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CHARACTERIZATION OF AVIAN POXVIRUS IN ANNA'S HUMMINGBIRD (*CALYPTE ANNA*) IN CALIFORNIA, USA

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ABSTRACT: Avian poxvirus (genus *Avipoxvirus*, family *Poxviridae*) is an enveloped double-stranded DNA virus that may be transmitted to birds by arthropod vectors or mucosal membrane contact with infectious particles. We characterized the infection in Anna's Hummingbird (*Calypte anna*; $n=5$ birds, $n=9$ lesions) by conducting diagnostic tests on skin lesions that were visually similar to avian poxvirus lesions in other bird species. Skin lesions were single or multiple, dry and firm, pink to yellow, with scabs on the surface, and located at the base of the bill, wings, or legs. Microscopically, the lesions were characterized by epidermal hyperplasia and necrosis with ballooning degeneration, and intracytoplasmic inclusions (Bollinger bodies) in keratinocytes. The 4b core gene sequence of avian poxvirus was detected by PCR in samples prepared from lesions. Nucleotide sequences were 75–94% similar to the sequences of other published avian poxvirus sequences. Phylogenetic analyses showed that the Anna's Hummingbird poxvirus sequence was distinguished as a unique subclade showing similarities with sequences isolated from Ostrich (*Struthio camelus*), Wild Turkey (*Meleagris gallopavo*), falcons (*Falco* spp.), Black-browed Albatross (*Diomedea melanophris*), Mourning Dove (*Zenaida macroura*) and White-tailed Eagle (*Haliaeetus albicilla*). To our knowledge this is the first published report of definitive laboratory diagnosis of avian poxvirus in a hummingbird. Our results advance the science of disease ecology in hummingbirds, providing management information for banders, wildlife rehabilitators, and avian biologists.

Key words: Anna's Hummingbird, avian poxvirus, California, *Calypte anna*, *Poxviridae*, skin lesions, *Trochilidae*.

INTRODUCTION

Avian poxviruses (genus *Avipoxvirus*, family *Poxviridae*) have a worldwide distribution affecting at least 3% of bird species (Bolte et al., 1999; Tripathy et al., 2000). Infections are characterized by two main disease syndromes: a diphtheritic and a cutaneous form. The diphtheritic form, commonly fatal, follows inhalation of virus and involves mucous membranes of the oral cavity, pharynx, and trachea (Tripathy and Reed, 2003). The cutaneous form is characterized by nodular lesions usually involving unfeathered areas near the eyes, feet, or legs (Hansen 1999). Commonly, the pathogen is transmitted by biting arthropods (vector-borne mechanical transmission; van Riper and Forrester, 2007). Direct contact between infected and susceptible birds and exposure to contaminated objects (fomites) are also potential sources of infection in domestic and free-ranging birds (van Riper and Forrester, 2007). Although less common, aerosol transmission has been also reported (Mete et al., 2001), especially under confinement situations such as aviaries or rehabilitation centers (van Riper and Forrester, 2007).

† This work is dedicated to Loreto Godoy and her passion for hummingbirds. A young Chilean veterinarian with a promising career as a conservation ecologist, she died in June 2013, a week after receiving her PhD. She is survived by her husband and two daughters.

In domestic poultry, the disease is associated with significant economic losses because of increased mortality and decreased production. In free-ranging birds, mortality rates associated with avian poxvirus vary depending on species susceptibility; however, in most bird families infections are described as mild and rarely resulting in mortality (van Riper and Forrester, 2007). Nevertheless, lesions associated with avian pox infection may predispose birds to predation, secondary infections, behavioral changes (e.g., change in call patterns), and reduction in reproductive success (Kleindorfer and Dudaniec, 2006; Laiolo et al., 2007). Immunologically naïve island bird populations have been shown to be more severely impacted and have higher prevalence of the disease than mainland species (van Riper and Forrester, 2007; Zylberberg at al., 2012). For example, avian pox has been implicated as a major contributor to the decline of endemic bird species in the Hawaiian Islands (van Riper et al., 2002).

Anna's Hummingbird (*Calypte anna*; family Trochilidae) is a medium-sized hummingbird with a breeding range centered primarily in California, USA. It feeds on insects and nectar from a wide range of cultivated urban and suburban plants and from artificial feeders (Clark and Russell, 2012). After the main nesting season (December to May) many individuals migrate; however, the nature of the migration is unclear: some move to higher altitudes and others migrate long distances to the south and east (Wethington and Russell, 2003). Some individuals remain resident in western North America, but more evidence is needed to establish whether the same individuals are present year round (Clark and Russell, 2012). Information about the presence and effects of infectious diseases in Anna's Hummingbird and in hummingbirds in general is scarce (Godoy et al., in press). Wart-like lesions (crusted and deformed feet and bills, here called "avian pox–like syndrome") have been observed in individuals of the species captured at hummingbird banding stations in western North America during the last 3 yr (Colwell, 2011). Bleitz (1957) described wart-like lesions similar to our recent observations in Anna's Hummingbirds as "foot pox" but no prevalence, numbers of afflicted birds, or definitive laboratory diagnosis of a pathogen were presented. Colwell (2011) evaluated leg injuries in banded female Anna's Hummingbirds and reported avian pox–like lesions on the legs and bill and around the eyes in 11 individuals captured during 2004; however, diagnostic tests were not conducted and syndrome prevalence was not determined.

Our goals were to 1) determine whether there was definitive evidence of avian poxvirus in tissues of Anna's Hummingbirds with avian pox–like syndrome by a) amplifying a diagnostic segment of the gene encoding the avian poxvirus 4b core protein using PCR or b) identifying the presence of viral particles using direct electron microscopy or histopathology; and 2) compare phylogenetic relationships of the hummingbird avian poxvirus with other published DNA sequences from avian poxvirus. To our knowledge, this is the first published detailed characterization of this virus in Anna's Hummingbird and in the family Trochilidae.

MATERIALS AND METHODS

As part of research to evaluate disease occurrence in hummingbirds, five carcasses with external lesions compatible with avian poxvirus (van Riper and Forrester, 2007) were selected for this study. Hummingbird carcasses were donated from the Lindsay Wildlife Museum Hospital (LWM) in Walnut Creek, California, the California Animal Health and Food Safety Laboratory (California West Nile virus dead bird surveillance program), and hummingbird banders (birds found dead; Table 1). Injured or ill hummingbirds were presented by the public to LWM for rehabilitation but many failed to survive because of the severity of their injuries. These samples were stored (frozen or refrigerated) until analysis.

TABLE 1. Individual identification (ID), date, and location where samples were collected, age, and sex of California Anna's Hummingbird (*Calypte anna*) carcasses that were included in this study of avian poxvirus infection in California, USA. Location of the lesions on the carcass and diagnostic tests performed are included.[a]

ID	Date collected	Location (city, county)	Age	Sex	Lesion location	Diagnostic tests
31239	14 October 2009	Concord, Contra Costa	Adult	Male	Base of bill and legs	PCR, EM
30928	21 July 2010	Davis, Yolo	Unknown	Male	Right wing	PCR, EM
32031	30 May 2011	Scotts Valley, Santa Cruz	Adult	Male	Right leg	PCR, EM
33409	9 August 2011	Walnut Creek, Contra Costa	Adult	Male	Base of bill, right wing	PCR,[b] histopathology
33406	13 November 2011	Oakland, Alameda	Adult	Male	Right side of bill, left leg	PCR, histopathology

[a] PCR = polymerase chain reaction; EM = electron microscopy.

[b] PCR diagnostic test was not positive for avian poxvirus.

Two of the five affected carcasses were submitted for histopathology. Sections of dermatologic lesions, feathered skin, trachea, esophagus, heart, bone marrow, bifurcation of the trachea, liver, skeletal muscle, intestine, heart, brain, beak, kidney, lungs, and spinal column were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin for microscopic examination. A small portion of crust lesion from the three other birds was removed and crushed with 2% phosphotungstate (PTA) for direct electron microscopic evaluation. Approximately 2 μL of the PTA-crust mix was applied to a mesh copper grid coated with Formvar (Ted Pella, Inc., Redding, California, USA) and backed with evaporated carbon. Samples were directly imaged with a Zeiss 906E transmission electron microscope (Carl Zeiss Inc., Peabody, Massachusetts, USA) at 100 KV accelerating voltage.

For viral DNA extraction and PCR, approximately 20 mg of exudate/tissue from external avian pox–like syndrome lesions (five birds, nine lesions; Table 1) were excised from hummingbird carcasses. Viral DNA was extracted using QIAamp DNA Micro Kit (Qiagen Inc., Valencia, California, USA) according to manufacturer's instructions. A segment of the gene encoding the fowl poxvirus 4b core protein was amplified using primers P1 forward, CAGCAGGTGCTAAACAACAA, and P2 reverse, CGGTAGCTTAACGCCGAATA (Lee and Lee, 1997). The PCR reaction was carried out in a MyCycler™ thermal cycler (Bio-Rad, Hercules, California, USA) at initial denaturation of 95 C for 5 min; 40 cycles of 95 C for 30 sec, 50 C for 30 sec, and 72 C for 1 min; and a final extension at 72 C for 7 min (Brower et al., 2010). The PCR products were separated by electrophoresis on 2% agarose gel and visualized under ultraviolet illumination. Amplicons of the expected size of 578 base pairs (bp) were excised from the gel and purified using a QIAquick Gel Extraction Kit (Qiagen). Purified DNA was sequenced at UC Davis DNA Sequencing Facility (University of California, Davis, California, USA). At least two DNA extractions and two PCRs per extraction for each specimen were performed.

Nucleotide sequences were aligned and manipulated in Sequencher® version 5.0 sequence analysis software (Gene Codes Corporation, Ann Arbor, Michigan, USA), then queried using the National Center for Biotechnology Information Basic Local Alignment Search Tool (BLAST) and GenBank® database. Nucleotides sequences of other avian poxviruses were compared with the Anna's Hummingbird consensus sequence. Multiple alignments were carried out using CLUSTAL_X 2 (Larkin et al., 2007). To ascertain phylogenetic relationships among the avian poxvirus strains infecting Anna's Hummingbirds and other bird species, a phylogenetic tree was constructed using the Jukes-Cantor genetic distance model, neighbor-joining method, and tree resampling using the bootstrap method and 1,000 replicates with a support threshold of 80% as implemented in Geneious Pro v5.6 (Drummond et al., 2012). The tree was rooted to *Molluscum contagiosum* sequence (GenBank accession U60315) as previously described (Jarmin et al., 2006).

RESULTS

Lesions presented in the five hummingbirds (Table 1) varied from <1 to 5 mm and were dry and firm, pink to yellow, and with scabs on the surface (Fig. 1). Post-

FIGURE 1. Photograph of adult male Anna's Hummingbird (*Calypte anna*) captured at a banding station in Scotts Valley, Santa Cruz County, California, USA, showing wart-like avian poxvirus lesions on the bill.

mortem gross examination and necropsy was performed on two of the affected birds. Microscopically, the lesions were characterized by epidermal hyperplasia and multifocal superficial necrosis. Epithelial cells in affected regions were swollen (ballooning degeneration); many had empty large round vacuoles in the cytoplasm and some had eosinophilic inclusions (Bollinger bodies, Fig. 2). Yeast and bacterial cocci

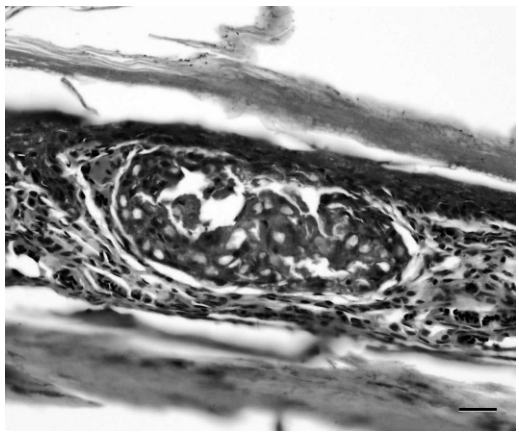

FIGURE 2. Epithelial proliferation with hyperkeratosis and intralesional epithelial cytoplasmic inclusions (Bollinger bodies) and bacteria from bill of Anna's Hummingbird (*Calypte anna*; ID 33406) in California, USA. Bar = 20 μm.

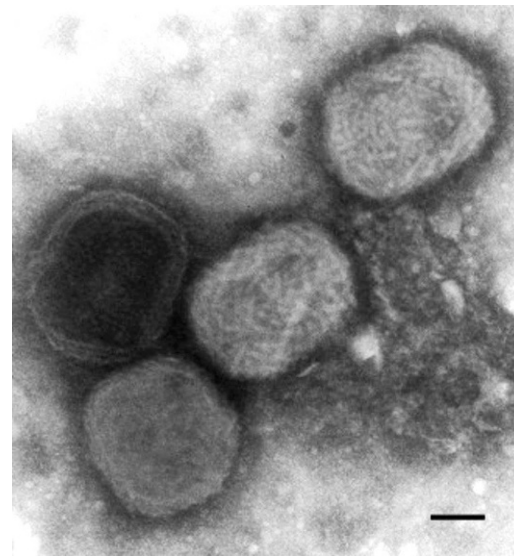

FIGURE 3. Poxvirus particles from skin lesions (leg) in Anna's hummingbird (*Calypte anna*) from California, USA, showing an intricate outer membrane and irregular spread-out surface. Bar = 100 μm.

were multifocally evident within hyperkeratotic layers of keratin in the bill lesion of one of the sampled birds (ID 33409). There were no lesions in any of the other tissues examined except the liver of one bird (ID 33406), which presented very mild portal hepatitis. Direct electron microscopic examination of negatively stained preparations of the lesions in three birds demonstrated oval, brick-shaped viral particles with an intricate outer membrane that were 221–271 nm (average 253 nm) by 281–336 nm (average 307 nm; Fig. 3), a result that is compatible with avian poxvirus.

PCR amplification of the 4b core poxvirus gene sequence was successful in four of five individuals (Table 1), resulting in products that averaged 575 bp (GenBank accession JX418296). All nucleotide sequences were identical. The BLAST search and comparison of other avian poxvirus published sequences in GenBank with Anna's Hummingbird poxvirus showed 75–94% similarity; however, no perfectly matching sequences were found. The avian poxvirus gene amplified from lesions of Anna's Hummingbirds clustered

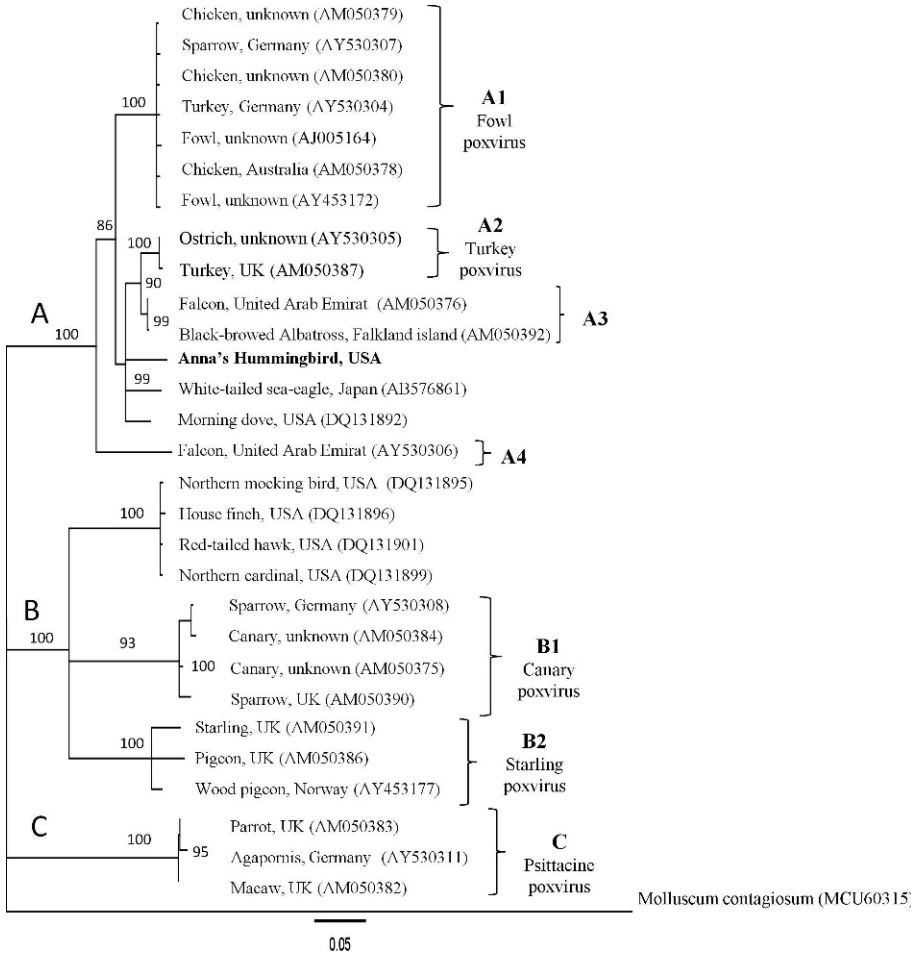

FIGURE 4. Neighbor-joining tree for avian pox sequences (4b core protein gene) constructed using the Jukes-Cantor genetic distance model. Numbers indicate bootstrap support (>80%). GenBank accession numbers are noted in parentheses. Avian clades A to C as in Jarmin et al. (2006) are shown. Anna's Hummingbird (*Calypte anna*) avian poxvirus sequence is in bold.

in clade A of the phylogenetic tree (Fig. 4) according to the phylogenetic classification reported previously (Jarmin et al., 2006). The Anna's Hummingbird avian poxvirus sequence did not cluster in any previously reported subclade, yet it showed high sequence similarity with avian poxviruses from species in subclade A2 (Ostrich [*Struthio camelus*], 94.3%, and Wild Turkey [*Meleagris gallopavo*], 94.1%) and in subclade A3 (falcon [*Falco* spp.], 94%, and Black-browed Albatross [*Diomedea melanophris*], 93.5%). The sequence also showed high similarity with Mourning Dove (*Zenaida macroura*, 93%) and White-tailed Eagle (*Haliaeetus albicilla*, 94.3%), species that were not previously reported in a specific subclade (Fig. 4).

DISCUSSION

To our knowledge, this is the first report of laboratory-diagnosed avian poxvirus infection in a hummingbird. The lesions on featherless parts of the legs, feet, and face were consistent with the cutaneous form of the poxvirus infection described in other bird species (van Riper and Forrester, 2007). In the birds we necropsied, there were no lesions compatible with the

diphtheritic ("wet" internal) form of avian pox. However, we did not test and evaluate the wet form, and future studies should be considered to examine whether this form occurs in hummingbirds. In our study, two of five birds had lesions on the wings (humeral and radial-metacarpal regions). Lesions on the wings have been observed in other free-ranging bird species (McClure, 1989; Gortazar et al., 2002; Medina et al., 2004); however, wing lesions seem to be less commonly reported than those on legs and around eyes and bill. On histopathology of our cases, eosinophilic intracytoplasmic inclusions were consistent with Bollinger bodies, and ultrastructural morphology was consistent with avian poxvirus (Tripathy and Reed, 2003).

In four of five tested hummingbirds we detected the avian poxvirus 4b core gene sequence by PCR. The hummingbird sequence clustered within clade A according to the phylogenetic classification proposed by Jarmin et al. (2006), yet our analysis did not place it in an existing subclade. Similar results have been shown for sequences isolated from other free-ranging bird species (Adams et al., 2005; Saito et al., 2009) and lead us to hypothesize that the Anna's Hummingbird sequence could comprise a new subclade within clade A. Additional characterization (antigenic, biologic, and genetic) is needed to determine if this sequence is a novel species in the genus of avian poxviruses and to determine if the Anna's Hummingbird pox variant descended from avian pox of sympatric bird species (e.g., Western Scrub Jay [*Aphelocoma californica*], House Finch [*Haemorhous mexicanus*]). Because of the inability to detect viral DNA from one of the hummingbirds with microscopic lesions compatible with avian poxvirus infection (Bollinger bodies), we recommend that more than one method of diagnosis be used to identify avian poxvirus. This is particularly important when the available volume of lesion sample is small, as is the case of the Trochilidae, birds that often weigh <5 g.

It is not clear if Anna's Hummingbirds are the predominant hummingbird host of the avian poxvirus identified in this study. Although cross-species transmission may occur (Thiel et al., 2005; Jarmin et al., 2006), host specificity of avian poxvirus can occur in free-ranging birds (Giddens et al., 1971; Cox, 1980). To date, the Black-chinned Hummingbird (*Archilochus alexandri*) is the only other hummingbird species sympatric with Anna's Hummingbird that has been anecdotally reported with avian pox–like lesions in the United States (B. Robinson, unpubl. data). Nonetheless, there have been few intensive capture and examination (banding) studies to evaluate health and disease in hummingbirds.

Morbidity and mortality rates associated with avian pox infection in any hummingbird species are unknown. Severe lesions (periocular, around bill, and on feet and legs) have been associated with the avian pox–like syndrome in Anna's Hummingbird; however, no laboratory tests were conducted to definitively diagnose the causative agent (Colwell, 2011). In field studies (unpubl. data), we have observed severe avian pox–like syndrome lesions in Anna's Hummingbirds, including those occluding eyes, wart-like encrusted lesions around the bill that would probably impact ability to eat, and leg and foot lesions likely to affect perching. We expect that these pathologies would lower survivorship and reproduction by reducing ability to forage, evade predators, and conduct the complex behavioral interactions that hummingbirds employ as mating rituals. Avian poxvirus infection in other species can cause severe injuries, including blinding due to severe eyelid lesions in gallinaceous birds (Forrester and Spalding, 2003), epithelial necrosis and sloughing that can lead to loss of toes and feet (van Riper et al., 2002) and severe debilitation affecting flight and sight (Docherty et al., 1991).

Under natural conditions, most hummingbirds are typically territorial birds

living primarily solitary lives (Buskirk, 1976; Hilton and Miller, 2003). They depend on flowering resources that are unlikely to act as a source of avian poxvirus infection. Urbanization and the resulting increase in exotic flowers and bird feeding stations have been correlated with higher density and diversity of hummingbirds in urban and suburban areas (Arizmendi et al., 2007; Clark and Russell, 2012). Increased hummingbird density might enhance the transmission of avian poxviruses, as has been suggested for other bird species (van Riper and Forrester, 2007). The presence of cultivated exotic flowers and hummingbird feeders has been associated with changes in Anna's Hummingbird geographic distribution in North America (Clark and Russell, 2012). Bird feeders could increase the fomite risk of pathogen transmission, as has been shown for other bird species (Hartup et al., 1998; Robb et al., 2008). Finally, anthropogenic environmental changes may also modify movement patterns of birds and vectors (particularly insects), increasing disease exposure (Daszak et al., 2001; Altizer et al., 2011). Deforestation and climate change have altered hummingbird distribution or shifted patterns of movement in several locations through the Americas (Hill et al., 1998; Hadley and Betts, 2009; Tingley et al., 2012).

Our findings advance the knowledge of diseases in the hummingbird family and provide a foundation for next steps in research to elucidate the impact of avian poxvirus infection in hummingbirds. This provides information to rehabilitation centers and banding stations where pathogen transmission and disease prevalence can be decreased by instituting proper preventive measures. Further research is needed to assess the individual- and population-level impacts, as well as the prevalence and virulence of this pathogen in hummingbirds.

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