Surveillance for Highly Pathogenic Avian Influenza Virus in Wild Birds during Outbreaks in Domestic Poultry, Minnesota, USA, 2015

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In 2015, a major outbreak of highly pathogenic avian influenza virus (HPAIV) infection devastated poultry facilities in Minnesota, USA. To understand the potential role of wild birds, we tested 3,139 waterfowl fecal samples and 104 sick and dead birds during March 9–June 4, 2015. HPAIV was isolated from a Cooper's hawk but not from waterfowl fecal samples.

Wild birds of the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls and shorebirds) are believed to be the predominant reservoir for avian influenza viruses (AIVs) (*1*), and most AIV subtypes are low pathogenicity (LPAIV) (*2*). Only subtypes H5 and H7 are commonly associated with highly pathogenic AIVs (HPAIVs), which sometimes arise from mutation after introduction of LPAIV in domestic poultry (*3*). The main transmission route of AIVs in birds is fecal-oral, with viral shedding in both feces and through the upper respiratory tract (*4*). Transmission involves direct or indirect contact between susceptible birds and infectious birds or fomites (*5*). A novel HPAIV (H5N2) strain discovered in North America in 2014, a reassortant with Eurasian (EA) and North American (AM) lineage genes (*6*), had been detected in domestic poultry and wild birds as far east as Kentucky, USA, through January 2016. Of 7,084 wild birds sampled by US federal and state agencies during December 2014–June 2015, a total of 98 (1.4%) tested positive for HPAIV (EA/AM H5N1, EA/AM H5N2, EA H5N8, or other EA H5); these birds were 68 dabbling ducks, 20 geese, 7 raptors, 2 passerines, and 1 diving duck (*7*).

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In Minnesota, USA, HPAIV subtype H5N2 was first confirmed in a poultry facility (hereafter termed facility) in Pope County on March 4, 2015. The scope of the outbreak in Minnesota was unprecedented, and by mid-June 2015, the virus had been found in 23 counties with confirmed cases at 104 sites (98 turkey facilities, 5 chicken facilities, 1 backyard flock). The outbreak resulted in the depopulation of 9 million birds (*8*) and an economic loss of at least $650 million (*9*). Given that wild waterfowl are reservoirs for AIVs and that their movement could contribute to HPAIV spread, we conducted surveillance to detect HPAIV in wild waterfowl feces, selected dead birds, and live birds displaying neurologic impairment.

The Study

On March 6, 2015, we conducted an aerial survey covering a 24-km radius around the Pope County facility and identified ≈100 resident mallards (*Anas platyrhynchos*) and 21 trumpeter swans (*Cygnus buccinator*). During March 9–12, 2015, we collected 148 representative waterfowl fecal samples, pooled in groups of up to 3, to determine whether wild birds were actively shedding HPAIV. We did not detect HPAIV, although 2 pooled samples contained LPAIV (detailed methods in the online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/7/15-2032-Techapp1.pdf).

In March 2015, we chose 5 counties with infected facilities (Kandiyohi, Lac Qui Parle, Meeker, Nobles, and Stearns) and 5 waterfowl production areas (online Technical Appendix 1) where facilities were uninfected (Figure 1) to test for a spatial difference in HPAIV shedding. Within these areas, we compiled a list of wetlands and lakes and scouted those areas for waterfowl activity and sampled feces. For each area, our goal was to collect 300 fecal samples. In counties with infected poultry, we choose sites within 16 km of infected facilities. We collected ≈20 samples from a given spatiotemporal point to obtain representation within a target area.

We solicited agency staff and the public to report any deceased wild birds or live birds exhibiting neurologic signs consistent with HPAIV infection, including raptors, wild turkeys, and groups of ≥5 dead birds from which we obtained samples. We refer to these as morbidity and mortality samples, and our collection efforts targeted birds that had died <24 h previously.

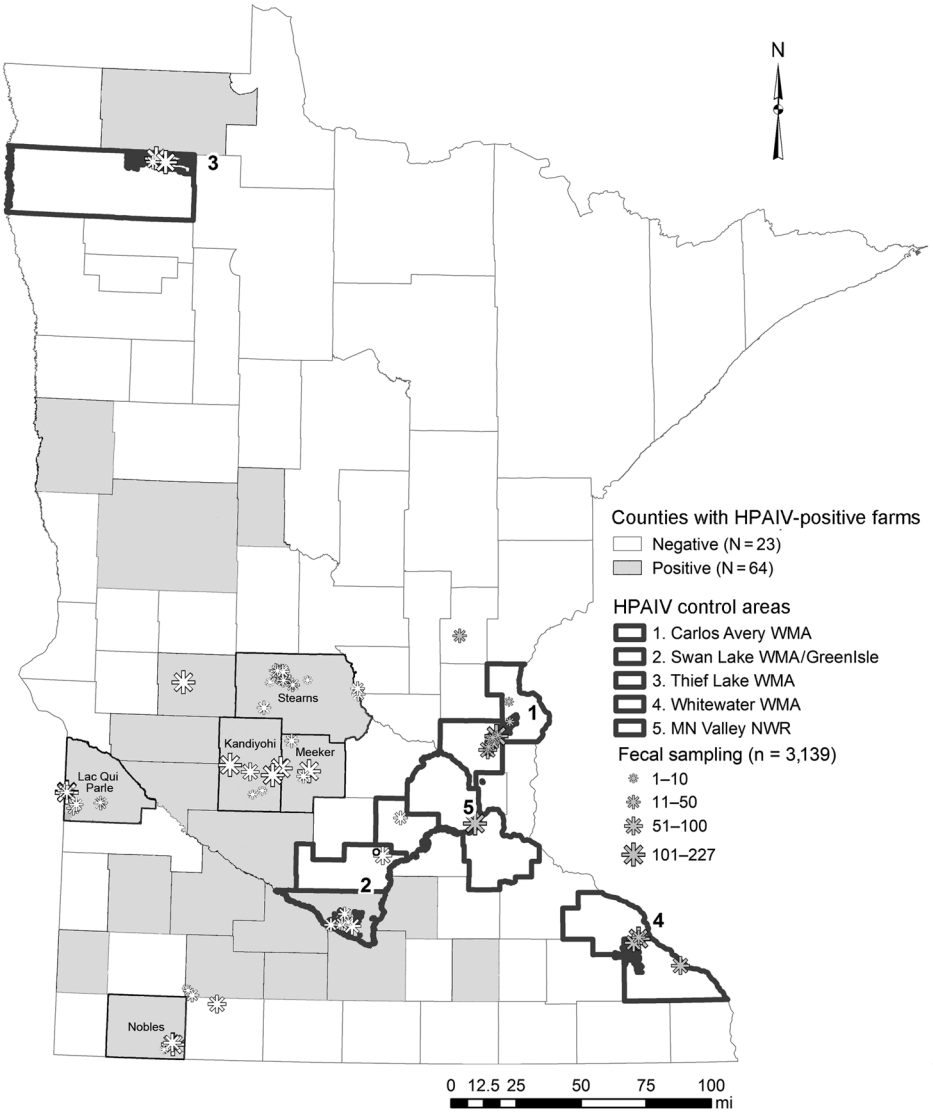

Figure 1. Minnesota collection sites for waterfowl feces sampled for highly pathogenic avian influenza virus (HPAIV) in spring 2015 (N = 3,139). Although HPAIV was confirmed in a Nicollet County poultry facility on May 5, 2015, our sampling occurred during April 22–April 27, 2015, and we consider this a control area (control no. 2). WMA, wildlife management area; NWR, national wildlife refuge.

In April 2015, which coincided with the peak rates of infection in Minnesota facilities (*8*), we collected 2,991 waterfowl fecal samples and pooled them into 1,027 brain-heart–infusion media vials; 1,591 samples (548 pooled) were obtained from counties with infected facilities, and 1,400 samples (479 pooled) were collected from waterfowl production areas without facilities (Figure 1). Although HPAIV was not detected in these samples, 30 pooled samples (representing 85 individual birds) tested positive for LPAIV. Apparent LPAIV fecal prevalence was 0.012 (95% CI 0.007–0.018) in counties with infected poultry, 0.008 (95% CI 0.004–0.014) in counties without infection, and 0.010 (95% CI 0.007–0.014) in the combined study area. Given that HPAIV was not detected and that we could not sample every individual bird in the waterfowl population, if HPAIV were present, there was a 95% probability that fecal prevalence was between 0 and 0.181% in areas with infection and 0 and 0.224% in areas without infection.

Through June 4, 2015 (last confirmed positive facility), we collected and tested 104 morbidity and mortality samples (Table) and detected a single HPAIV-positive bird, a Cooper's hawk (*Accipiter cooperii*) from Yellow Medicine County (20 km from an infected facility); this infection was confirmed on April 29, 2015 (Figure 2). We suspect that this woodland predator and opportunistic scavenger was exposed to HPAIV through a food item. Although not discovered as part of Minnesota Department of Natural Resources surveillance, 3 black-capped chickadees (*Poecile atricapillus*) were found in an urban neighborhood exhibiting neurologic signs and submitted to the University of Minnesota Veterinary Diagnostic Laboratory by the Minnesota Wildlife Rehabilitation Center in June

Table. Wild birds collected (n = 104) for highly pathogenic avian influenza virus screening as part of MNDNR morbidity and mortality sampling efforts, Minnesota, USA, March 9–June 4 2015

Order*	Family	Genus and species	Common name	Count
Anseriformes	Anatidae	*Branta canadensis*	Canada goose	8
		Cygnus buccinators	Trumpeter swan	3
		Aix sponsa	Wood duck	2
		Anas platyrhynchos	Mallard	2
Galliformes	Phasianidae	*Phasianus colchicus*	Ring-necked pheasant	8
		Meleagris gallopavo	Wild turkey	17
Pelicaniformes	Pelicanidae	*Pelicanus erythrorhynchos*	American white pelican	1
Accipitriformes	Cathartidae	*Cathartes aura*	Turkey vulture	1
	Accipitridae	*Haliaeetus leucocephalus*	Bald eagle	5
		Accipiter striatus	Sharp-shinned hawk	8
		Accipiter cooperii†	Cooper's hawk	6
		Buteo platypterus	Broad-winged hawk	1
		Buteo jamaicensis	Red-tailed hawk	3
Gruiformes	Rallidae	*Rallus limicola*	Virginia rail	1
		Porzana carolina	Sora	1
		Fulica americana	American coot	9
	Gruidae	*Grus canadensis*	Sandhill crane	1
Charadriiformes	Laridae	*Larus delawarensis*	Ring-billed gull	1
		Larus argentatus	Herring gull	1
Columbiformes	Columbidae	*Columba livia*	Rock pigeon	2
		Zenaida macroura	Mourning dove	1
Strigiformes	Strigidae	*Bubo virginianus*	Great horned owl	3
Caprimulgiformes	Caprimulgidae	*Chordeiles minor*	Common nighthawk	1
Passeriformes	Sturnidae	*Sturnus vulgaris*	European starling	10
	Parulidae	*Setophaga striata*	Blackpoll warbler	1
		Setophaga palmarum	Palm warbler	1
	Emberizidae	*Melospiza lincolnii*	Lincoln's sparrow	1
	Icteridae	*Euphagus carolinus*	Rusty blackbird	3
		Quiscalus quiscula	Common grackle	1

*1 sparrow not listed was identified to order Passeriformes.
†1 HPAIV-positive Cooper's hawk confirmed on April 29, 2015.

2015; in 1 bird there was weak detection of Eurasian H5 RNA, but no virus was recovered and no sequence could be obtained directly from the sample (*7*). All 3 birds demonstrated multifocal encephalitis, which was likely the cause for the neurologic signs (A. Armien, pers. comm.).

Conclusions

Morbidity and mortality samples yielded the only HPAIV detected in our surveillance of Minnesota wild birds, despite the relatively small number of samples. This sample type has proven valuable for HPAIV detection in wild birds in other states; 32% of HPAIV detections nationwide and 90% of HPAIV detections within the Mississippi flyway were derived from this source during December 2014–June 2015 (*7*). Evolving HPAIV strains can elicit clinical signs and death in young immunologically naive ducks (*10*), and targeted sampling of waterfowl postbreeding areas for dead or neurologically impaired hatch-year birds might prove useful for future HPAIV surveillance (*11*).

Careful thought has been given to the design of surveillance programs for avian influenza (*12*). The study objectives, coupled with the methodologic limitations of available approaches, drive the sampling tool ultimately applied. Although opportunistic sampling (e.g., morbidity and mortality surveillance) is accessible to most agencies, it is not suited for formal population-level inferences. For estimating AIV shedding prevalence, swab sampling of oropharyngeal and cloacal cavities in live birds or the trachea and cloaca in recently deceased birds is optimal because AIV replicates and sheds through the digestive tract (*13*) and the upper respiratory system (*14*). For investigating exposure history, sampling blood from live or recently dead birds for serologic testing would be more appropriate, although timing, location, and mechanism of exposure cannot be determined.

Most of our samples were obtained from waterfowl feces. The outbreak's speed required a quickly deployable method to collect adequate sample sizes and implement spatial design elements that would allow a meaningful comparison between known areas with infection and areas of the state apparently without infection. Modeling has shown that AIV maintenance in wild bird populations is mediated by environmental transmission (*15*), and the detection of LPAIV in waterfowl fecal samples supports that conclusion. No HPAIV was detected in waterfowl feces, although there was 95% probability of apparent fecal prevalence throughout the study area of 0 to 0.1%. Thus, we conclude that during the 2015 HPAIV (H5N2) outbreak in Minnesota poultry, HPAIV contamination in wild waterfowl feces was not widespread.

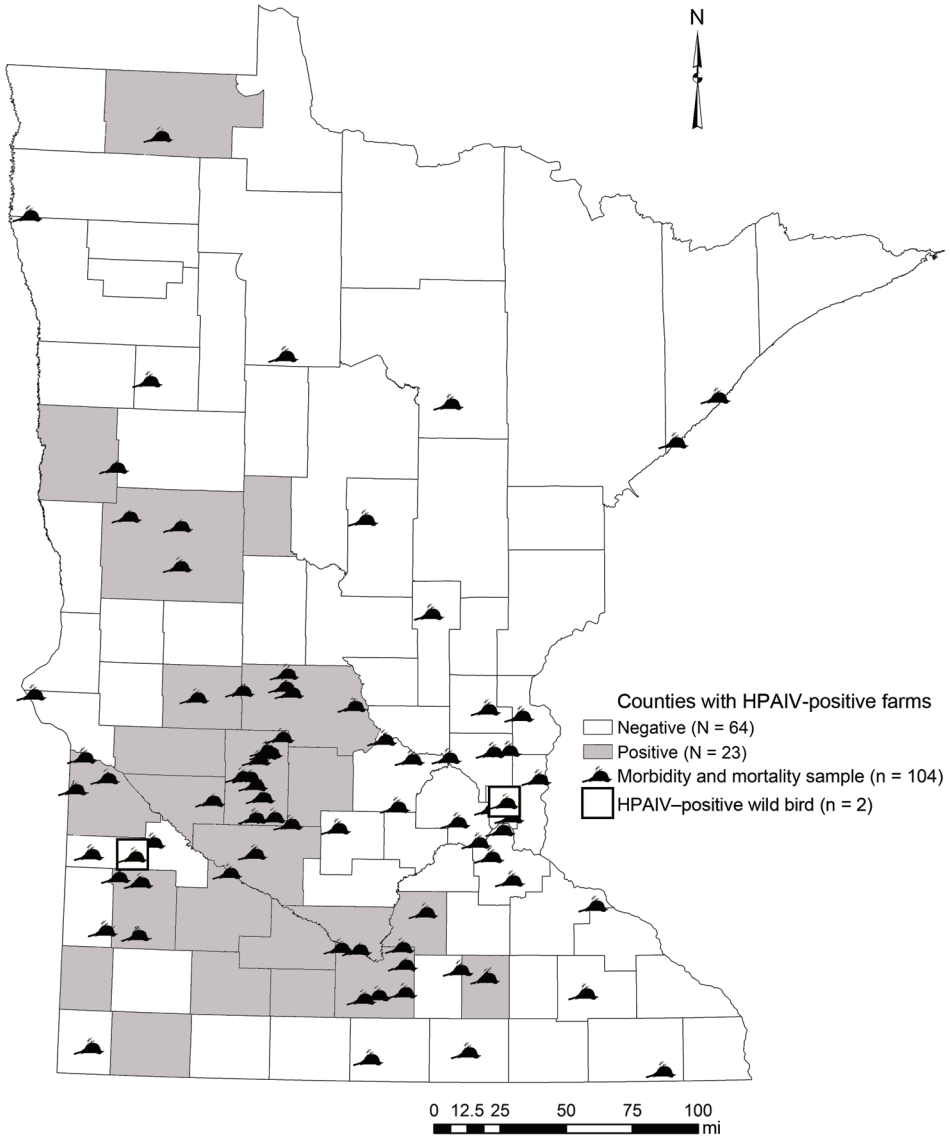

Figure 2. Wild bird morbidity and mortality samples (n = 104) screened for highly pathogenic avian influenza virus (HPAIV) in Minnesota through June 4, 2015. A Cooper's hawk was confirmed to be HPAIV positive in Yellow Medicine County on April 29, 2015, whereas weak titers of Eurasian H5 RNA were detected in a sampled black-capped chickadee from Ramsey County collected in June 2015.

Acknowledgments

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Dr. Jennelle is a research scientist in the Wildlife Health Program of the Minnesota Department of Natural Resources, Forest Lake, Minnesota, USA. He is interested in the ecology of wildlife diseases, quantitative methods for understanding wildlife disease dynamics, and conservation and management of wildlife populations.

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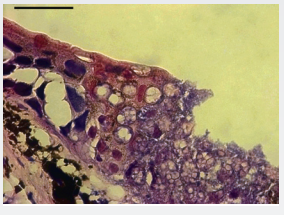
etymologia

Batrachochytrium salamandrivorans [bə-tray'-koh-kit"-ri-um sa"-la-man-dri-vo'rans]

Batrachochytrium salamandrivorans is a recently discovered fungus that kills amphibians. It is related to *B. dendrobatidis*, which also kills amphibians (from the Greek *dendron*, "tree," and *bates*, "one who climbs," referring to a genus of poison dart frogs). *Batrachochytrium* is derived from the Greek words *batrachos*, "frog," and *chytra*, "earthen pot" (describing the structure that contains unreleased zoospores); *salamandrivorans* is from the Greek *salamandra*, "salamander," and Latin *vorans*, "eating," which refers to extensive skin destruction and rapid death in infected salamanders.



Basal infection in skin of a fire salamander (*Salamandra salamandra*) characterized by extensive epidermal necrosis, high numbers of intra-epithelial colonial chytrid thalli, and loss of epithelial integrity. Photo by A. Martel and F. Pasmans, courtesy of Wikipedia.

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Technical Appendix

Methods

Fecal Sampling

There is a precedent established for avian influenza virus (AIV) surveillance in wild birds by sampling waterfowl feces, with comparable AIV prevalence estimates for fecal and oropharyngeal/cloacal swabbing (*1*–*5*). Given the rapid emergence of infected poultry facilities in Minnesota during April 2015, we focused on collecting waterfowl feces for the following reasons: 1) it afforded us the most control over sampling design elements and permitted hypothesis-driven surveillance (*6*), 2) a large sample size could be collected relatively quickly, and 3) the timing of the outbreak occurred when birds are not available for efficient live capture.

From March 9–March 12, 2015, we used polyester-tipped swabs to collect 148 representative waterfowl fecal samples, pooled in groups of up to 3, to determine whether ≈100 resident mallards (*Anas platyrhynchos*) and 21 trumpeter swans (*Cygnus buccinator*) were actively shedding high pathogenicity AIV (HPAIV) in the surveillance zone (≈1,830 km^2) around the Pope County index poultry facility. All fecal samples were submitted to the USDA National Wildlife Research Center (USDA-NWRC) in Fort Collins, Colorado, USA, for diagnostic testing.

For our designed sampling approach in areas of Minnesota with and without HPAIV-infected poultry, the waterfowl production areas we chose as control sites consisted of 5 wildlife management areas (WMA)/national wildlife refuges (NWR) without infected facilities (Carlos Avery WMA, Minnesota Valley NWR, Swan Lake WMA, Thief Lake WMA, and Whitewater

WMA). These areas are managed by state or federal agencies to sustain and enhance wildlife habitat (especially wild waterfowl game birds) for wildlife conservation. We used polyester-tipped swabs to collect fecal samples deposited in 1 of 17 location types (Technical Appendix Table) during April 8–April 30, 2015. We sampled what we perceived to be fresh waterfowl feces (<24 h) that were at least 2 m apart. We assumed that each feces pile represented a unique individual bird and pooled up to 3 samples per vial, which was filled with brain-heart infusion medium, and refrigerated. Samples were submitted to the USDA-NWRC in Fort Collins, Colorado, for diagnostic testing.

Morbidity and Mortality Sampling

We made no fixed goals for this sample type because of the opportunistic nature of discovery and reporting, and targeted birds that had been dead for <24 h. We used these data as an auxiliary source of information in our surveillance efforts and obtained samples statewide. Depending on the resources available for staff, we either collected whole carcasses (double-bagged and frozen) or used polyester-tipped swabs to separately obtain tracheal and cloacal specimens from sampled birds. Both swab samples from a bird were pooled in blood-heart infusion media and refrigerated. Whole carcasses were submitted to the USGS National Wildlife Health Center (USGS-NWHC) or the Minnesota Veterinary Diagnostic Laboratory in St. Paul MN, and swab samples were submitted to USDA-NWRC for diagnostic testing.

Sample Diagnostic Testing

At the USDA-NWRC, nucleic acid was extracted from 50 μL of pooled fecal swab samples using the MagMax-96 AI/ND Viral RNA Isolation Kit (ThermoFisher Scientific, Waltham, MA, USA). Five microliters of nucleic acid extracts were analyzed by real-time reverse transcription PCR (rRT-PCR) with primers and probes specifically designed to detect the influenza virus type A matrix gene (*7*) and the iTaq Universal Probes One-Step Kit (BioRad Laboratories, Hercules, CA, USA). The rRT-PCR conditions were the following: 50°C for 10 min, 95°C for 30 s, and 40 cycles at 95°C for 15 s and 60°C for 30 s. Samples with Ct values ≤38 were forwarded to the USDA National Veterinary Services Laboratory (USDA-NVSL) in Ames, Iowa, USA, for confirmation and further H5 and H7 testing and isolation.

Swabs from carcasses of diseased or dead birds were submitted to USGS-NWHC where diagnostic necropsies were performed. Tracheal and cloacal swab specimens were collected from

all carcasses and used to screen for HPAIV, when a necropsy was not performed. Tissue samples for AIV testing were homogenized in viral transport media and centrifuged at 1,000 × *g* for 30 min at 4°C. RNA from 50 μL of the supernatant of the tissue homogenate or swab material were recovered and tested for AIV by the current National Animal Health Laboratories Network protocols (*7*,*8*). Aliquots of samples for subtypes H5 and H7 were sent to USDA-NVSL for confirmation on the day of detection and were further characterized by additional tests, including rRT-PCR for virulence, sequence analysis, and virus isolation.

Data Analysis

For estimating apparent prevalence of low pathogenicity AIV) in fecal specimens, we used rRT-PCR matrix test results determined by the USDA-NVSL and applied a Bayesian approach (*9*), accounting for variable-sized pooled samples and imperfect test sensitivity and specificity. We used a binomial distribution to model the response variable (proportion of rRT-PCR positive test results) and used an uninformative prior distribution for low pathogenicity AIV prevalence. Because no published diagnostics are currently available for sensitivity and specificity of rRT-PCR matrix results from waterfowl fecal samples when specifically testing for the HPAIV(H5N2) Eurasian-American strain, we assumed unity (SE = Sp = 1). For calculating the detection threshold for HPAIV shedding in fecal samples given zero positive tests, we assumed independence among samples and used a Bayesian approach (*10*), again assuming an uninformative prior distribution beta ($\alpha = \beta = 1$) on HPAIV shedding prevalence.

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Technical Appendix Table. Description of 17 location types in Minnesota searched for waterfowl feces, April 2015

Location description
Foam baiting stations in ditches, ponds, and marshes
Mowed grass and gravel around ponds or along dikes
Top of gravel or grass dikes
Waste water ponds
Sand bars in lakes
Mud flats
Vegetation mats on water
Golf courses
Mowed ditches along roads
Gravel or grass shore along lakes
Upland hay meadows
Sandy beaches on lakes
Loafing rocks in open water and logs along lakes or pond shoreline
Cleared areas along lakes or ponds
Softball fields
Residence yards near ponds or lakes
Park grounds