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Detection of West Nile Virus in Stable Flies (Diptera: Muscidae) Parasitizing Juvenile American White Pelicans

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ABSTRACT Stable flies, *Stomoxys calcitrans* (L.) (Diptera: Muscidae), an economically important pest of livestock and humans, were observed parasitizing prefledged American white pelicans, *Pelecanus erythrorhynchos* (Pelecaniformes: Pelecanidae), in a pelican breeding colony in northeastern Montana where die-offs attributed to West Nile virus (family *Flaviviridae*, genus *Flavivirus*, WNV) have occurred since 2002. Engorged and unengorged flies were collected off nine moribund chicks. Of 29 blood-engorged flies testing positive for vertebrate DNA, all 29 contained pelican DNA. Virus isolation was performed on 60 pools (1,176 flies) of unengorged flies using Vero cell plaque assay. Eighteen pools were positive for WNV for an estimated infection rate of 18.0 per 1,000 flies. Fifty-four percent (36/67) of abdomens from blood-engorged flies tested positive for WNV. Pelican viremia levels from the blood-engorged fly abdomens revealed that at least one of the ill pelicans circulated a viremia capable of infecting *Culex* mosquito vectors. Stable flies may be involved in WNV transmission within the pelican breeding colony by serving as either a mechanical vector or as a source for oral infection if ingested by predators.

KEY WORDS West Nile virus, *Stomoxys*, detection, pelicans

Thousands of deaths of prefledged American white pelicans, *Pelecanus erythrorhynchos* (Pelecaniformes: Pelecanidae), were observed in 2002 and 2003 in several major breeding colonies in the northern Great Plains coincident with the arrival of West Nile virus (family *Flaviviridae*, genus *Flavivirus*, WNV) in the region. Mortality occurred during the late breeding season (mid-July to early August), a time when chick survival normally is high (≥95%). WNV was detected in a few representative chick carcasses tested in 2003 from each of four colonies located in North Dakota, South Dakota, Minnesota, and Montana. WNV was suspected as the etiologic agent for most of the deaths (Sovada et al. 2008). The landscape where breeding colonies are established is interspersed with wetlands that are prolific in producing mosquitoes, particularly *Culex tarsalis* Coquillett (Diptera: Culicidae), the primary vector of WNV in this region (Hale 2007). The potential population impacts of WNV on these pelicans generated concern because nearly half of the population of this colonial nesting bird breeds in four major colonies in the northern Great Plains (King and Anderson 2005, Sovada et al. 2008). Coordinated monitoring activities aimed at estimating late breeding season chick mortality, determining the seasonal occurrence and prevalence of WNV epizootics and examining other disease and nondisease factors that af-

fect prefledge pelican survival were started in 2004 by federal wildlife biologists at breeding colonies located in North and South Dakota and Montana (Sovada et al. 2008).

In 2007, the refuge manager at Medicine Lake National Wildlife Refuge in northeast Montana authorized the study of the Medicine Lake pelican flock to include monitoring weekly pelican chick mortality in the breeding colony and take possession of fresh carcasses for WNV testing of internal organ tissues, feathers, and skin (Johnson et al. 2010). While making mortality counts in mid-July, we observed numerous moribund pelican chicks with clusters of flies on the neck, gular pouch, and periocular region. A sample of flies was collected, and they were identified as stable flies, *Stomoxys calcitrans* (L.) (Diptera: Muscidae). Stable flies are obligate blood-feeders primarily associated with domestic livestock, particularly cattle, and in their absence will readily feed on companion animals and humans (Hogsette et al. 1987). Published reports of stable flies parasitizing domestic or wild birds are rare. Because of the uniqueness of this ectoparasite association, flies were collected directly from moribund pelicans for additional study. Blood-engorged flies were assayed to confirm the presence of pelican DNA in the bloodmeal. WNV infection rate in the flies was determined, and engorged flies were examined separately to estimate the virus titer encountered in blood of moribund pelicans and investigate whether the infecting virus is replicating in fly tissue. The potential role of stable flies as a mechanical vector, biological vector, or both of WNV is discussed.

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Materials and Methods

Medicine Lake National Wildlife Refuge (MLNWR) is located in Sheridan County in northeastern Montana (48° 27′ N, 104° 23′ W) in the Great Plains ecoregion (elevation 590 m). The refuge covers 13,000 ha and includes extensive wetlands, providing suitable breeding habitat for both mosquitoes and aquatic birds, including the fifth largest breeding colony of American white pelicans. Approximately 4,000 breeding pairs of pelicans nest on Bridgerman Point, a narrow peninsula that extends ≈500 m into the lake (Madden and Restani 2005).

In early June 2007, 1,000 pelican chicks were leg-banded to estimate prefledge mortality rates during the late breeding season (Sovada et al. 2008). Prefledged mortality counts were made weekly from 13 July to 15 August. Chick mortality rate was estimated by calculating the proportion of bands applied that were recovered from dead prefledged pelicans in the colony.

Stable flies were collected with a battery-operated insect vacuum (model 2820A, Bio-Quip, Rancho Dominguez, CA) from moribund pelicans. Nine moribund pelicans were collected on 1 August. After mechanically aspirating flies, the nine moribund chicks were euthanized by cervical dislocation by refuge personnel following National Wildlife Health Center Animal Care and Use Committee guidelines. Flies were placed on dry ice in the field, then stored for 2 d in a −20°C freezer at the refuge until transported to Montana State University (MSU), Bozeman, MT, where they were counted and categorized as blood-engorged (i.e., visible signs of blood in its midgut) or empty. Engorged flies were placed in individual cryovials; nonengorged flies were pooled in cryovials in groups of 20 or fewer. Flies were stored at −30°C. Pelicans were placed individually in plastic bags, and placed in a −20°C freezer at MLNWR for 7 d before transport to MSU on dry ice. Carcasses were stored frozen at −30°C until necropsy.

Homogenates of pools of unengorged flies, or individual abdomens and their respective individual bodies (without the abdomen) for engorged flies, were prepared following the procedures of Nemeth et al. (2007). Virus isolation was performed on stable fly homogenates by using Vero cell plaque assay as described previously (Panella et al. 2001). Viral plaques were confirmed as WNV by VecTest WNV antigen detection assay (Medical Analysis Systems, Camarillo, CA) as described previously (Nemeth et al. 2007). The prevalence of WNV infection in stable flies was estimated using PooledInfRate (http://www.cdc.gov/ncidod/dvbid/westnile/software.html), expressed in units of per 1,000 stable flies. When fly abdomens contained more than ≈1,000 plaque-forming units (pfu), stored supernatants were thawed and retested by titration to estimate the viral load in the bloodmeal.

Thirty blood-engorged stable flies were processed for bloodmeal identification as described previously (Kent et al. 2009). In brief, each stable fly abdomen was removed using forceps and homogenized as for the fly pools. DNA was extracted from homogenized fly tissues using a DNeasy blood and tissue kit (QIAGEN, Valencia, CA). After extraction, 2 μl of DNA template was amplified using primers specific for vertebrate mitochondrial cytochrome oxidase 1 (CO1). Polymerase chain reaction (PCR) products were run on a 2% ethidium bromide-stained gel, purified using a PCR purification kit (QIAGEN), and sequenced as described previously (Kent et al. 2009). Bloodmeals were identified using the DNA Barcode database (www.barcodinglife.org).

Results

Stable flies were found congregating mostly around the eye, neck, gular pouch, and crown of the head on moribund prefledged chicks (Fig. 1). Biting and intensive feeding resulted in blood pooling on the skin, alopecia, and perivascular dermatitis particularly in the periocular area (Figs. 1 and 2). Dried blood around the periocular region combined with the clusters of flies rendered the birds blind (Fig. 2). Defensive behaviors to dislodge flies, including head shake, head rub, wing flap, body fluff, and head swings, were weak and largely ineffective because dispersed flies quickly returned to the moribund bird and resumed probing and feeding. Healthy birds when attacked by stable flies exhibited similar defensive moves and were able to repeatedly dislodge flies once they landed and commenced biting. The persistent attacks of the biting flies sometimes forced many of the healthy birds into the water. Moribund birds were unable to move into the water and were forced to endure the persistent attacks of the flies. We did not observe stable flies feeding on recently deceased birds or on other moribund species (e.g., double-crested cormorants [Pelecaniformes: Phalacrocoracidae; *Phalacrocorax auritus* (Lesson)]; California gulls [Charadriiformes: Laridae; *Larus californicus* Lawrence]), which were fewer in number (<10 per species).

Overall, 1,243 stable flies were collected by aspiration directly from moribund pelicans, including 822 (mean 83.9 per bird ± 66.2 SD) from nine moribund pelicans that were collected, of which seven had confirmed acute WNV infection (Table 1). The ratio of male to female flies was ≈4:1. Most flies collected seemed unengorged (1,176; 95%). Thirty-nine pools containing 755 unengorged flies aspirated from the collected pelicans were tested and nine were positive for WNV for an estimated infection rate of 13.3 per 1,000 unengorged flies (95% confidence interval [CI], 6.6–24.5). Viral loads in these pools ranged from the minimum threshold of detection (10 pfu) to 80 pfu with one outlier containing 770 pfu (collected from pelican ML-20). An additional 21 pools of aspirated, unengorged flies ($n = 421$) taken from moribund pelicans of unknown WNV infection status yielded nine additional positive pools, with viral loads ranging from 10 to 320 pfu. The mean viral load of all 18 positive pools was 115 pfu (SD = 185, or excluding the outlier, SD = 91) and the estimated infection rate was 18.0 per 1,000 unengorged flies (95% CI, 11.1–28.0).

Fig. 1. Clusters of stable flies feeding on the head and neck of a moribund prefledged pelican. (Online figure in color.)

Engorged flies ($n = 67$ taken from moribund pelicans, including four from pelicans that tested negative for acute WNV infection) were not pooled but rather split into two parts (abdomen and head plus thorax) per fly for testing. Mostly abdomens ($n = 36, 54\%$) but also bodies ($n = 14, 21\%$) were positive for infectious

Fig. 2. Dried blood and scabbing in the periocular region due to stable fly feeding. (Online figure in color.)

Table 1. WNV detection from unengorged and engorged stable flies collected from moribund pelicans, 1 August 2007

Bird ID. no.	Pelican tissue assay[a]	Unengorged (♂:♀)	S. calcitrans tested/pools +	Engorged (♂:♀)	Abdomen, no. tested/no. +	Body, no. tested/no. +
ML-16	Pos	13 (11:2)	13/0	0 (0:0)	0/0	0/0
ML-17	Neg	193 (163:30)	193/0	2 (1:1)	2/0	2/0
ML-18	Pos	160 (130:30)	160/5	30 (24:6)	30/28	30/11
ML-19	Pos	37 (28:9)	37/2	12 (10:2)	12/8	12/3
ML-20	Pos	97 (75:22)	97/2	12 (10:2)	12/0	12/0
ML-21	Pos	55 (43:12)	55/0	3 (2:1)	3/0	3/0
ML-22	Pos	140 (118:22)	140/0	3 (3:0)	3/0	3/0
ML-23	Neg	40 (32:8)	40/0	2 (1:1)	2/0	2/0
ML-24	Pos	20 (15:5)	20/0	3 (1:2)	3/0	3/0
		755 (615:140)	755/9	67 (52:15)	67/36	67/14

[a] Pelicans testing positive had one or more of the following tissues (brain, heart, lung, kidney, spleen, skin, and feather pulp) positive for WNV infection by either virus isolation or detection of WNV RNA by reverse transcription-PCR.

WNV. Viral loads exceeded 1,000 pfu in several abdomens (maximum, 36,000 pfu) but were generally low among bodies (maximum, 75 pfu). Only one positive body (viral load, 10 pfu) lacked a positive counterpart abdomen. Of the 13 other positive bodies, 10 counterpart abdomens contained from 14 to >200-fold greater viral load, whereas three had four-fold greater viral load. All 37 WNV-positive engorged flies were among 42 engorged flies collected from just two (ML-18 and ML-19) of seven WNV-infected pelicans. The WNV content in the abdomens of the flies collected from ML-18 ($n = 30$) ranged from <5 pfu (i.e., undetectable by our plaque assay) to 36,000 pfu (median, 355). The range of infectious WNV content in abdomens of flies collected from ML-19 ($n = 12$) ranged from <5 to 650 pfu (median, 20 pfu). No WNV-positive engorged flies were collected from the two WNV-negative pelicans.

DNA was extracted from 29 blood-engorged abdomens (one additional abdomen failed to yield DNA) and DNA sequencing confirmed pelicans as the source for 29 of these bloodmeals. Seven of nine pelican carcasses collected had one or more tissues positive for WNV RNA; engorged and unengorged flies positive for WNV were collected from three of these positive pelicans (Table 1). Three hundred-one leg bands were recovered for an estimated mortality rate of 30% among prefledged pelican chicks.

Discussion

Stable flies are considered one of the most important veterinary arthropod pests in North America showing a preference for cattle (Anderson and Tempelis 1970, Hogsette et al. 1987). They readily attack humans and companion animals in the absence of livestock and are serious pests in outdoor environments such as recreational lakes, resorts and beaches, and agricultural premises with livestock (Hogsette et al. 1987). Immature stages of stable flies are found in decomposing feed and wet decaying vegetation mixed with manure and water or urine. Stable flies are diurnal with both sexes feeding at least once per day. Adults typically attack the lower front legs of large mammals and tips of ears on dogs, inflicting a painful bite using sharp prestomal teeth that pierce and tear

the host skin to obtain blood (Elzinga and Broce 1986). Livestock hosts react to stable fly attack by bunching and displaying numerous fly-repelling behaviors—foot stomping, head tossing, tail switching, and skin twitching (Mullens et al. 2006). Stable fly bites have been reported to cause necrotic and exudative dermatitis in cattle, horses, and dogs (Moorhouse 1972, Yeruham and Braverman 1995).

Published reports of stable flies feeding on birds are rare. Bird feeding may be hindered by limited non-feathered surface area available for bloodmeal acquisition and quick defensive movements by healthy birds that preempt or interrupt landing (Bishopp 1913, Golding 1946). Anderson and Tempelis (1970) collected blood-engorged stable flies on California poultry ranches and found 155 positive for bovine blood, two for equine and one for canine. Despite occasional endophily, none of the flies seemed to enter poultry houses and engorge on caged chickens. We observed stable flies attacking apparently healthy adult and prefledged pelicans and moribund prefledged pelicans in an environment devoid of unprotected large mammals. Healthy adult birds were quick to react with defensive behaviors (head shake, wing flap and bill snap) when stable flies attempted to land and feed. Healthy prefledged birds were not as quick to react, allowing flies to land and possibly initiate feeding Fly-repelling behaviors by the ill prefledged pelicans were weaker and ineffective, thus stable flies were able to engorge on sick and weakened animals with minimal disruption. Interestingly, we did not observe stable flies congregating on other moribund birds (e.g., double-crested cormorants or California gulls, although these birds were fewer in number [<10 per species]). Thus, stable flies were opportunistically selecting and feeding on moribund pelicans. Mitzmain (1913) reported fewer stable flies present on healthy horses compared with three weakened horses that were infected with *Trypanosoma evansi*, upon which the flies fed undisturbed on the weakened animals.

Although detection of arboviruses in various types of hematophagous arthropods is common, the capacity of these arthropods to serve as vectors requires them to be competent for transmission, an attribute determined by experimental evaluation, generally in controlled, laboratory-based studies. Biological vec-

tors require the pathogen to replicate to high levels and disseminate to a secretory pathway leading to infection of vertebrates (i.e., the salivary glands in culicine vectors). Stable flies are unlikely to function as biological vectors because higher flies rarely support efficient replication of arboviruses. Such a function could evolve under certain selective pressures. Black flies (Diptera: Simulidae) are biological vectors for vesicular stomatitis virus (*Vesiculovirus:* Rhabdoviridae) (Mead et al. 1997). Experimental infections have demonstrated replication in drosophila S2 cells for both Sindbis virus (*Alphavirus:* Togaviridae) and WNV (Mudiganti et al. 2006, Chotkowski et al. 2008.) WNV has been detected in other nonculicine arthropods in nature, including louse flies (Gancz et al. 2004, Farajollahi et al. 2005), biting midges (Naugle et al. 2004, Sabio et al. 2006), several genera of argasid and ixodid ticks (Hubalek and Halouzka 1999, Lawrie et al. 2004, Hutcheson et al. 2005, Mumcuoglu et al. 2005), and mesostigmatid mites (Mumcuoglu et al. 2005). Research studies have examined the role of some of these hematophagous arthropods in WNV epidemiology. Hutcheson et al. (2005) demonstrated experimental WNV transmission from infected to uninfected ducklings by a seabird argasid (soft) tick *Carios capensis* (Neumann) after a 35-d extrinsic incubation period. Lawrie et al. (2004) found the soft tick *Ornithodoros moubata* (Murray) capable of maintaining a WNV infection transstadially and transmitting a low level infection to a rodent host. The hard tick *Ixodes ricinus* (L.) became infected after feeding on a WNV-viremic host but was unable to maintain the infection (Lawrie et al. 2004). Any significant role of nonculicine arthropods in WNV ecology and epidemiology remains undiscovered.

In 2007, *Cx. tarsalis* seemed to be the major biological vector for WNV transmission to pelicans based on high vector and trap indices, and bloodmeal identification linking them to pelicans (Johnson et al. 2010). Although a role of biological vector is unlikely for the stable fly, a role of mechanical vector seems possible in specialized circumstances such as those occurring in the Medicine Lake pelican colony. Exposure of fly tarsi and mouthparts to open wounds contaminated with high-titered viremic blood clearly occurred at high frequency in the current study. WNV held in media or chicken feces lost 99% viability after 24 h; hence, exposure of flies to blood considered infectious to mosquitoes (at $>10^5$ pfu/ml serum) would probably render the fly a potential mechanical vector capable of contaminating an open wound of a vertebrate host (Langevin et al. 2001) for up to several days until the contaminating virions lose their viability. Turell and Knudson (1987) demonstrated that the stable fly mechanically transmits *Bacillus anthracis* with transmission rates similar at either <1 or 4 h after the initial exposure to a bacteremic animal. Mellor et al. (1987) found that the stable fly mechanically transmitted capripox virus and African swine fever virus both 1 h and 24 h after ingesting the virus. However, the stable fly was not an important mechanical vector of pathogens responsible for anaplasmosis (Scoles et al. 2005),

Potomac horse fever (Burg et al. 1994), lumpy skin disease (Chihota et al. 2003), or bovine leukosis (Weber et al. 1988). Failure of the stable fly to mechanically transmit pathogens may be due to an inadequate pathogen load, intracellular location of pathogens or loss of pathogen viability (Weber et al. 1988, Scoles et al. 2005).

Mechanical transmission of a pathogen by biting flies is enhanced when one of more of the following are noted: 1) a high percentage of interrupted feedings; 2) movement between infected and uninfected hosts; 3) feeding behavior that facilitates blood transfer from an infected to a naive animal; or 4) a high proportion of flies that carry pathogen-infected blood on their mouthparts, feet, or both. Schofield and Torr (2002) reported that 27% of stable flies landing on adult cattle fed to repletion with the majority leaving before completing feeding largely due to disturbance by the host's defensive behavior. Dislodged flies that immediately resumed feeding were reported to return to the same host reducing the amount of contact between infected and uninfected hosts (Weber et al. 1988). Stable flies have been shown to have the capacity to enclose 0.4 nl of blood in their mouthparts (Kloft 1992, Scoles et al. 2005). However, this volume of blood was inadequate for mechanical transmission of *Anaplasma marginale* (Scoles et al. 2005) and bovine leukemia virus (Weber et al. 1988). Butler et al. (1977) reported stable flies will occasionally regurgitate red blood cells and serum from a previously ingested bloodmeal, suggesting another potential mode of mechanical transmission.

Infected flies that are ingested by predators (e.g., birds or other insects) may also result in per os transmission (Komar et al. 2003). This type of transmission would be enhanced, and biological transmission would become possible, if viral replication were supported in the stable fly host. We observed one instance in fly abdomen tissue and four instances in fly nonabdomen tissue in which the viral load was greater than expected from contamination of alimentary or gut tissue or external body parts. Viral replication in the fly is one potential explanation for these observations, although contamination of these five flies from infections encountered in other birds the same day cannot be ruled out. However, if a portion of the flies derived from infectious pelicans were infected remotely, one would expect a similar rate of WNV detection in the flies collected from the other pelicans, but this was not the case.

We expected that the WNV titers in engorged stable fly abdomens collected from WNV-positive moribund pelicans would indirectly indicate the viremia levels circulating in these pelicans. We observed large variability in the viral loads of fly abdomens collected from each of two moribund pelicans that generated infected flies. The source of the variability may include bloodmeal size and stage of bloodmeal digestion in the wild-caught flies. Regardless, these moribund pelicans are clearly a potential source of WNV for *Culex* vectors because 27 of 64 blood-engorged flies collected from WNV-infected pelicans had imbibed at least 100 pfu

and eight of 64 had imbibed at least 1,000 pfu, the thresholds for infecting *Culex tarsalis* ($4.0 \log_{10}$ pfu/ml blood) and *Culex pipiens* ($5.0 \log_{10}$ pfu/ml blood) mosquitoes, respectively, assuming that the volume of blood ingested by the stable fly is 10 μl and that all of the pfu detected in these abdomens indeed derived from the recent bloodmeal (Turell et al. 2005). The high-titered bloodmeals all came from just one pelican. The failure of the other WNV-positive pelicans to produce high-titered bloodmeals is probably best explained by humoral immunity, i.e., the cocirculation of specific WNV-neutralizing antibodies. These antibodies are produced within the first week of infection. The WNV-infected pelicans probably circulated much higher viremia in the days before illness, and before the appearance of specific antibodies in the blood. These birds then would have been capable of infecting much greater numbers of *Culex* earlier in the infection process. Unfortunately, calculating the relative competence of pelicans for infecting mosquitoes with WNV requires knowledge of the viremia profile throughout the course of acute infection and thus remains unknown (Komar et al. 2003).

This study presents the novel observation that stable flies are becoming contaminated by WNV through feeding upon moribund American white pelicans. We observed many interrupted feedings on moribund pelicans and estimated a relatively high estimated infection rate in the flies, although no evidence of viral replication in the flies was observed. Detection of WNV in pools of unengorged flies is more likely explained by contamination rather than replication, because titers in these pools were consistently low. Further research is necessary to determine whether WNV replication occurs in stable flies and to evaluate their vectorial capacity.

Acknowledgments

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