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Research

Population and Community Ecology

Characterizing the seasonal abundance and reproductive activity of overwintering *Anopheles* (Diptera: Culicidae) mosquitoes

Hannah Dehus[1,*], **Alden Siperstein**[1], **Laura W. Pomeroy**[2,3], **Megan E. Meuti**[1]

[1]Entomology at the Ohio State University, Columbus, OH, USA, [2]College of Public Health at the Ohio State University, Columbus, OH, USA, [3]Translational Data Analytics Institute at the Ohio State University, Columbus, OH, USA *Corresponding author, mail: dehus.5@buckeyemail.osu.edu

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In temperate regions of the United States, female *Anopheles* mosquitoes respond to low temperatures and short photoperiods by entering an overwintering dormancy or diapause. Diapause in *Anopheles* results in reduced frequency of blood-feeding and reproductive arrest, indicating a period when pathogen transmission by these mosquitoes is unlikely. However, it is unclear precisely how late into the fall and how early in the spring these mosquitoes are biting, reproducing, and potentially transmitting pathogens. This is further complicated by the lack of clear markers of diapause in *Anopheles* (e.g., changes in egg follicle length). Our goal was to characterize the seasonal reproductive activity of female *Anopheles* in central Ohio, United States and evaluate egg follicle length as an indicator of *Anopheles* diapause. We used traditional mosquito traps and aspirators to collect *Anopheles* from urban woodlots and culverts, respectively, from late September 2021 through mid-May 2022 in central Ohio. By measuring their egg follicle length, reproductive status, and blood-feeding status, we found that egg follicle length is not a reliable indicator of *Anopheles* diapause. We also found that a small proportion of *An. punctipennis* (Say), *An. perplexens* (Ludlow), and *An. quadrimaculatus* (Say) continued to bite and reproduce into early November 2021 and that females of these species terminated reproductive dormancy and began biting by mid-March 2022. This period of reproductive activity extends beyond current mosquito surveillance and control in Ohio. Our findings suggest that within temperate regions of North America, *Anopheles* have the capacity to transmit pathogens throughout the spring, summer, and fall.

Key words: diapause, mosquito surveillance, host-seeking, seasonal response, ovarian development

Introduction

Malaria is one of the most dangerous mosquito-borne diseases (Hodge et al. 2021) and one of the most severe public health problems worldwide (CDC 2022). Specifically, there were an estimated 249 million human cases of malaria and 608,000 malaria deaths throughout the world in 2022 (WHO 2023). In the continental United States, malaria was eradicated in 1954 (Moreno-Madriñán and Turell 2018). However, in 2023, 7 locally acquired cases of human malaria were detected in 3 states (Florida, Texas, and South Carolina; CDC 2023b). Along with these locally acquired cases, several species of *Anopheles* mosquitoes in the United States can transmit malaria (Sinka et al. 2010, Hodge et al. 2021, CDC 2023a, 2023b), demonstrating that malaria has the potential to reemerge.

Two known vectors of malaria, *An. quadrimaculatus* Say and *An. punctipennis* Say, as well as *An. perplexens* Ludlow, have a broad range that covers most of the eastern and midwestern United States, including the state of Ohio (Fritz et al. 1991, Young et al. 2008, Noden et al. 2015, Hodge et al. 2021). Female *An. punctipennis* and *An. quadrimaculatus* are crepuscular and regularly feed on mammals and humans depending on host availability within the immediate environment (Jensen et al. 1996, Anderson et al. 2018). Both *An. quadrimaculatus* and *An. punctipennis* can transmit 2 major human malaria parasites, *Plasmodium vivax* and *P. falciparum* (Strickman et al. 2000), and *An. punctipennis* can also transmit the malaria parasite *P. odocoilei* that infects ungulates (Martinsen et al. 2016). In addition to malaria parasites, *Anopheles* may play a role in the transmission of human viruses in the United States, including Cache

Valley virus, eastern equine encephalitis virus, and West Nile virus, which have all been isolated from field-collected *An. punctipennis* and *An. quadrimaculatus* (Wallace and Grimstad 2002, Yaremych et al. 2004, Shepard et al. 2016, Anderson et al. 2018). Moreover, laboratory studies have demonstrated that *An. quadrimaculatus* and *An. punctipennis* are competent vectors of Cache Valley virus (Dieme et al. 2022, Hughes et al. 2023) and that *An. quadrimaculatus* is capable of transmitting eastern equine encephalitis (Shepard et al. 2016). As *An. perplexens* has been studied less frequently than other species, little is known about its biology (Jensen et al. 1995), and it is not currently considered a medically important species (Noden et al. 2015). However, like *An. punctipennis* and *An. quadrimaculatus*, *An. perplexens* regularly feeds on mammalian hosts and occasionally bites birds (Bingham et al. 2014) and therefore might also have the potential to transmit pathogens.

Both *An. punctipennis* and *An. quadrimaculatus* occupy rural and urban environments, and adults of these species rest in cool, shaded areas (e.g., overhangs, outdoor stairwells, culverts, trees, etc.; Carpenter and LaCasse 1955, Hitchcock 1968) near oviposition sites. Oviposition sites for these species include a variety of aquatic habitats, such as swamps, vegetated ponds and lakes, ditches, slow-moving streams, and, sometimes, rain barrels and other containers (Carpenter and LaCasse 1955, Jensen et al. 1993, Robertson et al. 1993, Chase and Knight 2003). Although less is known about the ecology of *An. perplexens*, larvae of this species have been collected from swamps and artificial containers (Jensen et al. 1995).

Throughout temperate regions of the United States, seasonal changes in daylength and temperature throughout spring (March 21–June 21), summer (June 22–September 21), fall (September 22–December 21), and winter (December 22–March 20; NOAA 2024) trigger seasonal responses, like diapause, in *Anopheles* mosquitoes. Short daylengths and lower temperatures that occur during late summer and fall in this region induce diapause in *Anopheles* mosquitoes, enabling nulliparous adult females (those that have not previously lain a batch of eggs) to survive low, winter temperatures (Vinogradova 2007, Denlinger and Armbruster 2014, Diniz et al. 2017). Diapause is defined by the cessation of reproductive activity (Denlinger and Armbruster 2014, Diniz et al. 2017) and thus diapausing female *Anopheles* consume blood less frequently than nondiapausing females (Vinogradova 2007). Therefore, when populations of *Anopheles* mosquitoes enter diapause, the risk of pathogen transmission decreases. Additionally, like *Culex* mosquitoes that serve as an overwinter reservoir for West Nile virus (Reisen and Wheeler 2019), diapausing female *Anopheles* might serve as an overwinter reservoir and thereby contribute to the local persistence of pathogens in temperate regions.

In central Ohio, daylength decreases from the onset of summer (from 15 h 28 min) to the beginning of winter (to 8 h 53 min; NOAA 2023) and average temperatures range from 30 °C in the summer to −6 °C in the winter (NWS 2023). Although the precise daylengths and temperatures that trigger the diapause response vary geographically (Depner and Hardwood 1966, Washino 1970, Ramsdale and Wilkes 1985, Vinogradova 2007), diapause in *Anopheles* mosquitoes initiates when larvae are exposed to short daylengths and low temperatures (Mer 1936, Depner and Hardwood 1966, Washino 1970, Ramsdale and Wilkes 1985). Adult females overwinter in protected shelters, like culverts, barns, tree holes, and cellars (Carpenter and LaCasse 1955, Hitchcock 1968, Washino and Bailey 1970, Magnarelli 1975). It is unclear how environmental signals influence diapause termination in *Anopheles* mosquitoes (Washino 1970, Case et al. 1977, reviewed by Denlinger and Armbruster 2016). However, adult females that survive the harsh winter months begin host-seeking and resume reproductive activity when daylengths and temperatures increase in the spring (Hitchcock 1968, Washino 1970).

Reduced or suspended blood-feeding behavior is characteristic of diapause in *Anopheles* mosquitoes. For some temperate species of *Anopheles* mosquitoes, such as *An. punctipennis*, diapause has been characterized by a complete cessation in blood-feeding behavior (Washino and Bailey 1970), while others, including *An. freeborni*, may continue to take bloodmeals but undergo gonotrophic dissociation wherein they divert nutrients from the bloodmeal to overwintering survival rather than maturing their egg follicles (Washino 1970, Ramsdale and Wilkes 1985). In species that undergo gonotrophic dissociation, the absence of ovarian development after consuming a bloodmeal is indicative of diapause (Depner and Hardwood 1966, Washino 1970). For many *Anopheles* species, such as *An. quadrimaculatus*, it is unclear whether they undergo gonotrophic dissociation during diapause (Hitchcock 1968). However, regardless of whether *Anopheles* mosquitoes blood-feed, all diapausing females fail to lay eggs (Hitchcock 1968, Washino 1970, Vinogradova 2007).

Assessing the diapause status of *Anopeheles* species that do not exhibit gonotrophic dissociation is more complicated as both diapausing and nondiapausing females can be nulliparous, and the developmental stage of egg follicle maturation in *Anopheles* can be the same for both diapausing and nonblood-fed, nondiapausing females (Washino 1970, Washino and Bailey 1970). Therefore, it is not possible to distinguish between field-collected diapausing and nondiapausing *Anopheles* females by using the stage of egg follicle development or parity alone. Unlike *Culex* mosquitoes, where egg follicle length serves as a reliable indicator of diapause status (Eldridge 1968, Sanburg and Larsen 1973, Spielman and Wong 1973), it is unclear whether reduced egg follicle length is indicative of diapause in *Anopheles* mosquitoes.

Diapause in *Anopheles* females is characterized by several physiological and behavioral changes, including reduced or suspended blood-feeding behavior and reproductive arrest; therefore, the characteristics associated with these changes (e.g., the absence of blood in the abdomen, nulliparity, and arrested egg follicle development) can help distinguish diapausing from nondiapausing females (Washino 1970, Vinogradova 2007). However, these characteristics are not exclusive of diapause in female *Anopheles* as both diapausing and recently emerged, nondiapausing females can be nulliparous and have undeveloped egg follicles (e.g., developed to Christophers' stage II; Hitchcock 1968, Washino and Bailey 1970). Additionally, in some *Anopheles* species both diapausing and nondiapausing females consume blood (Washino 1970, Washino and Bailey 1970, Vinogradova 2007). Therefore, while examining changes in the proportion of females that have blood-fed, are nulliparous, and have egg follicles in different developmental stages can be used to determine when populations of *Anopheles* mosquitoes initiate and terminate diapause, these features cannot be used to reliably determine whether an individual female *Anopheles* mosquito is in diapause.

Here, we characterize the seasonal abundance and reproductive activity of *Anopheles* mosquitoes in a temperate region of the United States throughout the fall, winter, and early spring, and evaluate whether egg follicle length can serve as a metric to distinguish diapausing females. The specific characteristics of diapause have not been thoroughly described for many *Anopheles* mosquitoes or across geographic locations. Because temperate, diapausing *Anopheles* mosquitoes either blood-feed at lower frequencies or do not blood-feed at all, determining when diapause initiation and termination occur can help us predict when *Anopheles* will be capable of transmitting pathogens.

Materials and Methods

Mosquito Collection

Adult *Anopheles* mosquitoes were collected between the fall of 2021 and the spring of 2022 from various locations in central Ohio as previously described in Siperstein et al. (2022). Adult mosquitoes were collected from 9 urban woodlots (hereafter referred to as above ground sites) and from 9 culverts (hereafter referred to as below ground sites; Supplementary Fig. S1). Above ground sites consisted of urban woodlots to standardize ecological traits like canopy cover and vegetation abundance. Culvert sites were selected if they contained mosquitoes and were accessible to researchers. Collections from each site took place weekly from 30 September 2021, to 30 November 2021, then once every 2 wk until 23 February 2022, at which point weekly collections resumed until 13 May 2022. One Biogents-sentinel 2 (BGS2) trap (Biogents), baited with CO_2 and a human pheromone lure, was placed at each above ground site and collected approximately 24 h later. BGS2 traps were used to collect mosquitoes throughout the entire collection period (30 September 2021–13 May 2022). From 23 February 2022 through 15 May 2022, 1 CDC light trap, baited with CO_2 and light, was also placed at each above ground site for approximately 24 h. Both BGS2 and CDC light traps were set in the same location at each site during each collection event. Throughout the entire collection period, either a mouth or mechanical aspirator was used by 1 researcher to aspirate mosquitoes from each below ground site (approximately 15 min per site) during each collection event. After collection, mosquitoes were euthanized by placing them on dry ice and transported to the lab where they were placed in individual 0.5 ml tubes, labeled, and stored at −20 °C.

To compare the egg follicle size of *Anopheles* that were reared under diapause-inducing, short day conditions or diapause-averting, long day conditions in the laboratory, larval *Anopheles* were collected from marshes, swamps, wetlands, and retention ponds ($n = 5$ total sites) in central Ohio on 16 June 2022, and 20 July 2022 using the larval dipping method. Surface water from each source was collected using a 400-ml dipping cup. The water collected in the dipping cup was inspected for *Anopheles* larvae, and when present, the larvae were transferred to a collection container using a disposable plastic pipette.

Species Identification

Adult female specimens, including those collected as adults and adults reared from field-collected larvae, were morphologically identified to species using a stereomicroscope (Leica) and a key by Craker and Collins (2014). The only exceptions were 13 female *Anopheles* that had been collected from a culvert and were too damaged to morphologically identify. Female *An. punctipennis* were distinguished from *An. perplexens* by visual estimation of the ratio between the length of the wings' subcostal pale scales and the preapical dark scales (≥1:2 and ≤1:3, respectively) according to Craker and Collins (2014; Supplementary Fig. S2). When the ratio fell between 1:2 and 1:3, the specimen was identified as *An. punctipennis* or *An. perplexens* depending on whether the ratio was closer to 1:2 or 1:3, respectively.

Genetic analyses of ITS2 sequences (Hodge et al. 2021) were used to confirm the morphological identifications of *An. perplexens*, *An. punctipennis*, and *An. quadrimaculatus* ($n = 21$ per species). Genomic DNA was extracted from mosquito legs using a Phire Tissue Direct kit (Thermo Scientific), and the purified gDNA concentration and quality was determined by using a Nanodrop spectrometer (Thermo Scientific). Forward and reverse primers designed by Hodge et al. (2021) were used to amplify the ITS2 fragment. PCR assays contained the following: 10 µl 2× Phire Tissue Direct PCR Master Mix (Thermo Scientific), 7 µl of molecular grade water, 500 nM of forward and reverse primers, and 1 µl of genomic DNA (20 µl total volume). PCR amplification was conducted in a thermocycler (Bio-Rad) with the following parameters: 98 °C for 5 min, followed by 40 cycles of 98 °C for 5 s, 54 °C for 5 s, and 72 °C for 20 s, and finally 72 °C for 1 min. PCR products were run on a 1% agarose gel at 150 V for 60 min. In preparation for genetic sequencing, electrophoresed PCR products were excised from agarose gel and then purified using Ultrafree-DA centrifugal filter devices (Millipore). Sanger Sequencing of the samples was performed at the James Comprehensive Cancer Center at the Ohio State University (Columbus, Ohio). The sequences were uploaded to MultAlin software (Corpet 1988) for analysis. Sequences of ITS2 that we obtained from field-collected *Anopheles* were compared with ITS2 sequences of *An. punctipennis* (accession number LR877261) and *An. quadrimacaultus* (accession number U32550) from GenBank (Altschul et al. 1990). As ITS2 sequences for *An. perplexens* were not available, sequences of ITS2 from mosquitoes that were morphologically identified as *An. perplexens* were compared to published ITS2 sequences of *An. quadrimaculatus* and *An. punctipennis* and analyzed for differences.

Ovarian Follicle Analysis

Ovaries were dissected from all nondamaged, nonblood-fed, adult female *Anopheles* mosquitoes that had been collected from the field ($n = 486$ dissected females/526 collected females) as well as all females reared from field-collected larvae ($n = 17$ disected females/17 laboratory-reared females).

The field-collected larvae were separated by developmental stage, and first instars were either reared under winter-like conditions (Light:Dark, 8 h:16 h, 18 °C) to induce diapause, or summer-like conditions (Light:Dark, 16 h:8 h, 27 °C) to avert diapause. First-instar larvae were reared under either diapause-inducing, short day conditions or diapause-averting, long day conditions. Because the diapause response is activated in most mosquitoes during a period of photosensitivity that occurs during juvenile development and well before the stage of developmental arrest (reviewed by Denlinger 2002, Denlinger and Armbruster 2016), all second-, third-, and fourth-instar larvae were reared under summer-like conditions to account for the chance that photoperiod had already stimulated a nonreversable, diapause-averting response in these mosquitoes. This is particularly important, as the specific developmental stage during which *An. punctipennis*, *An. perplexens*, and *An. quadrimaculatus* initiate or avert diapause is unclear. All larvae were reared in clear, plastic containers, and fed Tetramin tropical fish food. Adults were provided with reverse osmosis water and 10% sucrose solution, and 7 days after adult emergence, were euthanized at −20 °C.

Ovaries were dissected in 0.9% saline solution (NaCl). The parity status of each field-collected and laboratory-reared female was determined by observing the ovarian tracheoles according to Detinova (1962), where tightly coiled tracheoles indicated that the female was nulliparous (having never lain eggs) and unraveled, web-like tracheoles indicated that the female was parous (having previously lain a batch of eggs). Then egg follicles were separated and 5 follicles from each female were observed and measured under 200-fold magnification on an inverted microscope (Nikon) to determine the developmental stage and the average egg follicle length

Table 1. Total number and dates when female *Anopheles* mosquitoes were collected at different physiological stages, by species. Dashes indicate that no females were collected in this state

Measured variables	*Anopheles punctipennis*	*Anopheles quadrimaculatus*	*Anopheles perplexens*
Total number collected from culverts	322	98	66
Total number collected from woodlots	20	5	2
Date of last gravid mosquito collected in fall	30 September 2021	—	30 September 2021
Date of last blood-fed mosquito collected in fall	7 October 2021	28 October 2021	—
Date of first mosquito collected from above ground sites after winter	2 March 2022	16 March 2022	16 March 2022
Date of first gravid mosquito collected in spring	31 March 2022	—	25 March 2022
Date of first blood-fed mosquito collected in spring	6 April 2022	—	15 April 2022

for each female. For each specimen, the developmental stage of egg follicles was assessed according to modifications that Mer (1936) made to Christophers (1911; Supplementary Fig. S3). To evaluate whether egg follicle length could be used to distinguish diapausing female *Anopheles* mosquitoes, we measured the average egg follicle length of each nulliparous and parous female, and assessed whether egg follicle length changed in mosquitoes across our collection period.

Data Analysis

We fit a dose–response curve (*drc* package; Ritz et al. 2015) using 4-parameter log-logistics to estimate the time at which 50% of the *Anopheles* population resumed reproductive activity in the spring. To quantify the proportion of nulliparous females at collection sites over time, we included females collected from both above ground and below ground sites in our calculations to ensure that our estimates were representative of the entire population's reproductive state, as in early spring, the proportion of reproductively dormant females might be greater within overwintering hibernacula, like culverts, than outside of overwintering hibernacula, such as in urban woodlots. We calculated the proportion of females in reproductive dormancy as the counts of nulliparous mosquitoes divided by the sum of counts of nulliparous, parous, gravid, and blood-fed mosquitoes. Although it is unclear whether *An. quadrimaculatus* and *An. perplexens* undergo gonotrophic dissociation, we included blood-fed females in our calculations because ~76% of all blood-fed females were collected after 6 April 2022, and by this time undergoing gonotrophic dissociation would no longer be beneficial for overwinter survival. All analyses were conducted using R, version 4.1.2 or 4.2.2 (R Core Team 2021) via RStudio (RStudio Team 2022). Spatial and temporal analyses used the *lubridate* (Grolemund and Wickham 2011) and *maps* (Becker et al. 2022) packages.

Results

Species Identification

Of the female *Anopheles* we selected to genetically sequence, we determined that our morphological identifications were 100% accurate for *An. punctipennis* (*n* = 21/21; Supplementary Fig. S4a) and *An. quadrimaculatus* (*n* = 21/21; Supplementary Fig. S4c) based on the available sequences in Genbank (accession numbers LR877261 and U32550, respectively). We were unable to determine the accuracy of any of our female *An. perplexens* morphological identifications (*n* = 21) because the ITS2 sequences of *An. perplexens* were identical to that in *An. punctipennis* (Supplementary Fig. S4ab). As we were unable to distinguish female *An. perplexens* with molecular diagnostics, we instead relied on our morphological identifications to differentiate this species from *An. punctipennis*.

Seasonal Abundance

In total, we collected 526 adult female *Anopheles* from culverts (*n* = 486) and woodlots (*n* = 27), belonging to 3 different species: *An. punctipennis*, *An. perplexens*, and *An. quadrimaculatus* (Table 1). We collected a greater total number of female *An. punctipennis* (*n* = 342) than *An. perplexens* (*n* = 68) and *An. quadrimaculatus* (*n* = 103) with variation across sites (Table 1; Fig. 1). The number of female *An. perplexens*, *An. punctipennis*, and *An. quadrimaculatus* we collected drastically decreased by the week of 12 December 2021 (*n* = 1; *n* = 3; and *n* = 2, respectively), compared to previous collection weeks (mean ± SD = ~5 ± 3.60; ~26 ± 14.66; and ~8 ± 6.70 females per week, respectively, across 11 collection weeks), and very few female *Anopheles* mosquitoes of any species were collected during the winter and spring (*n* = 10; *n* = 55; and *n* = 9 total female *An. perplexens*, *An. punctipennis*, and *An. quadrimaculatus,* respectively, across 15 collection weeks; Fig. 1). However, by the week of 9 May 2022, there was an increase in the number of total female *An. punctipennis* collected from woodlots and culverts (*n* = 17 total females) compared to previous winter and spring collection weeks (mean ± SD = ~3 ± 1.98 females per week across 14 collection weeks). Females of *An. punctipennis* were consistently collected throughout the season (collected in 25/27 collection weeks), whereas female *An. perplexens* and *An. quadrimaculatus* were collected less frequently (collected in 20/27 and 17/27 collection weeks, respectively; Fig. 1). We did not collect any female *Anopheles* from BGS2 or CDC traps until the week of 28 February 2022, at which point we sporadically collected female *Anopheles* from these traps throughout the remainder of the collection period.

Seasonal Changes in Egg Follicle Length and Developmental Stage to Evaluate Diapause

We measured and observed seasonal changes in egg follicle lengths of individual females and found that both parous and nulliparous females had similar egg follicle lengths during the entire collection period (Fig. 2). From the beginning of the collection period through the week of 23 January 2022, most female *An. punctipennis*, *An. perplexens*, and *An. quadrimaculatus* (*n* = 252/273; *n* = 51/55; and *n* = 87/92 females, respectively) had egg follicle lengths between 50 and 100 µm (mean ± SD = 80.67 ± 11.57 µm; 79.02 ± 12.47 µm; and 67.08 ± 10.67 µm, respectively), albeit there were females of all 3 species (*n* = 18; *n* = 4; and *n* = 3, respectively) with egg follicle lengths greater than 100 µm (mean ± SD = 112. 83 ± 9.75; 109.75 ± 9.07; and 171.33 ± 114.03 µm, respectively), and some female *An. punctipennis* and *An. quadrimaculatus* (*n* = 3 each) had egg follicle lengths that were less than 50 µm (mean ± SD = 45.66 ± 4.93 and 45.33 ± 5.51 µm, respectively; Fig. 2a and c). By the week of 6 February 2022 (when our next collection event took place), and through the end of the collection period (the week of 8 May 2022), all dissected adult female *An. punctipennis*, *An. perplexens*, and *An.*

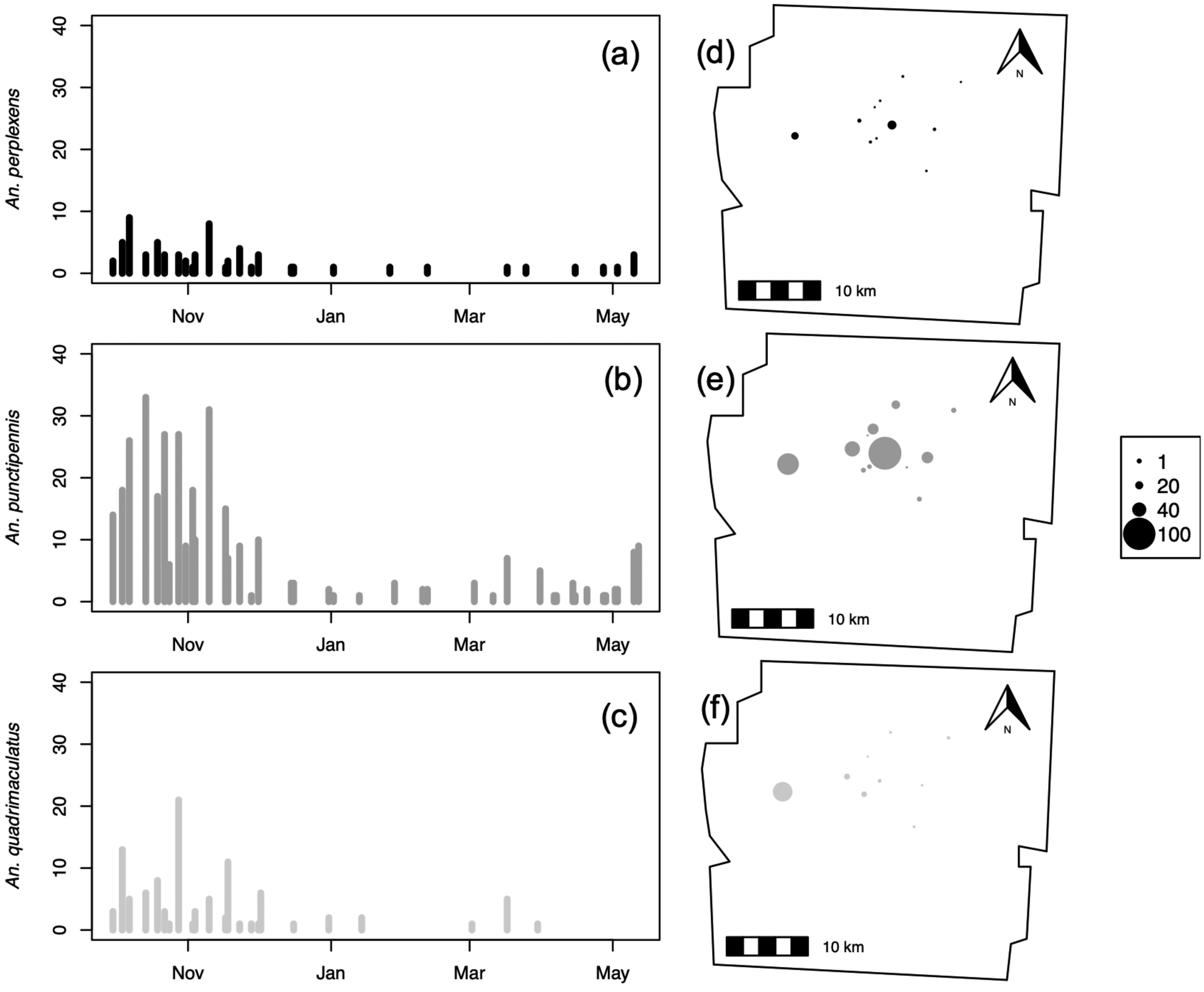

Fig. 1. Counts of *Anopheles perplexens*, *Anopheles punctipennis*, and *Anopheles quadrimaculatus* sampled over time in Franklin County, OH. The city of Columbus, OH, and nearby suburbs comprises the majority of Franklin County, OH. Counts of a) *An. perplexens*, b) *An. punctipennis*, and c) *An. quadrimaculatus* sampled by date in 2021–2022. Locations of d) *An. perplexens*, e) *An. punctipennis*, and f) *An. quadrimaculatus* sampled at multiple sites in Franklin County, OH, in 2021–2022. The size of the point for each site is proportional to the number of mosquitoes sampled at that site.

quadrimaculatus (*n* = 29; *n* = 4; and *n* = 7, respectively) had egg follicle lengths between 100 and 200 µm (mean ± SD = 157.07 ± 23.74 µm; 155.75 ± 13.67 µm; and 165.00 ± 12.49 µm, respectively) with the exception of 1 *An. punctipennis*, that had an egg follicle length of 205 µm (Fig. 2a), and 1 *An. quadrimaculatus*, that had an egg follicle length of 72 µm (Fig. 2c).

Of the adult females that were reared from larvae in the laboratory (*n* = 17 females), 100% of females reared under diapause-inducing, short day conditions (*n* = 7/7 females), had average egg follicle lengths less than 100 µm (mean ± SD = 70.85 ± 17.62 µm; Supplementary Fig. S5). Of the adult females reared under diapause-averting, long day conditions (*n* = 10), 70% of females (*n* = 7/10 females) also had egg follicle lengths less than 100 µm (mean ± SD = 93.3 ± 15.56 µm; Supplementary Fig. S5). While 30% of females reared in diapause-averting conditions (*n* = 3/10 females) had egg follicle lengths exceeding 100 µm (mean ± SD = 112.66 ± 2.08 µm), no females had egg follicle lengths greater than 115 µm (Supplementary Fig. S5).

We characterized seasonal changes in egg follicle maturation and found that female *Anopheles* collected in September, October, and most of November were in various stages of egg follicle development. All *An. perplexens* (*n* = 14) and all *An. punctipennis* (*n* = 44) collected from 21 November 2021 through mid-March 2022 had egg follicles at Christophers' stage II (Fig. 3); the only exception was 1 *An. punctipennis* collected during the week of 21 November 2021 whose egg follicles were in Christophers' stage I. In contrast, we observed various stages of egg follicle development in *An. quadrimaculatus*, consisting of Christophers' stage N, I, I–II, or II, from the beginning of the collection period through the week of 9 January 2022 (Fig. 3). Gravid (Christophers' stage V) females were collected in late September, and we collected gravid females again starting in late March (Fig. 3; Table 1).

Seasonal Changes in Physiological and Behavioral Indicators of Diapause

We collected both parous and nulliparous females of *An. punctipennis*, *An. perplexens*, and *An. quadrimaculatus* from late September until the middle of November, at which point all *An. quadrimaculatus* collected were nulliparous (Fig. 2). Parous *An. perplexens* and *An. punctipennis* were collected until the end of

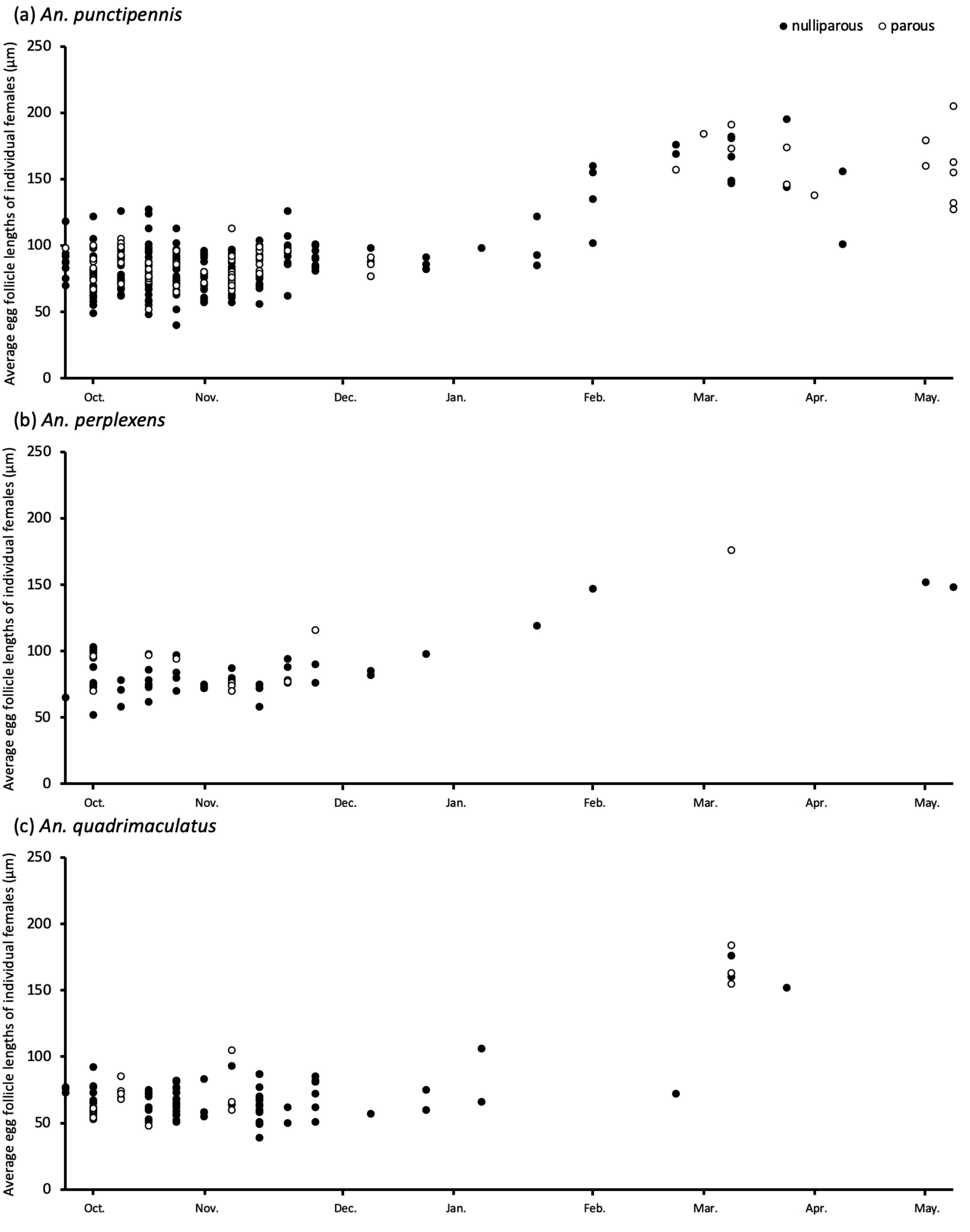

Fig. 2. Average egg follicle lengths of individual female a) *Anopheles punctipennis*, b) *Anopheles perplexens*, and c) *Anopheles quadrimaculatus* over time. Each data point represents the average ovarian follicle length (µm) of 5 primary follicles dissected from 1 female mosquito. Colors denote whether the individual was nulliparous (black) or parous (white).

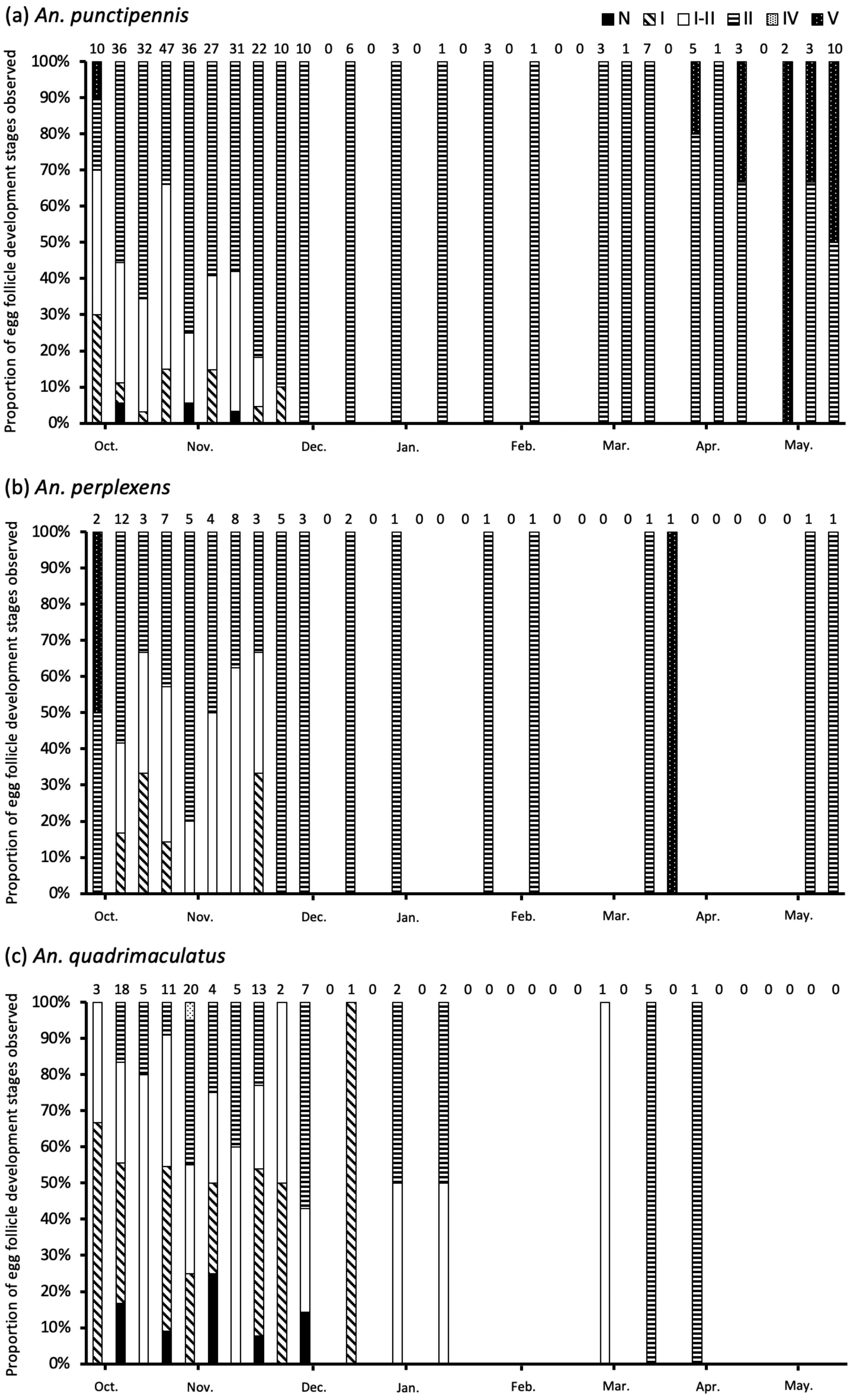

Fig. 3. Proportion of females in each ovarian development stage across the collection period for a) *An. punctipennis*, b) *An. perplexens*, and c) *An. quadrimaculatus*. Patterns represent each stage of egg follicle development (Christophers' stage N, Christophers' stage I, Christophers' stage I–II, Christophers' stage II, Christophers' stage III, Christophers' stage IV, and Christophers' stage V) according to modifications that Mer (1936) made to Christophers (1911). The total number of females that were collected and dissected each week is indicated above each column.

November and the middle of December, respectively. Parous *An. punctipennis* were collected again by early March, and parous *An. perplexens* and *An. quadrimaculatus* were collected again by the middle of March (Fig. 2).

By analyzing changes in the proportion of nulliparous females from individual sites over time, we found that 50% of *An. punctipennis* resumed reproductive activity between 1 March and 20 April 2022 in central Ohio (Fig. 4). We were unable to determine when 50% of *An. perplexens* and *An. quadrimaculatus* resumed reproductive activity by analyzing the proportion of nulliparous females from individual sites over time due to insufficient sample sizes (Supplementary Fig. S6). Instead, we analyzed the average proportion of nulliparous females collected from all sites over time and found that 50% of female *An. perplexens* and *An. quadrimaculatus* resumed reproductive activity by mid-March 2022 (Fig. 5).

The number of blood-fed *Anopheles* mosquitoes we collected varied throughout the collection period. While blood-fed *An. punctipennis* were collected during both the fall and spring, blood-fed *An. quadrimaculatus* were only collected in the fall and blood-fed *An. perplexens* were only collected in the spring (Supplemenytary Fig. S7). Furthermore, no blood-fed *Anopheles* were collected during the winter; the last blood-fed female was collected on 28 October 2021, and we did not collect blood-fed females again until 6 April 2022 (Table 1), at which point blood-fed females were collected weekly until the end of the collection period (Supplementary Fig. S7).

Discussion

By continuously collecting female *Anopheles* mosquitoes throughout the late fall, winter, and early spring, we discovered that egg follicle length and developmental state within field-collected female *Anopheles* are not reliable indicators of diapause status. This was further confirmed by collecting *Anopheles* larvae in the summer and rearing them under unambiguous long day or short day conditions. However, using multiple other lines of evidence, including seasonal changes blood-feeding and parity status, our results suggest that female *An. punctipennis*, *An. perplexens*, and *An. quadrimaculatus* had entered dormancy by late September 2021, as a high proportion of nulliparous females were collected during this time. Additionally, our results suggest that *Anopheles* females of all 3 species resume reproductive and host-seeking activity by mid-March 2022, well before current mosquito surveillance efforts begin (ODH: zoonotic disease program 2023). Notably, however, gonoactive and biting females of all 3 species persisted until late October 2021 and reappeared by late February 2022. These findings provide the foundation for future areas of investigation and have important implications for pathogen transmission and mosquito control.

One challenge that we encountered in this study was being able to accurately identify the *Anopheles* mosquitoes we collected to species, as our study revealed that the ITS2 sequence of *An. perplexens* is identical to that of *An. punctipennis*. Fortunately, in addition to having an ITS2 sequence that is distinct from other *Anopheles* species, *An. quadrimaculatus* also have a wing scale pattern that is different from *An. punctipennis* and *An. perplexens* (Craker and Collins 2014), allowing us to easily identify them (Supplementary Fig. S2). Unfortunately, however, the genome of *An. perplexens* has not yet been published and we were unable to use existing primers to genetically differentiate between *An. perplexens* and *An. punctipennis*. Therefore, we relied on morphological identification alone to differentiate between these 2 species.

We morphologically identified adult *An. punctipennis* and *An. perplexens* based on the ratio between the length of the wings' subcostal pale scales and the preapical dark scales according to Craker and Collins (2014). Specifically, adult female *An. punctipennis* had a ratio of ≥1:2 (Supplementary Fig. S2a) whereas *An. punctipennis* were identified when the ratio was ≤1:3 (Supplementary Fig. S2b). However, because the wing scale patterns are the only morphological difference between adult *An. punctipennis* and *An. perplexens* (Craker and Collins 2014), we had difficulty distinguishing female mosquitoes when the ratio between the length of subcostal pale scales and the preapical dark scales fell between these ranges.

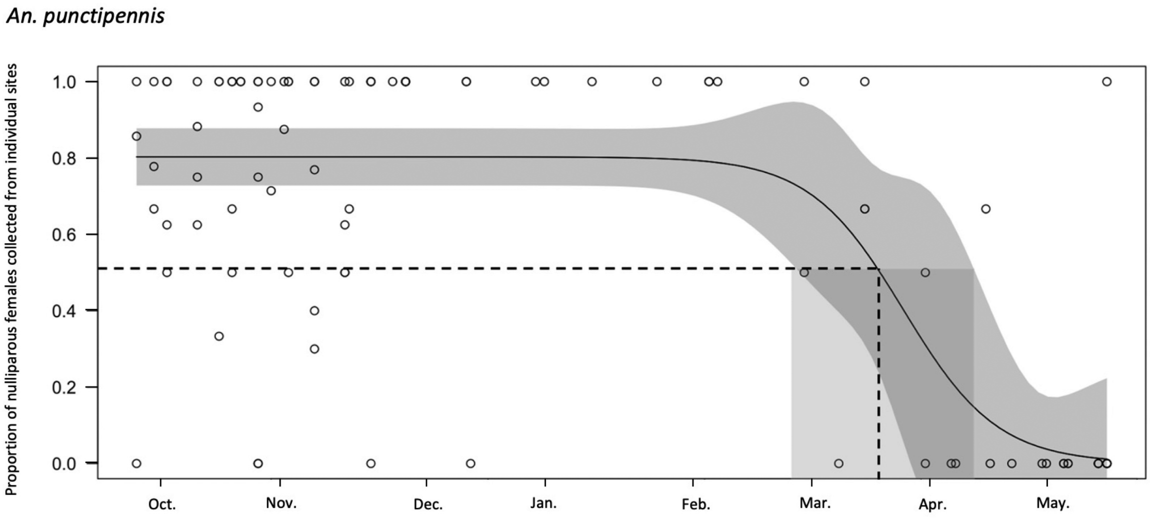
An. punctipennis

Fig. 4. Estimated time when 50% *Anopheles punctipennis* populations resumed reproductive activity in central Ohio in 2022. Each data point represents the proportion of nulliparous female *An. punctipennis* collected from an individual site each week (e.g., number of nulliparous females/total number of females collected from that site). A dose–response curve was fit using 4-parameter log-logistics and default arguments of the general model fitting function in the *drc* package in R (Ritz et al. 2015). The dark shaded area shows the 95% confidence interval determined by using the effective dose function in the *drc* package in R (Ritz et al. 2015). The dotted vertical line indicates when 50% of females resumed reproductive activity (26 March 2022), and the range in this time, based on standard error, is shown by the light shaded area (1 March 2022–20 April 2022).

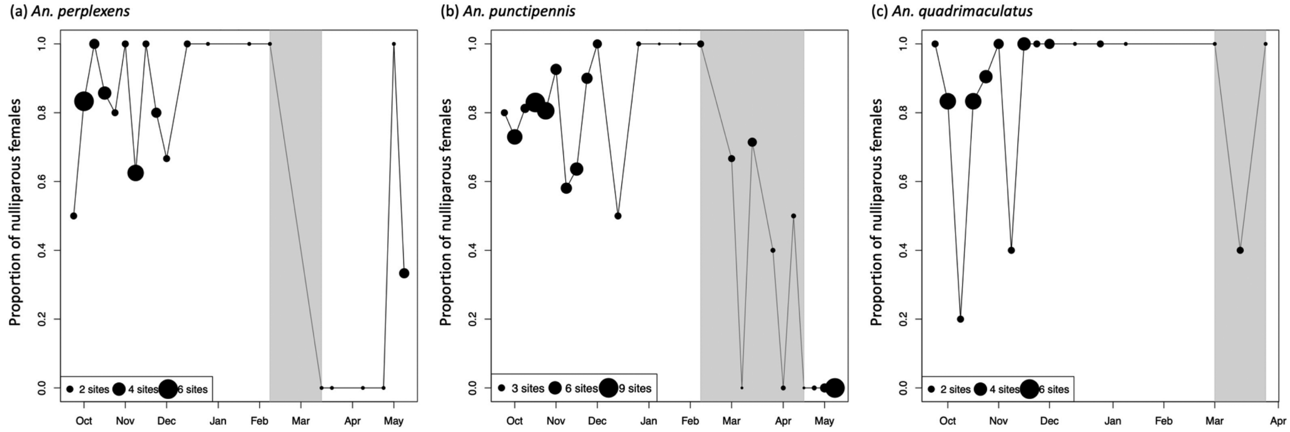

Fig. 5. Proportions of diapausing a) *Anopheles perplexens*, b) *Anopheles punctipennis*, and c) *Anopheles quadrimaculatus* collected from above and below ground field sites from fall 2021 through spring 2022. The size of each point is proportional to the number of culverts in which *Anopheles* spp. was found, even though all 18 sites were sampled during each collection event. Shaded areas highlight dates when there was a marked decrease in the proportion of nulliparous mosquitoes. a) The proportion of nulliparous *An. perplexens* rapidly decreased between 7 February 2022 and 14 March 2022. b) The proportion of nulliparous *An. punctipennis* decreased between 7 February 2022 and 18 April 2022. c) The proportion of nulliparous *An. quadrimaculatus* decreased between 28 February 2022 and 28 March 2022.

Previous studies have reported that wing scale patterns in *An. punctipennis* varies, making morphological distinction between *An. punctipennis* and *An. perplexens* unreliable (Fritz et al. 1991). Moreover, multiple studies have noted that the only way to definitively distinguish between *An. punctipennis* and *An. perplexens* is by observing differences in egg morphology (Kreutzer and Kitzmiller 1972, Fritz et al. 1991).

Previously, Kreutzer and Kitzmiller (1972) demonstrated that *An. punctipennis* and *An. perplexens* are separate species due to opposite banding patterns of different intensities in regions with paracentric inversions in the right arms of X chromosomes 2 and 3, albeit they are otherwise chromosomally very similar. Moreover, hybrid crosses of *An. punctipennis* and *An. perplexens* demonstrated that gene flow between these mosquitoes is not possible, further supporting the designation of *An. punctipennis* and *An. perplexens* as separate species (Kreutzer and Kitzmiller 1972). However, the close genetic and chromosomal similarities between *An. punctipennis* and *An. perplexens*, and their similar host preferences, could mean that *An. perplexens* might be able to transmit the same pathogens as *An. punctipennis*, although this has not yet been studied. Therefore, it is important to develop improved methods to distinguish adults of *An. punctipennis* and *An. perplexens* and to evaluate *An. perplexens*' capacity to carry and transmit pathogens.

We encountered further obstacles in determining when *Anopheles* initiate and terminate overwintering dormancy, in part because we were not able to determine whether an individual female *Anopheles* was in a diapause or nondiapause state. Unlike *Culex pipiens*, where Christophers' stage and egg follicle length can be used to distinguish diapausing and nondiapausing females (Spielman and Wong 1973; Robich and Denlinger 2005), neither Christophers' stage nor egg follicle length are reliable indicators of the diapause status of female *Anopheles*. Our observations re-emphasize that both diapausing and nondiapausing female *Anopheles* can have egg follicles that vary in length and are developed up to Christophers' stage II, as previously described for *An. punctipennis* (Washino and Bailey 1970) and *An. quadrimaculatus* (Hitchcock 1968). In contrast, only gonoactive females had egg follicles that had reached Christophers' stage III, IV, or V. Therefore, any stage of ovarian development preceding Christophers' stage III cannot be used to distinguish between diapausing and nondiapausing *An. punctipennis*, *An. perplexens*, and *An. quadrimaculatus*. Furthermore, our findings also demonstrate that egg follicle length cannot be used to distinguish between diapausing and nondiapausing female *Anopheles*; we observed similar changes in egg follicle length of both parous and nulliparous *An. punctipennis*, *An. perplexens*, and *An. quadrimaculatus* during fall, spring, and winter, and no difference in average egg follicle length was observed for *An. punctipennis* and *An. quadrimaculatus* that were reared as larvae under both summer-like and winter-like conditions in the laboratory. Moreover, our data demonstrate that the length and stage of egg follicles in female *Anopheles* are somewhat unrelated as nulliparous females collected from culverts in early spring have larger egg follicles than nulliparous females that were reared in the laboratory under long day, summer-like conditions. Unfortunately, we still lack a diagnostic method to determine the diapause status of individual field-collected *Anopheles*.

As the number of nulliparous, parous, and gravid adult female *An. punctipennis*, *An. perplexens*, and *An. quadrimaculatus* we collected varied throughout fall and into early winter, we could not determine the exact time when most females of each species arrested reproductive activity. Females collected in the late fall and early winter that exhibited characteristics associated with reproductive activity (i.e., being parous) could have emerged in late fall or early winter, averted diapause, and been reproductively active. Alternatively, it is possible that these females emerged in the late summer or early fall, completed their gonotrophic cycles, and simply had not yet succumbed to low winter temperatures. It is also unclear whether the nulliparous females that we collected in the fall had entered their overwintering dormancy or whether they were simply recently emerged and would have taken a bloodmeal and lain eggs if given the opportunity to do so. However, due to the high proportion of nulliparous females we collected at this time, we predict that diapause initiation for all 3 species likely occurred by late September 2021. By mid-December, all mosquitoes were in a state of reproductive arrest. Notably, Hitchcock (1968) reported that diapause initiation occurred in mid-September for a population of *An. quadrimaculatus* in northern Maryland, which is consistent with our results.

By analyzing changes in the proportion of nulliparous females over time, we determined that *An. perplexens* and *An. quadrimaculatus* likely terminated reproductive dormancy by mid-March in 2022.

Moreover we estimate that 50% of *An. punctipennis* mosquitoes resumed reproductive activity between early March and mid-April 2022 in central Ohio, comparable to a northern California population of this species that resumes reproductive activity around the middle of March (Washino and Bailey 1970). While we were unable to pinpoint precisely when any of the 3 species resumed reproductive activity, our estimates of when *An. quadrimaculatus*, *An. punctipennis*, and *An. perplexens* likely begin blood-feeding and laying eggs is still 1–2 months before mosquito surveillance and control begins in central Ohio (ODH: zoonotic disease program 2023), and therefore indicates a period of time that mosquito populations are likely increasing and potentially spreading pathogens.

As we collected very few blood-fed female *Anopheles* in late fall and early spring, it is still unclear precisely when most *Anopheles* species initiated and terminated host-seeking and blood-feeding behavior. However, no blood-fed *Anopheles* were collected from 1 November 2021 to 8 March 2022. Interestingly, while we collected blood-fed *An. punctipennis* in the fall and spring, blood-fed *An. quadrimaculatus* were only collected in the fall and blood-fed *An. perplexens* were only collected in the spring, suggesting that these 3 species might have different seasonal patterns of host-seeking and biting. As female *Anopheles* require a bloodmeal to develop mature eggs (Hitchcock 1968, Washino and Bailey 1970), the first and last collection of gravid females in the spring and fall, respectively, is also indicative of when females were actively host-seeking and consuming blood in 2021 and 2022. In the spring of 2022, we collected gravid *An. punctipennis* and *An. perplexens* before we were able to collect blood-fed females of these species, revealing that these species initiated host-seeking and blood-feeding sooner than our blood-fed collections alone would suggest. Furthermore, we collected parous *An. punctipennis*, *An. perplexens,* and *An. quadrimaculatus* in traditional mosquito traps, designed to collect host-seeking mosquitoes, before we collected any gravid or blood-fed females in the spring of 2022 (Table 1). As parous females must have previously taken a blood meal and lain a batch of eggs, our data suggest that some female *Anopheles* began host-seeking by late February 2022 and thus could have been transmitting pathogens at this time.

While our findings indicate that some female *Anopheles* were biting through late October 2021 and began biting by late February 2022, it is unclear whether females initiate and terminate host-seeking and biting behavior at the same time each year or if temperatures influenced seasonal host-seeking and biting behavior during our collection period. In October 2021, the average daily temperature in central Ohio was 16.8 °C, which is higher than the normal, or long-term (1991–2020) average, daily temperature of 12.9 °C (NWS 2023). It is possible that higher than normal average, daily temperatures during our collection period could have influenced the host-seeking and biting season of female *Anopheles* mosquitoes, as Ciota et al. (2011) demonstrated that *Culex* mosquitoes began blood-feeding earlier in years with higher winter temperatures. However, previous work has also demonstrated that short photoperiod (rather than low temperature) is responsible for the cessation of blood-feeding in field populations of *An. punctipennis* (Washino and Bailey 1970). In addition, field and laboratory studies have demonstrated that increasing photoperiod is more significant in influencing diapause termination (and thereby increased biting behavior) than temperature for some *Anopheles* species, as female *An. punctipennis* and *An. freeborni* terminate diapause well before the year's lowest temperatures (Washino 1970, Case et al. 1977). Our findings are similar in that the average daily temperature at in central Ohio in February 2022 was 0.3 °C, which is identical to the long-term daily temperatures (1991–2020). In March, the average daily temperature in central Ohio was 7.4 °C, which is above the normal average temperature (5.3 °C; NWS 2023). However, the average daily temperature in February and March 2022 was substantially lower than the average daily temperatures in October 2021, yet our data indicate that female *Anopheles* were host-seeking and biting during the late winter and early spring of 2022. Thus, it is unclear whether host-seeking and biting behavior of female *Anopheles* persists into late October and initiates in late February each year, or if this was unique to 2021–2022. Future studies that evaluate how temperature influences seasonal host-seeking and blood-feeding behavior of temperate *Anopheles* species would allow us to predict when *Anopheles* are biting and potentially spreading pathogens.

Our study identified a great amount of variation in the seasonal distribution of all 3 *Anopheles* mosquitoes and is the first to measure the overwintering population dynamics of *Anopheles* mosquitoes in the Midwestern United States. We observed that the abundance of *An. quadrimaculatus*, *An. punctipennis* and *An. perplexens* decreased by late November, which is consistent with what has previously been reported for a northern Maryland population of *An. quadrimaculatus* (Hitchcock 1968) and for a northern California population of *An. punctipennis* (Washino and Bailey 1970) that also decreased by late October and November respectively. Moreover, we determined that females terminate reproductive dormancy in early spring and therefore expected that shortly thereafter that populations would increase significantly. However, we did not observe a substantial increase in any of the 3 species throughout mid-May, a finding similar to Hitchcock (1968), whose previous study suggests that while *An. quadrimaculatus* resume reproductive activity by late March, populations of this species do not increase until the beginning of June. Precisely characterizing when *Anopheles* populations increase in the spring, combined with an estimated diapause termination time, could be useful in predicting when nondiapausing *Anopheles* populations are at their lowest, and accordingly, allow us to determine the best time to implement mosquito control efforts.

The time when *Anopheles* initiate and terminate diapause, as well as seasonal species abundance, might vary annually due to differences in environmental conditions such as temperature (Case et al. 1977, reviewed by Denlinger and Armbruster 2016). Elevated winter temperatures can increase energy consumption and deplete fat reserves that are necessary to support overwintering metabolism, and thereby result in increased mortality of overwintering female *Anopheles* (Hitchcock 1968, Sauer et al. 2022). Compared to normal average, or long-term average, daily temperatures from 1991 to 2020, average daily temperatures during our collection period (2021–2022) were slightly higher in October (2021 avg. = 16.8 °C; normal avg. = 12.9 °C), December (2021 avg. = 5.4 °C; normal avg. = 1.4 °C), and March (2022 avg. = 7.4 °C; normal avg. = 5.3 °C), and were the same or lower in November (2021 avg. = 5.4 °C; normal avg. = 6.4 °C), January (2022 avg. = –3.7 °C; normal avg. = –1.3 °C), and February (2022 avg. = 0.3 °C; normal avg. = 0.3 °C; NWS 2023). Thus, it is possible that higher than normal average daily temperatures in October and/or December 2021, resulted in increased energy consumption and mortality in diapausing *Anopheles* females during our study period. Previous work has suggested that increased energy consumption due to high winter temperatures might result in a shorter diapause period (Sauer et al. 2022), albeit it is unclear whether this was the reason diapause termination occurred for most *Anopheles* females in mid-March 2022 in central Ohio. In fact, it is still unclear what specific factors trigger diapause termination in *Anopheles* mosquitoes (Case et al. 1977, reviewed by Denlinger and Armbruster 2016). Therefore, future studies are important to fully characterize how different environmental conditions affect when *Anopheles* females arrest reproductive activity in the fall and resume host-seeking and egg-laying in the spring, so that mosquito control programs can better predict the best time to control these species.

Although *Anopheles* species are not the primary vectors of the most common and concerning mosquito-borne illnesses in the United States, they might currently facilitate in the spread of West Nile virus, Cache Valley virus, and eastern equine encephalitis (Wallace and Grimstad 2002, Yaremych et al. 2004, Shepard et al. 2016, Anderson et al. 2018, CDC 2023a), and are capable vectors of malaria, that might reemerge (Strickman et al. 2000, Martinsen et al. 2016), and Mayaro virus, that could become established in the future (Dieme et al. 2020). However, by characterizing seasonal changes in host-seeking, reproductive activity, and population abundance, as well as the specific environmental cues that signal these phenological changes, we can predict when *Anopheles* mosquitoes are biting and potentially transmitting pathogens. Although previous studies have characterized seasonal changes in abundance and reproductive activity of *Anopheles* mosquitoes, our study was the first to characterize these changes of *Anopheles* mosquitoes in the Midwest, an entirely temperate region of the United States wherein all *Anopheles* mosquitoes undergo reproductive dormancy during winter months (Wallace and Grimstad 2002). Our study not only allowed us to estimate when *Anopheles* mosquitoes initiated and terminated reproductive activity in central Ohio in 2022, but also revealed that *Anopheles* begin reproducing and host-seeking well before mosquito management efforts begin. Moreover, our study was the first to identify distinct seasonal changes in egg follicle lengths of female *Anopheles* that are independent of diapause state.

While our study revealed important information on the seasonal characteristics of *Anopheles* mosquitoes, our understanding of diapause in *Anopheles* mosquitoes will further benefit from additional research. Future studies should aim to determine the specific temperature and photoperiod necessary to initiate and terminate diapause, as well as how changes in environmental conditions other than temperature and photoperiod influence diapause initiation and termination time in *Anopheles* mosquitoes. Additionally, studies should evaluate the extent to which we can accurately use changes in environmental conditions to predict when and where *Anopheles* will be most abundant. Moreover, further research is needed to determine which *Anopheles* species undergo gonotrophic dissociation during diapause as these species are likely to continue to bite and transmit pathogens throughout the fall and winter. Future work should also compare the frequency at which diapausing and nondiapausing *Anopheles* species consume blood so that researchers can evaluate the relative contributions of these factors on seasonal cycles of pathogen transmission.

Overall, our study generated several novel findings that have important implications for mosquito control and pathogen transmission, while also laying the foundation for additional areas of research by identifying gaps in our scientific understanding. By measuring multiple aspects of reproductive development, our study revealed that egg follicle length is not a reliable indicator of diapause in *Anopheles* mosquitoes. Additionally, we found that a small proportion of *An. punctipennis*, *An. perplexens*, and *An. quadrimaculatus* continued to bite and reproduce into early November 2021 and that females of these species terminated reproductive dormancy by mid-March 2022. Our findings, along with those of future studies, will not only broaden our understanding of diapause in *Anopheles* mosquitoes, and therefore allow us better predict seasonal patterns in the abundance and behavior of these species, but will also provide necessary information for mosquito management programs to minimize the risk of *Anopheles*-borne diseases, and therefore, ultimately have the potential to protect human health.

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Author Contributions

Hannah Dehus (Conceptualization [Lead], Data curation [Lead], Formal analysis [Equal], Investigation [Lead], Methodology [Equal], Project administration [Lead], Validation [Equal], Visualization [Lead], Writing—original draft [Lead], Writing—review & editing [Equal]), Alden Siperstein (Conceptualization [Equal], Formal analysis [Supporting], Funding acquisition [Equal], Investigation [Supporting], Methodology [Equal], Project administration [Supporting], Resources [Equal], Validation [Equal], Visualization [Supporting], Writing—review & editing [Equal]), Laura Pomeroy (Data curation [Supporting], Formal analysis [Equal], Funding acquisition [Equal], Methodology [Equal], Resources [Equal], Software [Lead], Validation [Equal], Writing—review & editing [Equal]), and Megan Meuti (Conceptualization [Equal], Funding acquisition [Lead], Methodology [Equal], Resources [Lead], Supervision [Lead], Validation [Equal], Visualization [Supporting], Writing—review & editing [Equal])

Supplementary Material

Supplementary material is available at *Journal of Medical Entomology* online.

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