Phenology of *Coquillettidia perturbans* and *Culiseta melanura* (Diptera: Culicidae) in East-Central Georgia, USA: Implications for the Ecology of Eastern Equine Encephalitis Virus[1]

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Abstract We sampled mosquito larvae and adult females in east-central Georgia (Screven County), where two species of mosquitoes, *Coquillettidia perturbans* (Walker) and *Culiseta melanura* (Coquillett), believed to be important in the epidemiology of eastern equine encephalitis virus (EEEv) were common. The study site, being a wildlife management area, supported large numbers of birds and snakes that are believed to harbor EEEv, and EEEv had been historically reported from wild birds from Screven County. Thus, the location was conducive to studying aspects of the sylvatic cycle of EEEv. Adult traps (CO_2 baited) indicated that *Cs. melanura* adult females were only common in midsummer. In contrast, *Cq. perturbans* adult females were common for almost the entire summer, and this combined with larval sampling in local wetlands suggested that *Cq. perturbans* was bivoltine in east-central Georgia, which is much farther north than previously suspected. We did not detect EEEv in any mosquito samples, but the phenology of *Cq. perturbans* suggests that only the second generation of these mosquitoes would play an important role as bridge vectors of EEEv to humans and horses in eastern Georgia.

Key Words Carolina bays, *Culex*, epidemiology, voltinism, wetlands

Eastern equine encephalitis (encephalomyelitis) (EEE) is a highly virulent, although rare, mosquito-borne viral disease endemic to the eastern United States (Morris 1988). Limited information is available about the ecology of the disease in the natural environment (Morris 1988). The definitive hosts of EEE virus (EEEv) are birds, and the mosquito *Culiseta melanura* (Coquillett) cycles the virus between birds; however, *Cs. melanura* rarely bites humans or horses (which are dead-end hosts) and thus this mosquito poses little direct threat to people or horses. In inland freshwater areas, *Coquillettidia perturbans* (Walker) is widely believed to be the primary bridge vector for EEEv (Cupp et al. 2003), likely causing most infections in humans and horses. However, the dynamics of how EEEv cycles between *Cs. melanura* and *Cq. perturbans* is poorly understood. Further, Cupp et al. (2003) suspect that *Culex* mosquitoes may play some role in the ecology of EEE, and

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snakes (especially water moccasins, *Agkistrodon piscivorax* [Lacépéde], and copperheads, *Agkistrodon contortix* [L.]) are a suspected overwintering host for EEEv (Bingham et al. 2012), adding more twists to the disease's epidemiology.

In east-central Georgia (Screven County), we discovered a set of natural wetlands where large populations of *Cs. melanura* and *Cq. perturbans* larvae coexisted (as well as *Culex* spp.), and because the area was managed for wildlife, a diversity of potential reservoir birds and snakes (including water moccasins) were also abundant. EEEv has previously been isolated from wild birds of Screven County (Georgia Department of Health Services, unpubl. data). Thus, this location was conducive to examining the phenology of the two major mosquito vectors of EEEv, to provide a better understanding of the ecology of EEE in the natural environment (i.e., sylvatic cycle). Additionally, because we had identified specific breeding sites for *Cq. perturbans*, a mosquito notoriously difficult to study, the setting permitted us to focus attention on how the ecology of this species might influence EEEv cycling, a topic largely unaddressed.

Methods and Materials

Seasonal phenology. Tuckahoe Wildlife Management Area (WMA), Screven County, in east-central Georgia supports a large number of forested Carolina bay wetlands (small, acidic, depression wetlands; see Taylor et al. 1999) in and around its Dixon and Oak Orchard subunits. Previous samplings of aquatic invertebrates in these Carolina bays (Batzer and Murray 2018) established that numerous mosquito species used the wetlands as breeding sites, with *Cs. melanura* and *Cq. perturbans* both being abundant. We assessed relative abundances of mosquito larvae in March, July, and November from 2015 to 2017 in each of 10 Tuckahoe WMA Carolina bays using dip-net sampling (30-cm-diameter opening, 500-μm mesh netting). Four 1-m-long sweeps with the net were collected per sample, partitioned to sample from stands of aquatic emergent vegetation and their root masses (*Carex* and *Panicum*), along shallow wetland edges, and next to woody debris and tree trunks, as available. The net was used rather than devices (dippers) specifically designed to target mosquito larvae, including *Cq. perturbans* (see Batzer 1993), because this effort was part of a larger study of mosquito community ecology (Batzer and Murray 2018); that study found that the net performed similarly to a dipper. Changes in larval abundances for most mosquitoes among seasons were obvious (i.e., common versus rare), except for *Cq. perturbans*, which tended to maintain consistent populations levels. To ascertain if this persistence resulted from univoltinism or multivoltinism, *Cq. perturbans* larvae were classified to instars based on head capsule widths, and instar compositions across seasons were contrasted using a contingency test ($P < 0.05$).

Populations of adult mosquitoes were monitored in the Dixon and Oak Orchard WMA subunits from 1 April to 1 September 2017, using dry-iced-baited Centers for Disease Control and Prevention–type light traps. We set five or six traps monthly (on or near the first day of each month) spread across each of the two sample areas, adjacent to numerous wetlands known to be *Cs. melanura* breeding sites, and two wetlands known to be *Cq. perturbans* breeding sites (one in each WMA subunit). Combined, 10–12 total traps were used per date. Traps were set

approximately 2 h before sunset, and retrieved the next day approximately 2 h after sunrise. All adult mosquitoes collected were immediately killed on dry ice, and returned to the laboratory for processing (stored frozen). As for larvae, seasonal patterns (or the lack thereof) for adult mosquitoes were visually obvious using graphical examination; individuals of most species were only common in a few months, except for *Cq. perturbans*. Because virtually all *Cq. perturbans* captured at each WMA subunit were likely derived from single breeding sites, we viewed the multiple traps set per date as nonindependent subsamples, and averaged them for analysis.

Virus assays. In the laboratory, mosquitoes were identified and separated by species, over dry ice. For *Cs. melanura*, *Cq. perturbans*, and *Culex* spp., we created replicate pools for each location, date, and species, typically consisting of 25 or fewer individuals; if mosquito numbers were high, some pools of 30 or 50 individuals were created. Mosquitoes from each of the two study locations (Dixon and Oak Orchard subunits) were kept in separate pools for analyses.

Mosquito pools were assayed by virus isolation. Briefly, mosquito pools were homogenized in 1 ml virus isolation media (Minimum Essential Medium supplemented with 1,000 U penicillin G, 1 mg streptomycin, 0.25 mg gentamicin sulfate, 0.5 mg kanamycin monosulfate, 2.5 μg/mL amphotericin B, and 1% bovine serum albumin) using a Qiagen Mixer Mill 300 (Valencia, CA) then clarified by centrifugation (10 min at $9.6 \times g$). A 100-μL aliquot of the resulting supernatant fluid from each sample was inoculated onto a separate well of a 12-well plate with confluent 2-d-old Vero Middle America Research Unit (Vero M) cell culture monolayer and incubated at 37°C in a 5% CO_2 atmosphere. Cell cultures were examined daily for 7 d for evidence of cytopathic effects (CPE). Samples not displaying CPE within the first 7 d were subpassaged onto 2-d-old Vero M cells and observed for an additional 7 d. Samples were discarded and reported as negative if CPE were not evident within 14 d.

Results

Seasonal phenology. Overall collections of mosquito larvae from the Carolina bay wetlands ($n = 403$ larvae, from March, July, and November 2015–2017) were dominated by *Cq. perturbans* (40.2%) and *Cs. melanura* (31.8%) (see also Batzer and Murray 2018). *Culiseta melanura* were only common in July (77% of 128 total larvae). *Coquillettidia perturbans* larval numbers there were fairly similar across all three sampling periods (26% March, 47% July, 27% November, of 162 total larvae) in the two Carolina bays supporting them. In March, 88% of the *Cq. perturbans* larvae were mature fourth instars, in July, 68% of the larvae were fourth instars, but in November, only 21% of the larvae were fourth instars ($\chi^2 = 24.3$, df $= 2$, $P < 0.001$).

Over the 1 April to 1 September 2017 sampling period, we collected 11,273 adult female mosquitoes, from eight species. Collections from 1 April were overwhelming dominated (>95% of total) by *Anopheles crucians* Weidemann (Fig. 1A). Collections from 1 May were also dominated by *An. crucians*, although numbers had declined dramatically from the April peak; *Cq. perturbans* adults first became common in May (Fig 1B). In 1 June collections, *Cs. melanura* first appeared (Fig.

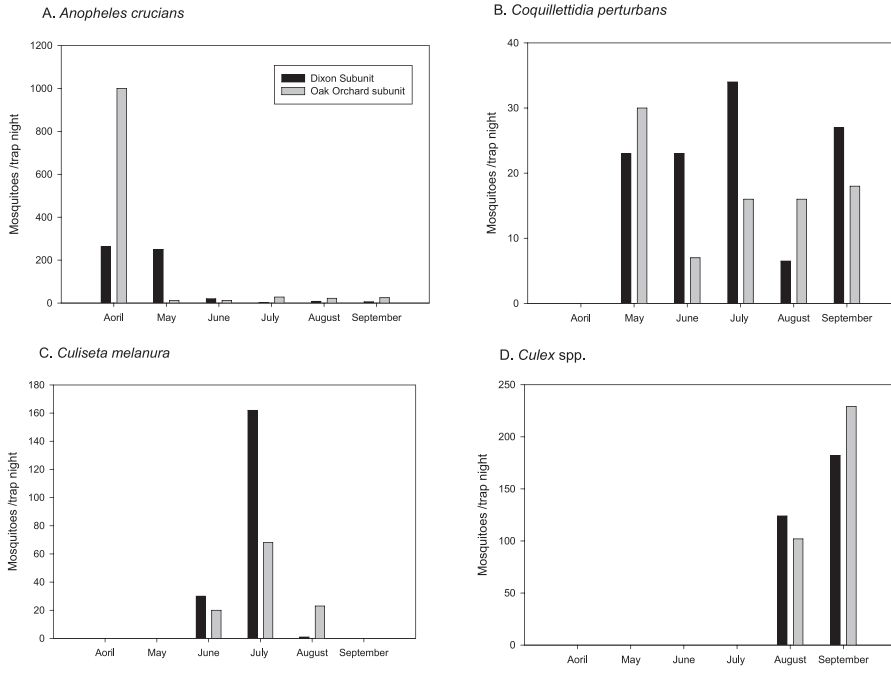

Fig. 1. Season patterns in adult mosquitoes at Tuckahoe Wildlife Management Area (Dixon and Oak Orchard subunits), Screven County, GA, from 1 April to 1 September 2017. Data (bars) represent the average of all traps used at a location on a specific date (females per trap night).

1C) and it was codominant with *Cq. perturbans* and *An. crucians*. The 1 July collections were dominated by *Cs. melanura*, and this was when that mosquito species had peak abundance; *Cq. perturbans* and *An. crucians* remained moderately abundant. In 1 August and 1 September collections, *Culex* spp. (*Culex erraticus* [Dyar and Knab], *Culex nigripalpus* Theobald, *Culex quinquefasciatus* Say) became very abundant (Fig. 1D), and *An. crucians* and *Cq. perturbans* remained moderately abundant, while *Cs. melanura* numbers declined to zero by September. Of the two species most relevant to the ecology of EEEv, *Cs. melanura* was not detected in April–May and was rare in September; it first appeared in June, and a clear peak in numbers occurred in July (Fig. 1C), while *Cq. perturbans* first appeared in May and their numbers remained stable over the next 4 mo (Fig. 1B).

Virus assays. We assessed occurrence of EEEv in 1,425 *Cs. melanura* females (61 pools), 983 *Cq. perturbans* females (91 pools), and 3,615 *Culex* spp. females (149 pools). We did not detect EEEv from any of these mosquitoes.

Discussion

A detailed description of the phenology of *Cq. perturbans* and *Cs. melanura* mosquitoes in east-central Georgia would require both more extensive (more sites,

more dates) and more intensive (more traps, more sweeps) sampling than available from this study, especially because both mosquito species are notoriously difficult to sample (see Batzer 1993, Morris 1988). However, some gross phenological patterns became evident, particularly the relative timing of *Cq. perturbans* versus *Cs. melanura* female availability, which likely has relevance to the natural cycling of EEEv in the area of study.

EEEv is believed to be cycled through mosquitoes by the virus first being amplified in bird populations by *Cs. melanura*, and then being transferred (or bridged) from birds to humans or horses by mosquito species like *Cq. perturbans* that have more catholic feeding habits (i.e., readily will bite birds, humans, and horses) (Morris 1988). While the role of *Cs. melanura* in the cycle of EEEv is well established (see Cupp et al. 2003), the roles of any bridge vectors are more ambiguous. The life cycle phenologies of *Cs. melanura* and *Cq. perturbans* at our study site suggest fairly complex interactions. First of all, *Cq. perturbans* began to emerge in late April and May, well before *Cs. melanura* began to emerge in late May and June. Thus, these early emerging *Cq. perturbans* likely have a minimal role in cycling EEEv.

The continued prevalence of *Cq. perturbans* adult females for over 4 mo was unexpected. *Coquillettidia perturbans* is considered to be univoltine throughout most of its range in North America (Batzer and Ranta 1994, Hagmann 1953). In Minnesota, quantitative samplings of univoltine populations of *Cq. perturbans* indicate that almost all emerge from individual wetlands over a 2–3-week period, and adult females at specific locations (likely derived from multiple wetlands) are common for less than 60 d (Batzer and Ranta 1994). In east-central Georgia, adult *Cq. perturbans* females were common at our study sites for twice that long, 123 + d, which is not consistent with univoltinism. The prevalence of four-instar larvae in July was also not consistent with a single univoltine cohort of *Cq. perturbans* in east-central Georgia; the larval populations in midsummer should have been dominated by early instars, reflecting a new cohort.

Bivoltine populations of *Cq. perturbans* have been previously reported from south Florida (Lounibos and Escher 1983). We suspect that *Cq. perturbans* may also be bivoltine in east-central Georgia, much farther north in the United States than was previously suspected possible. Overwintering larvae likely emerge as adults in late spring and early summer, and a second generation then develops from that cohort over early summer and emerges as adults in mid- to late summer; this second generation in turn produces a subsequent overwintering generation of larvae (the fact that early instars dominated larval collections in November supports this). If so, given that *Cs. melanura* was not prevalent until midsummer, any role of *Cq. perturbans* as a bridge vector of EEEv is likely restricted to the second generation in our study area.

Whether *Cq. perturbans* is univoltine or bivoltine, and the timing of their emergence relative to the active period *for Cs. melanura*, will undoubtedly affect the efficacy of *Cq. perturbans* as a bridge vector of EEEv across its natural range. Further, the apparent phenological plasticity of *Cq. perturbans* life cycles across latitudes (Batzer and Ranta 1994), presumably related to temperature, might suggest that the role of this mosquito to the local epidemiology of EEEv will change as the climate warms.

We were somewhat surprised that EEEv was not detected at our study site, as the ecological elements suspected to be important were all there, that is, *Cs. melanura*, *Cq. perturbans*, water moccasin snakes, and a diversity of birds co-occurred; virus had been previously detected in the county; and Cupp et al. (2003) had found a high incidence of the virus in these mosquitoes (and *Culex*) in Alabama. The unique findings between our effort and that of Cupp et al. (2003) may in part reflect procedural differences. Cupp et al. (2003) noted that their nested polymerase chain reaction technique could have detected even very low virus levels, such as may be present before the virus has disseminated from the gut to the salivary glands. Our methodology is more conservative and is likely to reflect the presence of infective mosquitoes. In addition, Cupp et al (2003) examined a larger sample of mosquitoes, including collections from resting boxes and vegetation that may have increased the representation of blood-fed individuals. Finally, Cupp et al (2003) collected *Cx. erraticus* throughout the season beginning in May, and they found 7.75% of the pools were positive for EEEv, which suggested this species may serve a role in EEE enzootic transmission dynamics in Alabama. In contrast, we did not detect *Culex* (dominated by *Cx. erraticus*) at our study site until July, and it became abundant only late in the season. Although methodological differences preclude a direct comparison between our study and that of Cupp et al. (2003), the fact that *Culex* spp. became very numerous only after *Cs. melanura* became numerous, may provide some indirect support for the contention of Cupp et al. (2003) that *Culex* may be a bridge vector of EEEv in some locations, including east-central Georgia. Clearly, how bridge-vectoring mosquitoes are involved with EEEv transmission needs additional characterization.

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