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Research Note—

An Adenovirus Linked to Mortality and Disease in Long-Tailed Ducks (Clangula hyemalis) in Alaska

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SUMMARY. An adenovirus was isolated from intestinal samples of two long-tailed ducks (*Clangula hyemalis*) collected during a die-off in the Beaufort Sea off the north coast of Alaska in 2000. The virus was not neutralized by reference antiserum against known group I, II, or III avian adenoviruses and may represent a new serotype. The prevalence of the virus was determined in live-trapped long-tailed ducks at the mortality site and at a reference site 100 km away where no mortality was observed. Prevalence of adenovirus antibodies in serum samples at the mortality site was 86% compared to 10% at the reference site. Furthermore, 50% of cloacal swabs collected at the mortality site and only 7% of swabs from the reference site were positive for adenoviruses. In 2001, no mortality was observed at either of the study areas, and virus prevalence in both serum and cloacal samples was low, providing further evidence that the adenovirus was linked to the mortality event in 2000. The virus was used to infect long-tailed ducks under experimental conditions and resulted in lesions previously described for avian adenovirus infections and similar to those observed in long-tailed duck carcasses from the Beaufort Sea. The status of long-tailed ducks has recently become a concern in Alaska due to precipitous declines in breeding populations there since the mid-1970s. Our findings suggest that the newly isolated adenovirus is a disease agent and source of mortality in long-tailed ducks, and thus could be a contributing factor in population declines.

RESUMEN. *Nota de Investigación*—Aislamiento de una cepa de adenovirus asociada a enfermedad y mortalidad en patos mavelda (Clangula hyemalis) en Alaska.

Se aisló una cepa de adenovirus a partir de muestras de intestino obtenidas de dos patos mavelda (*Clangula hyemalis*) provenientes del mar de Beaufort, en la costa norte de Alaska, durante un brote de enfermedad y mortalidad en el año 2000. El virus no pudo ser neutralizado con antisueros de referencia específicos para los grupos I, II y III de los adenovirus aviares, por lo cual puede representar un nuevo serotipo en patos. La prevalencia del virus se determinó *in vivo* en patos mavelda capturados en la zona de mortalidad y en patos obtenidos de una zona a 100 Km. de distancia en donde no se había reportado enfermedad, los cuales fueron usados como referencia. La prevalencia de anticuerpos específicos contra adenovirus fue de un 86% en la zona de enfermedad, mientras que la misma fue de solo 10% en las muestras obtenidas en la zona de referencia. El 50% de los hisopos cloacales obtenidos en la zona de mortalidad fueron positivos a la presencia de adenovirus, mientras que solo un 7% de las muestras obtenidas en la zona de referencia mostraron presencia del virus. En el año 2001 no se observaron brotes de enfermedad

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y mortalidad en ninguna de las zonas usadas en el estudio y la prevalencia de anticuerpos específicos contra el virus en muestras de suero e hisopos cloacales fue baja, lo cual apoya la teoría de que el brote de enfermedad y mortalidad del año 2000 fue ocasionado por el adenovirus. Este virus se utilizó para infectar patos mavelda en condiciones experimentales donde se observaron lesiones similares a las descritas previamente en infecciones por adenovirus y similares a las lesiones observadas en cadáveres de patos mavelda provenientes del mar de Beaufort. En años recientes, el estatus de esta especie de patos se ha convertido en motivo de preocupación en Alaska debido a la rápida disminución de la población reproductiva observada desde la década de los años 1970. Nuestros hallazgos sugieren que la cepa de adenovirus aislada es un agente causante de enfermedad y mortalidad en patos mavelda, por lo cual puede estar contribuyendo a la baja del número de individuos de la población.

Key words: adenovirus, *Clangula hyemalis*, long-tailed duck

Abbreviations: CPE = cytopathic effect; HBSS = Hanks balanced salt solution; IU = international unit; MDEF = muscovy duck embryo fibroblast; NWHC = National Wildlife Health Center; $TCID_{50}$ = tissue culture infectious dose

Long-tailed ducks (*Clangula hyemalis*) are small sea ducks inhabiting the marine environments of Palearctic and Nearctic regions, with wintering birds recorded as far south as in the Gulf of Mexico on the North American continent (14). In Alaska, long-tailed ducks are found along the arctic and subarctic coasts from as far north as Point Barrow to the southeastern panhandle. Long-tailed ducks are abundant and widespread in the nearshore lagoons of the Beaufort Sea, where tens of thousands of individuals have been estimated to congregate in July and August to undergo wing and tail feather molt (1,12,13,29). The wing molt of long-tailed ducks lasts approximately 3–4 weeks (15), during which time the flightless birds feed in the central portions of the lagoons or form roosts of up to several hundred individuals along the northern shores of the barrier islands. During the flightless period, the birds are susceptible to predation, adverse weather, and other disturbance, and have been listed as potential indicators for environmental impacts of industrial development in the area (13).

The status of long-tailed ducks has recently become of concern in Alaska due to precipitous declines in breeding populations there since the mid-1970s (8,28). In the barrier island lagoons between Oliktok Point and Brownlow Point, the densities of molting long-tailed ducks have decreased by 75% since 1990 (5). In Alaska, the long-tailed duck is currently designated *at risk,* and the U.S. Fish and Wildlife Service has been petitioned to list the species under the Endangered Species Act.

Apart from renal coccidia, identified in a male long-tailed duck found dead near the Teshukpuk Lake on the North Slope in the 1970s (6), little is known about infectious and parasitic diseases in long-tailed ducks in Alaska. Elsewhere in North America, long-tailed duck populations suffer from high mortality caused by various diseases. Avian cholera outbreaks have killed significant numbers of long-tailed ducks on at least three occasions in the Chesapeake Bay (16,17,21), and, in 2002, several thousand long-tailed duck carcasses were collected during a period of high mortality in the Great Lakes (primarily Lake Erie), which was attributed to avian botulism type E (Ward Stone, pers. comm.). Additionally, cestodes have been identified as intestinal parasites (25). Viral diseases have not been reported as a cause of mortality in long-tailed ducks, although viruses have been linked to die-offs in other species of waterfowl, including sea ducks (2,10). Viral diseases could be particularly significant when birds congregate in dense flocks during molting, and bird-to-bird contacts allow efficient transmission and perpetuation of an outbreak.

Evaluation of the effects of infectious diseases on populations of wild birds poses difficult challenges. However, recent studies suggest that diseases have significant impacts on species already in decline and, furthermore, that avian populations previously thought to be healthy and stable should be monitored for potential effects of new and rapidly spreading diseases (7). Because factors affecting survival of long-tailed ducks and the causes of recent population declines among sea ducks in Alaska are not well understood, our objectives were to evaluate long-tailed ducks for presence of viruses and viral diseases during molt in the Beaufort Sea. We evaluated viruses as a cause of death in long-tailed duck carcasses recovered in 2000, conducted an experimental inoculation study to characterize the pathogenicity of a virus isolated from these carcasses, and compared virus prevalences between

live-trapped long-tailed ducks at the mortality site and a reference area, where no mortality was observed.

MATERIALS AND METHODS

Field studies. Field study sites were located in the vicinity of Prudhoe Bay, AK, in the near-shore lagoons of the Beaufort Sea. The eastern study area was located in an unnamed lagoon (formed by the Stockton, Maguire, and Flaxman Island complexes) that lies approximately 80 km east of Prudhoe Bay, and the western study area was in the Simpson Lagoon (formed by the Jones and Return Island complexes) near Prudhoe Bay. Long-tailed ducks were trapped and sampled during molt in July and August of 2000 and 2001. Flightless birds were captured by driving flocks into net corrals, placed on beaches adjacent to roosting areas (23). In 2000, samples were collected from 40 birds at the eastern study site and from 30 birds at the western study site. In 2001, samples were collected from 30 and 20 birds at the eastern and western study sites, respectively. Cloacal samples were collected with Dacron-tipped swabs, transferred into virus transport media (Hanks balanced salt solution [HBSS] with 0.5% gelatin and 1500 international units [IU] penicillin, 1500 μg streptomycin, 100 μg gentamicin, and 100 IU mycostatin per ml), and stored in the gaseous phase of a liquid nitrogen dry shipper (Chart, Inc., New Prague, MN). Blood samples were collected by jugular venipuncture and transferred into Vacuette® (Greiner Meditech, Inc., Bel Air, MD) tubes without anticoagulant. The blood samples were allowed to clot for approximately 2 hr in a cooler with ice packs and centrifuged at 1500 × *g* for 10 min. Serum was harvested and stored in the dry shipper in the field at −80 C until analyzed.

Field work on other aspects of long-tailed duck molting ecology continued through August in 2000 and 2001. In August of 2000, glaucous gulls (*Larus hyperboreus*) were observed feeding on long-tailed duck carcasses floating on the water surface in the near-shore lagoons of the eastern study area. A total of 12 duck carcasses were found by mid-August; however, we were rarely in a position to observe scavenging or discover carcasses. Two intact carcasses were frozen at −20 C in the field and shipped to the USGS National Wildlife Health Center (NWHC) (Madison, WI).

Diagnostic laboratory analyses. The two carcasses were examined at necropsy, and samples of liver, spleen, lung, kidney, small intestine, and cloaca were collected for virus isolation. Moderate autolysis and freezing prevented histopathologic evaluation of tissues. Samples of liver and intestine were submitted to the microbiology laboratory of the NWHC for bacteriology, and samples of liver were submitted to the chemistry laboratory of the NWHC for lead analyses. Primary cultures of muscovy duck (*Cairina moschata*) embryo fibroblasts (MDEF) were used for virus isolation (3). Approximately 1 g of each tissue sample was homogenized in virus transport medium, and the homogenates were centrifuged at 800 × *g* for 30 min at 4 C. The cloacal swabs were mixed by vortexing and centrifuged at 800 × *g* for 15 min. Supernatants of each sample were inoculated separately onto MDEF monolayers and incubated at 37 C in 5% CO_2. The cell cultures were examined every other day for 7 days for viral cytopathic effects (CPEs). When no CPE was observed, samples were freeze thawed, blind passaged to fresh cell cultures, and monitored for an additional 7 days.

Virus identification. The nucleic acid type of the isolated viruses was determined by evaluating their infectivity to MDEF cells after 5-iodo-2′-deoxyuridine treatment. The presence or absence of lipoprotein envelope was evaluated with a chloroform lability assay (4). Positive cells cultures were prepared for electron microscopy by slow centrifugation at 800 × *g* for 30 min and by ultracentrifugation of the supernatant at 35,000 × *g* for 150 min. The viral pellets were resuspended in distilled water, placed on grids, negatively stained with 0.5% phosphotungstic acid, and examined with a Hitachi H-500 transmission electron microscope (Hitachi High Technologies, Tokyo, Japan). Representative isolates were tested against avian adenovirus I (serotypes 1, 3, and 5) (Spafas® Inc., Preston, CT), avian adenovirus II (hemorrhagic enteritis virus) (Spafas Inc.), and avian adenovirus III (egg drop syndrome virus, duck adenovirus serotype 1) (National Veterinary Services Laboratory, Ames, IA) antiserum using a standard virus neutralization assay (26).

Serology. Serum samples were heat inactivated at 56 C for 30 min, and serial twofold dilutions were tested in a standard virus neutralization assay (26) for antibodies against a representative isolate from the long-tailed ducks.

Experimental studies. Three long-tailed ducks (Dry Creek Waterfowl, Port Angeles, WA) were received at the NWHC on September 20, 2001, for a pilot virus infectivity trial. Ducks were housed in a biosafety level III room (5.7 m × 3.2 m) equipped with a pond liner and filled with water to a depth of 20 cm (experimental room). Water was exchanged in the pool at a rate of 4 liters/min, and the room was kept on a light cycle of 12 hr per day (0600 to 1800). All ducks were fed Mazuri® (PMI Nutrition International, Brentwood, MO) 5681 sea duck diet *ad libitum* throughout the experiment. Feeding stations consisted of two artificial islands per pool with open feeding pans placed on top. Titers of the virus stock used in the experiment were calculated by 50% tissue culture infectious doses ($TCID_{50}$). On September 22, two pilot ducks (one male and one female) were inoculated orally with approximately 7×10^4 $TCID_{50}$ in 10 ml of

Hanks balanced salt solution of the virus isolated from long-tailed ducks in 2000 and the second female was inoculated orally with approximately 3.5×10^5 $TCID_{50}$ in 10 ml of HBSS to evaluate the dose required in the main experiment. Based on the results of the pilot experiment, 13 additional long-tailed ducks were received on October 22 and placed in an identical biosafety level III room (control room). On November 5, eight ducks were moved to the experimental room and inoculated orally with 6.2×10^5 $TCID_{50}$ in 10 ml of HBSS. The three ducks from the pilot study were moved into the room with the rest of the infected birds, and the five ducks remaining in the control room were dosed orally with sterile virus transport media. Preinoculation blood samples and cloacal swabs were collected from all ducks. Postinoculation blood samples were collected at intervals of approximately 1 week, and cloacal samples were collected on the day after inoculation and every 3 days thereafter from the virus-inoculated ducks and weekly from the control ducks. The weight of each duck was recorded each time a cloacal sample was taken. During the first 2 weeks postinoculation, the feeding pans of the ducks were periodically swabbed for virus isolation. On December 7, three experimental ducks (second inoculation) and three controls were inoculated orally with approximately 1.6×10^6 $TCID_{50}$ in 10 ml of HBSS. Ducks were euthanatized and necropsied 6 to 32 days postinoculation, based on weight changes in conjunction with data on virus activity. The three ducks from the pilot study were euthanatized on January 5, 2002. Blood samples were collected prior to euthanasia and centrifuged at $1500 \times g$ for 10 min, and serum was harvested for antibody testing and serum biochemistries. At necropsy, the following tissues were collected for both virus isolation and histopathology: duodenum, jejunum, ileum, large intestine, cloaca, cecum, liver, spleen, kidney, lung, and gonad. Additionally, heart and skeletal muscle were collected for histopathology. Virus isolation was performed as described for samples collected from the wild birds.

RESULTS

Necropsy findings. Both long-tailed ducks collected from the eastern study site in 2000 were males and in poor to moderate body condition (reduced pectoral muscles and subcutaneous fat reserves). Gross lesions of enteritis (dilated small intestines, mucoid and hemorrhagic intestinal contents) were observed at necropsy in both individuals. No pathogens were isolated in bacterial cultures, and lead levels in liver samples were not considered elevated. Small intestinal and cloacal samples from carcasses were positive in virus cultures, and the isolates were characterized as nonenveloped DNA viruses. By electron microscopy, the viral capsomeres were arranged in equilateral triangles and the isolates were identified as adenoviruses (11). The virus was not neutralized by reference antiserum against avian adenovirus group I, II, or III viruses.

Virus prevalence in the field. In 2000, virus was isolated from 50% of cloacal swabs collected at the mortality site (eastern study area) and from 7% of cloacal swabs from the western study area, where no mortality was observed. Prevalence of serum antibody titers $\geq$1:64 was 86% at the eastern study area and 10% at the western study area. In 2001, no mortality was observed at either of the study areas. Virus was recovered from 7% and 0% of the cloacal swabs collected at the eastern and western study areas, respectively. Seroprevalence was 20% in the eastern study area and 0% in the western study area.

Experimental study. No mortality occurred during the experimental study. Clinical signs indicative of gastrointestinal disease were observed in ducks inoculated with adenovirus and included watery consistency of feces and blood in feces. Serologic testing indicated that all inoculated ducks became infected with the virus. Titers of >1:256 were detected at 7 to 14 days postinoculation. After 3 weeks, the circulating antibody levels dropped and exhibited an undulating pattern in the three ducks that were monitored for 3.5 months. Most inoculated birds were shedding viruses from their cloaca for approximately 2 weeks, and the three ducks that were monitored for 3.5 months were shedding periodically throughout the entire period. In the 11 ducks that were necropsied during days 6–32 postinoculation, virus was isolated most frequently from the ileum and large intestine (73%) but was also isolated from duodenum (63%), jejunum (55%), cloaca (55%), cecum (55%), liver (36%), kidney (27%), lung (9.1%), testis (14% of males), and ovary (14% of females). At necropsy, pinpoint and paintbrush hemorrhages were noted in the proximal intestine of eight (73%) birds. Histological findings included widespread lymphoid hyperplasia and necrosis (in 91% of the ducks), mild to moderate multifocal nonsuppurative enteritis (91%), mild to moderate multifocal nonsuppurative typhlocolitis (36%), multifical nonsuppurative hepatitis (36%), and hepatic intranuclear inclusions (14%) (Fig. 1). Lymphoid and enteric lesions were seen throughout the 6–32 day period, and hepatic lesions were seen in ducks euthanatized on days 6, 7, and 12 postinoculation. Virus was reisolated from the spleen of two of three individuals monitored for 3.5 months. One of these

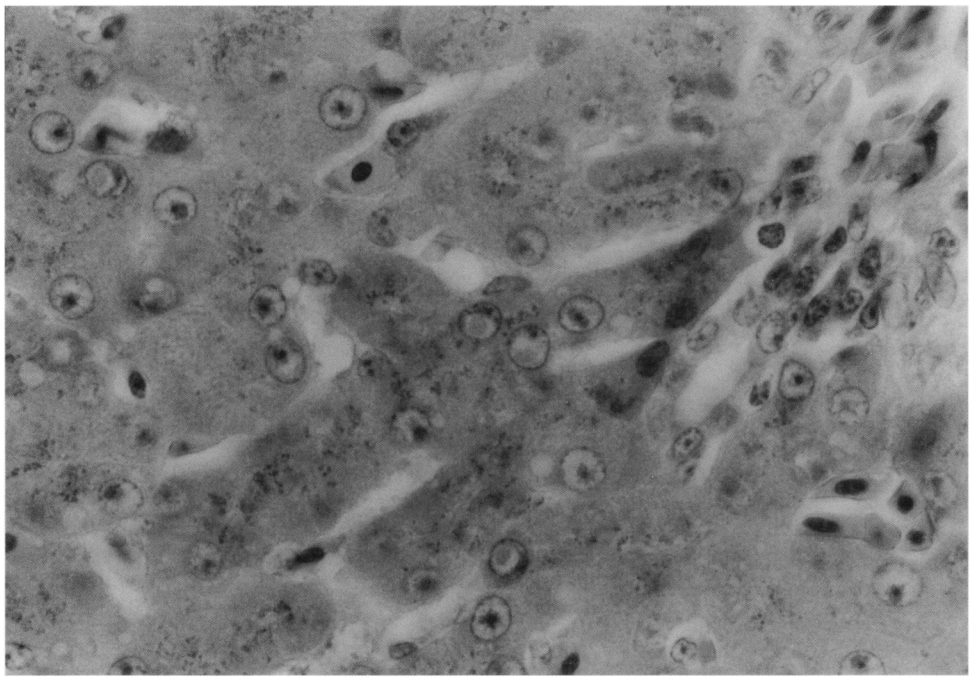

Fig. 1. Intranuclear inclusion bodies in the liver of a long-tailed duck inoculated orally with the adenovirus. Two inclusion bodies are visible in the center of the liver section.

ducks showed lymphoid hyperplasia and necrosis of spleen and small intestine and had multifocal nonsuppurative enteritis. Viruses were also isolated from materials swabbed from the feeding pans during the first 2 weeks of the study.

DISCUSSION

We isolated an adenovirus from intestinal tissues of long-tailed ducks found dead in the Beaufort Sea in Alaska in 2000. The virus was not neutralized by antisera against previously known avian group I, II, or III adenoviruses and probably represents a new adenovirus serotype. Although no mortality occurred in the inoculated long-tailed ducks, the virus was infectious under experimental conditions and resulted in lesions previously described for avian adenovirus infections, including intestinal hemorrhage, inclusion body hepatitis, and lymphoid tissue pathology (19,20,24). Based on the pathology observed in long-tailed ducks, the newly isolated adenovirus appears to share some characteristics with both group I and II avian adenoviruses. Inclusion body hepatitis has been previously reported in chickens, pigeons, and some species of raptors, with the etiologic adenovirus belonging to group I. Hemorrhagic enteritis virus has been previously reported from turkeys, is classified as a group II adenovirus, and has also been associated with immune system pathology. Reproductive pathology has been linked to group III avian adenoviruses, and virus-associated effects include reductions in egg production and egg abnormalities (18,20). In our experimental study, the adenovirus was isolated from gonad tissues of one male and one female long-tailed duck, but the potential of the newly isolated virus to affect reproductive parameters was not studied in long-tailed ducks.

The carcasses collected from the original die-off in the Beaufort Sea showed lesions indicative of enteric pathology. Because the tissues were not suitable for a histological evaluation, we were unable to determine whether liver inflammation and hepatic intranuclear inclusion bodies were present. Based on the serology and virus isolation results from live-trapped long-tailed ducks, high prevalence of both serum antibodies and viruses in cloacal samples was strongly linked to the observed mortality in 2000. The prevalence of antibodies and viruses was significantly lower in birds trapped at the reference site 100 km away, where no mortality was observed in 2000. Furthermore, in 2001, the year following

the die-off when no mortality was observed, virus prevalences were also very low. These results support the hypothesis that the virus was either responsible for or a significant contributing factor in the mortality observed in 2000.

Experimentally infected long-tailed ducks were shedding viruses from their cloaca for approximately 2 weeks postinoculation and intermittently for at least an additional 3 months. Viruses were isolated from feeding pans of the experimental pools for 2 weeks postinoculation. Because all ducks were shedding viruses and virus persisted in the feeding pans, transmission via a fecal–oral route seems likely for the long-tailed duck adenovirus. In the wild, molting long-tailed ducks roost in dense flocks on shore during the nights, allowing for close bird-to-bird and bird-to-feces contacts and, thus, potential virus transmission.

Animals inoculated with viruses and housed under experimental conditions often exhibit less severe disease than that which occurs in natural infections (27), and a similar scenario may explain our experimental results in captive birds that were fed *ad libitum* and not stressed by other environmental factors. The virus caused moderate intestinal, hepatic, and lymphoid pathology in long-tailed ducks under experimental conditions, and effects on organs can be more significant in the wild because birds undergoing molt may be nutritionally stressed due to energy demands of feather growth and decreased insulation (9,22). Indeed, Howell (12) documented declines in body mass and lipid levels in long-tailed ducks undergoing wing molt in the Beaufort Sea during the same time that our study was conducted. Additionally, birds in the eastern areas, where the prevalence of virus was higher, were in poorer condition than birds in the western area. Birds that experience nutritional stress during molt may be more susceptible to viral infection, and, therefore, the molt may have been a contributing factor to the mortality event in 2000. Furthermore, an infectious agent interfering with intestinal function could increase the level of nutritional stress and lead to a cycle of deteriorating body condition during molt. Both carcasses that were examined in 2000 were in relatively poor body condition, suggesting that interactions between nutrition and intestinal adenovirus infections contributed to the mortality.

Additional indirect health effects of the virus isolated from long-tailed ducks may relate to immunopathology and reproductive disorders. If the adenovirus induces immunosuppression as a consequence of the lymphoid depletion and necrosis noted in our experimental study, interactions between the virus and other pathogens or environmental contaminants may be important. Other than gonadotropism, we have no evidence for the potential of the newly isolated virus to cause reproductive problems in its host, but because previously reported strains of adenoviruses associated with ducks have been linked to decreased egg production and egg abnormalities, the possibility of reproductive consequences should be evaluated in long-tailed ducks during nesting. If the newly isolated adenovirus is associated with impaired nutrition, reproduction, and immunocompetence in addition to causing direct mortality, its overall effects may be more subtle but result in a long-term reduction in population size and viability.

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