

ages were available. If the birds tested were representative of the populations sampled, then there has been a general increase in infection rates in the geese of the Mississippi flyway that has been reflected and amplified in the two nonmigratory populations.

It is probable that the exposure risk is greater for geese in the nonmigrant populations, which generally associate more closely with one another throughout the year, than for birds in the migrant populations; this is particularly true at Crex Meadow, where captive birds are restricted to the confines of a small 2.4-ha pen. The environmental factors that promote dissemination of influenza among birds in a flock or between flocks are not well understood, but intimacy of contact is unquestionably an important factor.

There was no significant difference ($P > 0.05$) between the distribution of reactors by age–sex groups, or by age or sex groups separately. There was a trend towards higher reactor rates in the adult groups. High reactor rates were seen in adult females at Green Bay and Crex Meadow and in the group for which the sex was not determined at Crex Meadow.

There was good correlation between the levels of HI and virus neutralizing antibody in the small numbers of goose sera tested in the serological survey. In one serum both HI and neutralizing antibodies were found against the turkey/Wisconsin/66 and turkey/Wisconsin/68 viruses. This suggests exposure to more than one virus, since no cross-relationship has been shown between these two viruses.

The presence of anti-RNP antibody is generally considered to be an indication of recent infection. If so, there is no significant difference ($P > 0.05$) in onset of infection between winter and summer, although the sample size was very small.

The absence of reactors to A/Hong Kong/68 and to duck/Ukraine/1/63 virus suggests that the birds had not been exposed to these or to closely related viruses. No reactors to turkey/Wisconsin/68 were found in sera collected before 1968. This suggests that this virus did not circulate in the sampled goose populations before it had been isolated from domestic fowl.

The complete absence of clinical disease in all the birds in the three exposure experiments indicates that these two viruses are not likely to cause morbidity and mortality in the same species in nature. However, the effects of extrinsic variables such as the stress of inclement weather were not introduced in the experiments and might alter the hosts' response to infection with these agents.

The demonstrable HI antibody in the experimentally exposed geese was usually short-lived; in every case antibody appeared between the fourth and twelfth day after exposure, and in all but two individuals persisted less than 35 days. The public health significance of the demonstration of influenzaviruses in wild birds remains unknown. However, the increasing amount of evidence linking antigens of avian viruses to antigens of human viruses (Pereira et al., 1967) suggests that the avian viruses in general may be more closely related to human isolates than was previously suspected. Before the significance of the present results can be adequately assessed it will be necessary to confirm the presence of influenzaviruses in wild goose populations by virus isolation.

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RÉSUMÉ

LA GRIPPE CHEZ LES OIES DU CANADA

On a mené une étude sérologique, à l'aide des épreuves d'inhibition de l'hémagglutination (IH) et de précipitation en gel de gélose, afin de rechercher la présence d'anticorps grippaux de type A chez des oies sauvages du Canada. Les oiseaux ont été capturés, saignés, puis remis en liberté dans 8 endroits de repos, aux Etats-Unis d'Amérique, abritant soit des populations sauvages migratrices soit des populations sédentaires semi-domestiquées. Quinze séries de prélèvements (1401 sérums) ont été effectuées de 1966 à 1969. Les échantillons ont été recueillis en hiver et en été chez des oiseaux adultes ou jeunes, mâles ou femelles. On a utilisé pour les épreuves IH les antigènes A/Hong Kong/

68, turkey/Wisconsin/66, turkey/Wisconsin/68, turkey/Canada/63, turkey/Alberta/6962/66 et duck/Ukraine/1/63; pour les épreuves de précipitation, un antigène ribonucléoprotéinique (RNP) non différencié de type A.

Des sérums positifs ont été trouvés dans 12 des 15 séries de prélèvements. Sur les 1401 sérums examinés, 66 (4,7%) renfermaient des anticorps IH contre les virus grippaux. On n'a pas relevé de différences notables des taux de positivité selon les catégories d'oiseaux, mais les femelles étaient plus fréquemment porteuses d'anticorps. La proportion des sérums positifs s'est accrue sensiblement de 1966 à 1969. Les anticorps ont été trouvés avec une fréquence maximale chez les oies appartenant à des populations sédentaires.

On a enregistré des réactions IH positives en présence des antigènes turkey/Wisconsin/66, turkey/Wisconsin/68, turkey/Canada/63 et turkey/Alberta/6962/66; aucun sérum n'a réagi avec les antigènes A/Hong Kong/68 et duck/Ukraine/1/63. Un tiers environ des sérums positifs contenaient des anticorps pour plusieurs des antigènes testés. Dans 8 (0.6%) des 1359 sérums examinés en épreuve de précipitation, on a décelé la présence d'anticorps actifs contre l'antigène RNP de type A.

On a exposé des oies et des canards sauvages à des aérosols de virus turkey/Wisconsin/66 et turkey/Wisconsin/68, tandis que d'autres étaient mis en contact avec ces animaux d'expérience. La plupart des oiseaux directement exposés et un certain nombre de contacts ont élaboré des anticorps IH contre les virus d'épreuve. Des anticorps RNP ont été trouvés chez une faible proportion des premiers. Tous les essais de réisolement de virus ont échoué et aucun animal n'a présenté de signe clinique de maladie.

La mise en évidence d'anticorps IH chez des oies du Canada appartenant à diverses populations sauvages donne à penser que l'infection naturelle par des virus grippaux est fréquente dans cette espèce. On n'a découvert aucun indice d'une infection par le virus A/Hong Kong/68, avant ou après son apparition chez l'homme, ni par la souche exotique duck/Ukraine/1/63. Par ailleurs, rien n'indique que l'infection grippale est une cause de morbidité ou de mortalité dans les deux expèces d'oiseaux étudiées.

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