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Prevalence of filarioid nematodes and trypanosomes in American robins and house sparrows, Chicago USA

Gabriel L. Hamer [a,*], Tavis K. Anderson [b,c], Garrett E. Berry [a], Alvin P. Makohon-Moore [d], Jeffrey C. Crafton [e], Jeffrey D. Brawn [f], Amanda C. Dolinski [g], Bethany L. Krebs [f], Marilyn O. Ruiz [h], Patrick M. Muzzall [d], Tony L. Goldberg [b], Edward D. Walker [a]

[a] Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI, USA
[b] Department of Pathobiological Sciences, University of Wisconsin, Madison, WI, USA
[c] Virus and Prion Diseases Research Unit, National Animal Disease Center, USDA-ARS, Ames, IA, USA
[d] Department of Zoology, Michigan State University, East Lansing, MI, USA
[e] College of Veterinary Medicine, Michigan State University, East Lansing, MI, USA
[f] Department of Natural Resources and Environmental Sciences, University of Illinois, Urbana, IL, USA
[g] College of Veterinary Medicine, University of Illinois, Urbana, IL, USA
[h] Department of Pathobiology, University of Illinois, Urbana, IL, USA

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ABSTRACT

Hosts are commonly infected with a suite of parasites, and interactions among these parasites can affect the size, structure, and behavior of host–parasite communities. As an important step to understanding the significance of co-circulating parasites, we describe prevalence of co-circulating hemoparasites in two important avian amplification hosts for West Nile virus (WNV), the American robin (*Turdus migratorius*) and house sparrow (*Passer domesticus*), during the 2010–2011 in Chicago, Illinois, USA. Rates of nematode microfilariemia were 1.5% of the robins ($n = 70$) and 4.2% of the house sparrows ($n = 72$) collected during the day and 11.1% of the roosting robins ($n = 63$) and 0% of the house sparrows ($n = 11$) collected at night. Phylogenetic analysis of nucleotide sequences of the 18S rRNA and cytochrome oxidase subunit I (COI) genes from these parasites resolved two clades of filarioid nematodes. Microscopy revealed that 18.0% of American robins ($n = 133$) and 16.9% of house sparrows ($n = 83$) hosted trypanosomes in the blood. Phylogenetic analysis of nucleotide sequences from the 18s rRNA gene revealed that the trypanosomes fall within previously described avian trypanosome clades. These results document hemoparasites in the blood of WNV hosts in a center of endemic WNV transmission, suggesting a potential for direct or indirect interactions with the virus.

1. Introduction

Recent awareness of parasite community ecology has led to a shift from a focus on one-host–one-parasite framework to multi-host–multi-parasite systems (Cox, 2001; Pedersen and Fenton, 2007; Cattadori et al., 2008; Telfer et al., 2010). This rapidly emerging field has led to important discoveries about how parasite interactions can be synergystic or antagonistic (Cox, 2001; Tompkins et al., 2010). Interactions can occur directly (e.g. through competition for resources), or indirectly, as modulated through the host or vector immune system (Graham, 2008). Additionally, ecological interference among co-circulating parasites can occur when one parasite removes susceptible hosts which are then unavailable to a second parasite (Rohani et al., 2003). These interactions have potential fitness consequences for the host (morbidity and mortality) and for the parasite (transmission potential). Further, these individual level effects may influence population dynamics of hosts and parasites (Graham et al., 2007). Consequently, understanding interaction patterns at the level of the individual and population may have dramatic consequences on understanding pathogen transmission, preventing disease, and aiding in control programs (Lello et al., 2004).

In vector-borne disease systems, parasite interactions in the arthropod vector can facilitate or reduce pathogen dissemination (Mellor and Boorman, 1980; Vaughan and Turell, 1996; Aliota et al., 2011). This study focuses on parasites that co-circulate and possibly interact with West Nile virus (WNV), one of the most widely distributed arboviruses in the world (Weaver and Reisen, 2010). WNV is maintained in an enzootic cycle between *Culex* spp. mosquitoes and birds. In Colorado, USA, a study documented

* Corresponding author. Address: Department of Entomology, Texas A&M University, 2475 TAMU, College Station, TX 77843, USA. Tel./fax: +1 011 979 862 4067.
E-mail address: ghamer@tamu.edu (G.L. Hamer).

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a positive association between trypanosomes and WNV in *Culex* mosquitoes (Van Dyken et al., 2006), and in Illinois, USA, a study documented a positive association between *Culex* flavivirus and WNV in *Culex* mosquitoes (Newman et al., 2011). Both studies provide support for the idea that parasite–parasite interactions can influence disease transmission.

Among the hemoparasites that could potentially interact with viruses such as WNV, filarioid nematodes have received the most attention because of a mechanism known as microfilarial enhancement of arboviruses (Mellor and Boorman, 1980; Turell et al., 1984; Vaughan and Turell, 1996; Vaughan et al., 2009). This phenomenon arises when an arthropod vector concurrently ingests microfilariae and an arbovirus. Microfilarial penetration of the midgut allows the virus to enter the hemocoel and disseminate to the rest of the mosquito. Importantly, this process could increase vector competence as the virus bypasses the midgut barrier. Additionally, microfilarial penetration of the midgut shortens the extrinsic incubation period (Turell et al., 1987; Vaughan and Turell, 1996). Filarioid nematodes include species that are the causative agents of filariasis in humans, along with a diversity of other species that infect birds as the definitive hosts, and arthropods as intermediate hosts and vectors (Bartlett, 2008).

In this study, we describe co-circulating hemoparasites in the avian hosts responsible for the WNV enzootic cycle in suburban Chicago, Illinois, USA, an urban "hot spot" of transmission (Ruiz et al., 2004; Hamer et al., 2008). The American robin (*Turdus migratorius*) and house sparrow (*Passer domesticus*) have been implicated as the most important hosts contributing to the amplification of WNV in this study region (Hamer et al., 2011), and are the focal species of this study. Specifically, we describe infections of filarioid nematodes and trypanosomes which might be capable of interacting with WNV. We also present data on WNV infection in *Culex* sp. mosquitoes and bird blood and the detection of WNV antibodies in bird blood collected in the same study region.

2. Materials and methods

2.1. Study area and mosquito and bird collections

Sampling sites were in southwest suburban Chicago, Illinois and included residential and semi-natural sites in the municipalities of Alsip, Evergreen Park, Oak Lawn, and Palos Hills (87°44′W, 41°43′N; Loss et al., 2009). In 2010, mosquitoes were trapped using CDC gravid traps in 40 locations and CDC miniature light traps in 100 locations. In 2011, mosquitoes were trapped using gravid traps in 39 locations and light traps in 96 locations. Each location in each year was trapped one night per week from June to October. Female mosquitoes were identified to species (Andreadis et al., 2005) and up to 50 individuals were pooled by species and trap location and stored at −20 °C or −80 °C.

In 2010 and 2011, birds were captured during the day using mist-nets (Hamer et al., 2008) and roosting birds were captured at night using mist-nets, an extension net, and by hand. Roosting birds were flushed into 12 m long mist-nets stretched between conduit pipes held above the ground by two personnel. Flashlights were used to temporarily disorient the roosting birds (i.e. 'dazzled'; Hudson, 1986), which facilitated the extension net and hand capture techniques. The extension net consisted of a black nylon mesh net in a 45 cm^2 hoop and the telescopic pole extended from 2.1 m to 5.5 m (Tomahawk Live Traps, Hazelhurst, Wisconsin, USA). Captured birds were identified, weighed, sexed, aged, banded, and a blood sample was obtained by jugular venipucture using a 28 gauge insulin syringe within 15 min of being captured. The blood was added to a tube containing 1.0 mL of BA-1 diluent and centrifuged within 5 h. Serum and BA-1 was pipetted off the clot and placed in a 2.0 mL cryovial; clots and the serum were stored separately at −20 °C or −80 °C prior to diagnostics. Samples from common grackles (*Quiscalus quiscula*) and red-winged blackbirds (*Agelaius phoeniceus*) recovered from Holt, Michigan in March of 2010 were used during the development of the molecular diagnostics. All field work was carried out under appropriate collecting permits (Illinois Department of Natural Resources Scientific Permit # NH10.5379, Federal Fish and Wildlife Scientific Collecting Permit mb # MB13235A-0) with approval from the Institutional Animal Care and Use Committee at Michigan State University (Animal Use Form # 08/08-125-00).

2.2. Field microscopy

A hematocrit centrifuge technique was used to screen blood for microfilariae and trypanosomes (Bennett, 1962). A portion of the blood sample was transferred from the syringe to a heparinized capillary tube (70 μL). One end of the capillary tube was sealed with clay and spent 5 min in a hematocrit microcentrifuge (International Equipment Co. IEC MB Centrifuge) at 14,000 rpm (12,700 G). The capillary tube was examined for motile microfilariae and trypanosomes for at least 5 min using a compound microscope. These parasites were concentrated at the interface of the white blood cells and plasma (i.e. buffy coat) and were screened at 100× with closer inspection at 400× (Woo, 1970). After microscopy, the hematocrit was broken with a nail clipper about 2 mm below the buffy coat layer and a paper clip was used to push the clay plug and express the buffy coat region into 100 μL of BA-1 diluent. The hematocrit microcentrifuge and compound scope were connected directly to a field vehicle battery using an Enercell 350 W High-Power Inverter. We tested for significant differences in microfilariae and trypanosome detection between robin blood collected during the day and night using a logistic regression in the computer program R (R Development Core Team, 2011).

2.3. Necropsy

Using a test-and-cull approach, we euthanized birds that were microfilaremic to increase the probability of recovering the adult filarial worms. We also euthanized a subset of birds that were negative for microfilariae because adult worms could still be present during the prepatent period and because microfilariae may exhibit periodicity (Bartlett, 2008). Necropsies were performed on American robins, house sparrows, northern cardinals (*Cardinalis cardinalis*), and house finches (*Carpodacus mexicanus*). After euthanasia, birds were stored at 4 °C and necropsied within 12 h, or sooner. During necropsy, tissues and organs were placed in petri dishes containing 0.85% physiological saline and parasites were recovered using a stereomicroscope. We examined the brain, heart, trachea, esophagus, crop, lungs, and body cavity for adult filarioid nematodes. Adult nematodes were stored in 70% ethanol, cleared with glycerin, and identified using keys and primary literature (Anderson and Freeman, 1969; Bartlett and Anderson, 1980, 1985; Anderson et al., 2009; Gibbons, 2010). Voucher specimens of a male and female *Chandlerella quiscali* and a male and female *Splendidofilaria* sp. collected from an American robin were deposited in the United State National Parasite Collection, Beltsville, Maryland (*C. quiscali*, 105668; *Splendidofilaria* sp., 105669).

2.4. Molecular diagnostics

Mosquito pools and bird serum were screened for WNV using quantitative RT-PCR as previously described (Hamer et al., 2008, 2011). One exception was that the viral RNA purification from bird serum and mosquito pools in this study occurred with the use of the MagMAX Total RNA Isolation Kit run on a MagMAX Express

Magnetic Particle Processor (Applied Biosystems, Foster City, California). We used maximum likelihood estimates for *Culex* spp. mosquito infection rates using the Pooled Infection Rate version 3.0 add-in (Biggerstaff, 2006) in the program Excel (Microsoft, Redmond, Washington). Bird serum was tested for WNV antibodies using an inhibition ELISA (Hamer et al., 2008).

2.5. Avian filarioid nematode and trypanosome phylogenetics

We extracted parasite DNA following the Animal Tissue Protocol (DNeasy Tissue Kits; Qiagen, Valencia, California). For the extraction of DNA from the adult filarioid nematodes, we utilized about 1 mm of material. For the blood clot or the buffy coat region expressed out of the capillary tube, we used 10 μL of material. For the birds recovered from Holt, Michigan, we extracted DNA from homogenized tissue (lungs, heart, brain). We used polymerase chain reaction (PCR) to amplify a 580 bp region of the filarial nematode 18s rRNA gene using the following primers: ChandFO-5′-GAGACCGTTCTCTTTGAGGCC-3′ and ChandRO-5′-GTCAAGGCG-TANNTTTACCGCCGA-3′ (J. Vaughan personal communication). The cycling profile consisted of denaturation at 94 °C for 2 min, followed by 39 cycles of 94 °C denaturation for 30 s, 57 °C annealing for 30 s, and 72 °C for 2 min, and a final extension at 72 °C for 7 min. We also targeted a 688 bp region of the filarial nematode mitochondrial cytochrome c oxidase subunit I (COI) gene using the following primers: COIintF 5′-TGATTGGTGGTTTTGGTAA-3′ and COIintR 5′-ATAAGTACGAGTATCAATATC-3′ (Casiraghi et al., 2001; Merkel, 2008). The touchdown cycling profile consisted of denaturation at 94 °C for 2 min, followed by 8 cycles of 94 °C for 45 s, 51 °C for 45 s (reduced by 0.5 °C for each cycle), and 72 °C for 1.5 min, followed by 25 cycles of 94 °C for 45 s, 45 °C for 45 s, and 72 °C for 1.5 min, and a final extension of 72 °C for 7 min.

We targeted a 326 bp region of the trypanosome 18s rRNA gene using a nested PCR using the following primers: outer forward S-762, GACTTTTGCTTCCTCTAWTG; outer reverse S-763, CATA-TGCTTGTTTCAAGGAC; nested forward S-755, CTACGAACCCTTTAA-CAGCA; nested reverse S-823, CGAAYAACTGCYCTATCAGC (Maslov et al., 1996; Sehgal et al., 2001; Van Dyken et al., 2006). The initial cycling profile consisted of denaturation at 95 °C for 5 min followed by 5 cycles at 95 °C for 1 min, 45 °C for 30 s, 65 °C for 1 min, and 35 cycles at 95 °C for 1 min, 50 °C for 30 s, 72 °C for 1 min, and a final extension at 65 °C for 10 min. One μL of the first reaction was added to the nested PCR which consisted of 96 °C for 3 min, 35 cycles at 96 °C for 30 s, 58 °C for 1 min, 72 °C for 30 s, and a final extension at 72 °C for 7 min. Negative controls were included in each batch of DNA extractions and in each PCR. We used the Failsafe PCR System (Epicentre Biotechnologies, Madison, Wisconsin) and 1 μL of template DNA for all PCRs. Amplicons were visualized by electrophoresis (agarose gels or the E-gel system; Invitrogen, Carlsbad, California) and purified (QIAquick PCR Purification Kits; Qiagen). Nucleotide sequences were obtained by direct sequencing in the forward and reverse directions (ABI Prism 3700 DNA Analyzer; Applied Biosystems, Foster City, California).

Sequences were aligned using ClustalW with manual correction. Forty-three sequences from this study were deposited in the NCBI GenBank (Accessions: JQ867025–JQ867067). We constructed neighbor-joining phylogenetic trees, accounting for gaps using pairwise deletion, and using Kimura's two-parameter substitution model using MEGA 5.0 (Tamura et al., 2011). Statistical support for phylogenetic groupings was estimated using bootstrap analysis. The topology of trees constructed using alternative methods (e.g. maximum likelihood) were congruent. We included in the analysis the only known avian filarioid nematode sequences in the NCBI Database, which were obtained from Galapagos penguins (*Spheniscuc mendiculus*) and flightless cormorants (*Phalacrocorax harrisi*) (Merkel, 2008). We included *Chandlerella quiscali* recovered from a common grackle in Ohio (Muzzall et al., 2011) and North Dakota. We also included *Caenorhabditis elegans* and *Thelazia lacrimalis*, an eyeworm found in horses, as outgroups for the filarioid nematode analysis (Casiraghi et al., 2001) and *Bodo caudatus*, a single-cell flagellate protozoan (Kinetoplastida), as an outgroup for the trypanosome analysis (Maslov et al., 1996)

3. Results

3.1. Culex infection rates

In 2010, we collected 2255 *Culex* spp. mosquito pools (23,068 individuals); 166 pools were positive for WNV with a peak infection rate of 42.6 per 1000 individuals (95% confidence interval of 27.9–63.3) at the end of August. In 2011, we collected 1954 *Culex* spp. mosquito pools (11,637 individuals) and 6 pools were positive for WNV.

3.2. Microscopy and necropsy

We screened 59 American robins and 38 house sparrows by the hematocrit centrifuge technique in 2010. We detected microfilariae in five robins (8.5%) and two house sparrows (5.2%), and we detected trypanosomes in 14 robins (23.7%) and four house sparrows (10.5%; Table 1). In 2011, we screened 74 American robins and 45 house sparrows and detected microfilariae in three robins (4.1%) and one house sparrow (2.2%), and we detected trypanosomes in 11 robins (13.5%) and 10 house sparrows (22.2%).

For the 2 years combined; a total of 70 robins were screened during the day and one (1.4%) was positive for microfilariae and 11 (15.7%) were positive for trypanosomes. A total of 63 robins were screened at night and seven (11.1%) were positive for microfilariae and 13 (20.6%) were positive for trypanosomes. Significantly more microfilaremic robins were detected at night than during the day ($P = 0.047$, df = 131, $Z = 1.99$), but no significant difference was found for trypanosome positive birds ($P = 0.339$, df = 131, $Z = 0.96$). A total of 72 sparrows were screened during the day; three (4.2%) were positive for microfilariae and eight (11.1%) were positive for trypanosomes. A total of 11 house sparrows were screened during the night; none were positive for microfilariae and six (54.5%) were positive for trypanosomes.

We necropsied 11 adult and 19 juvenile American robins and recovered *C. quiscali* from the brain of one juvenile robin and recovered *Splendidofilaria* sp. from the heart of two adult robins. We necropsied seven adult and three juvenile house sparrows and recovered *Splendidofilaria* sp. in the heart of one adult house sparrow.

3.3. Avian filarioid nematode taxonomic classification

We screened 157 samples using the primers targeting the 18S rRNA gene for filarioid nematodes and produced 20 sequences (Fig. 1A). Two major clades of our field collected filarioid nematode sequences emerged; one belonging to the putative *C. quiscali* (98% bootstrap support) and the other belonging to *Splendidofilaria* sp. (79% bootstrap support). Four of the six sequences in the *C. quiscali* clade are from adult worms collected from the brain of the host (two common grackles, one American robin, and one northern cardinal), three of which were morphologically identified to species. One of the sequences in the *C. quiscali* group was obtained from the microfilariae from the northern cardinal that also had adult worms in its brain. Three of the 9 sequences belonging to the *Splendidofilaria* sp. group were from adult worms collected from

Table 1
Prevalence of microfilariae and trypanosomes in American robins and house sparrows using the hematocrit centrifuge technique in suburban Chicago, Illinois, 2010, 2011.

Species	Year	Month	Time	Age	*n*	Microfilariae Number positive (%)	Trypanosomes Number positive (%)
American robin (*Turdus migratorius*)	2010	June	Day	AHY	8	0	0
	2010	June	Day	HY	5	0	1 (20.0)
	2010	June	Night	HY	7	0	2 (28.6)
	2010	July	Day	AHY	3	0	0
	2010	July	Day	HY	15	0	5 (33.3)
	2010	July	Night	AHY	8	3 (37.5)	3 (37.5)
	2010	July	Night	HY	13	2 (15.4)	3 (23.1)
	2011	June	Day	AHY	7	0	1 (14.3)
	2011	June	Day	HY	8	0	0
	2011	June	Night	AHY	4	1 (25.0)	1 (25.0)
	2011	June	Night	HY	4	0	0
	2011	July	Day	AHY	6	1 (16.7)	1 (16.7)
	2011	July	Day	HY	18	0	3 (16.7)
	2011	July	Night	AHY	7	0	1 (14.3)
	2011	July	Night	HY	15	0	3 (20.0)
	2011	Aug	Night	AHY	2	1 (50.0)	0
	2011	Aug	Night	HY	3	0	1 (33.3)
House sparrow (*Passer domesticus*)	2010	June	Day	AHY	5	0	1 (20.0)
	2010	June	Day	HY	3	0	0
	2010	July	Day	AHY	12	1 (8.3)	0
	2010	July	Day	HY	18	1 (5.6)	3 (16.7)
	2011	June	Day	AHY	8	1 (12.5)	1 (12.5)
	2011	June	Day	HY	7	0	2 (28.6)
	2011	June	Night	AHY	8	0	6 (75.0)
	2011	July	Day	AHY	10	0	0
	2011	July	Day	HY	9	0	1 (11.1)
	2011	July	Night	AHY	3	0	0

AHY, after hatch year (adult) bird; HY, hatch year (juvenile) bird.

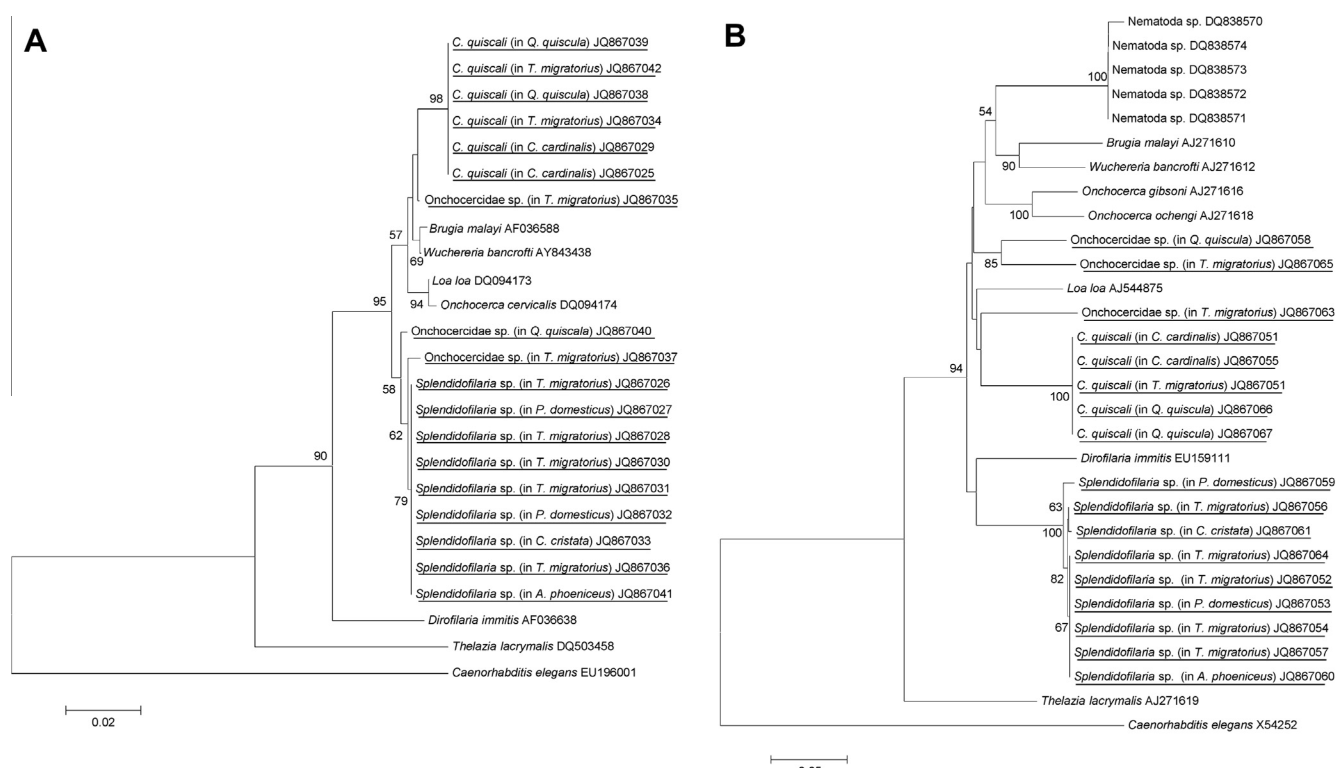

Fig. 1. Neighbor-joining phylogenetic trees for a 475 bp region of the 18S rRNA gene for filarioid nematodes (A) and a 529 bp region of the filarial nematode mitochondrial cytochrome c oxidase subunit I gene (B). Sequences were obtained from bird blood clots, bird tissues, or adult nematodes recovered from birds. Underlined sequences are from this study. Additional sequences for filarial nematode species were downloaded from NCBI Genbank for comparison and *Thelazia lacrimalis* and *Caenorhabditis elegans* were used as outgroups. Numbers by branches indicate statistical bootstrap support of ⩾50%.

the heart of the host (two American robins and one house sparrow) and the others were from microfilariae, including an adult male red-winged blackbird recovered near Holt, Michigan.

We screened a total of 46 samples using the primers targeting the COI gene for filarioid nematodes and produced 17 sequences (Fig. 1B). The COI gene shows a similar phylogeny as the 18S rRNA gene, with two major clades for *C. quiscali* (100% bootstrap support) and *Splendidofilaria* sp. (100% bootstrap support). We also observed several 18S rRNA and COI sequences in clades that appear to belong to unknown filarioid nematodes. The sequences from this study did not group with the avian filarioid sequences obtained from Galapagos penguins and flightless cormorants (Merkel, 2008; Nematode sp. in Fig. 1B).

To confirm the adult filarioid nematodes recovered during necropsies were responsible for the microfilariae observed during microscopy, we compared the sequences obtained from extracted DNA from the adult worm and microfilariae in the blood clot from the same birds. The sequence from the adult *C. quiscali* recovered from the brain of a northern cardinal was identical to the sequence obtained from the microfilariae from the same bird for 18S rRNA (JQ867025 and JQ867029, respectively) and COI (JQ867051 and JQ867055, respectively; Fig. 1). The sequence for the adult *Splendidofilaria* sp. nematode recovered from the heart of an American robin was identical to the sequence obtained from the microfilariae from the same bird for 18S rRNA (JQ867028 and JQ867031, respectively) and COI (JQ867054 and JQ867057, respectively; Fig. 1). The sequences obtained from the adult *Splendidofilaria* sp. nematode recovered from the heart of an American robin was identical to the sequence obtained from the microfilariae from the same bird for 18S rRNA (JQ867026 and JQ867030, respectively) and had one single nucleotide polymorphism in the COI gene (JQ867052 and JQ867056, respectively; Fig. 1). The sequences obtained from the adult *Splendidofilaria* sp. nematode recovered from the heart of a house sparrow was identical to the sequence obtained from the microfilariae from the same bird for 18S rRNA (JQ867027 and JQ867032, respectively) and had one single nucleotide polymorphism in the COI gene (JQ867053 and JQ867059, respectively; Fig. 1).

3.4. Trypanosome taxonomic classification

We screened a total of 58 samples using the primers targeting the 18s rRNA gene for trypanosomes and produced eight sequences (Fig. 2). Five sequences fell into a clade with 91% bootstrap support that included *Trypanosoma bennetti*. We also generated a sequence from a northern cardinal that grouped closely with *Trypanosoma corvi*. None of our sequences grouped with the *Trypanosomatida* clade discovered in *Culex* mosquitoes in Colorado (Van Dyken et al., 2006).

3.5. WNV and antibody detection in bird blood

We detected 10 WNV positive birds out of 557 tested (1.8%) using RT-PCR in 2010 and zero positive birds out of 294 tested in 2011. We did not document concomitant infections with WNV and microfilariae or trypanosomes. No American robins had concomitant infections with microfilariae and trypanosomes and one house sparrow had both parasites.

We detected 37 birds positive for WNV antibodies out of 560 tested (1.8%) in 2010 and 21 antibody positive birds out of 294 tested (7.1%) in 2011. One American robin collected on 30, August 2011 was positive for WNV antibodies and had a microfilariae infection implying a previous concomitant infection was possible. One Northern cardinal was positive for WNV antibodies and had a *Trypanosoma* sp. infection in 2010.

4. Discussion

Many studies have investigated microfilarial nematode prevalence in birds, reporting prevalences up to 20% (Greiner et al., 1975; Bennett et al., 1991; Rodriguez and Matta, 2001; Dusek and Forrester, 2002; Hauptmanova et al., 2004; Sehgal et al., 2005; Akinpelu, 2008; Benedikt et al., 2009). However, the majority of these studies screened blood smears for the presence of microfilariae using blood collected during the day, which could result in an underestimate of prevalence due to the periodicity of microfilariae. Additionally, most studies obtained blood from peripheral circulation, such as the brachial vein, which has been demonstrated to result in underestimates of microfilariae prevalence (Holmstad et al., 2003). The current study reports prevalence for birds captured at night and using blood from the deep circulatory system (i.e. jugular vein), which provides an ecologically relevant measure of prevalence from the perspective of nocturnal hematophagous arthropod vectors. Specifically, of the American robins collected during the day, 1.4% were microfilaremic and of the roosting robins collected at night, 11.1% were microfilaremic.

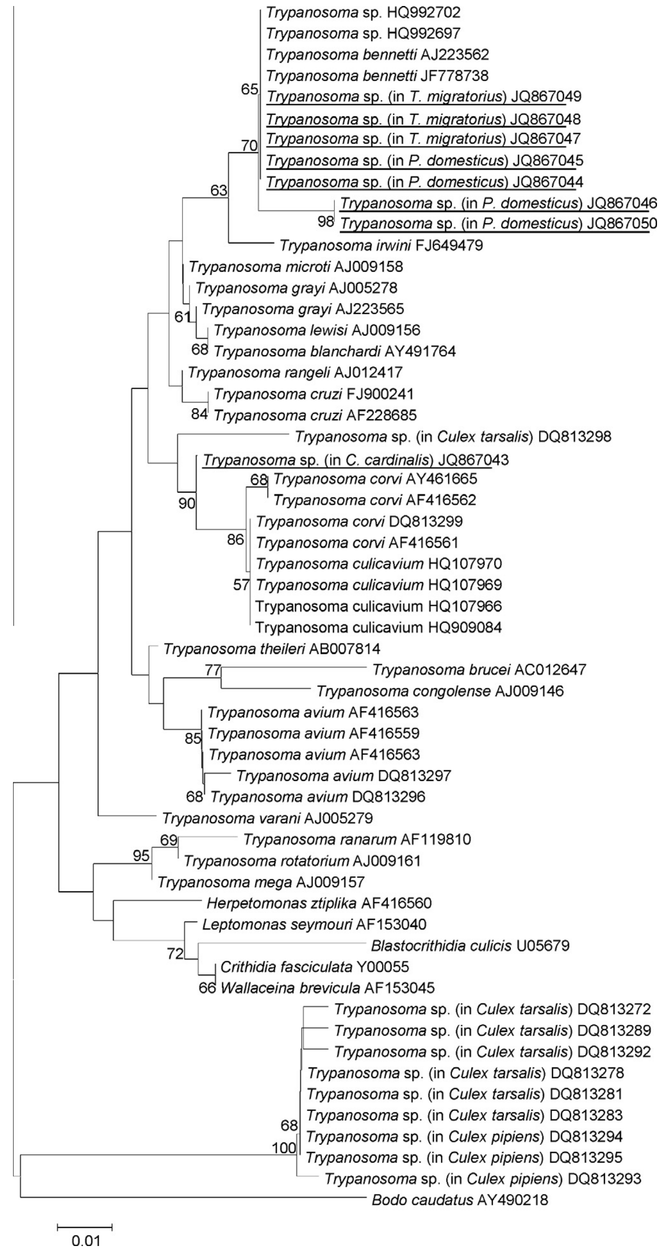

Fig. 2. Neighbor-joining phylogenetic tree for a 219 bp region of the trypanosome 18s rRNA gene. Underlined sequences are from this study. Sequences for additional *Trypanosoma* spp. were downloaded from NCBI Genbank for comparison and *Bodo caudatus* was used as an outgroup. Numbers by branches indicate statistical bootstrap support of ⩾50%.

These data demonstrate circadian periodicity in these parasites. We infer from these data that, like many species of microfilarial nematodes, the nematodes in our system enter peripheral blood during the night and congregate in deep circulation during the day, especially the lungs where the rate of blood flow is slow (Robinson, 1955; Hibler, 1963; Holmstad et al., 2003).

Our data also suggest temporal and age-structured heterogeneity in prevalence of microfilariae. The presence of microfilaremic juvenile robins and house sparrows in 2010 but not 2011 suggests that more transmission occurred in our study region in 2010 than in 2011. The reasons for this are unknown but spatial and temporal heterogeneity in microfilariae in birds in Ohio, USA, have been previously observed and it has been hypothesized that vector distribution and bird nesting habits are major determinants of transmission (Robinson, 1961). Little is known about the arthropod vectors of microfilarial nematodes, which include lice (order Phthiraptera) and flies (order Diptera, families Simuliidae, Culicidae, and Ceratopogonidae), but the most likely vectors of the species of filarioid nematodes in this study are *Culicoides* midges based on abundance and infection status observed in previous studies (Robinson, 1971; reviewed by Bartlett, 2008).

Adult filarioid nematodes are notoriously difficult to locate and identify and the identification of microfilariae to species is not possible based on morphological features (Bartlett, 2008). Because of this, we adopted a combined morphological and molecular approach to identify the species present in our study sites (McKeand, 1998). We did not recover adult filarioid nematodes from every microfilaremic individual, which could have been due to error or to the ephemeral nature of the adult worms, which sometimes die and are resorbed soon after producing microfilariae (Bartlett, 2008). The two most abundant clades of filarioid nematodes at our study sites were *C. quiscali* and *Splendidofilaria* sp. We also detected unique 18S rRNA and COI sequences from DNA that we didn't have adult specimens for morphological identification, suggesting additional species could be present. The sequences from this study submitted to the NCBI database represent the third study to submit sequences for avian filarioid nematodes.

We found 18.0% (n = 133) of American robins and 16.9% (n = 83) of house sparrows infected with trypanosomes. These prevalence rates are difficult to compare to studies screening birds for trypanosomes using blood smears or blood culture due to differences in sensitivity, but the results are within reported ranges (Greiner et al., 1975; Kirkpatrick and Lauer, 1985; Bennett et al., 1991; Sehgal et al., 2001; Dusek and Forrester, 2002; Holmstad et al., 2003; Akinpelu, 2008). The taxonomy and systematics of avian *Trypanosoma* are poorly understood (Valkiunas et al., 2011; Votypka et al., 2012). This study used light microscopy to visualize motile trypanosome parasites, with subsequent molecular diagnostics on a subsample of these individuals. Although the conserved 18s rRNA gene has limitations for inferring phylogenetic relationships among avian trypanosomes (Sehgal et al., 2001; Valkiunas et al., 2011), our results suggest the presence of multiple *Trypanosoma* lineages. The majority of the *Trypanosoma* sequences from this study fell in a clade with sequences identical to *Trypanosoma anguiformis* collected from Olive sunbirds (*Cyanomitra olivacea*) in Ghana (Valkiunas et al., 2011) and *T. bennetti* collected from a Lesser-spotted Eagle (*Aquila pomarina*) in the Czech Republic (Votypka et al., 2002, 2012). Further genetic data including more variable regions of genes will be necessary to distinguish these *Trypanosoma* spp. based on DNA sequences.

Many types of hematophagous arthropods have been implicated as vectors for avian trypanosomes, including black flies (Diptera: Simuliidae), midges (Diptera: Ceratopogonidae), and mosquitoes (Diptera: Culicidae) (Bennett, 1962). Trypanosomes have been detected in *Culex* spp. mosquitoes, black flies, hippoboscid flies, and biting midges (Votypka et al., 2002; Van Dyken et al., 2006), but the presence of the parasites doesn't imply involvement in transmission. In Colorado, USA, trypanosome prevalence was 17.7 per 1000 *Culex pipiens* and 27 per 1000 *Culex tarsalis* (Van Dyken et al., 2006). Several mechanisms of transmission have been described for different arthropods and trypanosomes. Volf et al. (2004) reported that trypanosomatid parasites block the stomodeal valve in *Culex quinquefasciatus* causing regurgitation which facilitates parasite transmission to the vertebrate host during blood feeding by a mechanism similar to that for *Leishmania*. In other experiments, *Trypanosoma avium* was transmitted to canaries (*Serinus canaria*) by black flies (*Eusimulium latipes*) by ingestion of infected black flies and by contamination of host conjunctiva (Votypka and Svobodova, 2004). Additionally, *Trypanosoma culicavium* was successfully transmitted from *C. quinquefasciatus* to birds by ingestion of infected mosquitoes but not the bite of infected mosquitoes (Votypka et al., 2012). The vectors responsible for transmission of the trypanosomes observed in this study remain unknown.

Our data, combined with evidence from other studies, suggests that a substantial proportion of birds and mosquitoes have trypanosome infections in our study region prior to exposure to WNV. When this occurs, indirect immune-mediated interactions could occur in the avian or mosquito host. Although host immunity has been extensively explored for human trypanosomiasis (Tabel et al., 2008; Junqueira et al., 2010), little attention has been given to avian host responses. Within the mosquito host, dissemination of a virus throughout the body by trypanosomes could result from penetration of the midgut wall, as occurs with the microfilariae. Alternatively, the stimulation of regurgitation by blocking the stomodeal valve could alter vector behavior by increasing probing on multiple hosts and lead to epidemiologically important consequences.

Overall, this study documents a suite of hemoparasites in the same avian hosts responsible for the amplification of WNV in suburban Chicago, Illinois, USA. Although we found little evidence of concomitant infection with WNV and a second parasite, we demonstrate that these parasites are co-circulating in a region known as a hotspot for WNV transmission (Ruiz et al., 2004). In our long-term study of WNV in this region from 2005 to 2011, we captured and tested 5728 birds for the presence of WNV and only 27 (0.05%) were RT-PCR positive (Hamer et al. unpublished data). Capturing live WNV positive birds is a rare event when the viremic period is typically less than 7 days (Komar et al., 2003) during a period of reduced activity (Yaremych et al., 2004) which means these birds are less likely to be captured by standard ornithological techniques. Additionally, the birds screened for microfilariae and trypanosomes in this study were captured in June and July, while most of the WNV positive birds occur in August in this study region (Hamer et al., 2008). For these reasons, future studies investigating the consequences of direct and indirect parasite interactions in the WNV system will require controlled laboratory infection experiments to gain a mechanistic understanding. We suggest an approach that considers the host community composition (i.e. Hamer et al., 2011), alongside the composition and dynamics of the parasite community, which will further understanding of the consequences of parasite interactions and provide better predictions of disease emergence to aid in the development of control programs.

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References

Akinpelu, A.I., 2008. Prevalence and intensity of blood parasites in wild pigeons and doves (Family: Columbidae) from Shasha Forest Reserve, Ile-Ife Nigeria. Asian J. Anim. Vet. Adv. 3, 109–114.

Aliota, M.T., Chen, C.C., Dagoro, H., Fuchs, J.F., Christensen, B.M., 2011. Filarial worms reduce plasmodium infectivity in mosquitoes. PLoS Neglect. Trop. Dis. 5, e963.

Anderson, R.C., Freeman, R.S., 1969. *Cardiofilaria inornata* (Anderson 1956) from woodcock with a review of Cardiofilaria and related genera (Nematode Filarioidea). Trans. Am. Microsc. Soc. 88, 68.

Anderson, R.C., Chabaud, A.G., Willmott, S., 2009. Commonwealth institute of helminthology. Keys to the Nematode Parasites of Vertebrates, Archival volume. CABI, Wallingford.

Andreadis, T.G., Thomas, M.C., Shepard, J.J., 2005. Identification guide to the mosquitoes of connecticut. In: This Mosquito ID Guide was Produced by the Connecticut Agricultural Experimental Station and is Bulletin No. 966., New Haven, CT. http://www.ct.gov/caes/lib/caes/documents/publications/bulletins/b966b996.pdf.

Bartlett, C.M., Anderson, R.C., 1980. Filarioid nematodes (Filarioidea: Onchocercidae) of *Corvus Brachyrhynchos Brachyrhynchos* Brehm in Southern Ontario, Canada and a consideration of the epizootiology of avian filariasis. Syst. Parasitol. 2, 77–102.

Bartlett, C.M., Anderson, R.C., 1985. On the filarioid nematodes (*Plendidofilaria* spp.) from the pulmonary arteries of birds. Can. J. Zool. 63, 2373–2377.

Bartlett, C.M., 2008. Filarioid nematodes. In: Atkinson, C.T., Thomas, N.J., Hunter, D.B. (Eds.), Parasitic Diseases of Wild Birds. Blackwell Publishing, Ames, Iowa.

Benedikt, V., Barus, V., Capek, M., Havlicek, M., Literak, I., 2009. Blood parasites (*Haemoproteus* and microfilariae) in birds from the Caribbean slope of Costa Rica. Acta Parasitol. 54, 197–204.

Bennett, G.F., 1962. Hematocrit centrifuge for laboratory diagnosis of hematozoa. Can. J. Zool. 40, 124–125.

Bennett, G.F., Garvin, M., Bates, J.M., 1991. Avian hematozoa from west central Bolivia. J. Parasitol. 77, 207–211.

Biggerstaff, B.J., 2006. Pooled Inf Rate. In: Version 3.0: a Microsoft Excel Add-In to compute prevalence estimates from pooled samples. Centers for Disease Control and Prevention. Fort Collins, CO.

Cattadori, I.M., Boag, B., Hudson, P.J., 2008. Parasite co-infection and interaction as drivers of host heterogeneity. Int. J. Parasitol. 38, 371–380.

Casiraghi, M., Anderson, T.J.C., Bandi, C., Bazzocchi, C., Genchi, C., 2001. A phylogenetic analysis of filarial nematodes: comparison with the phylogeny of Wolbachia endosymbionts. Parasitology 122, 93–103.

Cox, F.E.G., 2001. Concomitant infections, parasites and immune responses. Parasitology 122, S23–S38.

Dusek, R.J., Forrester, D.J., 2002. Blood parasites of American crows (*Corvus brachyrhynchos*) and fish crows (*Corvus ossifragus*) in Florida. USA Comp. Parasitol. 69, 92–96.

Gibbons, L.M., 2010. Keys to the Nematode Parasites of Vertebrates, Supplementary volume. CABI, Wallingford, Oxfordshire, UK/Cambridge, MA, USA.

Graham, A.L., Cattadori, I.M., Lloyd-Smith, J.O., Ferrari, M.J., Bjornstad, O.N., 2007. Transmission consequences of coinfection: cytokines writ large? Trends Parasitol. 23, 284–291.

Graham, A.L., 2008. Ecological rules governing helminth-microparasite coinfection. Proc. Natl. Acad. Sci. USA 105, 566–570.

Greiner, E.C., Bennett, G.F., White, E.M., Coombs, R.F., 1975. Distribution of the avian hematozoa of North America. Can. J. Zool. 53, 1762–1787.

Hamer, G.L., Walker, E.D., Brawn, J.D., Loss, S.R., Ruiz, M.O., Goldberg, T.L., Schotthoefer, A.M., Brown, W.M., Wheeler, E., Kitron, U.D., 2008. Rapid amplification of West Nile virus: the role of hatch-year birds. Vector Borne Zoonotic Dis. 8, 57–67.

Hamer, G.L., Chaves, L.F., Anderson, T.K., Kitron, U.D., Brawn, J.D., Ruiz, M.O., Loss, S.R., Walker, E.D., Goldberg, T.L., 2011. Fine-scale variation in vector host use and force of infection drive localized patterns of West Nile virus transmission. PLoS One 6, e23767.

Hauptmanova, K., Barus, V., Literak, I., Benedikt, V., 2004. Haemoproteids and microfilariae in hawfinches in the Czech Republic. Helminthologia 41, 125–133.

Hibler, C.P., 1963. Onchocercidae (Nematoda: Filarioidea) of the American Magpie, *Pica pica hudsonia* (Sabine), in northern Colorado. In: Colorado State University, Fort Collins, Colorado, pp. 189.

Holmstad, P.R., Anwar, A., Lezhova, T., Skorping, A., 2003. Standard sampling techniques underestimate prevalence of avian hematozoa in willow ptarmigan (*Lagopus lagopus*). J. Wildl. Dis. 39, 354–358.

Hudson, P.J., 1986. The effect of a parasitic nematode on the breeding production of red grouse. J. Anim. Ecol. 55, 85–92.

Junqueira, C., Caetano, B., Bartholomeu, D.C., Melo, M.B., Ropert, C., Rodrigues, M.M., Gazzinelli, R.T., 2010. The endless race between *Trypanosoma cruzi* and host immunity: lessons for and beyond Chagas disease. Expert Rev. Mol. Med. 12, e29.

Kirkpatrick, C.E., Lauer, D.M., 1985. Hematozoa of raptors from southern New Jersey and adjacent areas. J. Wildl. Dis. 21, 1–6.

Komar, N., Langevin, S., Hinten, S., Nemeth, N., Edwards, E., Hettler, D., Davis, B., Bowen, R., Bunning, M., 2003. Experimental infection of North American birds with the New York 1999 strain of West Nile virus. Emerg. Infect. Dis. 9, 311–322.

Lello, J., Boag, B., Fenton, A., Stevenson, I.R., Hudson, P.J., 2004. Competition and mutualism among the gut helminthes of a mammalian host. Nature 428, 840–844.

Loss, S.R., Hamer, G.L., Goldberg, T.L., Ruiz, M.O., Kitron, U.D., Walker, E.D., Brawn, J.D., 2009. Nestling passerines are not important hosts for amplification of West Nile virus in Chicago Illinois. Vector Borne Zoonotic Dis. 9, 13–17.

Maslov, D.A., Lukes, J., Jirku, M., Simpson, L., 1996. Phylogeny of trypanosomes as inferred from the small and large subunit rRNAs: implications for the evolution of parasitism in the trypanosomatid protozoa. Mol. Biochem. Parasitol. 75, 197–205.

McKeand, J.B., 1998. Molecular diagnosis of parasitic nematodes. Parasitology 117, S87–S96.

Mellor, P.S., Boorman, J., 1980. Multiplication of bluetongue virus in *Culicoides nubeculosus* (Meigen) simultaneously infected with the virus and the microflariae of O*nchocerca cervivalis* (Railliet and Henry). Ann. Trop. Med. Parasitol. 74, 463–469.

Merkel, J., 2008. Microfilarial in galapagos penguins (*Spheniscus mendiculus*) and flightless cormorants (*Phalacrocorax harrisi*): Genetics, morphology and prevalence. J. Parasitol. 94, 190–196.

Muzzall, P.M., Cook, J., Sweet, D.J., 2011. Helminths of belted kingfishers, *Megaceryle alcyon* Linnaeus, 1758, from a fisher hatcher in Ohio. USA. Comp. Parasitol. 78, 367–372.

Newman, C.M., Cerutti, F., Anderson, T.K., Hamer, G.L., Walker, E.D., Kitron, U.D., Ruiz, M.O., Brawn, J.D., Goldberg, T.L., 2011. *Culex* flavivirus and West Nile virus mosquito co-infection and positive association in Chicago USA. Vector Borne Zoonotic Dis. 11, 1099–1105.

Pedersen, A.B., Fenton, A., 2007. Emphasizing the ecology in parasite community ecology. Trends Ecol. Evol. 22, 133–139.

Robinson, E.J., 1955. Observations on the epizootiology of filarial infections in two species of the avian family Corvidae. J. Parasitol. 41, 209–214.

Robinson, E.J., 1961. Incidence of microfilariae in some Ohio birds and data on the habits of a possible vector. J. Parasitol. 47, 441–444.

Robinson, E.J., 1971. *Culicoides crepuscularis* (Malloch) (Diptera Ceratopogonidae) as a host for *Chandlerella quiscali* (Von Linstow, 1904) comb. n. (Filarioidea Onchocercidae). J. Parasitol. 57, 772–776.

Rodriguez, O.A., Matta, N.E., 2001. Blood parasites in some birds from eastern plains of Colombia. Mem. Inst. Oswaldo Cruz 96, 1173–1176.

Rohani, P., Green, C.J., Mantilla-Beniers, N.B., Grenfell, B.T., 2003. Ecological interference between fatal diseases. Nature 422, 885–888.

Ruiz, M.O., Tedesco, C., McTighe, T.J., Austin, C., Kitron, U.D., 2004. Environmental and social determinants of human risk during a West Nile virus outbreak in the greater Chicago area, 2002. Int. J. Health Geograph. 3, 11.

Sehgal, R.N.M., Jones, H.I., Smith, T.B., 2001. Host specificity and incidence of Trypanosoma in some African rainforest birds: a molecular approach. Mol. Ecol. 10, 2319–2327.

Sehgal, R.N.M., Jones, H.I., Smith, T.B., 2005. Molecular evidence for host specificity of parasitic nematode microfilariae in some African rainforest birds. Mol. Ecol. 14, 3977–3988.

Tabel, H., Wei, G., Shi, M., 2008. T cells and immunopathogenesis of experimental African trypanosomiasis. Immunol. Rev. 225, 128–139.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.

R Development Core Team., 2011. R: A language and environment for statistical computing. In: R Foundation for Statistical Computing, Vienna, Austria.

Telfer, S., Lambin, X., Birtles, R., Beldomenico, P., Burthe, S., Paterson, S., Begon, M., 2010. Species interactions in a parasite community drive infection risk in a wildlife population. Science 330, 243–246.

Tompkins, D.M., Dunn, A.M., Smith, M.J., Telfer, S., 2010. Wildlife diseases: from individuals to ecosystems. J. Anim. Ecol. 80, 19–38.

Turell, M.J., Rossignol, P.A., Spielman, A., Rossi, C.A., Bailey, C.L., 1984. Enhanced arboviral transmission by mosquitoes that concurrently ingested microfilariae. Science 225, 1039–1041.

Turell, M.J., Mather, T.N., Spielman, A., Bailey, C.L., 1987. Increased dissemination of Dengue-2 virus in *Aedes aegypti* associated with concurrent infection of microfilariae of *Brugia malayi*. Am. J. Trop. Med. Hyg. 37, 197–201.

Valkiunas, G., Lezhova, T.A., Carlson, J.S., Sehgal, R.N.M., 2011. Two new Trypanosoma species from African birds, with notes on the taxonomy of avian trypanosomes. J. Parasitol. 97, 924–930.
Van Dyken, M., Bolling, B.G., Moore, C.G., Blair, C.D., Beaty, B.J., Black, W.C., Foy, B.D., 2006. Molecular evidence for trypanosomatids in *Culex* mosquitoes collected during a West Nile virus survey. Int. J. Parasitol. 36, 1015–1023.
Vaughan, J.A., Turell, M.J., 1996. Dual host infections: enhanced infectivity of eastern equine encephalitis virus to *Aedes* mosquitoes mediated by *Brugia* microfilariae. Am. J. Trop. Med. Hyg. 54, 105–109.
Vaughan, J.A., Focks, D.A., Turell, M.J., 2009. Simulation models examining the effect of Brugian filariasis on dengue epidemics. Am. J. Trop. Med. Hyg. 80, 44–50.
Volf, P., Hajmova, M., Sadlova, J., Votypka, J., 2004. Blocked stomodeal valve of the insect vector: similar mechanism of transmission in two trypanosomatid models. Int. J. Parasitol. 34, 1221–1227.
Votypka, J., Obornik, M., Volf, P., Svobodova, M., Lukes, J., 2002. *Trypanosoma avium* of raptors (Falconiformes): phylogeny and identification of vectors. Parasitology 125, 253–263.
Votypka, J., Svobodova, M., 2004. *Trypanosoma avium*: experimental transmission from black flies to canaries. Parasitol. Res. 92, 147–151.
Votypka, J., Szabova, J., Radrova, J., Zidkova, L., Svobodova, M., 2012. *Trypanosoma culicavium* sp. nov., an avian trypanosome transmitted by *Culex* mosquitoes. Int. J. Syst. Evol. Microbiol. 62, 745–754.
Weaver, S.C., Reisen, W.K., 2010. Present and future arboviral threats. Antiviral Res. 85, 328–345.
Woo, P.T., 1970. The haematocrit centrifuge technique for the diagnosis of African trypanosomiasis. Acta Trop. 27, 384–386.
Yaremych, S.A., Novak, R.J., Raim, A.J., Mankin, P.C., Warner, R.E., 2004. Home range and habitat use by American Crows in east-central Illinois. Wilson Bull 116, 232–239.