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DIPLOTRIAENA OBTUSA (NEMATODA: DIPLOTRIAENIDAE) FROM BARN SWALLOWS (*HIRUNDO RUSTICA*) AND CLIFF SWALLOWS (*PETROCHELIDON PYRRHONOTA*) COLLECTED DURING MORTALITY EVENTS IN THE UPPER MIDWEST, USA

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KEY WORDS

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Partial *18s* rRNA
Diplotriaena obtusa
Diplotriaena tricuspis
Diplotriaena anthreptis
Air sac
Nematode
Infection

ABSTRACT

Several mortality events involving barn swallows (*Hirundo rustica*) and cliff swallows (*Petrochelidon pyrrhonota*) were reported in the Upper Midwestern states in 2017 and 2018. Barn swallow mortality followed unseasonal cold snaps, with the primary cause of death being emaciation with concurrent air sac nematodiasis. Lesions in cliff swallows were consistent with blunt force trauma from suspected car impacts. Examination of air sac nematodes from both bird species revealed morphological characters consistent with *Diplotriaena obtusa*. Sequence analysis of the partial *18S* rRNA gene indicated the samples clustered with other species in the genus *Diplotriaena*. These nematodes provide a link between morphological specimens and DNA sequence data for *D. obtusa*.

Diplotriaenoidea is a superfamily of spirurid nematode that is characterized by large body size, ovipary, and tropism of the adults for the air sacs of Sauropsids worldwide (Anderson et al., 2009). Species of *Diplotriaena*, Railliet & Henry, 1909 (Nematoda: Diplotriaenidae), infect the air sacs of birds (Anderson et al., 2009), and the same host individual may be infected with more than 1 species (Vicente et al., 1983). *Diplotriaena* spp. are widely distributed geographically (Anderson, 2000) and reported from species of Anseriformes, Apodiformes, Galliformes, Charadriiformes, Piciformes, and Passeriformes (Anderson, 2000). Predation or consumption of infected arthropods such as orthopterans has been documented in life cycle studies (Chabaud, 1955; Anderson, 1957, 1962; Cawthorn and Anderson, 1980) where the nematode larvae develop in the bird's hepatic portal system and migrate to the lungs via the heart and pulmonary arteries, finally invading the air sacs of the definitive host. The migration of adult and larval worms and the deposition of eggs can cause lesions such as hemorrhage and fibrosis in the liver, edema of the trachea, and air sac fibroplasia. Infections are often subclinical but can include clinical signs such as lethargy, labored breathing, stunting, and feather loss (Sterner and Cole, 2008).

Since 1996, the U.S. Geological Survey (USGS) National Wildlife Health Center (NWHC) has documented 19 barn swallow (BS) (*Hirundo rustica*) and 10 cliff swallow (CS) (*Petrochelidon pyrrhonota*) mortality events (USGS, https://www.nwhc.usgs.gov/whispers, Search 19 April 2021). Thirteen of the 19 BS events and 2 of the 10 CS events had a primary event diagnosis of emaciation from suspect starvation. Herein, we report the diagnostic findings on 4 temporally clustered mortality events in BS and CS in the Midwestern United States. Both bird species are widely distributed in the United States but are suffering local declines in some areas. Their diet consists mainly of flying insects, which they capture and consume in the air, often while flying low over water (https://www.allaboutbirds.org/guide/Barn_Swallow/lifehistory#conservation).

In 2017 and 2018, mortality events in various species of swallow following sudden temperature drops were reported to the NWHC. A BS mortality event from 20 to 22 May 2017 (Case BS1) estimated at 20–25 birds was reported in a grassland campground within the Lewis and Clark State Recreation Area, Nebraska (42°50′11.292″N, 97°34′24.816″W). Daily temperature data from the University of Nebraska Automated Weather Data Network (Sioux City AP station 42°23′28.68″N, 96°22′44.76″W and Yankton 2E Station 42°52′42.96″N, 97°21′48.96″W) indicated that temperatures leading up to the mortality event were 26.6–29.4 C and dropped to 10 C. A second BS mortality event from 20 to 23 May 2017 (Case BS2) was reported southwest of Sioux Falls, South Dakota (43°32′59.28″N, 96°42′01″W), with the entire colony of 20 BS found dead on the ground and in their nests. Temperature changes were similar to those noted above (https://hprcc.unl.edu/onlinedataservices.php Sioux Falls Foss Field). On 4 August 2017, an estimated 50 CS (Case CS1) were reported dead after a moderate temperature decrease from 29.4 to 21.1 C in the daytime to 10 C at night (https://hprcc.unl.edu/onlinedataservices.php) in 2 locations 4 miles apart in Pleasant Valley Township, Illinois (42°13′33″N, 90°1′37″W). Citizens

Table I. National Wildlife Health Center or University of Wisconsin Oshkosh (in bold) necropsy results from mortalities of barn swallows (*Hirundo rustica*) (BS) and cliff swallows (*Petrochelidon pyrrhonota*) (CS) with case, estimated total dead, individual bird number, age, sex, body condition, carcass weight, and determination of cause of death (COD) with number of *Diplotriaena obtusa* observed. Individual birds in bold were examined at the University of Wisconsin, Oshkosh for helminths only, not determination of cause of death.

Case	No. Dead	Bird	Age and sex	Condition	Carcass weight (g)	COD	No. of *D. obtusa*
BS1	25	1	A F	Poor	11.77	E	1
		2	A M	Poor	12.35	E	0
		3	A F	Poor	12.43	E	9
		4	A U	Poor	15.37	E	7
		5	A F	Poor	13.60	E	1+*
		6	**A M**	**Poor**	**14.00**	**U**	**0**
		7	**A M**	**Fair**	**13.94**	**U**	**9**
		8	**U M**	**Fair**	**14.51**	**U**	**3**
		9	**A F**	**Poor**	**13.00**	**U**	**0**
		10	**I F**	**Poor**	**13.81**	**U**	**4**
		11	**I F**	**Poor**	**13.35**	**U**	**5**
BS2	20	1	A F	Poor	12.80	E	1
		2	A M	Poor	13.70	E	4
		3	A M	Poor	13.15	E	2
		4	A M	Poor	14.07	E	1+*
		5	**I M**	**Poor**	**12.07**	**U**	**0**
		6	**A M**	**ND**	**13.08**	**U**	**0**
BS3	40	1	I F	Poor	10.43	E	2
		2	I F	Poor	12.13	E	0
		3	A F	Poor	11.93	E	0
		4	A F	Poor	11.67	E	0
		5	A F	Poor	11.26	E	0
CS1	50	1	I M	Fair	19.66	T	2
		2	I U	Good	15.82	T	0
		3	I U	Good	18.37	T	0
		4	I M	Good	18.99	T	0
		5	I M	Good	21.49	T	0
		11	I M	Good	20.53	T	0
		12	I M	Good	17.02	T	0
		13	A U	Good	18.95	T	0
		17	A U	Good	17.39	T	0
		18	I F	Good	19.64	T	0

* Pathologist's report noted large number of lung nematodes but were not enumerated.

Abbreviations: BS, barn swallow; CS, cliff swallow; A, adult; I, immature; F, female; M, male; U, undetermined; E, emaciation; T, trauma; ND, no data.

reported seeing birds ground roosting on an oil/chip road surface and flying reluctantly for short distances when approached. On 15 October 2018, a third BS mortality event (Case BS3) of approximately 40 birds was reported near Grand Lake in Cold Spring, Minnesota (45°26′9.24″N, 94°20′20.4″W), where birds were found on door ledges and building facades. This event also occurred following sudden and brief temperature declines; daytime temperatures were 7.2 C with nighttime temperatures dropping to –6.6 C (https://hprcc.unl.edu/onlinedataservices.php Kimball 3N station). Carcasses in the best postmortem condition from each mortality event were frozen and shipped by overnight courier from state wildlife agencies to the NWHC for determination of the cause of death (COD). A necropsy examination was conducted by a board-certified veterinary pathologist. Birds not necropsied for COD were sent to the University of Wisconsin, Oshkosh, for dissection and recovery of nematodes (Table I).

Upon necropsy, a selection of tissues based on postmortem condition and observed gross lesions were fixed in 10% neutral buffered formalin for histopathology. Paraffin-embedded tissues were cut in 5-μm sections, stained with hematoxylin and eosin (H&E), and examined using light microscopy. Tracheal and cloacal swabs were collected and submitted for avian influenza matrix RT-PCR screen (Ip et al., 2012). One bird each from both BS1 and BS2, and 4 birds from CS1 were tested for brain cholinesterase inhibition (Hill and Fleming, 1982) to rule out toxicosis from organophosphate or carbamate. Nematodes were collected in physiological saline, then preserved in 70% ethanol, with a subset preserved in 100% molecular grade ethanol. All nematodes were cleared and mounted in lactophenol and taxonomically relevant characters (Anderson, 1959; Vieira et al., 2017) were examined using light microscopy (Olympus BX 50F microscope, Olympus Corporation, Center Valley, Pennsylvania). Relevant structures were photographed using a microscope-mounted Insight CMOS camera with SPOT 5.2 digital imaging software (Diagnostic Instruments, Sterling Heights, Michigan). Measurements of parasites are presented in microns (μm), with means and standard deviations in parentheses (Table II). Vouchers of nematodes and extracted DNA were deposited in the Museum of Southwestern Biology, the University of New Mexico, Albuquerque, New Mexico (MSB:Para: 30694–30724). A 2-mm piece from the midsection from a subset of nematodes

Table II. Comparative measurements listed as minimum/maximum (mean ± standard deviation) of *Diplotriaena* spp. samples recovered from *Hirundo rustica* and those reported for *Diplotriaena obtusa* (Sonin, 1968), *Diplotriaena tricuspis* (Anderson, 1959; Sonin, 1968), *Diplotriaena bargusinica* (Vieira et al., 2017) and *Diplotriaena anthreptis* (Dewi and Zhang, 2010). Measurements in microns; ND = No data; Ant-nerve ring = distance from anterior end to middle of nerve ring; Ant-vulva = distance from anterior end to vagina.

	Worms this study	*D. obtusa*	*D. tricuspis*	*D. anthreptis*	*D. bargusinica*
Male (n = 16)					
Trident length	107–150 (129 ± 11)	100–140	90–120	100	100–140 (120 ± 10)
Ant-nerve ring	173–427 (256 ± 58)	220	200–300	140	ND
Right spicule	622–970 (719 ± 85)	596–690	960–1,100	560	410–520 (460 ± 30)
Left spicule	1,013–1,596 (1288 ± 121)	1,064–1,232	1,140–2,000	910	680–760 (680 ± 40)
Female (n = 25)					
Trident length	92–165 (127 ± 15)	130	90–120	120–130 (123)	110–130 (118 ± 6)
Ant-nerve ring	177–420 (240 ± 51)	290	190–200	220–240 (220)	ND
Ant-vulva	585–1,075 (816 ± 141)	590	590–760	560–750 (673)	330–460 (390 ± 50)
Trident apex	convex	convex	concave/flat	concave	sharply convex

collected in 100% molecular grade ethanol (17 from BS1; 1 each from CS1, BS3, and BS2) was removed, and genomic DNA extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, California) per manufacturer's instructions. Amplification of the partial *18S* small subunit ribosomal RNA (rRNA) gene used primers and cycling conditions as reported in Floyd et al. (2005) and Vieira et al. (2017), respectively. PCR reaction consisted of 5 μl of nuclease-free water, 12.5 μl of 2× GoTaq® Green Master Mix (Promega, Madison, Wisconsin) 1.25 μl of each primer (final concentration 0.5 μM each), and 5 μl DNA template for a total reaction volume of 25 μl. Five microliter samples of the PCR products were examined on 1.0% agarose gels containing 0.0001% Gel Red (Thomas Scientific, Swedesboro, New Jersey) along with a 100 bp ladder (Promega) by gel electrophoresis. Primers and nucleotides were removed from the PCR products using ExoSap-IT (1 μl) (Phenix, Candler, North Carolina). Products were sequenced at the University of Wisconsin—Madison Biotechnology Center's DNA Sequencing Facility using the BigDye Terminator v3.1 3730xl automated DNA sequencing instrument (Applied Biosystems, Foster City, California). Estimates of evolutionary divergence between the sequences of the partial *18S* rRNA gene based on 638 positions in the final dataset was zero (Kimura, 1980 and Kumar et al., 2018) (GenBank MT_129505–129524). Sequences were examined and aligned using SeqMan Pro (DNASTAR Lasergene 17, DNASTAR, Madison, Wisconsin). Molecular analyses were conducted with MEGAX (Molecular Evolutionary Genetics Analysis version 10 (Kumar et al., 2018)). Sequences were aligned with sequences from GenBank (www.ncbi.nlm.nih.gov/genbank/) using Clustal W in MEGA X and manually trimmed. A maximum likelihood tree was built using Kimura parameter model + G. Statistical support for groupings was estimated by bootstrap analysis with 1000 replications. Sequences from species in Diplotriaenoidae and closely related Desmodocercidae, which are all respiratory parasites of birds, were included and the tree was rooted using *Oxyuris equi*. (EF180062) (Fig. 1).

The cause of death (COD) in 100% (14/14) of BS necropsied at NWHC was emaciation. Cause of death was not determined for BS examined at UW Oshkosh (Table I in bold) as the intent of those examinations was only to record gross necropsy observations and to enumerate air sac nematodes. In total, based upon gross exam, 59% (13/22) of BS were infected with *D. obtusa* (Fig. 2). The majority of BS had poor body condition with empty stomachs. Only 10% (1/10) of CS were infected with *D. obtusa*. The major COD of CS was trauma consistent with vehicular impact, with 90% (9/10) of birds in good body condition and food items in their stomachs.

Histopathology of tissues from BS were mostly unremarkable. Some infected birds had evidence of chronic gastrointestinal hemorrhage; however, autolytic condition of the tissues prevented full histological evaluation. Nematode eggs were seen in some histological sections of the parabronchi of birds with *D. obtusa* infection, although no significant inflammatory lesions were noted. Histopathology from tissues of CS were characterized by multifocal areas of hemorrhage in major organs consistent with trauma. Brain cholinesterase was normal for 6 tested birds, suggesting that carbamate or organophosphate toxicosis was unlikely. Avian influenza matrix RT-PCR testing was negative for all birds tested (n = 24).

In total, 51 nematodes from the air sac or abdominal cavity were collected from 14 of 32 birds (44%). Nematodes possessed a cephalic pair of lateral pores opening to a cuticularized trident beside the anterior end of the esophagus, which is characteristic of species of *Diplotriaena* (Anderson et al., 2009). Differentiation of species is based on the lengths of the spicules and tridents, shape of the apex of the manubria, and form or shape of the spicules (Seibert, 1944; Anderson, 1959). Based on the convex or rounded shape of the apex of the manubria of the trident (Fig. 3A), the sharply twisted right spicule with the most distal third twist occurring closer to the distal end of the spicule (Fig. 3B), and other measurements (Table II), the nematodes were identified as *D. obtusa* (Table II). We found the trident characters to be the most reliable regardless of host postmortem condition. The vulva in a few specimens was protruding, which we attributed to freeze-thaw artifact or poor postmortem condition of hosts and specimens.

Analysis for the estimate of evolutionary divergence between sequences was performed in MEGA X (Kumar et al., 2018) using a final dataset including 20 nucleotide sequences with a total of 638 positions and revealed that all samples collected in the present study were identical. Therefore, 1 sequence was used in phylogenetic analysis. The number of base pair substitutions per site (Kumar et al., 2018) between *D. obtusa* (MT129519 this

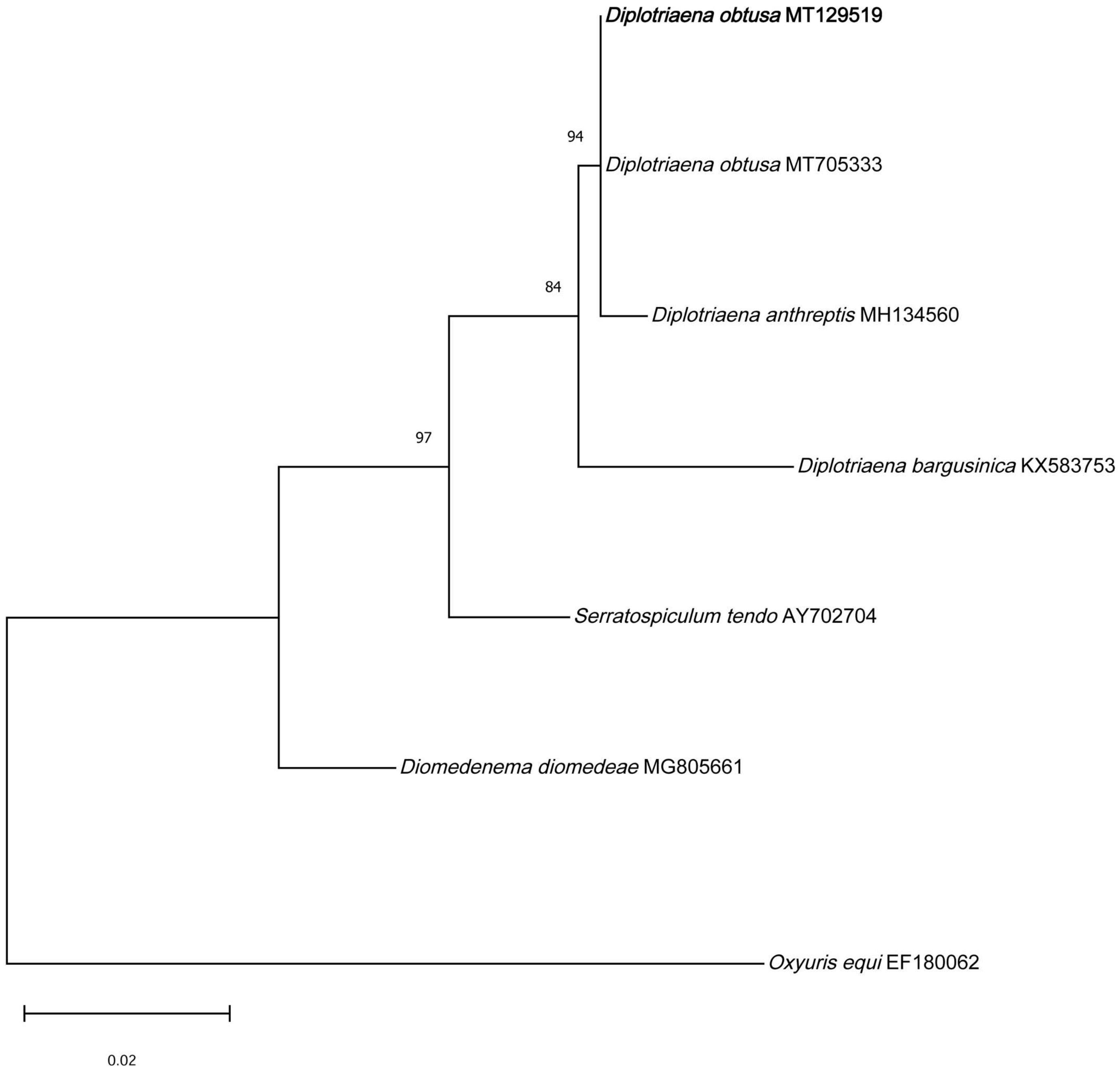

Figure 1. Molecular phylogenetic analysis by maximum likelihood method based on the Kimura 2 – parameter model + G with 1,000 bootstrap replications based on partial *18S* rRNA gene sequences of *Diplotriaena obtusa* (bold this study) and other species of *Diplotriaeana* and species from the suborder Spirurina along with *Oxyuris equi* as an outgroup. Tree is drawn to scale with branch lengths measure in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X.

study) and other sequences used in the analysis are in Table III. Phylogenetic analysis by maximum likelihood method showed sequences from our study grouped with *D. obtusa 18S* rRNA gene sequences from a Eurasian blue tit (*Cyanistes caeruleus*) collected in Germany (Fig. 1) (Rentería-Solís et al., 2021). For these specimens, however, only female anterior and posterior anatomy was described, and no physical vouchers were available. In our analysis, *Diplotriaena anthreptis* (MH134560) collected from the Eurasian Jay (*Garrulus glandarius*) is not referenced in a publication nor linked to a morphological voucher; therefore, a confirmation that the sequence is actually from *D. anthreptis* was not possible. *Diplotriaena anthreptis* (Dewi and Zhang, 2010) was described from the brown throated sunbird (*Anthreptis malacensis malacensis*) from Kangean Island, Indonesia, based on the morphology of 3 female and 1 male specimens with no molecular data provided. Dewi and Zhang (2010) identified several features which separate *D. anthreptis* from *D. obtusa*, including apex of the manubrium of the trident–concave (vs. convex) and median

Figure 2. Photograph of barn swallow (*Hirundo rustica*) with *Diplotriaena obtusa* removed from air sac. Color version available online.

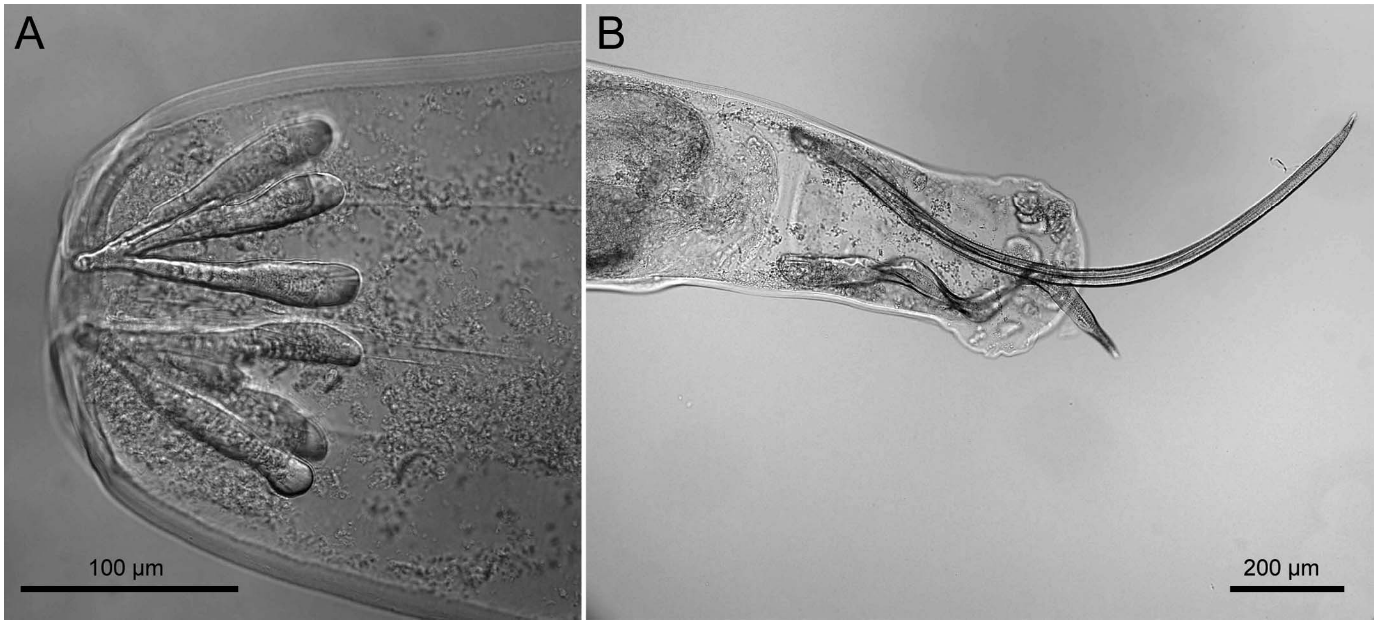

Figure 3. *Diplotriaena obtusa* (Nematoda: Diplotriaenidae) infecting air sac of barn swallow (*Hirundo rustica*) and cliff swallow (*Petrochelidon pyrrhonota*) in upper Midwest USA. (**A**) Anterior end of *D. obtusa* with convex apex of manubrium of trident. (**B**) Spicules of *D. obtusa* with curved right spicule and spiraled left spicule.

Table III. The number of base substitutions per site between 6 partial sequences of the 18S rRNA gene from *Diplotriaena obtusa, Diplotriaena bargusinica, Diplotriaena anthreptis, Diomedenema diomedeae, Serratospiculum tendo,* and *Oxyuris equi* with a total of 504 positions in the final dataset.

Diplotriaena obtusa MT129519						
Diplotriaena obtusa MT705333	0.0000					
Diplotriaena bargusinica KX583753	0.0186	0.0186				
Diplotriaena anthreptis MH134560	0.0040	0.0040	0.0272			
Diomedenema diomedeae MG805661	0.0359	0.0359	0.0495	0.0362		
Serratospiculum tendo AY702704	0.0250	0.0250	0.0250	0.0359	0.0359	
Oxyuris equi EF180062	0.1204	0.1204	0.1392	0.1242	0.1066	0.1146

branch of trident with flat posterior (vs. not flat). Dewi and Zhang (2010) specifically differentiate *D. anthreptis* from *D. obtusa* based on the shorter left spicule (910 µm versus 1064–1232 µm) (Sonin, 1968). However, Anderson (1959) noted that the minimum–maximum length of the left spicule for *D. obtusa* was 600–1,100 µm, a range overlapping with *D. anthreptis*. In addition, Boulenger (1928) gave a range of 950–1,200 µm for the left spicule of *D. obtusa*. Dewi and Zhang (2010) do not compare *D. anthreptis* with *Diplotriaena tricuspis*, which has a concave apex of the manubrium of the trident but a much longer left spicule. Anderson (1959) noted that the species of *Diplotriaena* exhibited highly variable morphological characters within the same species of worm and within and between different host species. Finding *D. anthreptis* in an Indonesian sunbird (Dewi and Zhang, 2010) and an Asian corvid (*G. glandarius*) is unusual, since most corvids are host to *D. tricuspis*. Molecular exploration is limited by the dearth of publicly available sequences for different species of *Diplotrieana* and other spirurid parasites of the respiratory tract. Our specimens provide a link between morphological and sequence data for nematodes collected in North America.

The leading COD of all swallows reported herein was either emaciation (14 of 22 BS with 8 not assessed for COD) or blunt force trauma (10 of 10 CS). Eighteen of the 22 BS were grossly emaciated as evidenced by a prominent keel, with 9 of the 18 having concurrent lung nematodiasis. Weather events or extension of cold temperatures have been reported to affect migration, nesting and reproduction success, and foraging in insectivorous birds (Brown and Bomberger-Brown, 2000; Arbeiter et al., 2016; Cox et al., 2019, 2020; Yang et al., 2021). Daily weather also affects abundance of airborne insects (Grüebler et al., 2008). Winkler et al. (2013) reported 18.5 C as the critical temperature below which flying insect availability begins to decline in areas studied in New York. The cold snaps reported in the immediate days preceding the mortality events were most likely a driving factor for the starvation, affecting perhaps both bird and insect activity. The role of *D. obtusa* is less clear and may have been incidental or a co-factor in some individuals. Cawthorn and Anderson (1980) noted clinical signs of disease were only seen in 1 of 44 nestling crows (*Corvus brachyrhynchos*) experimentally infected with *D. tricuspis*. Santoro et al. (2016) reported signs of disease in falcons (*Falco peregrinus*) and goshawk (*Accipiter gentilis*) naturally infected with *Serratospiculum tendo* (Diplotriaenoidea). In all cases, raptors exhibited cachexia and traumatic impact injuries that lead to death. The authors suggested that limited respiratory performance could impair hunting and increase accidental impacts. Similarly, free-ranging raptors in North America infected with *Serratospiculoides amaculata* (Diplotriaenoidea) can show clinical signs such as dyspnea, poor flight performance, cachexia, lethargy, and vomiting (Kocan and Gordon, 1976; Santoro et al., 2016). A space-occupying mass, such as *D. obtusa*, in the air sac may affect daily activities and foraging of small aerial insectivores.

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