

Avian Influenza Surveillance in Wild Birds Presenting to Tri-State Bird Rescue & Research, Inc.

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Abstract: Public health officials must continue to explore and improve methods for highly pathogenic avian influenza surveillance because of its growing worldwide distribution and ability to adapt to many host species. The sampling of birds presenting to rehabilitation facilities for sickness and injury is a promising, yet underutilized surveillance method. In this cross-sectional prevalence study, designated species presenting to Tri-State Bird Rescue and Research, Inc. (Tri-State), Newark, DE were sampled and the type A isolation prevalence from these birds was compared to results from published studies of healthy free-ranging wild birds. One hundred cloacal swabs from waterfowl, shorebirds, and raptors were submitted for virus isolation and antigen capture ELISA of type A avian influenza at Dr. Richard Slemons' laboratory at Ohio State University College of Veterinary Medicine, Department of Preventive Veterinary Medicine. None of the samples submitted were positive for type A avian influenza virus. This limited study suggests that virus prevalence from rehabilitating wild birds is not higher than the prevalence in free-ranging wild birds. This paper describes the methodology used in sampling wild birds presenting for rehabilitation, the laboratory methods used to identify the presence of type A avian influenza, the results of this study, and implications for future surveillance projects.

Keywords: type A avian influenza, surveillance, wild birds, rehabilitation center, virus isolation, antigen capture ELISA

INTRODUCTION

Many species of wild birds are asymptomatic carriers of the low pathogenic avian influenza (LPAI), making them efficient at spreading the disease to both wild and domestic birds (Normille 2005). A member of the Orthomyxoviridae virus family, AI can cause acute systemic respiratory and intestinal disease with accompanying high mortality in wild birds if the strain is highly pathogenic (HPAI) (Saif 2003). Several type

A avian influenza (AI) surveillance studies of wild birds had been published long before the Hong Kong H5N1 outbreak in 1997. These studies primarily involved free-ranging wild bird populations, rather than sick and injured wild birds presenting to rehabilitation facilities. Previous research has not attempted to determine if any correlations exist between sick and injured wild birds presenting to rehabilitation centers and the prevalence of avian influenza. The purpose of this cross-sectional prevalence study was to determine if the virus isolation frequency found in Tri-State's rehabilitation birds were higher than the values from published free-ranging wild bird surveillance studies. The results could be used to determine whether AI circulating in wild bird populations would more likely cause carrier birds to present to a rehabilitation center.

This type A AI surveillance study of designated species in Delaware, Maryland, and New Jersey was conducted by collecting samples from birds presenting to Tri-State for the purpose of rehabilitation. Tri-State's location along the Atlantic Coast migratory flyway, as well as its diversity of species treated, makes it a promising location for sample collection. As the virus has many hosts and is capable of rapid mutation from LPAI to HPAI strains, a broad species sampling at Tri-State was necessary. The author compared the surveillance results from the rehabilitation birds to previously recorded studies involving healthy birds sampled in the field. Additionally this study was intended to help public health officials consider whether wild bird rehabilitation centers are a good place to focus efforts when structuring avian influenza surveillance programs.

There is wide variation in data generated from previous surveillance studies of free-ranging birds. The United States Department of Agriculture (USDA)

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and Department of Interior (DOI) are coordinating a National HPAI Early Detection Data System. Between 1 April 2006 and 1 September 2006, they had 113 positive type A AI samples from 25,000 predominantly free-ranging wild birds (USDA and DOI 2006). Table 1 shows results from two previous studies that provided type A AI prevalence values of wild bird species sampled from free-ranging healthy populations (Olsen 2006, CCWHC 2006). The premise of the current study was to test the hypothesis that the virus isolation frequency values from birds sampled at Tri-State (i.e., those that were ill or injured) would be higher than these frequency values of healthy, free-ranging birds.

[*Editor's Note:* The 2006 Canadian Cooperative Wildlife Health Center (CCWHC) Wild Bird Survey results are now complete and available on the website. Due to the time in which this article was written, the format of the results has changed and they are now presented in groups of live birds and dead birds surveyed.]

MATERIALS AND METHODS

Sample Collection. Due to the diverse host adaptability of AI, a variety of species were sampled to reach a quota of 100 cloacal samples. Samples were selected based on the availability of previous surveillance data on free-ranging wild bird populations of those species; no distinction was made based on gender, age, or location found within the designated states. Prevalence comparisons could then be made between the previous data and any positive results from this study.

Wild birds present to Tri-State with a variety of injuries. Injury prognosis and the bird's time of presentation to the clinic influenced when a cloacal swab was taken. If the prognosis was poor and the bird was to be euthanized, a swab was taken immediately after euthanasia. If the prognosis was good, a swab was taken from the bird during handling for the initial physical exam, thus minimizing handling and decreasing stress to the bird. If the bird presented when an individual involved with this research project was not present, a swab would be taken later during the bird's daily treatments, or in the case of dead or euthanized birds, within four days from the time of death, after the bird had been frozen at -20°C (-4°F).

Tri-State regularly houses waterfowl and gulls together after their health has stabilized, and prior to release to the wild. In naturally infected birds, 3 days elapse from the time of infection to the time of virus shedding (Saif 2003). In the few samples taken from such birds, no bird sampled had been in group housing for longer than 2 days. The birds' health status at the time of sampling was: 47 dead (recently euthanized or frozen), 29 injured, 14 sick, and 10 healthy.

Fisherbrand Sterile Swabs[®] (Fisher Scientific

Table 1. Summary of AI results from two previously published field surveillance studies (Olsen 2006 and CCWHC 2006).

Study	Mallards		Gulls		Raptors	
Olsen (2006)	1,965	13%	199	1.40%	2	1%
	15,250		14,505		192	
CCWHC (2006)	1,166	43%			3	8%
	2,695				37	

Company, L.L.C., Pittsburgh, PA) were used to swab the bird's cloacal mucosa. The swab was then vortexed by hand in a vial containing penicillin and streptomycin fortified brain heart infusion broth with tryptose, supplied by Dr. Richard Slemons' laboratory at the Ohio State University College of Veterinary Medicine, Department of Preventive Veterinary Medicine. The vials were then sealed with Parafilm M[®] laboratory film (Alcan Inc., Montreal, Quebec) and placed on dry ice until transport to a -80°C (-112°F) freezer. One hundred cloacal samples were collected and sent stored on dry ice to Dr. Slemons' laboratory.

Diagnostic Methods. Dr. Slemons and a team of technicians ran diagnostic testing for the 100 samples. Techniques included virus isolation and agglutination, and antigen capture ELISA. Virus isolation involved incubating chicken eggs at 37°C (98.6°F) for 10 days. The eggs were then candled using a Maglite[®] (Mag Instrument, Inc., Ontario, CA) to determine if the embryos were viable, as indicated by visible movement and vasculature. Each viable egg was removed from incubation and, under a sterile hood, the dome above the air sac was disinfected using an iodine swab. Using a sterile nail, the dome of the egg was then tapped to make inoculation easier. The vials containing the virus samples and swab were removed from the -80°C (-112°F) freezer, thawed, and centrifuged at 6,000 rpm for five minutes. 0.15 cc of the centrifuged supernatant from the vials was syringe-injected into

the allantoic sac of each of the corresponding eggs. The eggs were again incubated for 48 hours at 37°C (98.6°F). After 48 hours, the eggs were removed from incubation and refrigerated for 12 hours to ensure that the embryos were non-viable. The eggs were then ready for harvesting the chorioallantoic fluid (CAF). Egg crackers and sterile forceps were used to remove the shell's dome. A sterile pipet was used to remove the CAF.

After harvesting, the CAF was used to check for an agglutination reaction that is typical of ortho- and paramyxoviruses. This reaction occurs if hemagglutinin surface glycoprotein is present in the sample and binds to chicken red blood cells (CRBCs). Each sample well of a 96-well microtiter plate was filled with 0.025 ml phosphate buffered saline (PBS) and the control well was filled with 0.05 ml PBS. 0.05 ml of a half-percent suspension of CRBCs in PBS solution (0.5 ml CRBCs in 99.5 ml PBS) was then added to each sample well. Using a sterile disposable pipet, one drop CAF was transferred from the egg to each well. The wells were then covered with aluminum foil. After 45 minutes at room temperature, each well was checked for agglutination. If there was a foggy pink appearance in the well, the CRBCs had agglutinated with the sample's hemagglutinin molecules, forming a lattice structure indicative of a positive agglutination reaction. If a collection of CRBCs gathered at the well bottom and streaked when the wells were tilted, the sample was negative for agglutination. For each positive reaction, the remaining CAF from that egg was collected and placed in a 14-ml sterile tube to be used for checking hemagglutinin titers and type A testing.

For the hemagglutinin titer test, a 96-well microtiter plate was filled with 0.05 ml PBS and the rows were labeled 2, 4, 8, 16, 32, 64, 128, and 256, starting at the second row. 0.05 ml CAF from every 14-ml tube was added to the first row of wells. In order to make sample dilutions, 0.05 ml of the mixture in row one was pipetted into the second row labeled two. That mixture was then pipetted into the third row labeled 4, and so on until the last row. Then 0.05 ml of the half-percent suspension of CRBCs was added to each well and the tray sat for 45 minutes at room temperature. The first row was always positive for agglutination because it had the highest dilution of hemagglutinin molecules. Eventually, each sample was negative for agglutination at a certain dilution, as marked by the CRBC appearance that streaked when the tray was tilted. The titer value read the inverse of the last row's number in which there was a positive reaction. This information is used by the National Veterinary Services Laboratory (NVSL) to determine

the approximate levels of antibody present.

Using the Avian Influenza Virus type A Antigen Test Kit (Synbiotics Corporation, San Diego, CA), based on Rapid Immuno-Migration technology, each positive agglutinating sample would be tested for type A virus. Eight drops of the extraction buffer were placed into the provided test tubes. Then 0.25 ml CAF of the positive agglutination sample was added to the buffer. A test strip coated with one antibody specific for p56 nucleoprotein present in all AI type A viruses binds the corresponding antigen in the sample CAF. This antigen-antibody complex migrates along the strip until captured by a secondary antibody which causes a purple line to develop in positive type A samples after about 15 minutes. This can be confirmed by a positive control band on the test strip. If the sample was positive for agglutination and negative for type A AI testing, a differential diagnosis such as Exotic New Castle Disease would be considered

Agar gel immunodiffusion is another method used by Dr. Slemmons' laboratory to detect the presence of type A avian influenza virus; immunodiffusion was not used in this study but is presented here as an alternative screening option. This method is inexpensive, easy, and fast, and can be used for any bird species. This serology test is used to indirectly test for type A avian influenza antibodies to the ribonucleoprotein and matrix 1 proteins. Advantages of this method are that it can test a large sample size in a short time period, detect all subtypes of type A, be used on all species, and only takes 24 hours to read. The main disadvantage is that its interpretation is subjective and the precipitin lines may be difficult to read. Using an agarose gel in a petri dish, six holes are punched on the perimeter and one hole is punched in the middle. The test antigen obtained from NVSL is placed in the center hole. The sample serum and positive control serum are placed in alternating holes on the perimeter. After 24 hours, immunoprecipitation lines form at the antigen-antibody interface. The dish can be evaluated by illuminating it and looking for the precipitate line. If the line is continuous at the same level as the positive control, there is type A AI virus present in the sample serum. One must be careful to not misinterpret any non-specific line or partial lines as positive results. Also, one must be careful to use the correct reagent balance.

RESULTS

Samples from 48 raptors and 52 non-raptors yielded no positive results when subjected to virus isolation and agglutination. Raptor species sampled included red-tailed hawks (*Buteo jamaicensis*) (11), osprey

(*Pandion haliaetus*) (7), great horned owl (*Bubo virginianus*) (9), turkey vulture (*Cathartes aura*) (6), American kestrel (*Falco sparverius*) (2), black vulture (*Coragyps atratus*) (2), bald eagle (*Haliaeetus leucocephalus*) (3), eastern screech owl (*Megascops asio*) (2), red-shouldered hawk (*Buteo lineatus*) (1), and Cooper's hawk (*Accipiter cooperii*) (5). Non-raptor species sampled included laughing gull (*Larus atricilla*) (23), mallard (*Anas platyrhynchos*) (9), American crow (*Corvus brachyrhynchos*) (6), great blue heron (*Ardea herodias*) (7), ring-billed gull (*Larus delawarensis*) (2), great egret (*Casmerodius albus*) (1), herring gull, (*Larus argentatus*) (1), black-crowned night heron (*Nycticorax nycticorax*) (1), greater black backed gull (*Larus marinus*) (1), green heron (*Butorides virescens*) (2), and mute swan (*Cygnus olor*) (1).

DISCUSSION

The 2006 USDA and DOI *Early Detection System for Highly Pathogenic H5N1 Avian Influenza in Wild Migratory Birds US Interagency Strategic Plan* recorded 113 type A samples in 25,000 birds tested (<0.5%) between 1 April 2006 and 1 October 2006 (USDA and DOI 2006). The Interagency Plan sampled more free-ranging birds in comparison to this research study which focused on birds presenting to a rehabilitation facility. The conclusion can be made that from this limited study the virus isolation prevalence in birds presenting to Tri-State is not significantly higher than the virus isolation prevalence of the Interagency Plan. The same conclusion can be made when comparing this study's results to the individual species referenced in both Bjorn Olsen's study and the Canadian Cooperative Wildlife Health Centre report (Table 1).

Several project limitations influenced data collection and should be considered when structuring future avian influenza surveillance projects. First, this was a summer study. Krauss reports that duck isolation frequencies are highest in the fall southern migration due to the increased concentration of young, susceptible birds (Krauss 2004). Slemons further argues that free-flying, non-migratory ducks in Maryland served as effective sentinels for the migratory populations as they began their fall southern migration (Slemons 2003). The lack of any AI positive mallards (0/9) detected in this summer study affirms the effect that the time of year has on surveillance results.

Wild birds are often asymptomatic for the low pathogenic strains of AI (Webster 2006). It is possible that wild birds infected with the low path strains will not present to rehabilitation centers with a frequency higher than non-infected birds. However, one should not make this conclusion in birds infected with high

path strains. There have been reports of massive die-offs in wild birds due to highly pathogenic strains, including 6,000 migratory birds that died from an HPAI H5N1 outbreak at Qinghai Lake in China (Normille 2005).

There is also the possibility that using a -20°C (-4°F) freezer to store carcasses before sampling could have resulted in degradation of the virus were there only a mild infection in that bird. Swayne (1998) reports that when testing can not occur immediately, samples should be stored at -70°C (-94°F). Because Tri-State did not have a -70°C (-94°F) freezer, some carcasses were stored in the -20°C (-4°F) freezer up to 4 days before sampling could occur. However, once the samples were taken, all swabs were stored at -70°C (-94°F).

Sampling limitations imposed by diagnostic laboratories further diminished the sample size, species variety, and states that permitted sampling. The relative short duration of this study, as well as only sampling birds from one rehabilitation center also contributed to a small sample size. Ideally this study would have involved repeated studies at the same time of year over several years. DeMarco (2003) emphasizes the importance of long-term monitoring as it helps place results in the context of yearly repeated patterns, which is far more useful than a study over the period of one summer.

Some studies, even though they were long-term, isolated the AI virus at frequencies similar to this study. Graves (1992) reports that through a 33-month study of 5,013 birds, virus isolation was a mere 0.4 percent. Though his technique of sampling feces is different from cloacal swabbing, it illustrates that patterns of surveillance results are often unpredictable, prompting the need for further surveillance studies. A larger sample size and further surveillance efforts are necessary to draw further conclusions about whether or not rehabilitation centers are a good place to launch surveillance efforts.

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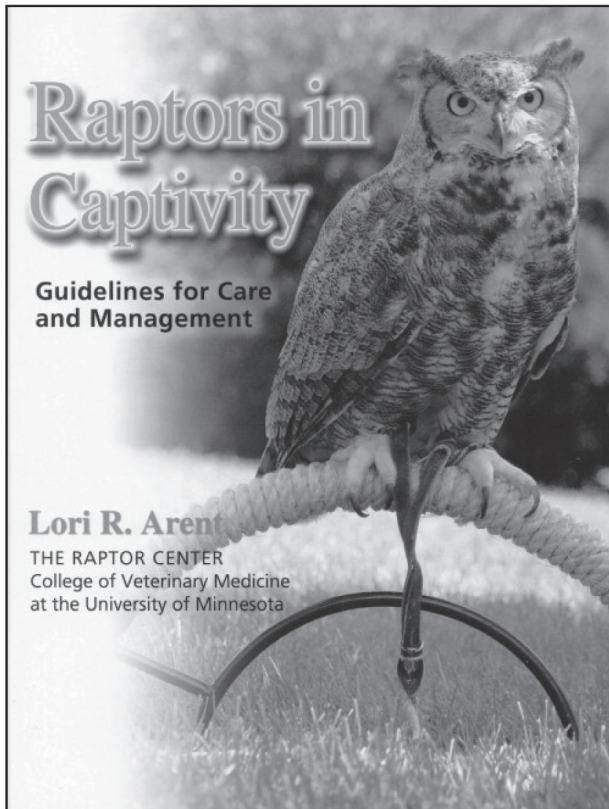
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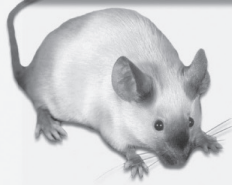
Appendix E: Maintenance and Medical Supplies

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Mice



Size	Less than 500	500	1000	2500	5000+	Length(inches)	Weight(grams)	Count
X-Small Pinkies:	\$0.16	\$0.15	\$0.14	\$0.13	\$0.12	0.50 - 1.00	1.30 - 1.80	100
Small Pinkies:	\$0.16	\$0.15	\$0.14	\$0.13	\$0.12	0.50 - 1.00	1.90 - 2.40	100
Large Pinkies:	\$0.16	\$0.15	\$0.14	\$0.13	\$0.12	0.50 - 1.00	2.50 - 3.00	100
Peach Fuzzies:	\$0.19	\$0.18	\$0.17	\$0.16	\$0.15	1.00 - 1.25	3.10 - 4.40	100
Fuzzies:	\$0.19	\$0.18	\$0.17	\$0.16	\$0.15	1.25 - 1.50	4.50 - 7.00	100
Hoppers:	\$0.29	\$0.27	\$0.25	\$0.23	\$0.21	1.50 - 2.00	8.00 - 12.00	100
Weanlings:	\$0.39	\$0.37	\$0.35	\$0.33	\$0.31	2.00 - 2.50	13.00 - 19.00	50
Large Adults:	\$0.44	\$0.42	\$0.40	\$0.38	\$0.36	2.50 - 3.00	20.00 - 29.00	50
X-Large Adults:	\$0.49	\$0.47	\$0.45	\$0.43	\$0.41	3.00 - 3.75	30.00 - 50.00	25

* We offer combined quantity discount mouse pricing. * Measurement does not include tail length.

Rats



Size	Less than 500	500	1000+	Length (inches)	Weight (grams)	Count
Pinkies:	\$0.34	\$0.29	\$0.24	1.50 - 2.00	3.00 - 8.00	100
Fuzzies:	\$0.44	\$0.39	\$0.34	2.00 - 2.50	9.00 - 19.00	100
Pups:	\$0.54	\$0.49	\$0.44	2.50 - 3.50	20.00 - 29.00	25
Weaned:	\$0.69	\$0.64	\$0.59	3.50 - 4.50	30.00 - 44.00	25
Small:	\$0.79	\$0.74	\$0.69	4.50 - 6.00	45.00 - 84.00	20
Medium:	\$1.19	\$1.14	\$1.09	6.00 - 8.00	85.00 - 174.00	10
Large:	\$1.24	\$1.19	\$1.14	8.00 - 9.00	175.00 - 274.00	5
X-Large:	\$1.49	\$1.44	\$1.39	9.00 - 11.00	275.00 - 374.00	3
XX-Large:	\$1.74	\$1.69	\$1.64	11.00 - 13.00	375.00 - 474.00	2
XXX-Large:	\$1.99	\$1.94	\$1.89	11.00 - 13.00	475.00 - 600.00+	2

* We offer combined quantity discount rat pricing. * Measurement does not include tail length.

Coturnix Quail



Size	Less than 500	500	1000+	Grams	Oz.	Count
1 Day:	\$0.34	\$0.29	\$0.24	7.50 - 10.00	.25	100
1 Week:	\$0.64	\$0.59	\$0.54	30.00 - 40.00	1.0	25
2 Week:	\$0.84	\$0.79	\$0.74	50.00 - 75.00	2.5	10
3 Week:	\$1.04	\$0.99	\$0.94	100.00 - 125.00	4.0	10
6 Week:	\$1.34	\$1.24	\$1.14	130.00 - 150.00	5.0	5
8 Week:	\$1.44	\$1.34	\$1.24	155.00 - 185.00	6.5	5
10 Week:	\$1.64	\$1.54	\$1.44	190.00 - 225.00	8.0	5

* Sizes of quail can be ordered in any combination to qualify for quantity discounts.

Rabbits



Size	Our Price	Weight (lbs.)	Count
X-Small:	\$3.00	0.50 - 0.75	1
Small:	\$4.00	1.00 - 1.75	1
Medium:	\$5.00	2.00 - 3.75	1
Large:	\$6.00	4.00 - 5.75	1
X-Large:	\$7.00	6.00 - 7.75	1
XX-Large:	\$8.00	8.00 - 9.75	1
XXX-Large:	\$9.00	10.00 - 11.75+	1

Chicks

Size	Less than 500	500	1000	5000	10000+	Grams	Ounces	Count
Small:	\$0.25	\$0.20	\$0.15	\$0.12	\$0.10	30.00 - 35.00	1.0	25

* Additional discounts available for large wholesale orders.



Guinea Pigs

Size	Less Than 500	500	1000+	Inches	Grams	Count
Medium:	\$1.19	\$1.14	\$1.09	6.00 - 8.00	85.00 - 174.00	10
Large:	\$1.24	\$1.19	\$1.14	8.00 - 9.00	175.00 - 274.00	5
X-Large:	\$1.49	\$1.44	\$1.39	9.00 - 11.00	275.00 - 374.00	3
XX-Large:	\$1.74	\$1.69	\$1.64	11.00 - 13.00	375.00 - 474.00	2
XXX-Large:	\$1.99	\$1.94	\$1.89	11.00 - 13.00	475.00 - 600.00	2
XXXX-Large:	\$2.24	\$2.19	\$2.14	13.00 - 15.00	601.00 - 900.00+	1

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**US FISH AND WILDLIFE SERVICE SEEKS COMMENT:
PROPOSAL TO ALLOW FALCONERS TO REMOVE AND POSSESS
MIGRATORY PEREGRINE FALCONS FROM THE WILD**

The US Fish and Wildlife Service released 13 November 2007 for public comment a Draft Environmental Assessment (DEA) and Management Plan that proposes to allow limited removal and possession of migrant first-year Northern (predominantly Arctic subspecies) peregrine falcons from the wild for use in falconry. The falcons could be captured in areas and at times where removal would have no significant impact on the population.

“A few decades ago, the peregrine falcon in North America was on the verge of extinction due to the effects of DDT, which affected both the American and Arctic peregrine falcon subspecies,” said Service Director Dale Hall. “We recognize that falconers have long sought protection of wild raptor populations and played a significant role in the species’ comeback. They were among the first to report the decline in peregrine populations and, in fact, contributed peregrines held for falconry to captive propagation efforts. Now that peregrine populations are healthy, the Service is considering once again allowing the traditional capture of migrant peregrine falcons for use in falconry.”

There are three recognized subspecies of peregrine falcons in North America: the Arctic peregrine which nests in Alaska, northern Canada and Greenland and migrates south to Central and South America; the American peregrine which nests in parts of southern Canada, Alaska, and the conterminous US, some of which migrate south; and the non-migratory Peale’s peregrine which resides on the Pacific coast from Alaska to Oregon.

In the DEA, the Service considers six alternatives for removal and possession of migrant peregrine falcons in the US. Four alternatives allow capture and possession in different locations and at different times. The Service found one alternative for take that was initially considered reasonable would not allow take under the rigorous restrictions adopted. Finally, the no-action alternative would mean that current prohibition on take of migrating peregrines would remain in place.

The preferred alternative is to allow annual removal of up to 105 first-year peregrine falcons split evenly between males and females, between September 20 and October 20, from southern Georgia, Florida, and the Gulf of Mexico coastal area, and expand authorization in Alaska to include migrants and fledged young of all subspecies. Because both American and Arctic peregrines nest in Alaska, the DEA considers take of nestlings, recently fledged young, and migrants. However, take in Alaska is factored into the alternatives that allow take of migratory first-year peregrines elsewhere in the US. The Service has concluded that any take that may be allowed is unlikely to effect populations of peregrine falcons negatively in North America or Greenland.

The majority of peregrine falcons that migrate from North America to Central and South America pass along the Atlantic coast and over the Gulf of Mexico. However, many other peregrines in the eastern US and southeastern Canada do not migrate far south. This difference in migration allows the Service to consider take of migrants. The alternatives that allow take of migrants are restricted so as to protect the continuing recovery of the eastern US and southeastern Canada American peregrine falcon population. The Service has considered only levels of take that would ensure the continued growth of the population in this region.

Copies of the DEA and Draft Management Plan can be obtained from the US Fish and Wildlife Service Division of Migratory Bird Management, 4401 North Fairfax Drive, Mail Stop 4107, Arlington, VA 22203-1610. Written comments on the DEA can be sent to the same address, noting Attention—Migrant Peregrine EA. The Draft EA also is available at <<http://www.fws.gov/migratorybirds/>>. Comments on the DEA may also be submitted electronically via the Division of Migratory Bird Management web site at <<http://www.fws.gov/migratorybirds/>>, where a link for comments is available. The due date for comments is February 11, 2008.