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Original Contribution

Using Bloodmeal Analysis to Assess Disease Risk to Wildlife at the New Northern Limit of a Mosquito Species

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Abstract: The historically southeastern mosquito species *Culex erraticus* has over the last 30 years undergone a marked expansion north. We evaluated this species' potential to participate in local disease cycles in the northeastern USA by identifying the vertebrate sources of blood in *Cx. erraticus* specimens from New Jersey. We found that the majority of bloodmeals (92.6%) were derived from birds, followed by 6.8% from mammals (of which half were human), and a single amphibian bloodmeal from a spring peeper (0.56%). Medium- and large-sized water birds from the order Pelecaniformes made up 60.4% of the bird species and 55.9% of all identified hosts. This group of birds is known enzootic hosts of arboviruses such as eastern equine encephalitis virus, for which *Cx. erraticus* is a competent vector. Additionally, we screened blooded mosquitoes for avian malaria parasites and identified three different lineages of *Plasmodium*, including what may represent a new *Plasmodium* species (likely a wetland bird specialist) in bloodmeals from Green Herons, a Great Egret, and a Double-Crested Cormorant. Our results support the utility of mosquito bloodmeals as sources of information about circulating wildlife pathogens and reveal the potential of range-expanding species to intensify local zoonoses and bridge enzootic pathogens to humans.

Keywords: Pelecaniformes, Eastern equine encephalitis virus, Xenosurveillance, *Plasmodium*, Climate change

INTRODUCTION

Even as global climate change promotes the expansion of endemic and invasive species into northern latitudes (Rochlin et al. 2013) relatively little empirical evidence has been gathered about the potential impact of southern vector species on the epidemiology of zoonotic disease cycles in the north (Mills et al. 2010, but see Purse et al. 2005). *Culex erraticus* (Dyar & Knab) is one of the more widespread members of the primarily tropical subgenus *Melanoconion*, with records in the USA, the Caribbean, Central America, and South America, as far south as Paraguay (Pecor et al. 1992; Mendenhall et al. 2012). In the USA, *Cx. erraticus* has been historically common and often highly abundant in southeastern states (Cupp et al. 2003; Hassan et al. 2003; Oliveira et al. 2011). In recent years, however, it has been

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detected as far north as New Jersey and New York (Kulasekera et al. 2001; Farajollahi and Crans 2012) as well as the Canadian province of Ontario (Hunter et al. 2015). In New Jersey, *Cx. erraticus* was first collected in Cape May County in the southernmost part of the state in 1969 (Crans 1970). It remained relatively localized and collections sporadic until 1986, when it became commonly detected in several nearby counties, still in the southern part of the state (Crans and McCuiston 1987). By 2011, it was present in all 21 NJ counties and at higher densities than previously reported (Farajollahi and Crans 2012). Larval habitat appears to be the shallow edges of ponds and wetlands with emergent vegetation or dense root mats (Carpenter and LaCasse 1955; McNelly and Crans 1989).

This expansion has been met with alarm, as *Cx. erraticus* has been implicated in the transmission of several human and animal pathogens (Hunter et al. 2015) including the highly pathogenic eastern equine encephalitis virus (EEEV, Chamberlain et al. 1954; Cupp et al. 2003; Bingham et al. 2016). EEEV primarily circulates between wild birds and bird-biting mosquitoes and does not usually cause illness in the infected birds. However, in infected mammals (such as horses or humans), there is a high likelihood of death or permanent neurological damage in survivors (Scott and Weaver 1989). A mosquito that bites both birds and mammals allowing an arbovirus circulating in the former to then infect the latter is known as a bridge vector (Armstrong and Andreadis 2010) and is often key to epidemics (Farajollahi et al. 2011). In the southeastern USA, *Cx. erraticus* is known to be a generalist in its feeding patterns, acquiring bloodmeals from birds, mammals, and reptiles (Table 1 and associated references). Based on its broad host usage, high abundance at EEE foci, and frequent detection of EEEV-infected specimens, researchers hypothesize that *Cx. erraticus* acts as a bridge vector of EEEV by becoming infected when feeding on amplifying hosts (birds) and then transmitting the virus to human and horse populations, particularly in areas where land use changes have brought humans in close proximity to wetlands (Cupp et al. 2003; Cohen et al. 2009). This scenario is underscored by a recent study showing that *Cx. erraticus* is a highly competent vector of EEEV (Bingham et al. 2016). By contrast, in the northeastern USA, relatively little is known about the role of *Cx. erraticus* in the epidemiology of EEEV. In fact, while *Culiseta melanura* (Coquillett) is accepted as the primary enzootic vector, bridge vectors appear to vary across regions and habitat contexts (Scott and Weaver 1989; Armstrong and Andreadis 2010). Early work conducted in NJ identified *Aedes sollicitans* (Walker) as the bridge vector in coastal areas (Crans et al. 1986), while this role was filled by *Coquillettidia perturbans* (Walker) further inland (Crans and Schulze 1986). However, due to the expansion of *Cx. erraticus* in subsequent years, this epidemiological scenario may have changed. Surveillance testing for pathogens of medical and veterinary importance has detected pools of *Cx. erraticus* in NJ occasionally infected with EEEV first in southern counties (Cape May, Salem) but recently also in central counties (Monmouth, Ocean, http://vectorbio.rutgers.edu/reports/vector/, accession date Dec 11, 2017). In years with positive test results, EEEV minimum field infection rates (MFIR) have varied between 0.217 and 1.84 infected mosquitoes per 1000. In addition to EEEV, *Cx. erraticus* pools in NJ have also tested positive for West Nile virus (WNV), albeit only rarely (three pools in 2002, then no activity until 1–2 pools each year between 2013 and 2016, http://vectorbio.rutgers.edu/reports/vector/, accession date 11 Dec 2017).

Analyzing bloodmeals from *Cx. erraticus* within NJ can provide information on its potential role in disease transmission cycles by revealing which hosts it may acquire viruses from or, conversely, infect with viruses. Furthermore, assaying the host blood present in the mosquito for wildlife pathogens can provide information on the health of wildlife populations (Leighton et al. 2008). Several recent studies have taken advantage of the host-seeking abilities of hematophagous insects for conservation purposes, such as assaying biodiversity in remote areas (Schnell et al. 2012; Calvignac-Spencer et al. 2013; Rovie-Ryan et al. 2013). Many groups of wildlife needing disease surveillance and study are threatened or endangered, necessitating the use of noninvasive sampling methods (Waits and Paetkau 2005). Collection of blood-engorged mosquitoes is often easier than capturing and bleeding hosts and does not stress the animal beyond the mosquito bites they would experience naturally. Indeed, a growing number of studies have demonstrated the utility and validity of using mosquitoes to screen for and even discover human and animal pathogens (Leighton et al. 2008; Barbazan et al. 2009; Ng et al. 2011; Mehus and Vaughan 2013; Grubaugh et al. 2015; Martinsen et al. 2016).

While parasites that circulate between birds and hematophagous dipterans primarily manifest as low-level chronic infections in coevolved species (Valkiunas 2005), they can cause acute disease and high mortality when introduced to naïve birds such as captive species or endemic avifauna encountering exotic mosquitoes (Lapointe

Table 1. Comparison of *Culex erraticus* Vertebrate Blood Hosts Identified by Genetic Analysis in Several Southeastern US States (Prior Studies) with Results from the Current Study.

Location	Florida	Alabama	Tennessee	North Carolina Zoos	North Carolina	New Jersey
Year(s)	2011–2013	2001–2004; 2006–2008	2006	2009–2011	1985–1986	2002; 2005–2007
Avian	305 (61.0)	414 (35.6)	13 (15.9)	14 (58.3)	51 (30.2)	164 (92.6)
Accipitriformes	1 (0.33)					
Anseriformes	2 (0.66)	16 (3.9)				1 (0.61)
Caprimulgiformes		1 (0.24)				
Charadriiformes	1 (0.33)					
Ciconiiformes	28 (9.2)					
Columbiformes				3 (21.4)	39 (76.5)	
Cuculiformes		4 (0.97)	1 (7.7)			
Galliformes		5 (1.2)			1 (2.0)	22 (13.4)
Gaviiformes						7 (4.3)
Gruiformes	6 (2.0)			1 (7.1)		5 (3.0)
Passeriformes	6 (2.0)	100 (24.2)	9 (69.2)	6 (42.9)	10 (19.6)	
Pelecaniformes	217 (71.1)	258 (62.3)	1 (7.7)			99 (60.4)
Phoenicopteriformes				2 (14.3)		
Piciformes				1 (7.1)		
Podicipediformes	2 (0.66)	7 (1.7)				
Strigiformes	1 (0.33)	21 (5.1)	2 (15.4)		1 (2.0)	
Struthioniformes				1 (7.1)		
Suliformes	41 (13.4)	2 (0.48)				30 (18.3)
Mammalian	175 (35.0)	696 (60.2)	63 (76.8)	7 (29.2)	80 (47.3)	12 (6.8)
Human	51 (29.1)	31 (4.5)		3 (42.9)	6 (7.5)	7 (58.3)
Nonhuman	124 (70.9)	665 (95.5)	63 (100)	4 (57.1)	74 (92.5)	5 (41.7)
Reptilian	16 (3.2)	43 (3.7)	6 (7.3)	3 (12.5)	34 (20.1)	
Amphibian	4 (0.80)	3 (0.26)			4 (2.4)	1 (0.56)
Total	500	1156	82	24	169	177
References	Bingham et al. (2014)	Hassan et al. (2003, Cupp et al. (2004) and Burkett-Cadena et al. (2011)	Cohen et al. (2009)	Tuten (2011)	Robertson et al. (1993)	This study

Avian meals are broken down by Order. Numbers in parentheses represent percentage of total in the case of broad groups (e.g., percentage of all bloodmeals derived from birds) or percentage of group in the case of avian orders (e.g., percentage of avian bloodmeals derived from Passeriformes). For all studies, results are only reported for samples where a host species was successfully identified.

et al. 2012). Unlike arboviruses, infection rates with *Plasmodium* can be high both in the vectors and the hosts (Ferraguti et al. 2013).

To examine the potential of *Cx. erraticus* to participate in enzootic disease cycles at the northern front of its expansion, we (1) identified their primary blood hosts, determining first if they feed on bird species that are known reservoirs for arboviruses, and secondly, if they could act as a bridge vector by occasionally feeding on mammals; additionally, we (2) screened blooded mosquitoes for lineages of *Plasmodium* parasites that could be circulating in wildlife host populations.

METHODS

Mosquito Collections

The majority of blooded *Cx. erraticus* were obtained from resting box collections from May through October across 4 years (2002, 2005, 2006, and 2007) and in four NJ counties. These counties include Mercer (Latitude 40.195253, Longitude − 74.733534; area 593 km^2; human population density 627/km^2), Atlantic (39.301603, − 74.739062; 1740 km^2; 191/km^2), Cape May (39.182751, − 74.851765; 1607 km^2; 147.3/km^2), and Salem (39.515443, − 75.140829; 964 km^2; 75.5/km^2). Resting boxes are small wooden frames open on one side that take advantage of a female mosquitoes' desire to find a dark, shady area to digest a bloodmeal (Crans 1989). To obtain a representative sample, Mercer County collections were obtained from red maple, *Acer rubrum*, freshwater swamps abutting suburban neighborhoods while collection sites in Atlantic and Cape May were situated in rural habitats within dense pine plantations in the vicinity of permanent freshwater swamps containing primarily white cedars, *Chamaecyparis thyoides*. Salem County collections were conducted from a sparsely populated area bordered by salt marshes and dredge spoils.

Resting boxes were placed in shaded areas at the beginning of the mosquito season each year (which is officially June 1 across all twenty-one NJ county mosquito control programs) and sampled weekly during the morning hours. Mosquito specimens were collected using a handheld aspirator and kept on dry ice until they could be sorted and identified using standard keys (Darsie and Ward 2005). *Culex erraticus* specimens with a visible bloodmeal were set aside and preserved at − 80°C for later genetic analysis. A small number of additional blooded specimens caught in other vector surveillance traps (gravid, NJ light, CO_2-baited CDC) as part of routine disease surveillance in the abovementioned counties were also included in the analyses. This approach, using blooded specimens identified during routine surveillance or unrelated research and set aside by members of the extended NJ mosquito control community, has been used very successfully to study mosquito host feeding in the past (Apperson et al. 2004; Molaei et al. 2009; Egizi et al. 2014; Faraji et al. 2014).

Mosquito Bloodmeal Identification

Dissections were first performed on the specimens to separate the blooded abdomen from the head and thorax. DNA was isolated from the abdomens using DNeasy Blood and Tissue 96 Well Plate Kits (Qiagen, Valencia, CA) following the manufacturer's specifications. The blood host was identified using PCR protocols for cytochrome b that are known to work well on *Culex* species, as previously described (Egizi et al. 2014; Faraji et al. 2014). This approach involves amplification with a combination of primers that target mammals, birds, reptiles, and amphibians (Kocher et al. 1989; Cicero and Johnson 2001; Cupp et al. 2004). PCR products were purified (ExoSAP-IT, Affymetrix, Santa Clara, CA) then sequenced, and sequences sharing 98% or higher identity with a vertebrate host sequence in GenBank were recorded (Kent 2009). Low-quality sequences, which may have included mixed bloodmeals that would require cloning to obtain clean sequences (e.g., Egizi et al. 2014), were removed from further analysis.

Detection of Avian Blood Parasites

Samples with an avian blood host successfully identified were next screened for avian blood parasites using the primers and nested PCR protocol from Waldenström et al. (2004). Successful amplification of a PCR product as detected by gel electrophoresis indicated the presence of blood parasites. PCR products were subsequently cleaned, sequenced, and aligned by eye using Sequencher version v5.4.6. By BLAST analysis, each sequence was then compared to all other published *Plasmodium* sequences from the MalAvi (http://mbio-serv2.mbioekol.lu.se/Malavi/, Bensch et al. 2009) and Genbank databases (accession date November 10, 2017). To better understand the evolutionary relationships of *Plasmodium* lineages from the current study with prior published lineages we conducted phylogenetic analyses with other lineages from the MalAvi database. These included lineages identified to morphospecies, closely related lineages, and lineages isolated from birds in the same genus as the birds fed upon by infected mosquitoes in our study. Taxa from closely related genera to *Plasmodium* (*Haemoproteus* and *Leucocytozoon*) were included as outgroup taxa and a total of 479 base pairs were aligned by eye for all taxa. Phylogenies were estimated by maximum likelihood methods using GARLI 2.01 (Zwickl 2006) and Bayesian inference using MrBayes 3.2 (Ronquist and Huelsenbeck 2003). For all analyses, a GTR + I + G model was selected with estimated parameters. For the Bayesian analysis, ten million generations (two independent runs, four chains) were run with sampling every 100 generations and the first 25% of trees discarded as burn-in. Nodal support was estimated by bootstrap analysis (1000 replicates) and Bayesian posterior probability values.

RESULTS

Mosquito Collections and Bloodmeal ID

Out of 337 blooded specimens of *Cx. erraticus*, 177 produced a clean DNA sequence and were confirmed to match a host species with $\geq$ 98% identity. The majority of identified hosts were avian-derived (92.6%), with a small number of mammals (6.8%), of which about half were derived from humans (58.3% of mammalian meals; 3.9% of all meals). Only a single amphibian (0.56%) bloodmeal was detected (spring peeper, *Pseudacris crucifer*, Table 2). The birds were almost exclusively water-associated species: e.g., Pelecaniformes made up 60.4% of the birds and more than half of all hosts (55.9%, Tables 1, 2).

Nearly 80% of the samples for which a vertebrate host was successfully identified were collected from resting boxes, followed by 15.3% from CO_2-baited light traps (CDC), 1.1% from gravid traps, and 0.56% from NJ light traps. Mosquitoes that fed on Pelecaniformes were found in all trap types except the CO_2 baited CDC traps.

Spatial and Temporal Comparisons

Blood-feeding patterns were similar between counties with coastal (saltwater) and inland (freshwater) marshes: both were dominated by water birds (Table 2A). Surprisingly, the county with the highest human population density (Mercer) lacked human-derived bloodmeals, and the southern counties lacked bloodmeals from nonhuman mammals (Table 2B). Samples were also pooled across counties and across years to decipher seasonal differences (Fig. 1). Broadly, patterns appeared relatively consistent across months, with the exception that mammalian bloodmeals were highest in August. However, this could be a sampling artifact as August is when the *Cx. erraticus*

Table 2. Blood Hosts Identified from *Cx. erraticus* Specimens Collected in Four NJ Counties: (A) Avian Hosts, (B) Mammalian Hosts, (C) Reptile/Amphibian Hosts.

Species	Mercer	Atlantic	Cape May	Salem	All
(A)					
Great Egret (*Ardea alba*)	16	1	42	1	60
Green Heron (*Butorides virescens*)	0	0	34	1	35
Double-Crested Cormorant (*Phalacrocorax auritus*)	2	2	19	7	30
Chicken (*Gallus gallus domesticus*)	2	0	20	0	22
Common loon (*Gavia immer*)	0	0	7	0	7
Common moorhen (*Gallinula chloropus*)	0	1	4	0	5
Great blue heron (*Ardea herodias*)	0	0	1	1	2
Black-crowned night heron (*Nycticorax nycticorax*)	0	0	1	0	1
Canada goose (*Branta canadensis*)	0	0	1	0	1
Snowy egret (*Egretta thula*)	0	0	1	0	1
Total Avian	20	4	130	10	164
(B)					
Human (*Homo sapiens*)	0	0	6	1	7
White-tailed deer (*Odocoileus virginianus*)	4	0	0	0	4
North American river otter (*Lontra canadensis*)	1	0	0	0	1
Total Mammalian	5	0	6	1	12
(C)					
Spring peeper (*Pseudacris crucifer*)	1	0	0	0	1
Total Amphibian	1	0	0	0	1
Total for dataset	26	4	136	11	177

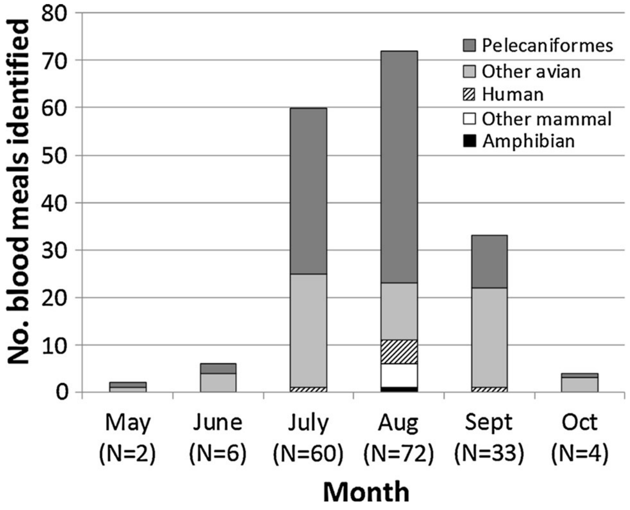


Figure 1. Number of vertebrate hosts in each group identified from *Cx. erraticus* bloodmeals by month (each month is summed across counties and years). N = number of specimens collected in that month that produced a blood host result. Shades of gray = avian bloodmeals, stippled = human, white = other mammalian bloodmeals, and black = amphibian bloodmeal.

population commonly peaks in NJ (McNelly and Crans 1989).

Blood Parasite Infections

Although 13 specimens out of 177 tested positive for *Plasmodium* parasites (7.34%), several of these were collected on the same day in the same location (with the same host ID), so conservatively (i.e., minimally) seven different birds were infected (Table 3). The majority (6/7) of infected birds were sampled in Cape May County, with a single infected Great Egret in Mercer County (Table 3). Three different *Plasmodium* parasite lineages were recovered (Fig. 2). A single infection from a Great Egret (E14T) matched 100% to the *Plasmodium* lineage GRW6 (morphospecies *P. elongatum*), a lineage previously documented from a diversity of birds (numerous orders) worldwide including a Great Blue Heron (*Ardea herodias*) from the USA (Beadell et al. 2006). Another single infection (D02S2) from a Green Heron (*Butorides virescens*) matched 100% to the *Plasmodium* lineage TUMIG03, a lineage isolated from wild songbirds (order Passeriformes) in North and South America.

The majority of *Plasmodium* sequences recovered from bloodfed *Cx. erraticus* including those from Green Herons, a Great Egret (*Ardea alba*), and a Double-Crested Cormorant (*Phalacrocorax auritus*) (Table 3) represented a novel lineage that did not match closely any other *Plasmodium* lineages in the MalAvi and GenBank databases (Fig. 2). Its closest relatives (98% genetic similarity or 2% genetic divergence) were three other *Plasmodium* lineages: lineage SPMAG10 isolated from a captive penguin (*Spheniscus magellanicus*) in Brazil and from a Common Loon (*Gavia immer*) in New York, lineage IXOMIN03 isolated from a Little Bittern (*Ixobrychus minutus*) in Turkey, and lineage CXINA01 isolated from *Culex inatomii* mosquitoes in Japan (Kim and Tsuda 2015, Vanstreels et al. 2015). Querying the MalAvi database for *Plasmodium* parasites shown to infect bird species in the same genus as the birds *Cx. erraticus* fed on revealed three lineages: (1) lineage BUTSTR01 from Striated Herons (*Butorides striata*) in Guyana, (2) lineage ARDALB01 from Great Egrets (*Ardea alba*) in Uruguay, and (3) lineage ORW1/PHACAR01/SW2 isolated from Great Cormorants (*Phalacrocorax carbo*) in Mongolia (Durrant et al. 2006, Seimon et al. 2016).

Phylogenetic analyses placed the three *Plasmodium* lineages isolated from *Cx. erraticus* within different clades (Fig. 2). The other lineages isolated from congeneric host species, i.e., other water birds, were non-identical and also scattered throughout the phylogeny underscoring the fact that a diversity of unrelated *Plasmodium* parasites infect water birds. Of note, the novel lineage detected in this study and closely related lineages from the MalAvi database (SPMAG10, IXOMIN03, and CXINA01) formed their own distinct clade within the phylogeny (Fig. 2).

Discussion

We found distinct patterns of blood-feeding behavior in *Cx. erraticus* from NJ, a state in the US northeast where based on state-wide records this species has only recently become widespread and abundant. Prior studies from its historical range in the southeast USA identified a much larger proportion of mammalian feedings ranging from 29.2 to 76.8% as opposed to the 6.8% we observed, and between 3.2 and 20.1% feeding on reptiles, of which we observed none (Table 2). New Jersey is home to 71 species of reptiles and amphibians including snakes, turtles, salamanders, frogs, and toads (Schwartz and Golden 2002) and supports populations of *Culex territans*, an amphibian/reptile specialist (Burkett-Cadena et al. 2008). Not detecting reptile bloodmeal in *Cx. erraticus* in NJ is therefore unlikely to be due to a lack of amphibians and reptiles to

Table 3. *Cx. erraticus* Samples That Tested Positive for Avian Blood Parasites.

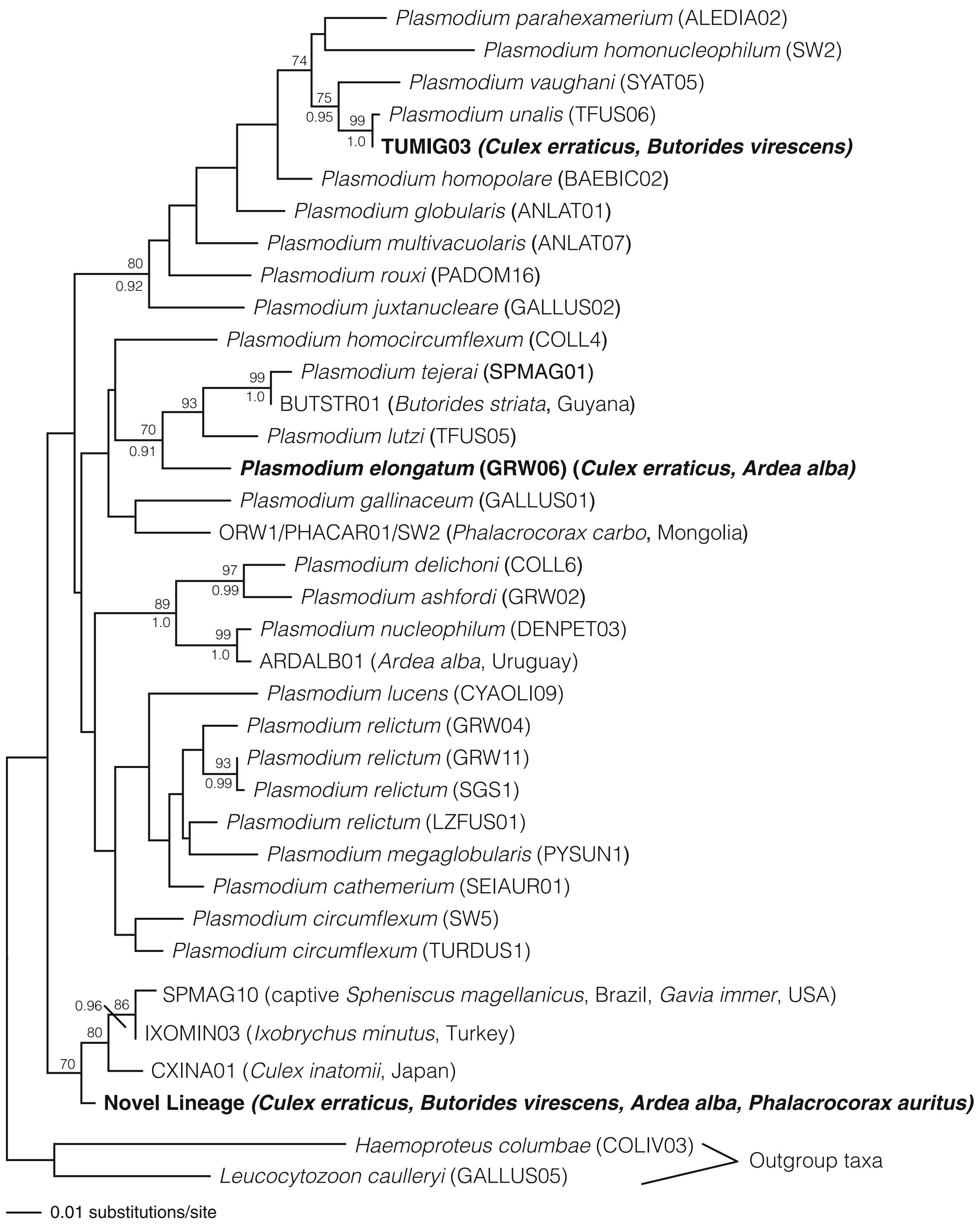
Specimen#	Blood host ID	County	Day	Month	Year	Individual
E-14 T	Great Egret	Cape May	24	July	2006	1
D02S2	Great Egret	Cape May	8	August	2007	2
E02S2	Great Egret	Cape May	8	August	2007	2
E-005 B	**Green Heron**	**Cape May**	**27**	**July**	**2007**	**3**
E-005 C	**Green Heron**	**Cape May**	**27**	**July**	**2007**	**3**
E-007 A	**Green Heron**	**Cape May**	**27**	**July**	**2007**	**3**
E-008 J	**Green Heron**	**Cape May**	**31**	**July**	**2007**	**4**
E-008 L	**Green Heron**	**Cape May**	**31**	**July**	**2007**	**4**
E-008 N	**Green Heron**	**Cape May**	**31**	**July**	**2007**	**4**
E-009 B	**Green Heron**	**Cape May**	**31**	**July**	**2007**	**4**
G06S1	**Great Egret**	**Mercer**	**24**	**August**	**2007**	**5**
E-007 G	Chicken	Cape May	27	July	2007	6
G01S2	**Double-Crested Cormorant**	**Cape May**	**31**	**July**	**2007**	**7**

Bold samples indicate ones with the ‘new’ *Plasmodium* species. Specimens collected in the same location on the same day are given the same number in the ‘Individual’ column, as they likely all fed on the same infected bird.

bite during the very warm summer months. Of further note, although the overall proportion of mammalian meals identified by our study was lower than in previous work, the proportion of human-derived bloodmeals was actually higher (58.3% of mammalian meals, Table 1). Studies in the southeast USA typically found the largest proportion of mammalian meals derived from white-tailed deer (41.4–97.5%, Robertson et al. 1993, Cupp et al. 2004, Bingham et al. 2014), and some found no human-derived bloodmeals at all (Cohen et al. 2009). Interestingly, the counties where we observed human bloodmeals are some of the least densely populated counties in the state (e.g., Salem county, 190 persons/km^2) as opposed to Mercer county where we observed no human bloodmeals (1664 persons/km^2; http://worldpopulationreview.com/states/new-jersey-population/accessed 1 June 2018).

The specific avian hosts associated with *Cx. erraticus* in NJ also differed somewhat from studies in other regions of the USA. Although wading birds and other waterfowl are important blood hosts (Hassan et al. 2003; Estep et al. 2011; Oliveira et al. 2011; Bingham et al. 2014) most prior studies also found that passerine birds make up a significant portion of avian meals in *Cx. erraticus* (as much as 69%, Table 1). In some studies, *Cx. erraticus* feeding on passerines surpassed estimates of the bird species’ field abundance indicating preferential feeding (Hassan et al. 2003; Estep et al. 2011). Unfortunately, we lack estimates of host abundance at our specific collection sites, and therefore cannot distinguish opportunistic vs. preferential feeding (Mendenhall et al. 2012). However, we do have data from other mosquito species collected in the same resting boxes at the same site, and these reveal a wide array of blood host patterns (Apperson et al. 2004; Molaei et al. 2009). For example, the ornithophilic mosquitoes *Culiseta melanura* and *Culex pipiens* fed predominantly on songbirds, rather than wading birds as we saw with *Cx. erraticus* (Apperson et al. 2004), while *Anopheles quadrimaculatus* and *An. punctipennis* fed almost exclusively on mammals, including humans (Molaei et al. 2009).

While passerines are particularly competent enzootic hosts for arboviruses like WNV and EEEV (Scott and Weaver 1989; Komar et al. 1999, 2003; Kilpatrick et al. 2006), wading birds (Ciconiiformes and Pelecaniformes) have also tested positive for EEEV in the southeastern USA, including several species identified in our study such as Great Egrets, Green Herons, Great Blue Herons, Snowy Egrets (*Egretta thula*), and Black-Crowned Night Herons (*Nycticorax nycticorax*) (Stamm 1958; Spalding et al. 1994). In one Louisiana site, over 80% of Yellow-Crowned Night Herons (*Nyctanassa violacea*) were seropositive for EEEV (Stamm 1958). Additionally, laboratory experiments revealed that Snowy Egrets and Glossy Ibises (*Plegadis faclinellus*) captured in southern NJ and experimentally infected with EEEV, maintained viremias high enough to infect mosquitoes (McLean et al. 1995). As a result, wading birds can be important hosts in the dynamics of EEEV


Plasmodium parahexamerium (ALEDIA02)
Plasmodium homonucleophilum (SW2)
74
Plasmodium vaughani (SYAT05)
75
0.95
99
Plasmodium unalis (TFUS06)
1.0
TUMIG03 (Culex erraticus, Butorides virescens)
Plasmodium homopolare (BAEBIC02)
Plasmodium globularis (ANLAT01)
Plasmodium multivacuolaris (ANLAT07)
80
0.92
Plasmodium rouxi (PADOM16)
Plasmodium juxtanucleare (GALLUS02)
Plasmodium homocircumflexum (COLL4)
99
Plasmodium tejerai (SPMAG01)
1.0
BUTSTR01 (Butorides striata, Guyana)
93
70
0.91
Plasmodium lutzi (TFUS05)
Plasmodium elongatum (GRW06) (Culex erraticus, Ardea alba)
Plasmodium gallinaceum (GALLUS01)
ORW1/PHACAR01/SW2 (Phalacrocorax carbo, Mongolia)
97
0.99
Plasmodium delichoni (COLL6)
Plasmodium ashfordi (GRW02)
89
1.0
99
1.0
Plasmodium nucleophilum (DENPET03)
ARDALB01 (Ardea alba, Uruguay)
Plasmodium lucens (CYAOLI09)
Plasmodium relictum (GRW04)
93
0.99
Plasmodium relictum (GRW11)
Plasmodium relictum (SGS1)
Plasmodium relictum (LZFUS01)
Plasmodium megaglobularis (PYSUN1)
Plasmodium cathemerium (SEIAUR01)
Plasmodium circumflexum (SW5)
Plasmodium circumflexum (TURDUS1)
0.96
86
SPMAG10 (captive Spheniscus magellanicus, Brazil, Gavia immer, USA)
80
IXOMIN03 (Ixobrychus minutus, Turkey)
70
CXINA01 (Culex inatomii, Japan)
Novel Lineage (Culex erraticus, Butorides virescens, Ardea alba, Phalacrocorax auritus)
Haemoproteus columbae (COLIV03)
Leucocytozoon caulleryi (GALLUS05)
Outgroup taxa
0.01 substitutions/site

◀**Figure 2.** Phylogeny of *Plasmodium* lineages sequenced from bloodfed *Cx. erraticus* (shown in bold with bloodmeal host indicated in parentheses), lineages previously identified to morphospecies, lineages isolated from congeneric bird species as those identified in the mosquito bloodmeals, and lineages closely related to the novel lineage found in this study. Phylogeny estimated by both maximum likelihood and Bayesian methods. Maximum likelihood bootstrap values greater than 70% and Bayesian posterior probability values greater than 0.90 are shown (bootstrap values above nodes and posterior probability values below nodes).

within wetland habitats if adequate vectors are present (Bingham et al. 2014). This possibility should be more closely examined, given that their feeding behavior (i.e., sit and wait predators) may make them highly vulnerable to bites (Edman and Kale 1971; Edman et al. 1984) such that a single viremic bird may infect a large number of mosquitoes.

We observed the greatest number (proportion) of mammalian meals in August (Fig. 1), the month with the largest number of samples. Fittingly, this is when populations of *Cx. erraticus* are at their peak in NJ (McNelly and Crans 1989) which is later than endemic mosquito species, possibly due to the toll exerted by winter temperatures on this primarily southern species. In the southeast USA where *Cx. erraticus* is a year-round species, studies have documented seasonal changes in their feeding behavior (Bingham et al. 2014; Oliveira et al. 2011; Burkett-Cadena et al. 2011, 2012). For example, in Alabama, *Cx. erraticus* shifted from feeding primarily on birds in May to primarily mammals in August (Oliveira et al. 2011; Burkett-Cadena et al. 2012), although the exact dates of this crossover appeared to vary as a function of the previous winter's chill accumulation. Also, in Alabama Burkett-Cadena et al. (2011) found that *Cx. erraticus* showed preference for host species, mammals or birds, during their respective breeding seasons. By contrast, in NJ, peak abundance of *Cx. erraticus* does not overlap with the bird breeding season in early spring, nor with the deer rutting season, in the late fall. Species endemic to NJ such as *Cx. restuans* are, however, able to target local birds during their peak nesting season (Egizi et al. 2014). Therefore, vectors who have expanded into new climate ranges may have unexpected host associations which could alter their participation in pathogen transmission cycles.

The detection of *Plasmodium* infections in several *Cx. erraticus* bloodmeals indicates this mosquito may also be involved in multiple vector-borne disease cycles. Indeed, *Culex* species are known to vector several *Plasmodium* species worldwide (Valkiunas 2005; Carlson et al. 2015). We identified three *Plasmodium* lineages from blooded *Cx. erraticus* including a cosmopolitan generalist (GRW06), a New World lineage that prefers thrush species (TUMI03), and critically, a novel lineage not previously identified that likely represents a new species. The fact that all closest relatives of this novel lineage were all isolated from water birds suggests it may be a wetland bird specialist, a type of parasite difficult to sample due to the reclusiveness of their hosts (Kim and Tsuda 2015; Vanstreels et al. 2015). In fact, many of these birds are notoriously difficult to observe in the wild, Bingham et al. (2014) remarked that they seldom saw wading birds in their bird counts despite their presence in *Cx. erraticus* bloodmeals. Further, several water birds are of conservation concern in NJ (NJDEP 2012) highlighting the need to explore the health threats posed by these blood parasites. Unfortunately, based on the possibility that oocysts in the mosquito midgut (dissected with the blood) might have contaminated the bloodmeal during DNA extraction, we are not sure if the infection was limited to the mosquito, the host blood, or both, restricting our conclusions about the role of *Cx. erraticus* in the transmission of these *Plasmodium* lineages. Future studies examining this system in more detail should carefully dissect out the bloodmeal from the midgut as well as the salivary glands of fresh specimens to disentangle parasite-host and parasite-vector associations (Kim et al. 2009; Mehus and Vaughan 2013).

In conclusion, our examination of host feeding patterns in the mosquito *Cx. erraticus* across coastal and inland locations in the northern part of its new range revealed their willingness to feed on wild wetland birds. In contrast to exotic vectors that specialize in anthropogenic landscapes (Lounibos 2002), native invaders (sensu Simberloff et al. 2011) such as *Cx. erraticus* can reach high densities in less developed habitats that put local wildlife at risk. Due to a paucity of information on the distribution and prevalence of wildlife diseases (Rhyan and Spraker 2010), the gradual effects of expanding vectors are hard to demonstrate, unlike sudden introductions of new disease agents (LaDeau et al. 2007). Of note, a Common Loon, *Gavia immer,* was recently found dead in New Hampshire with clinical signs consistent with malaria and a mixed infection of two *Plasmodium* strains (Martinsen et al. 2017). The loon's death made headlines because prior surveys had reported the absence of *Plasmodia* in loons, as well as in Green

Herons and Double-Crested Cormorants (Haefele et al. 2005; Valkiunas 2005). Our identification of multiple bloodmeals from these species in *Cx. erraticus* supports the idea that changing vector distributions could impact wildlife health. In an era of ongoing climate and landscape change, bloodfed mosquitoes should be considered valuable sentinels of ecosystem health.

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