ENVIRONMENTAL TOLERANCE OF A H4N6 AVIAN-ORIGIN TYPE A INFLUENZA VIRUS

A Thesis

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By

Charles F. Schwarten

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Honors Examination Committee:

Dr. Robert J. Gates, Advisor

Dr. Richard D. Slemons, Advisor

Dr. Roger A. Williams

Approved by

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Advisor
Honors Undergraduate Program in Natural Resources

Environmental Tolerance of a H4N6 Avian-Origin Type A Influenza Virus

Abstract:

Detection of avian-origin type A influenza virus infections has been well documented within domestic and wild bird populations. However, research focused on the ability of these viruses to remain viable under naturally fluctuating environmental conditions, which we have termed "environmental tolerance" (ET), has led to untested hypotheses about the existence of an environmental viral reservoir. By measuring ET, we can determine the potential importance of an environmental reservoir. We developed three microcosms (soil, water, and water-soil interface) using soil, water, and field data collected from Waterfowl Lake at the Cleveland Metroparks Zoo during the summer of 2008 to construct a controlled environmental model quantifying the ET of influenza A viruses. Focusing on the effect of mean, minimum and maximum summer temperatures recorded in Waterfowl Lake, we spiked three replicates of each microcosm with a known quantity of a H4N6 avian influenza virus. An aquarium with soil and water that was not spiked served as a negative viral control for the microcosms. We hypothesized that there would be both qualitative and quantifiable differences in virus viability among our microcosms and temperature treatments. Samples were collected over four weeks and tested for the presence of viable virus using virus isolation in 10-day old embryonating chicken eggs. Surprisingly, viral infectivity generally surpassed four weeks. Therefore, we demonstrated that type A influenza viruses have sufficient ET to remain viable for at least 30 days in a pond under summer environmental conditions and concluded that ponds could serve as an environmental reservoir for influenza A viruses and should therefore be considered when managing bird collections. Currently, we are determining the mean egg infective dose

concentration (EID_{50}) of virus in our samples to attempt to detect quantitative changes in virus concentration over time.

Introduction:

Avian influenza infections can be caused by any number of antigenically diverse type A influenza viruses maintained in wild birds. These viruses are commonly referred to as avian influenza viruses (AIVs) and can be classified as either highly pathogenic (HP), causing severe infection and high mortality, or low pathogenic (LP), causing only subclinical or mild disease in chickens following intravenous inoculation (Alexander 2004). Contained in a host-derived lipid bilayer envelope, AIVs have eight segments of negative sense, single stranded RNA which code for at least ten different structural and non-structural proteins (International Committee on Taxonomy of Viruses Database Management 2006). AIVs are subtyped based on the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), which serve as major surface antigens for these viruses. Specifically, HA mediates the fusion between the viral envelope and the host endosomal membrane, and NA mediates viral release by cleaving the host cell sialic acid receptors that are bound to the virus hemagglutinin. (Garcia-Sastre and Whitley 2006; Webster et al. 1992). There are a total of 16 HA subtypes and nine NA subtypes, allowing for a total of 144 HA-NA combinations (Webster et al. 1992).

While AIVs have historically infected birds, these avian-origin type A influenza viruses have also been known to genetically reassort with other influenza viruses (Perdue and Swayne 2005). Importantly, AIVs appear to have contributed genomic material in the emergence of the three human influenza pandemic viruses of the 20th century and consequently are the focus of increased interest. While the biological origin of the 1918 (H1N1) “Spanish Influenza” human pandemic virus remains uncertain, both the 1957 (H2N2) and 1968 (H3N2) human pandemic

viruses have been linked to genetic reassortment involving AIVs and human influenza viruses. Specifically, the 1957 human pandemic virus was derived from a human H1N1 influenza virus that acquired both avian-origin HA and NA encoding RNA segments, coding for a H2 hemagglutinin and a N2 neuraminidase, during a reassortment event with an AIV. Also, the 1968 human pandemic virus was derived from a H2N2 descendent of the 1957 human pandemic virus, which acquired a different HA encoding RNA segment, coding for a H3 hemagglutinin, after reassorting with another AIV (Scholtissek et al. 1978). These changes in surface proteins created novel viruses, which led to increased rates of infection due to a naïve human immune system (Kilbourne 2006).

The severity of infection from past influenza pandemics prompted serious concern when the Asian lineage highly pathogenic H5N1 AIV first surfaced in 1997, and again in 2003. While the other three major human pandemic viruses arose from the genetic reassortment of an AIV and a human influenza virus, this virus appeared to be completely avian in origin, being able to jump the species barrier to infect humans via direct contact with infected birds (Perdue and Swayne 2005). Also, as stated above, the H5N1 AIV exhibited high pathogenicity, with the virus causing a sustained mortality of 60% over seven years (World Health Organization 2009, Table 1). While the potential for the HP H5N1 AIV to become a human pandemic virus should be seriously considered, it is important to note that all of the major influenza pandemic viruses have originated from the reassortment of human influenza viruses with LP AIVs. Therefore, it is important to continue to monitor and further the scientific knowledge of both HP and LP AIVs.

Currently, the natural history of AIVs is of interest to management agencies and organizations, such as zoos, who want to develop emergency influenza protocols in the event of

	2003		2004		2005		2006		2007		2008		2009		Total	
Country	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths
Azerbaijan	0	0	0	0	0	0	8	5	0	0	0	0	0	0	8	5
Bangladesh	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0
Cambodia	0	0	0	0	4	4	2	2	1	1	1	0	0	0	8	7
China	1	1	0	0	8	5	13	8	5	3	4	4	7	4	38	25
Djibouti	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0
Egypt	0	0	0	0	0	0	18	10	25	9	8	4	25	4	76	27
Indonesia	0	0	0	0	20	13	55	45	42	37	24	20	0	0	141	115
Iraq	0	0	0	0	0	0	3	2	0	0	0	0	0	0	3	2
Lao People's Democratic Republic	0	0	0	0	0	0	0	0	2	2	0	0	0	0	2	2
Myanmar	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0
Nigeria	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1
Pakistan	0	0	0	0	0	0	0	0	3	1	0	0	0	0	3	1
Thailand	0	0	17	12	5	2	3	3	0	0	0	0	0	0	25	17
Turkey	0	0	0	0	0	0	12	4	0	0	0	0	0	0	12	4
Viet Nam	3	3	29	20	61	19	0	0	8	5	6	5	4	4	111	56
Total	4	4	46	32	98	43	115	79	88	59	44	33	36	12	431	262

Table 1. Cumulative number and distribution of laboratory confirmed human cases and deaths from the H5N1 AIV (WHO.int).

another pandemic (USDA 2006). However, to understand the natural history of AIVs, we must first understand the life history of the host species. Migratory waterbirds (Anseriformes and Charadriiformes in particular) are known to be a major reservoir for these viruses (Webster et al. 1992). These waterfowl migrate twice a year, flying south to their respective wintering grounds in the autumn, and returning to their breeding grounds in the spring. These migrations are seasonal, predictable and are repeated each year (Sibley 2001). Despite remaining questions about the evolutionary costs and benefits of this migratory behavior, many of these birds are known to be transcontinental migrants.

Waterfowl begin to cluster together in large concentrations during seasonal migrations, reaching numbers in the thousands at migratory stopover sites. The population of birds includes both adults and juveniles. With high densities of birds inhabiting the same area, disease transmission is much higher at this time than non-migratory periods (Wobeser 1997). When these birds congregate, AIVs infect many immunologically naïve juvenile birds, temporarily increasing the prevalence rate of these viruses. These infections can then be exacerbated if the birds become stressed. Generally, the prevalence of AIVs during this time can reach levels as high as 60% (Olsen et al. 2006).

Migratory waterfowl generally use pre-established routes to arrive at their respective destinations. These routes are collectively known as "flyways," and act as highways for migrating birds. In North America, four primary flyways have been recognized, which include the Eastern, Mississippi, Central, and Pacific Flyways. These flyways extend from the arctic (Figure 1), down through the United States (Figure 2) and into Central and South America. However, waterfowl do not always conform to such a simple migration scheme, leading to the

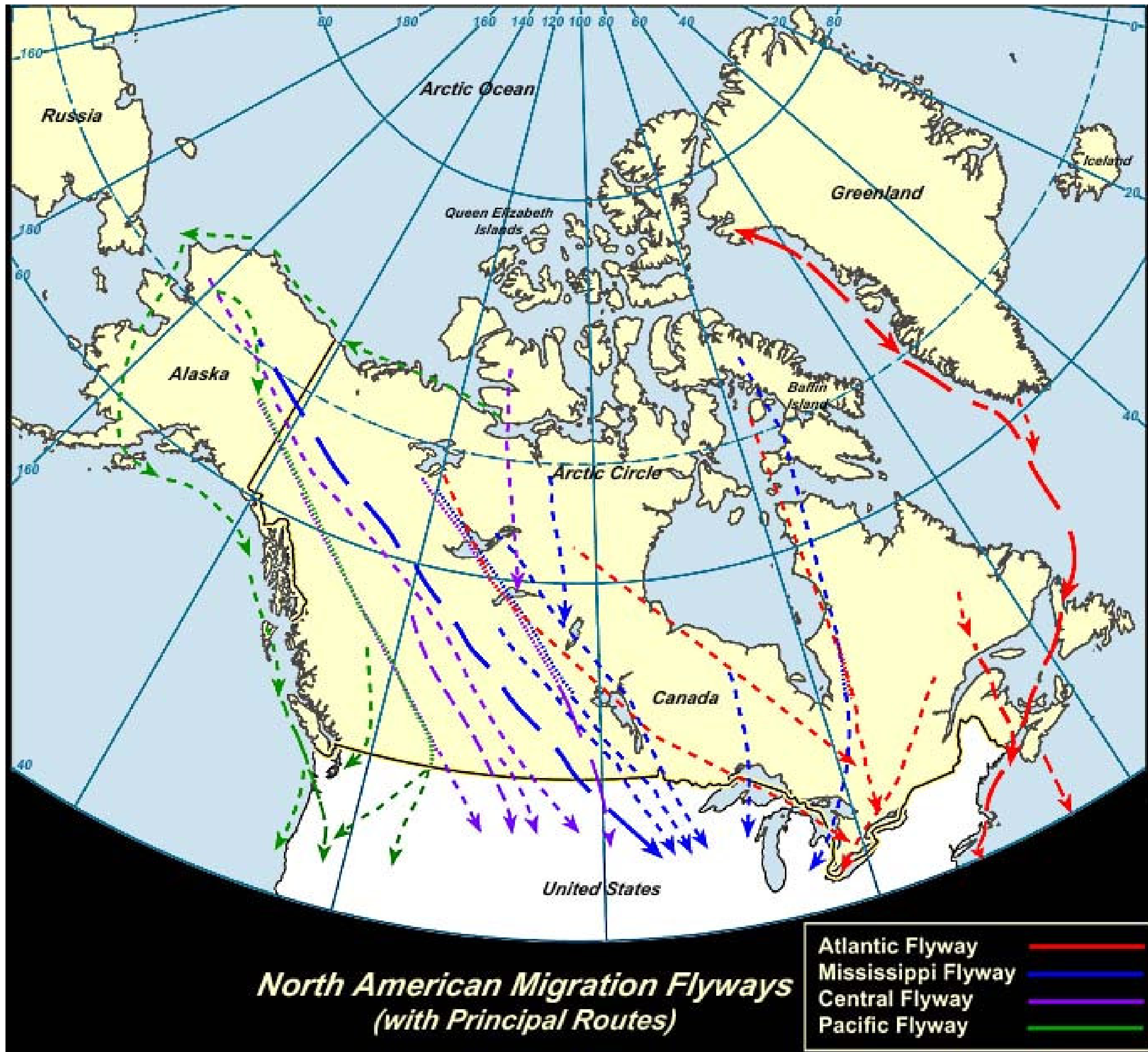

Figure 1. The northern limits of the North American flyways. (Image courtesy of www.birdnature.com)

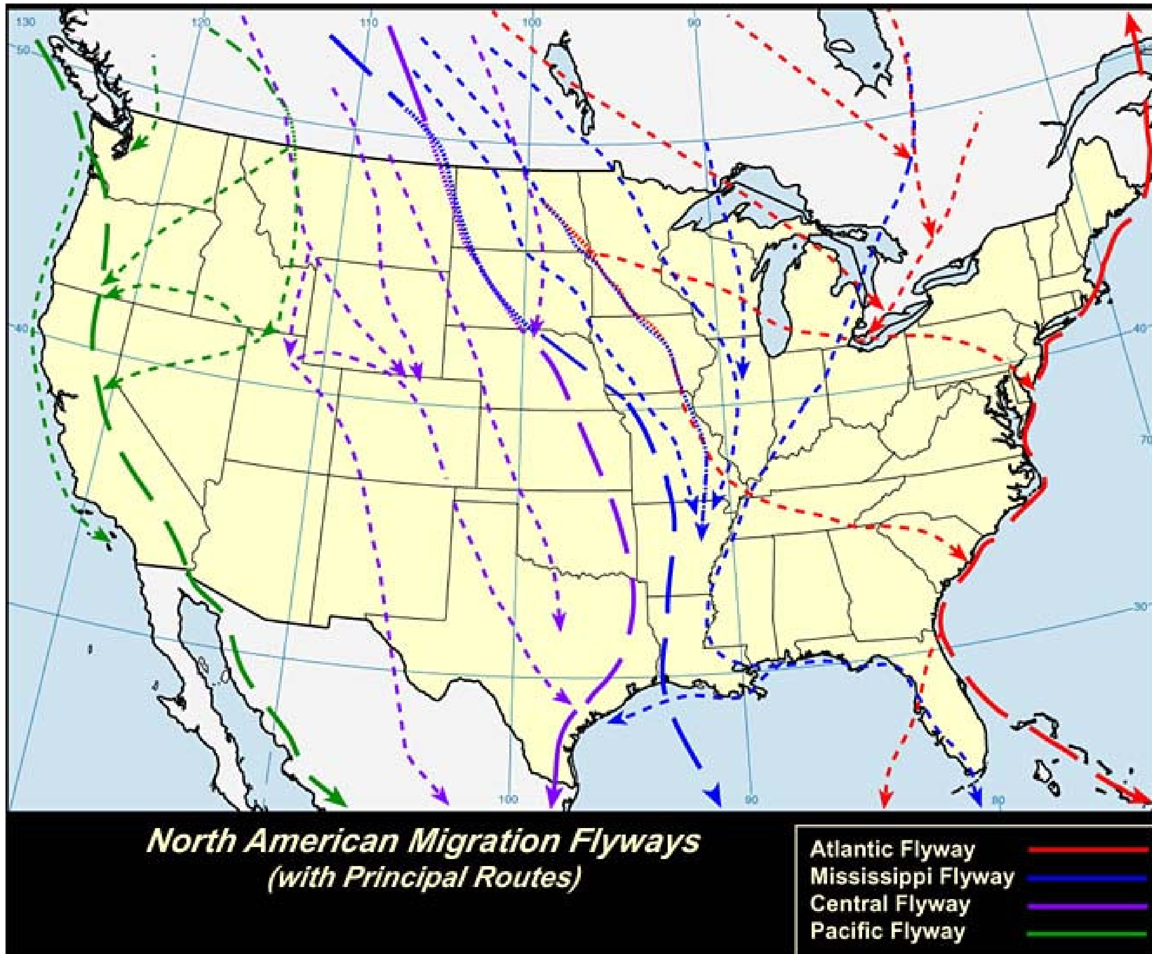

Figure 2. The boundaries and overlap of the North American Flyways in the United States.

(Image courtesy of www.birdnature.com)

significant overlap of many flyways. This overlap of continental flyways leads to the intermingling of otherwise separate waterfowl populations (Sibley 2001).

Movement of birds between flyways during migration has been a major source of concern because of the potential for rapid and widespread disease transfer of AIVs to previously uninfected waterfowl populations. The North American Flyways also overlap with other world flyways. Importantly, Alaska is located at the intersection of the North American Flyways and the East Asia – Australian Flyway (Figure 3). Here, North American waterfowl populations share breeding grounds with waterfowl from Asia and Australia. Intermingling of Asian and North American waterfowl has led to concern about the possible entry of Asian lineage, HP H5N1 into North America (Olsen et al. 2006).

While AIVs are known to be transmitted indirectly from bird to bird, there is speculation that AIVs may persist in the environment long after infected birds have left. These viruses may be shed by infected migratory birds into wetlands they inhabit, where they could persist for varying lengths of time in some environments. The longer persisting viruses may reach infective doses and infect immunologically naïve or stressed birds during migration by indirect transmission. The ability of AIVs to persist under natural environmental conditions, which we have defined as *environmental tolerance* (ET), has been inadequately examined; however, Stallknecht et al. (1990a) showed that AIVs can persist for extended periods in laboratory water. (Stallknecht et al. 1990b) also showed that AIVs can persist for varying periods under different chemical conditions. These results suggest that heavily used waterfowl habitats, such as the Alaskan breeding grounds, may be able to serve as a potential environmental reservoir for AIVs, spreading infection via indirect transmission to birds from a source of viruses persisting for longer periods of time in nature (Ito et al. 1995).

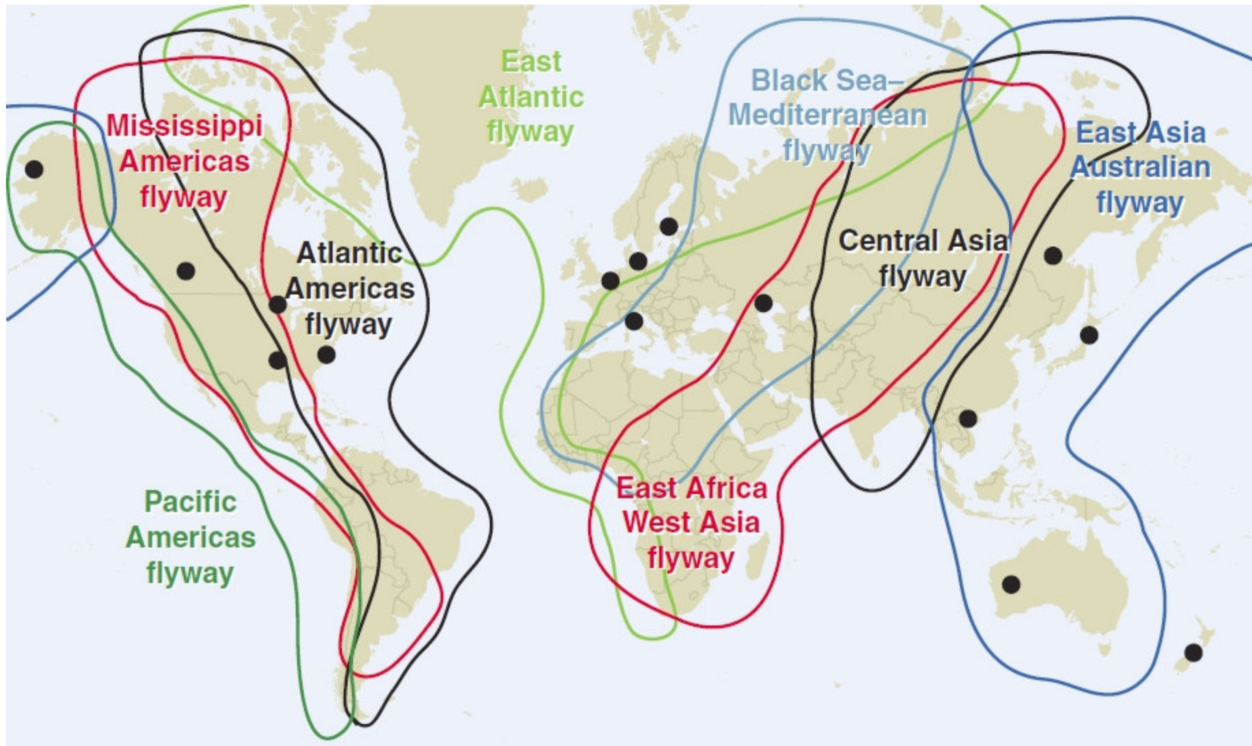

Figure 3. A world map showing the principle flyways of wild migratory birds. Notice the overlap of the North American flyways with the East Asia – Australian flyway (Olsen et al. 2006).

This study was designed to evaluate the possibility of an environmental reservoir for AIVs. To determine if an AIV reservoir could be maintained in the environment, we examined the virus ET of an AIV under natural conditions. To achieve this objective, we developed a simulated environmental model to examine virus ET in the laboratory. This objective was crucial, because our analysis of virus ET required a high level of control over environmental variables, and also required that the natural environmental conditions were being realistically represented. Therefore, we used soil and water collected from the environment in our model, and collected pH data from our model to determine its effectiveness at simulating the actual environmental conditions.

After establishing our model system, we were able to pursue our second objective, which was to determine the ET of an AIV under summer temperature conditions. We hypothesize that higher temperatures will lead to lower AIV ET, while lower temperatures will lead to higher AIV ET. We also explored the impact that microenvironments have on AIV ET, by separating our environmental samples into three categories: humic soil, water, and the soil-water interface. We hypothesize that the high organic matter content in the humic soil will stabilize the virus, leading to an increased ET in comparison to the water.

Study Area:

Our model was based on conditions existing in the large pond, known as Waterfowl Lake, at the Cleveland Metroparks Zoo in Cleveland, Ohio. While this pond was initially a natural formation, it was significantly altered at some point in the past. A majority of the shoreline was replaced by a rock wall, and a large portion of the natural mud bottom was overlain with gravel. However, Waterfowl Lake has remained relatively undisturbed for the past

20 years, with the exception of aerators and propellers used to circulate the water, and represents a homogonous study area to examine virus ET.

Waterfowl Lake is used by the Cleveland zoo during the summer to house a number of their bird species, including their Chilean flamingo collection. However, due to its size, the pond also acts as suitable habitat for wild migratory waterfowl. Waterfowl Lake is heavily used by numerous waterfowl species, with mallard ducks and Canada geese being the most prevalent species which come in direct contact with the zoo's bird collections. This situation puts the zoo's bird collections at risk of exposure to avian pathogens, including AIVs, from the wild birds inhabiting the pond.

Methods:

Characterizing Ambient Environmental Conditions

We established four sampling sites at Waterfowl Lake. One site was located near vegetation, and was shaded during part of the afternoon. Another site was located where zoo employees scattered corn for the zoo's resident birds and wild waterfowl. Two other sites were located near vegetation, and were not shaded during the afternoon. All four sites were spaced apart from each other and were located three to five feet from the shore, where the water was approximately 18 inches deep, to allow for dabbling ducks to feed (Figures 4 and 5). From each sampling site, we collected temperature, conductivity, pH, dissolved oxygen, and oxidation-reduction potential data. Also, we collected humic and mineral soil samples and water samples from each sampling site to create our model system in the laboratory. Furthermore, we used a temperature logger to collect temperature data every nine hours from Waterfowl Lake. Using these data to define the environmental parameters of Waterfowl Lake, we created a model

A

B

C

D

Figure 4. The four sampling locations at Waterfowl Lake, Cleveland Metroparks Zoo, where chemical parameters and soil and water samples were collected every month from May 2008 to September 2008. (A) Sampling site 1, located in shallow water near shrubby vegetation and not shaded. (B) Sampling site 2, also located in shallow water near shrubby vegetation and not shaded. (C) Sampling site 3, located in shallow water with no vegetation where zoo employees scatter corn for resident birds. (D) Sampling site 4, located in shallow water near dense shrubby vegetation and is mostly shaded.

Figure 5. Map of Waterfowl Lake at the Cleveland Metroparks Zoo, in Cleveland, Ohio, Cuyahoga County, marking our four sampling sites. Notice the aerators in the center and at both ends of the pond. (Image courtesy of Google Earth)

system that simulated the environment in the laboratory. All field data were collected from May 2008 through September 2008, which we have defined as summer.

Laboratory Model System

For our laboratory model examining ET, we used the AIV A/feces/Ohio/zoo11/2006 (H4N6) (A/feces (H4N6)), which was isolated from a fecal sample at the Cleveland Metroparks Zoo in 2006. Then, using the field data from our study site, we designed a microcosm model that compared the persistence of A/feces (H4N6) under different temperatures and in different microenvironments. A total of 27 microcosms were used, and each contained 35 ml of substrate in a 50 ml, aerated conical.

First, we designed three microcosm conditions to examine the effects the environment has on virus ET. The three conditions included humic soil, water, and the soil-water interface. The humic soil microcosms were filled with 25 ml of wet soil and ten ml of centrifuged water from the study site was added to create a 35 ml soil slurry. The water microcosms consisted of 35 ml of centrifuged water from Waterfowl Lake. The soil-water interface microcosms consisted of 35 ml of uncentrifuged water that contained particulate soil matter. A negative control was also established in an aquarium using soil and water from Waterfowl Lake.

Three temperature treatments were created for each of the three microcosm conditions, totaling nine different microcosm combinations. The three temperatures used were the maximum (27°C), minimum (15°C) and mean (23°C) summer temperatures recorded for Waterfowl Lake. These temperatures were maintained for the duration of the experiment.

Finally, three replicates of each microcosm construction were used to provide statistical significance to our results. After creating our microcosms and preparing the treatments, each microcosm was spiked with A/feces (H4N6), resulting in a final concentration in each

microcosm of 1 x $10^{3.8}$ EID_{50}/ml of microcosm substrate. Our negative control (the aquarium) was not spiked with virus.

Environmental Tolerance

A total of seven samples were collected from each of the 27 microcosm replicates. The microcosms were initially sampled after being spiked with virus, then they were sampled again after 24 hours, three days, and then on a weekly basis for four weeks. 1.8 ml of substrate was removed during each sampling period from each microcosm replicate and was transferred to cryovials containing 1.8 ml of brain heart infusion broth (BHIB), which resulted in a 1:2 dilution of the substrate. Each sample was frozen at -86°C until tested for virus persistence.

Expecting a qualitative difference in virus persistence between our microcosms, we initially tested each sample for the presence of virus, using virus isolation. We inoculated 0.15 ml of the 1:2 diluted substrate into 10 day old commercial embryonating chicken eggs. Each sample was inoculated into four separate eggs. We then harvested the amnio-allantoic fluid following 48 hours of incubation in a humidified incubator and 24 hours of chilling in a refrigerator. Using 96-well microtiter plates, a drop of amnio-allantoic fluid from each sample was added to separate wells containing 25 µl phosphate buffered saline (PBS). To test for the presence of virus, 50 µl of 0.7% chicken red blood cells (CRBCs) was added to each well, and the agglutination of the CRBCs in a well corresponded to a positive result for infectious virus.

Following our qualitative methods, we have also begun testing each microcosm sample for a quantifiable difference in virus concentration. Using 10 day old commercial embryonating chicken eggs, we are determining the egg infective dose concentration (EID_{50}) of virus in our samples. Each sample is first diluted by a series of ten-fold dilutions using BHIB with penicillin and streptomycin. Then, 0.10 ml from each dilution is inoculated separately into four

commercial embryonating chicken eggs, and allowed to first incubate for 48 hours and then chill for 24 hours. We are then harvesting the amnio-allantoic fluid, and mixing a drop of the fluid with 50 μl 0.7% CRBCs and 25 μl PBS in 96-well microtiter plates. The agglutination of the CRBCs in a well corresponds to a positive result for virus persistence. The number of positives per sample is used to then calculate the EID_{50} of the sample.

Results:

Characterizing Ambient Environmental Conditions

Interestingly, our field data collected for conductivity, pH, and dissolved oxygen in the water, and pH and Oxidation-Reduction Potential (ORP) in the humic and mineral soil layers during May 2008 varied greatly from the general trend in the data that began in June. The results from the month of May were much higher than the results from June, and were more similar to the results obtained in September (data not shown). Due to this unexplained discrepancy, we excluded May 2008 data from our results and focused our analysis on the months from June through September.

The chemical parameters of water from Waterfowl Lake suggest that trends exist for conductivity, pH, and dissolved oxygen. The conductivity of the water declined steadily from June to September (r^2=0.97, Figure 6 A). However, the pH of the water increased steadily over the course of our data collection (r^2=0.95, Figure 6 B). Dissolved oxygen levels in the water also increased steadily over the period of the study (r^2=0.92, Figure 6 C). In addition, by collecting water temperature data every nine hours over the course of the study, we were able to follow the summer temperature trend, which initially rose from May through June, held steady from June until the beginning of August, and then gradually fell from August through September, as the lake began to cool (Figure 7).

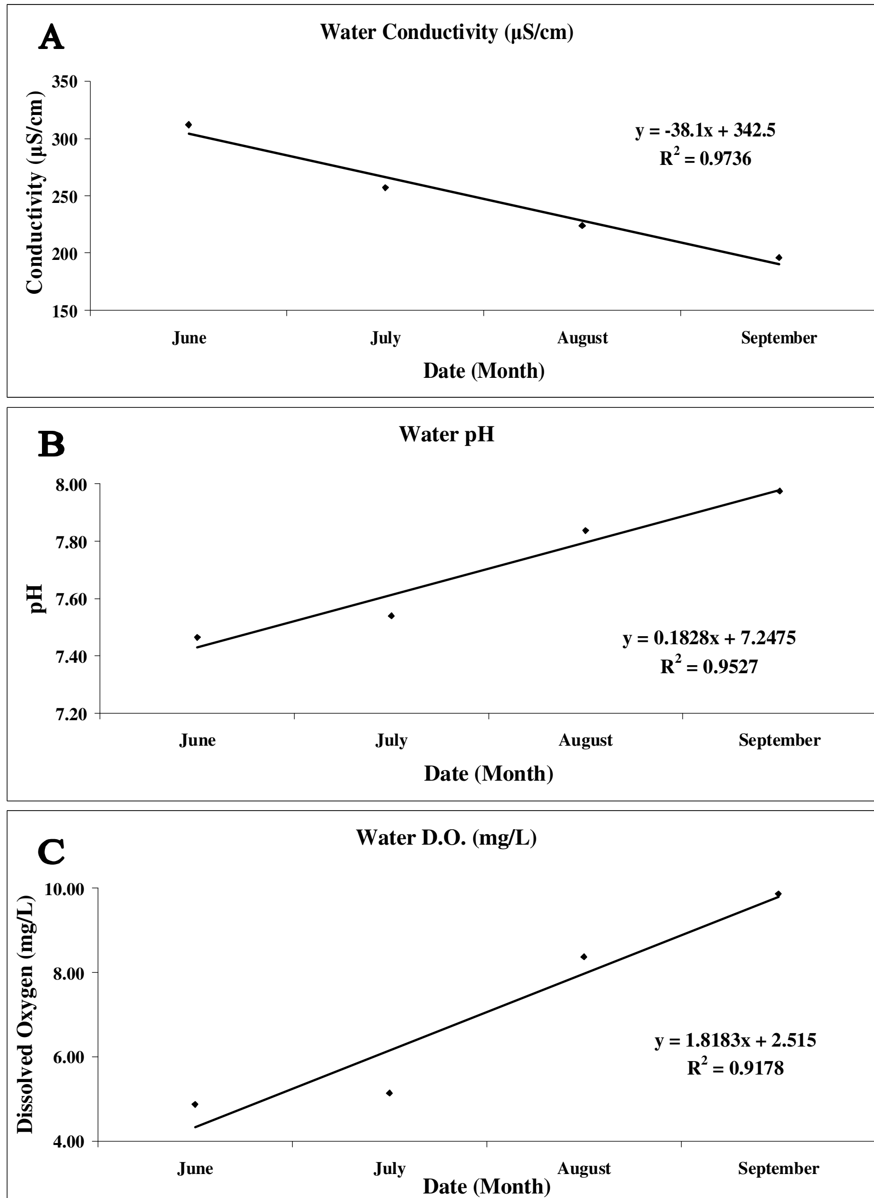

Figure 6. Water chemical data collected from June 2008 to September 2008 from Waterfowl Lake, Cleveland Metroparks Zoo, Cleveland, Ohio, Cuyahoga County. (A) The mean conductivity of water from Waterfowl Lake, measured in microSiemens per centimeter, collected monthly from all four sampling sites. (B) The mean pH of water from Waterfowl Lake collected monthly from all four sampling sites. (C) The mean level of dissolved oxygen from Waterfowl Lake, measured in milligrams per liter, collected monthly from all four sampling sites.

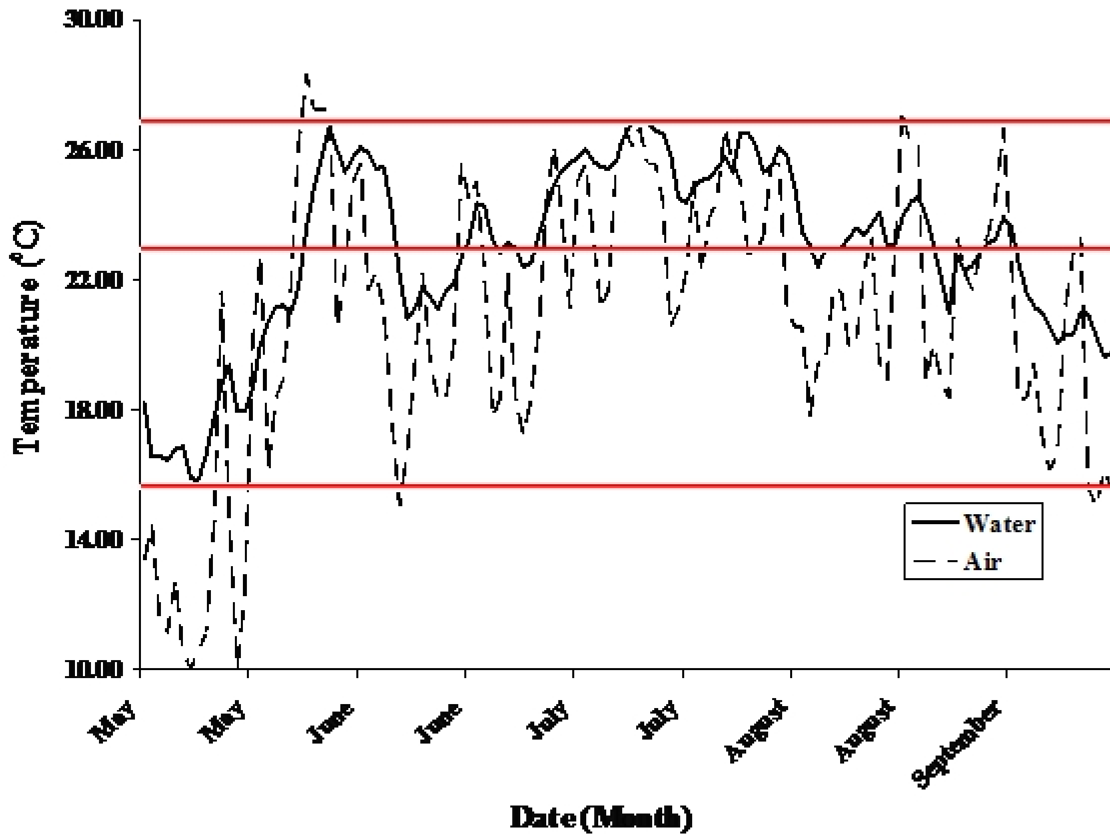

Figure 7. Temperature data recorded from Waterfowl Lake at the Cleveland Metroparks Zoo, Cleveland, Ohio, Cuyahoga County, during the months of May 2008 through September 2008. The solid line represents the water temperature within Waterfowl Lake at a depth of approximately 18 inches. The red horizontal lines represent the maximum (27°C), minimum (15°C) and mean (23°C) summer temperatures recorded from Waterfowl Lake. The dotted line represents the air temperature in Cleveland, Ohio from May 2008 through September 2008. Air temperature data was obtained from the NOAA Daily Climate Summary (2008).

Trends were also observed in our soil data collected from Waterfowl Lake. The pH of the humic soil slowly increased from June to September (r^2=0.98, Figure 8 B). Of interest however, the pH of the mineral soil slowly decreased from June to September (r^2=0.33, Figure 9 B). The humic soil's ORP gradually declined over the course of the study (r^2=0.999, Figure 8 A). However, the ORP of the mineral soil increased from June to September (r^2=0.66, Figure 9 A). Finally, the temperature of both the humic and mineral soils gradually declined from May through September(r^2=0.78, Figure 8 C; r^2=0.68, Figure 9 C).

Based on the data recorded from Waterfowl Lake at the Cleveland Metroparks Zoo, we chose temperature as our treatment variable and defined our three microcosm treatments using the maximum (27°C), minimum (15°C) and mean (23°C) summer temperatures recorded from Waterfowl Lake.

Laboratory Model System

Due to technological limitations, we were only able to test the pH of our microcosms. The pH of each microcosm was logged during each sampling period. In order to compare our simulated environmental model with the natural environment, we compared the pH from our field work to the pH values recorded in the lab. In the field, the mean water pH was 7.81, and ranged from monthly means of 7.47 to 8.21. The mean humic soil pH over the course of the study was 6.97, and ranged from 6.87 to 7.10 on a monthly basis. Finally, the mean mineral soil pH from our field work was 6.88, and the monthly means ranged from 6.73 to 7.07.

Table 2 compares the averaged microcosm pH values to the field data collected from Waterfowl Lake. Considering the water microcosm treatments, both the 15°C and 27°C treatments had pH values outside those obtained in the field. However, the mean pH for the 23°C water microcosm treatment was within the boundaries defined by the field data. When

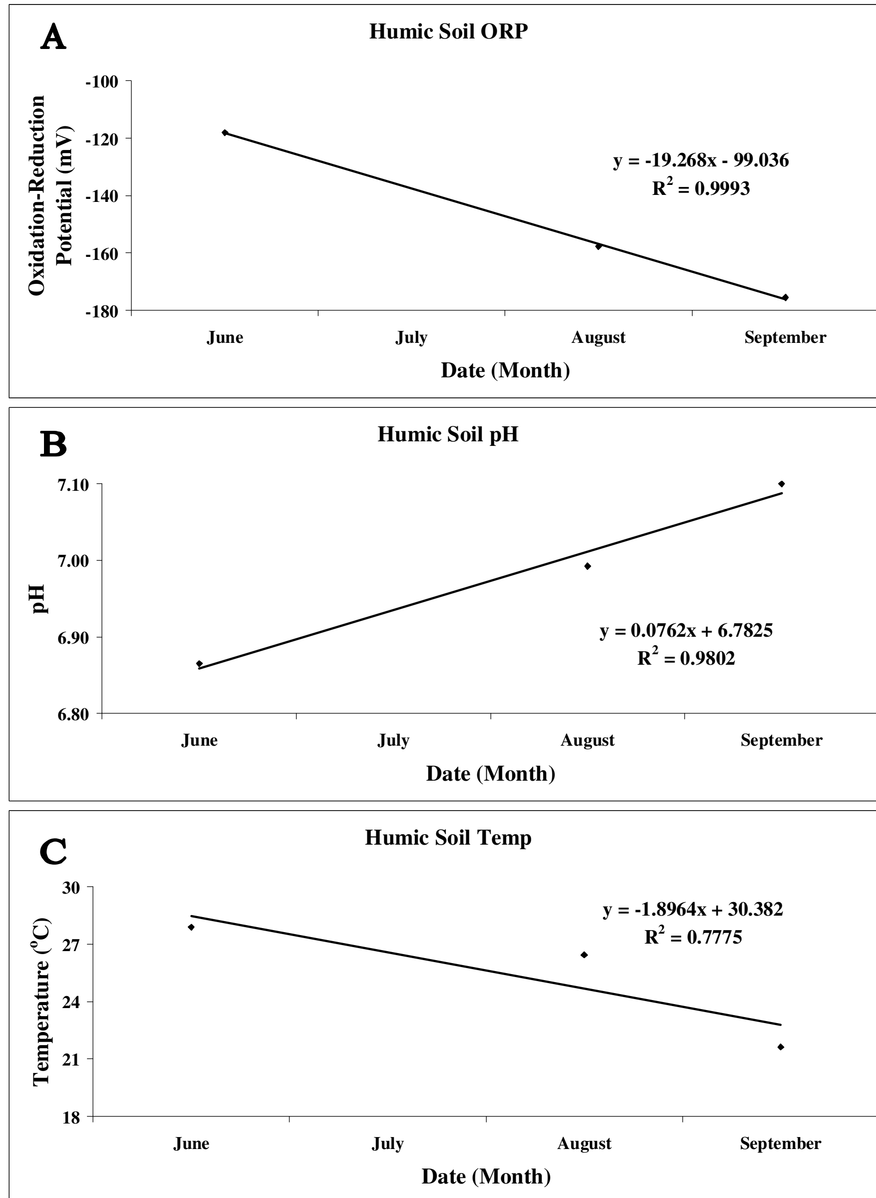

Figure 8. Humic soil chemical data collected from June 2008 to September 2008 from Waterfowl Lake, Cleveland Metroparks Zoo, Cleveland, Ohio, Cuyahoga County. (A) The mean Oxidation-Reduction Potential (ORP) of humic soil from Waterfowl Lake, measured in millivolts, collected monthly from all four sampling sites. (B) The mean pH of humic soil from Waterfowl Lake collected monthly from all four sampling sites. (C) The mean temperature of humic soil from Waterfowl Lake, collected monthly from all four sampling sites.

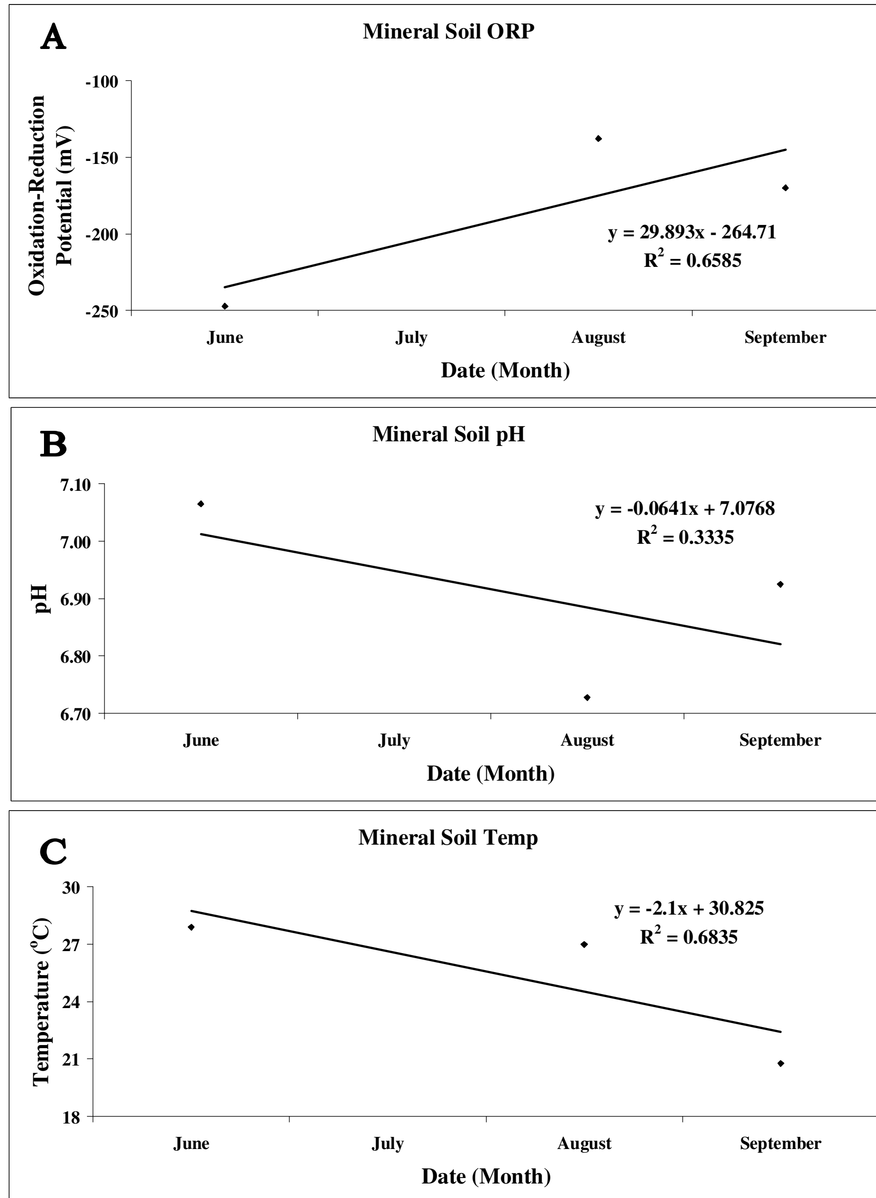

Figure 9. Mineral soil chemical data collected from June 2008 to September 2008 from Waterfowl Lake, Cleveland Metroparks Zoo, Cleveland, Ohio, Cuyahoga County. (A) The mean Oxidation-Reduction Potential (ORP) of mineral soil from Waterfowl Lake, measured in millivolts, collected monthly from all four sampling sites. (B) The mean pH of mineral soil from Waterfowl Lake collected monthly from all four sampling sites. (C) The mean temperature of mineral soil from Waterfowl Lake, collected monthly from all four sampling sites.

	15°C	23°C	27°C
Soil	7.04[a]	6.49[b]	6.79[b]
Soil-Water Interface	7.52[a]	7.70[a]	7.36[b]
Water	7.46[b]	7.60[a]	7.34[b]

[a]mean pH values within the boundaries defined by the field data
[b]mean pH values outside the boundaries defined by the field data

Table 2. Mean pH values recorded from our microcosms.

Values were calculated using all seven weeks of pH data.

comparing the soil microcosm treatments to the field data for humic soil, both the 23°C and 27°C treatments had pH values outside the range obtained in the field. However, the 15°C soil microcosm treatment had a mean pH within the boundaries defined by our field data. When comparing the soil-water interface mean pH values to the mean water pH data, both the 15°C and 23°C water microcosm treatments had mean pH values within the boundaries defined by the field data. However, of note, the mean pH values collected from the 27°C soil-water interface microcosm treatments fell outside the boundaries established by the field data. Next, comparing the soil-water interface to the humic soil shows that the all the mean soil-water interface data is outside and above the boundaries established by the mean humic soil pH field data.

Qualitative analysis

After four weeks of exposing our virus-spiked microcosms to our set summer temperatures, all microcosm treatments tested positive for virus recovery (Table 3). However, four separate microcosm replicates tested negative by the end of the experiment. The same water microcosm replicate at 23°C tested negative during both the sixth and seventh sampling periods. In addition, one replicate of the soil-water interface microcosm at 23°C and two soil microcosms at 27°C tested negative for virus during the seventh sampling period. No microcosm replicates tested negative for virus from the 15°C treatment. Due to the fact that none of the microcosms had all three replicates test negative for AIVs, no end point was reached.

Quantitative analysis

After obtaining the above results, we began to quantify the amount of virus in each microcosm. To achieve this, we are currently determining the mean egg infective dose concentration (EID_{50}) of virus in our first and last microcosm samples. Preliminary results have shown variation in the level of infective virus across temperatures (Table 4). While microcosm

	15°C			23°C			27°C		
Sampling Period	Soil	Water	Interface	Soil	Water	Interface	Soil	Water	Interface
1	3	3	3	3	3	3	3	3	3
2	3	3	3	3	3	3	3	3	3
3	3	3	3	3	3	3	3	3	3
4	3	3	3	3	3	3	3	3	3
5	3	3	3	3	3	3	3	3	3
6	3	3	3	3	2[a]	3	3	3	3
7	3	3	3	3	2[a]	2	1	3	3

Table 3. Qualitative results for A/feces (H4N6) - spiked microcosms. The numbers represent the number of microcosm replicates testing positive for A/feces (H4N6) during each sampling period.

[a]The same water microcosm replicate at 23°C tested negative for both sampling periods six and seven.

Microcosm	Temperature	Sampling Period 1	Sampling Period 7	Difference
water	27°C	$1 \times 10^{4.67}$	$1 \times 10^{1.87}$	$1 \times 10^{2.81}$
	23°C	$1 \times 10^{4.33}$	$1 \times 10^{0.50}$	$1 \times 10^{3.83}$
	15°C	$1 \times 10^{6.73}$	$1 \times 10^{3.93}$	$1 \times 10^{2.80}$
soil	27°C	$1 \times 10^{4.20}$	$1 \times 10^{0.22}$	$1 \times 10^{3.98}$
	23°C	$1 \times 10^{3.50}$	0.00	$1 \times 10^{3.50}$
	15°C	$1 \times 10^{5.23}$	$1 \times 10^{2.23}$	$1 \times 10^{3.00}$

Table 4. Preliminary quantitative results for A/feces (H4N6) – spiked microcosms. The numbers represent the mean 50% egg infective dose concentrations (EID_{50}/ml) for each microcosm combination. The soil and water samples have not yet all been examined quantitatively. The soil-water interface samples have not been examined quantitatively at this time.

samples from sampling period one vary, the amount of virus degraded over the course of the experiment appears to be similar between the soil and water microcosms. Currently, all of the microcosms from the seventh sampling period have shown a marked decrease in infectious virus, with the drop in virus EID_{50} concentrations varying from $1 \times 10^{2.80}$ EID_{50}/m to $1 \times 10^{3.83}$ EID_{50}/ml.

Discussion:

Due to the discrepancies between the May and June data collected and the low sample size of most parameters, we chose temperature as our treatment variable. Our data logger, which logged temperature every nine hours in Waterfowl Lake, produced the most consistent and reliable trend. Using this data, we calculated the maximum (27°C), minimum (15°C), and mean (23°C) summer temperatures for the pond.

Interestingly, the dissolved oxygen levels and pH of the water actually increased over the course of the study, while the conductivity decreased. The increase in dissolved oxygen and pH may be attributed to the effective use of aerators and propellers to circulate the water in the pond. While the conductivity was initially high, it may be attributed to the residual levels of salt left in the pond due to runoff from salting the walkways during the winter.

Considering the soil data, the humic soil appears to be negatively correlated with the mineral soil underneath. While the humic soil ORP increased, the mineral soil ORP actually decreased. These results suggest that as the summer progresses, the humic soil becomes more reduced, while the mineral soil underneath becomes more oxidized. Also, while the pH of the humic soil increases, the pH of the mineral soil decreases. This relationship between the humic soil layer, which contains a high level of organic matter, and the mineral soil layer, which

contains a lower level of organic matter, in Waterfowl Lake demonstrates how even a near stagnant pond can contain a dynamic environment.

By collecting data from both Waterfowl Lake and our microcosms, we attempted to evaluate the effectiveness of our simulated environmental model by comparing data sets. Unfortunately, due to equipment limitations, we were only able to obtain pH data from our microcosms to compare with the pH data collected from our study site (Table 2). Of interest is the soil-water interface. At both the 15°C and 23°C temperatures, the microcosms maintained a mean pH that was within the boundaries of the natural environment. The soil and water microcosms only maintained a mean pH within the boundaries of the natural environment at 15°C and 23°C, respectively. However, no microcosm at 27°C maintained a mean pH within the boundaries of the field data, and therefore suggests that this microcosm may need to be buffered if used in future studies. Our data suggests that natural pH values can be maintained without the use of buffers at lower temperatures.

Also of note, by the end of the four week experiment, our microcosms represented anoxic conditions, and the presence of dihydrogen sulfide could be detected by smell (data not shown). This condition could be associated with the pH values obtained over the course of the experiment. This condition arose because of the design of the microcosms. By using conicals, only a small surface area of substrate was in contact with the air, which allowed only minimal diffusion of oxygen into the microcosms. Future microcosm designs should use containers with a larger surface area open to the air and may require agitation to allow for a more aerobic environment.

Based on our results, we have determined that A/feces (H4N6) can persist for at least 28 days within an environmental model simulating Waterfowl Lake under summer conditions at the

Cleveland Metroparks Zoo. These results suggest that the length of the microcosm experiment should be increased to 60-90 days in future trials, pending our quantitative results. Also, these results suggest that ponds, such as Waterfowl Lake, could serve as a possible environmental reservoir for AIVs. Therefore, the prolonged persistence of AIVs under natural conditions should be considered in preventive medicine programs and when managing bird collections.

While our qualitative results did not show a difference in persistence between microcosms, our preliminary quantitative results suggest that some microcosms experience a three-fold loss in infectivity over four weeks. This loss of infectivity is equivalent to a 1000X decrease in virus concentration over four weeks. While only a small set of samples have been examined quantitatively, we are expecting to detect differing levels of virus persistence among microcosms. These data will help us to determine the preferred temperature and microenvironment of A/feces (H4N6), which will allow us to define its ET.

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