

Overwintering Biology of *Culex* (Diptera: Culicidae) Mosquitoes in the Sacramento Valley of California

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ABSTRACT At temperate latitudes, *Culex* (Diptera: Culicidae) mosquitoes typically overwinter as adult females in reproductive arrest and also may serve as reservoir hosts for arboviruses when cold temperatures arrest viral replication. To evaluate their role in the persistence of West Nile virus (WNV) in the Sacramento Valley of California, the induction and termination of diapause were investigated for members of the *Culex pipiens* (L.) complex, *Culex tarsalis* Coquillett, and *Culex stigmatosoma* Dyar under field, seminatural, and experimental conditions. All *Culex* spp. remained vagile throughout winter, enabling the collection of 3,174 females and 1,706 males from diverse habitats during the winters of 2010–2012. Overwintering strategies included both quiescence and diapause. In addition, *Cx. pipiens* form molestus Forskäl females remained reproductively active in both underground and aboveground habitats. Some blood-fed, gravid, and parous *Cx. tarsalis* and *Cx. pipiens* complex females were collected throughout the winter period. Under both field and experimental conditions, *Cx. tarsalis* and *Cx. stigmatosoma* females exposed to autumnal conditions arrested primary follicular maturation at previtellogenic stage I, with primary to secondary follicular ratios <1.5 (indicative of a hormonally induced diapause). In contrast, most *Cx. pipiens* complex females did not enter reproductive diapause and ovarian follicles matured to ≥stage I–II (host-seeking arrest) or were found in various stages of degeneration. Diapause was initiated in the majority of *Cx. tarsalis* and *Cx. stigmatosoma* females by mid-late October and was terminated after the winter solstice, but host-seeking seemed limited by temperature. An accrual of 97.52 ± 30.7 and 162.85 ± 79.3 degree-days after the winter solstice was estimated to be necessary for diapause termination in *Cx. tarsalis* under field and seminatural conditions, respectively. An increase in the proportion of blood-fed *Culex* females in resting collections occurred concurrently with diapause termination in field populations based on ovarian morphometrics. WNV RNA was detected in one pool of 18 males and in a single blood-fed female *Cx. tarsalis* collected during winter. Therefore, both vertically and horizontally infected *Culex* females may persist through winter and possibly transmit WNV after diapause termination in late winter or early spring in the Sacramento Valley of California.

KEY WORDS diapause, quiescence, West Nile virus, degree-day, molestus

West Nile virus (WNV, *Flaviviridae: Flavivirus*) invaded California in 2003 and then overwintered successfully and amplified to epidemic levels during 2004 (Reisen et al. 2006b). Viral infection of the primary vectors is one mechanism proposed for arbovirus persistence in temperate climates where temperatures become too cold for virus replication and the primary vectors enter dormancy (Reeves 1961, Rosen 1987, Reisen et al. 2006b, Reisen and Brault 2007). *Culex* (Diptera: Culicidae) mosquitoes are the primary summer vectors of WNV when there is active horizontal

transmission between birds and mosquitoes, and they also may serve as overwintering reservoir hosts. This hypothesis is supported by the finding of infected *Culex* mosquitoes during winter (Nasci et al. 2001, Bugbee and Forte 2004, Farajollahi et al. 2005, Andreadis et al. 2010).

Short daylength and cooling water temperature experienced by the immature stages induce diapause in adult *Culex* females of some species (Eldridge 1987). Emerging females that are programmed for diapause typically develop hypertrophy of the fat body (Schaefer and Washino 1970), arrest hormonal secretions to retain ovarian follicles at the prereesting stage (Spielman and Wong 1973b, Skultab and Eldridge 1985, Reisen et al. 1986b), and seek protected hibernacula (Mitchell 1979, Farajollahi et al. 2005). Because *Culex* females that enter diapause purportedly do not feed on blood (Mitchell and Briegel 1989) and the majority of diapausing females collected from hiber-

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naacula are nulliparous (Mitchell 1979, Vinogradova 2000), the most likely means by which a diapausing female may become infected with WNV is through vertical transmission, that is, the passage of the virus from an infected female to progeny destined for diapause. In agreement, WNV has been detected in nulliparous overwintering *Culex* mosquitoes in North America (Nasci et al. 2001, Bugbee and Forte 2004, Farajollahi et al. 2005), although none have been found in California. As proof of principle in support of the vertical infection hypothesis, Anderson and Main (2006) demonstrated that naturally infected *Culex pipiens* L. females could transmit WNV vertically to their F₁ progeny, which can then overwinter and horizontally transmit virus after diapause termination. In contrast, *Culex* mosquitoes that undergo quiescence (i.e., a period of temperature-induced inactivity not requiring a preparatory phase) or remain gonotrophic may acquire virus either by taking an infectious bloodmeal or by becoming infected vertically.

The overwintering strategies in *Culex* mosquitoes seem to differ with respect to latitude, and therefore temperature or photoperiod (Reisen 1995). Throughout California, *Culex tarsalis* Coquillett overwinter as inseminated adult females in reproductive diapause; however, a few blood-fed, gravid, and parous individuals can be collected during winter in the south, indicating that field populations may represent assemblages of diapausing and nondiapausing forms (Bellamy and Reeves 1963, Kliewer et al. 1969, Reisen et al. 1986a). Another competent vector of WNV, *Culex stigmatosoma* Dyar, has not been extensively studied, but undergoes diapause in Oregon (Skultab and Eldridge 1985). The southern member of the *Cx. pipiens* complex, *Culex quinquefasciatus* Say, does not enter diapause, but rather may overwinter in a temperature-induced quiescence (Eldridge 1968, Reisen et al. 1986a) or remain gonotrophically active throughout the winter in underground habitats (Su et al. 2003, Kluh et al. 2005). Although the overwintering strategies of the northern member of the *Cx. pipiens* complex, *Cx. pipiens*, has not been extensively studied in California, they apparently undergo diapause in the eastern United States (Eldridge 1968, Spielman and Wong 1973b, Spielman 2001) and Oregon (Eldridge 1987). In addition, *Cx. pipiens* exists as two identical morphological forms that exhibit distinct behavioral and physiological characteristics. *Cx. pipiens* form molestus Forskål are autogenous (oviposit the first batch of eggs without a bloodmeal), stenogamous (mate within confined spaces), and remain gonotrophically active throughout the winter in underground habitats (Spielman 1964, Vinogradova 2000, Spielman 2001). In contrast, *Cx. pipiens* f. *pipiens* require a bloodmeal to initiate egg development (anautoyeny), mate at swarms in open areas (eurygamous), and undergo reproductive diapause during winter. There is extensive genetic introgression among members of the *Cx. pipiens* complex in California (Tabachnick and Powell 1983, Urbanelli et al. 1997, Cornel et al. 2003), which may lead to variation in their purported overwintering strategies (Strickman and Fonseca 2012).

The termination of diapause in *Culex* has been attributed to temperature accrual after the winter solstice (Kliewer et al. 1969; Reisen et al. 1986b, 1995). These environmental changes are thought to terminate hormonal constraints on ovarian development, gradually initiate vitellogenesis to the host-seeking stage I-II, and stimulate host-seeking avidity (Spielman 1974, Mitchell 1981). Previous studies evaluating diapause termination were conducted at southern latitudes where termination occurred immediately at or after the winter solstice (Reisen et al. 1995), precluding evaluation of the effects of cool temperatures on termination. In addition, at northern latitudes, diapause termination has been correlated with changes in soil temperature during spring (Bennington et al. 1958, Bennington and Sherman 1960, Bellamy and Reeves 1963), when females were found to emerge from diapause and seek a bloodmeal (Mitchell 1979). Further studies are needed to delineate the relative contributions of temperature and photoperiod to the termination of diapause in *Culex* females.

Our research addressed the overarching hypothesis that WNV overwinters within infected *Culex* females and that the mechanism, effectiveness, and timing of this overwintering is strongly linked with the winter biology of the vector species. The present work addressed this hypothesis by 1) comparing the overwintering strategies of members of the *Cx. pipiens* complex, *Cx. tarsalis* and *Cx. stigmatosoma*, vectors of WNV in the Sacramento Valley; 2) delineating the environmental cues necessary for the onset, maintenance, termination of diapause or quiescence, and the onset of blood feeding in the field; and 3) attempting to detect WNV RNA in natural populations of overwintering *Culex* mosquitoes. The initiation of gonotrophic activity by *Culex* mosquitoes after winter dormancy determines the earliest date for potential mosquito-borne encephalitis virus transmission (Reisen et al. 2006b), and therefore, forecasting the termination of diapause or quiescence would signify the potential onset of the virus transmission season and inform the timing necessary for vector control.

Materials and Methods

Field Areas. Study sites were chosen at various habitats in Sacramento and Yolo Counties of the Sacramento Valley of California during 2010–2012. Sites included rural, suburban, and urban locations known to support populations of *Culex* mosquitoes and recent WNV activity, as detected by positive mosquito pools during summer surveillance. Two sites were sampled in Yolo County during both winters, including one rural site at a farmstead supporting a large stand of Eucalyptus trees that was ≈4 km NE Davis (38.603 N, –121.711 W) and ≈3 km W of the Yolo bypass of the Sacramento River, a large rice-farming area. Most of the overwintering mosquitoes collected from the farmstead most likely came from the bypass, as rice fields flooded for fall waterfowl provided ideal habitat for larval *Culex*. In addition, this site was home to a large nesting colony of herons and egrets and had a

history of WNV activity (Reisen et al. 2009). The second site in Yolo County was a suburban residence in Woodland (38.673 N, -121.782 W) located near a cemetery where *Cx. pipiens* complex mosquitoes have been collected. During the summer of 2010 and 2011, WNV was isolated from *Culex* mosquitoes collected from a park \approx 1 km from collection sites in Woodland. In Sacramento County, a rural site \approx 30 km SE of Sacramento near the town of Wilton surrounded by small cattle and horse ranches was sampled during the winter of 2011–2012. This residence (38.385 N, -121.219 W) had numerous WNV-positive mosquitoes from July to September 2011. A suburban residence in north Sacramento (38.661 N, -121.447 W), with dense foliage and a woodpile known to harbor resting *Culex* mosquitoes, was sampled during both winters. WNV was detected in summer populations of *Cx. pipiens* complex females at this site in 2010. Urban locations, including manholes in Old Sacramento (38.584 N, -121.504 W) and downtown Sacramento (38.585 N, -121.491 W), and catch basins in downtown Sacramento (38.577 N, -121.496 W) and the Sacramento Zoo (38.540 N, -121.505 W), were found to harbor *Cx. pipiens* complex mosquitoes. These locations were sampled during both winters, but collections were intermittent because of mosquito control operations. In addition, during 2011–2012, collections of overwintering *Culex* females were attempted from Laguna Creek in Elk Grove (38.418 N, -121.357 W), a location identified as a focus of both horizontal and vertical virus transmission (Fechter-Leggett et al. 2012) during the 2011 season. After repeated attempts, no resting *Culex* adults were collected. However, *Culex* larvae were collected from Laguna Creek and used in experiments in the semi-natural enclosures, described further.

Field Collections. *Culex* mosquitoes were collected by using a handheld aspirator (Ryobi 18 Volt One+ Hand Vac, One World Technologies, Inc., Anderson, SC) on a biweekly basis from October until 21 December (the winter solstice) and then weekly until most females had terminated diapause. Collections were made during daylight hours from various resting sites, including woodpiles, sheds, barns, large resting boxes (Meyer 1987), foliage, storm drains, manholes, and other locations in and around people's homes. After collection, mosquitoes were immediately transported to the laboratory, where they were anesthetized, identified to species, and enumerated under a dissecting microscope. All samples were frozen at -80°C until dissection. For each sampling occasion, up to 25 *Culex* females of each species were dissected to determine diapause status. Both dissected and undissected whole mosquitoes were sorted into pools of ≤ 50 mosquitoes according to sex, species, location, gonotrophic status, and date, and stored at -80°C until later virus testing. Additional females were saved for microsatellite analysis as part of a larger study that genetically characterized *Cx. pipiens* complex populations throughout California (Kothera et al. 2013).

Seminatural Studies. To delineate diapause induction and termination cues used by cohorts of *Culex*

mosquitoes of known age and emergence site, a semi-natural outdoor enclosure (96 by 46 by 96 cm) was constructed from plywood to house overwintering *Culex* during the winters (October–March) of 2010–2012. This “mosquito house” was elevated from the ground by using 50-cm 2-by-4 wooden supports, and each was moated with water to prevent ant infestation. The house was naturally illuminated through Plexiglas windows, exposed to outdoor temperatures by using window screen on two opposing sides of the enclosure, and equipped with plastic roofing to deflect rainfall. Mosquito houses were located in Davis, CA (38.521 N, -121.755 W), during 2010–2011 and in Woodland, CA (38.673 N, -121.782 W), during winter 2011–2012, and positioned under vegetation to simulate natural resting sites.

Gravid, blood-fed, or both *Culex* females were collected from natural populations in the Sacramento Valley just before the autumnal equinox (23 September) and into October, separated by species, and placed into cartons with water for oviposition. Eggs were hatched and immatures reared under natural conditions inside or adjacent to the mosquito house. During 2011–2012, *Cx. pipiens* egg rafts from Shasta County were included in the mosquito house as a *Cx. pipiens* diapause control. All larvae were reared in 50.8 by 38.1 by 12.7-cm (length \times width \times height) dark-colored pans containing 2 gallons (7.6 liters) of water and fed a 1:1 by volume mixture of rabbit chow and fish food, as needed, until pupation. Emerging adults were partitioned into cohorts based on species and emergence date. Weather loggers (UA-002-08 HOBO Pendant, Onset Computer Corp., Bourne, MA) recorded both water and ambient conditions inside the mosquito house. Adult mosquitoes were held in 1-gallon (17.0 by 16.8 [diameter \times height] cm) paper cartons covered with window screen and offered water-moistened cotton balls and Craisins (Ocean Spray Cranberries Inc., Lakeville–Middleboro, MA) for the duration of the overwintering period. Biweekly, 10–15 females from each cohort were dissected to track changes in the morphology of the ovarian follicles. Only 5–10 females were dissected from cohorts with < 100 females. After the winter solstice, females were sampled on a weekly basis until the deposition of yolk granules (stage I–II to IIb) in the majority of primary follicles indicated the termination of diapause.

Environmental Chamber Studies. To evaluate the genetic potential for diapause induction, a subsample of *Cx. pipiens* complex larvae from Woodland, Elk Grove, Old Sacramento, and Shasta County; and *Cx. tarsalis* larvae from Davis and Elk Grove collected for seminatural studies in 2011–2012 (previously described) were transferred to a bioenvironmental chamber and reared under midwinter diapause conditions of 10:14 (light:dark [L:D]) h and 16°C , known to induce diapause in *Cx. tarsalis* (Reisen et al. 1986b). Larvae that were third instars or smaller were reared in 21 by 12-cm (height \times diameter) “Mosquito Breed-ers” (BioQuip Products Co., Gardena, CA) and fed as previously described. Emerging mosquitoes were held on water-moistened cotton balls and Craisins. Females

were removed at 21 d postemergence and frozen at -80°C until dissection.

Dissection Protocol. Ovarian dissection procedures were modified from Giglioli (1963). The ovaries were excised with forceps into a drop of distilled water under a dissecting microscope. One ovary was rinsed and dried on a template microscope slide and viewed under a phase-contrast compound microscope at $100\times$ to determine parity by examining coiling of the ovarian tracheoles (Detinova 1962). The second ovary was placed in a small drop of 1:1 of Gentian Violet and physiological saline (0.9%) under a dissecting microscope. At $40\times$ magnification, two pin vises holding 0.15-mm minutin pins were used to separate the ovary into its individual ovarioles. Individual follicles were examined under a compound microscope at $200\text{--}400\times$. Primary follicles were classified morphologically by size and the degree of vitellogenesis in the most mature follicles (Kawai 1969, Clements and Boocock 1984). For unfed and nulliparous females, the lengths of five representative primary and secondary follicles were measured by using a compound microscope at $200\times$. The length of the primary follicle was measured from the base of the ovariole to the joining of the secondary follicle. The length of the secondary follicle was then measured to the distal tip of the germarium. The primary follicle length and the ratio of the primary to secondary follicle length were used to morphologically determine diapause status (Spielman and Wong 1973a, Reisen et al. 1986b). Fully degenerated or autogenously developed follicles (>stage II) and the follicles of parous females were not measured. The number of dilatations was determined for parous females by using the methods of Polovodova (1949).

Follicles in diapausing mosquitoes were at stage I (stages N-Ib of Kawai (1969)), whereas anautogenous females in host-seeking arrest were at stage II (stage I-II, IIa, b). For experimental studies where females were not blood-fed, females with follicles at stages III-V, and therefore developed past resting stage II, were considered autogenous regardless of insemination status. For field collections, spermathecae were removed, crushed by using forceps in a drop of distilled water, and spermatozoa visualized under a compound microscope at $400\times$ to determine insemination. Only inseminated females without a bloodmeal and with follicles matured to stages III-IV and up to stage V if noninseminated were considered to be autogenous.

Virus Diagnostics. All viral assays were done under BSL-3 conditions at the University of California, Davis, Center for Vectorborne Diseases laboratory, in accordance with BUA protocol 0873 approved by Environmental Health and Safety of the University of California, Davis. Mosquito pools containing two 5-mm glass beads and 1.0 ml of mosquito diluent (Dulbecco's modified eagle medium, containing 5% penicillin, 0.4% amphotericin, and 20% fetal bovine serum) were homogenized by mixer mill (MM300, Retsch; Haan, Germany) and total RNA extracted by using a MagMAX Express-96 system following manufacturer protocols

(Life Technologies: Applied Biosystems (ABI); Carlsbad, CA). Each extraction plate contained a positive control well generated from cultured virus of known titer (PFU/ml) and at least two negative control wells (mosquito diluent). RNA was analyzed for the presence and quantity of WNV RNA by TaqMan One-Step (ABI) and SensiFAST Probe Lo-ROX (Bioline USA Inc., Taunton, MA) real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) by using an ABI ViiA 7 real-time PCR platform. Initial screening was done by using the TaqMan assay per manufacturer protocols in singleplex. Plates containing positive samples were retested by using the SensiFAST Probe Lo-ROX assay per manufacturer protocols in duplex (two primer per probe sets). The singleplex reaction used primers per probe specific for the envelope region of the viral genome (WN1) (Lancioti et al. 2000): (forward) 5'-TCA GCG ATC TCT CCA CCA AAG -3', (reverse) 5'-GGG TCA GCA CGT TTG TCA TTG -3', and (probe) 6FAM-TGC CCG ACC ATG GGA GAA GCT -BHQ-1. The duplex reaction included primers per probes for WN1 and the nonstructural (NS1) region of the viral genome (WN2) (Shi and Kramer 2003): (forward) 5'-GGC AGT TCT GGG TGA AGT CAA -3', (reverse) 5'-CTC CGA TTG TGA TTG CTT CGT -3', and (probe) Quasar670-TGT ACG TGG CCT GAG ACG CAT ACC TTG T -BHQ-2. Samples with a cycle threshold (Ct) score <40 were considered positive. All qRT-PCR plates contained standards generated from a 10-fold dilution series of cultured virus of known titer (PFU/ml) and negative water controls.

Positive mosquito pools also were tested for infectious WNV by plaque assay by using Vero cells before and after passage in C6/36 *Aedes albopictus* Skuse cells. Two hundred microliters from each sample was added to a 25-cm² tissue culture flask containing 70% confluent C6/36 cells from which all but 2.0 ml of tissue culture media (Dulbecco's modified eagle medium, containing 10% fetal bovine serum and 0.2% Primocin) had been removed. An additional 2.0 ml of culture media was added after an absorption period of 1 h at 28°C and 5% carbon dioxide. After 7 d incubation, the tissue culture media was collected and tested for the presence of infectious virus by Vero cell plaque assay.

Statistical Analysis. The lengths of five primary and secondary follicles were averaged per female and used to calculate the length of the primary follicle and the primary to secondary follicular ratio for each female. Values per female were averaged by collection week with 95% CI calculated for all empty nulliparous females per species. Ovarian maturation stage also was averaged for all nulliparous (including autogenous) *Culex* females by collection week with 95% CI. Data on follicle stage, primary follicle length, and follicular ratio from dissected *Culex* females were tested for significant effects among species, seasons (October-December and January-March), and years (2010-2011 and 2011-2012) by general linear model-analysis of variance. Tukey's honestly significant difference (HSD) multiple-comparison tests were used to deter-

Table 1. Number of male and female *Culex* species mosquitoes collected, pooled, and tested for WNV RNA, including adults reared from field-collected larvae, during the winters (October–February) of 2010–2012 from the Sacramento Valley, CA

Year	<i>Culex</i> species	Method	Sex	No. collected ^a	No. tested	No. pools
2010–2011	<i>pipiens</i> complex	Vacuum	M	714	667	23
		Vacuum	F	896	746	45
		Rear	M		20	1
	<i>stigmatosoma</i>	Rear	F		535	12
		Vacuum	M	1	0	0
		Vacuum	F	11	11	7
	<i>tarsalis</i>	Vacuum	M	274	218	9
		Vacuum	F	467	486	37
		Rear	M		19	1
		Rear	F		111	5
2011–2012	<i>pipiens</i> complex	Vacuum	M	334	288	18
		Vacuum	F	1,055	933	98
		Rear	M		870	31
	<i>stigmatosoma</i>	Rear	F		1,157	48
		Vacuum	M	22	20	1
		Vacuum	F	44	40	11
	<i>tarsalis</i>	Rear	M		2	2
		Rear	F		22	7
		Vacuum	M	361	341	19 ^b
		Vacuum	F	701	590	69 ^c
<i>Culex</i> spp.	Rear	M		60	11	
	Rear	F		378	27	
	Rear	M		45	2	
Totals				4,880	7,559	484

^a Wild-caught adults collected by vacuum aspiration from various resting sites.

^b One pool positive by qRT-PCR, virus isolation negative.

^c One pool positive by qRT-PCR, virus isolation negative.

mine which means were significantly different from one another at the 0.05 significance level. Diapause induction and termination rates were estimated for each overwintering *Culex* species by dividing the number of diapausing and nondiapausing females, respectively, by the total number of unengorged nulliparous females collected for each collection week. Diapause induction or termination dates were delineated when >50% of the females dissected had entered or terminated diapause, respectively. Temperature data generated by using the Terrestrial Observation and Prediction System provided by the Ecological Forecasting Lab at NASA Ames Research Center for Sacramento and Yolo Counties were used to calculate cumulative degree-days (DD) in field studies and chart mean monthly values for maximum and minimum air temperatures. Temperature data gathered from a weather station at the University of California, Davis (38.533 N, –121.783 W), and downloaded from the University of California Integrated Pest Management Web site (<http://www.ipm.ucdavis.edu>) was used to backfill missing temperature values. Because average daily maximum and minimum air temperatures from Terrestrial Observation and Prediction System and the Davis weather station were highly correlated, but the means were significantly different (paired *t*-test, $P < 0.001$), backfilled values were adjusted by using the regression functions (maximum, $y = 0.987x + 0.616$; minimum, $y = 0.980x + 0.584$), as these regressions provided good fits for the data (maximum, $R^2 = 0.99$; minimum, $R^2 = 0.94$). Temperature data from the weather logger inside the mosquito house were used to calculate cumulative DD in seminatural studies. DD values were calculated by

using the horizontal cutoff method, single sine model, and the winter solstice as the summation start date by using methods available at the Integrated Pest Management Web site. A lower threshold of 8°C was chosen as the minimum developmental point estimate for *Cx. tarsalis* based on previous laboratory studies (Reisen et al. 1992). All statistical analyses were done by using Minitab 15 (Minitab Statistical Software, Inc., State College, PA, 2007).

Results

Mosquito Collections and WNV Detection. The number of *Cx. tarsalis*, *Cx. stigmatosoma*, and *Cx. pipiens* complex mosquitoes collected, pooled, and tested for WNV RNA during the winter periods of 2010–2011 and 2011–2012 are summarized in Table 1. Overall, 3,174 *Culex* females and 1,706 *Culex* males were collected resting at study areas in the Sacramento Valley. Predominance of *Cx. pipiens* complex mosquitoes reflected the urban nature of some of the productive sampling sites in the city of Sacramento. Mosquitoes remained vagile throughout the winter, and resting sites were repopulated rapidly after removal sampling by vacuum aspiration. In addition, 5,009 *Culex* females and 2,550 *Culex* males were pooled and tested for WNV RNA, including adults reared from field-collected larvae (Table 1). Few mosquitoes tested positive. WNV RNA was detected by qRT-PCR in a pool containing 18 *Cx. tarsalis* males and in a single blood-fed *Cx. tarsalis* female. The pool of 18 *Cx. tarsalis* males was collected from the farmstead at Davis on 18 November 2011, had a screening WN1 Ct score of 36.8, and was confirmed by using WN2 with a Ct score of

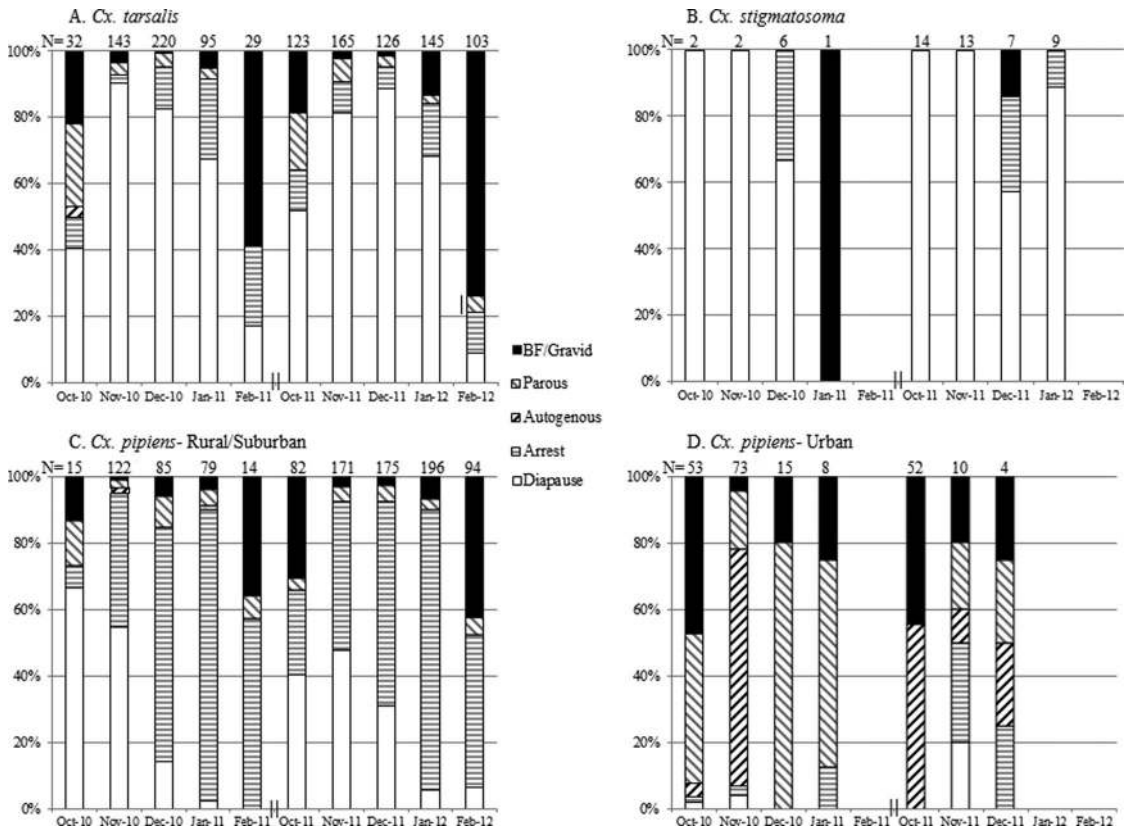


Fig. 1. Percentage of total female (A) *Cx. tarsalis*, (B) *Cx. stigmatosoma*, (C) rural and suburban *Cx. pipiens*, and (D) urban *Cx. pipiens* that were collected diapausing, in host-seeking arrest, autogenous, unfed and parous, and blood-fed/gravid from the Sacramento Valley, CA.

38.0. The blood-fed *Cx. tarsalis* female was collected from a suburban residence in North Sacramento on 25 January 2012, had a screening Ct score of 37.2, but did not confirm with WN2. Infectious virus was not isolated from either pool after three passages in C6/36 cells, followed by Vero-cell plaque assay.

Most *Culex* females collected during winter were inseminated, with 91.9% (679/739) and 94.7% (1,351/1,426) of females containing spermatozoa in their spermathecae during 2010–2011 and 2011–2012, respectively. Although male abundance during winter declined by December, one *Cx. pipiens* male and five *Cx. tarsalis* males were collected during January. These findings agreed with previous studies (Bellamy and Reeves 1963, Farajollahi 2005) and indicated that males typically do not survive the winter at northern latitudes.

Field Studies. The physiological status of overwintering *Culex* females collected from the Sacramento Valley during 2010–2012 is summarized by month in Fig. 1. Females were grouped into five overwintering classes: diapause, no yolk granules apparent in the primary follicles (stage I); arrest, yolk granules apparent, primary follicles in various stages of degeneration, or both (stage II); autogenous, inseminated females without a bloodmeal and with follicles matured to

stages III–IV or up to stage V if noninseminated; parous, uncoiled ovarian tracheoles and follicles with one or more dilatations; and blood-fed or gravid, all stages of blood digestion up to no visible bloodmeal with follicles at stage V and inseminated. In addition, for each nulliparous unengorged (stage I–II) *Culex* female, stages of follicular development, the primary follicle length, and the primary to secondary follicular ratio were evaluated for each species, sampling season, and sampling year (Fig. 2; Table 2). Only follicular stages of development were recorded for autogenous females.

Diapause Induction. More than 50% of *Cx. tarsalis* females had entered reproductive diapause when collected on 5 October 2010 (10.6°C minimum, 23.9°C maximum, 11.6 h daylength) and 12 October 2011 (11.8°C minimum, 26.9°C maximum, 11.3 h daylength). Similarly, the first *Cx. stigmatosoma* females in diapause were collected on 20 October 2010 (10.7°C minimum, 24.4°C maximum, 11.0 h daylength) and 19 October 2011 (11.0°C minimum, 25.3°C maximum, 11.1 h daylength), and 100% of these had arrested development at stage I. The percentage of diapausing *Cx. tarsalis* females collected during 2010 increased from 41% (13/32) in October to 90% (129/143) in November. A similar increase occurred during 2011,

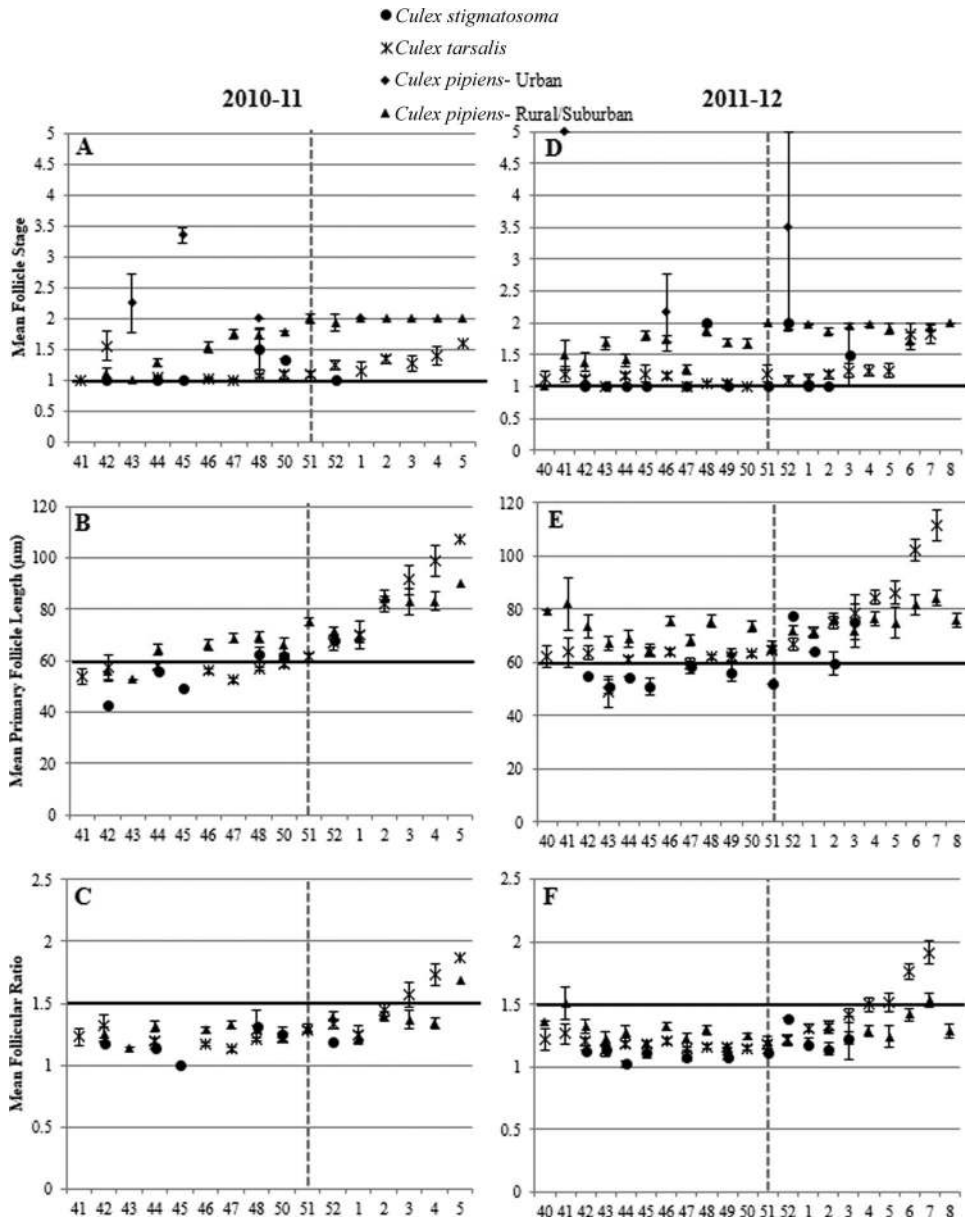


Fig. 2. Temporal changes in the mean follicle stage (A and D), mean primary follicle length (B and E), and mean follicular ratio (C and F) among field-collected overwintering *Culex* females in the Sacramento Valley, CA. Represented on the x-axis are week numbers for 2010–2011 and 2011–2012. Dark horizontal lines denote diapause thresholds, and dashed vertical lines denote the occurrence of the winter solstice.

with 52% (64/123) in diapause during October and 81% (134/165) during November. Diapause was maintained in >60% of *Cx. tarsalis* females through January during both winters, but declined to <20% by February. The renewal of blood feeding at collection sites in February during both winters (>50%) was concurrent with diapause termination in *Cx. tarsalis* based on ovarian morphometrics (Fig. 1). Although the majority of females entered and maintained a reproductive diapause, blood-fed, gravid, and parous females were collected during every winter month.

Although relatively few females were collected, similar trends were observed in *Cx. stigmatosoma*, with diapausing females collected in October and November during both winters. The percentage of diapausing *Cx. stigmatosoma* in December was 67% (4/6) and 57% (4/7) for 2010 and 2011, respectively. Only one blood-fed *Cx. stigmatosoma* female was collected in January 2011, whereas 89% (8/9) of females were still in diapause during January 2012. No females were collected in February during both winters.

Table 2. Reproductive status of *Cx. tarsalis*, *Cx. stigmatosoma*, and rural and suburban and urban *Cx. pipiens* complex females collected during the overwintering period in the Sacramento Valley, CA, from 2010 to 2012

Species	<i>Cx. tarsalis</i>		<i>Cx. stigmatosoma</i>		Rural and suburban <i>Cx. pipiens</i>		Urban <i>Cx. pipiens</i>	
	Oct.-Dec.	Jan.-Feb.	Oct.-Dec.	Jan.-Feb.	Oct.-Dec.	Jan.-Feb.	Oct.-Dec.	Jan.-Feb.
No. evaluated ^a	360	99	10	2010-2011	201	80	61	—
Mean follicle stage (95% CI)	1.10 (1.07-1.14)	1.30 (1.21-1.40)	1.20 (0.90-1.50)	—	1.58 (1.50-1.66)	2.00 (1.94-2.06)	3.23 (2.97-3.49)	—
No. evaluated ^b	358	99	9	—	200	79	—	—
Mean 1° follicle length, μm (95% CI)	59.2 (58.2-60.1)	88.9 (84.6-93.2)	56.5 (46.9-66.1)	—	66.5 (64.8-68.2)	81.0 (78.1-83.9)	—	—
Mean 1°/2° follicle ratio (95% CI)	1.23 (1.21-1.24)	1.55 (1.48-1.62)	1.20 (1.07-1.33)	—	1.30 (1.27-1.33)	1.37 (1.33-1.42)	—	—
No. evaluated ^a	349	144	33	2011-2012	374	225	37	—
Mean follicle stage (95% CI)	1.11 (1.08-1.15)	1.25 (1.18-1.32)	1.06 (0.97-1.15)	9	1.69 (1.64-1.73)	1.92 (1.89-1.96)	4.46 (4.04-4.88)	—
No. evaluated ^b	342	142	32	9	344	193	—	—
Mean 1° follicle length, μm (95% CI)	62.5 (61.4-63.6)	80.2 (77.4-82.9)	54.0 (50.8-57.2)	63.6 (54.3-73.0)	70.5 (69.1-71.9)	75.9 (74.0-77.9)	—	—
Mean 1°/2° follicle ratio (95% CI)	1.18 (1.17-1.20)	1.43 (1.39-1.47)	1.12 (1.08-1.16)	1.16 (1.06-1.27)	1.25 (1.23-1.27)	1.31 (1.27-1.34)	—	—

^a The mean follicle stage was evaluated for nulliparous unengorged females.
^b Autogenous females and those with degenerated follicles were not measured.

In contrast, *Cx. pipiens* complex females collected from rural and suburban locations did not maintain a reproductive diapause during either winter, with >50% of females remaining at arrest or gonotrophically active during November and >70% during December. In addition, blood-fed, gravid, and parous females were collected in every month during winter. Urban autogenous populations of *Cx. pipiens* mosquitoes were discovered resting in manholes and catch basins in urban Sacramento. These populations remained gonotrophically active throughout winter, with autogenous, blood-fed, gravid, and parous females collected through January 2011 and December 2012. Interestingly, mosquitoes disappeared from these collection sites during late winter.

Oviparity. *Culex* females that were parous by the ovarian tracheation method (Detinova 1962) were confirmed by examining follicular dilatations (Polovodova 1949). Only empty females with one or more dilatations were recorded as parous. The majority of parous females collected during the winter season were uniparous, showing only one follicular dilatation. During the 2010-2011 and 2011-2012 winter seasons, only one *Cx. tarsalis* and two *Cx. pipiens* complex females and two *Cx. tarsalis* and four *Cx. pipiens* complex females were multiparous, respectively, each having two dilatations. During both fall and winter periods, parity rates for *Cx. tarsalis* were highest in October (25% [8/32] and 17% [21/123], respectively), but declined to <7% during November through February (Fig. 1). In suburban and rural locations, *Cx. pipiens* complex females were mostly nulliparous, with parity rates ranging from 2 to 15%. In contrast, 36% (54/149) of unfed and nongravid females were parous when collected from urban areas in Sacramento during 2010-2011. Fewer parous females were collected during winter in 2011-2012 (5% [3/66]), but sample sizes were small. No parous *Cx. stigmatosoma* were collected during either winter.

Ovarian Morphometrics. Unfed *Culex* females were grouped by seasons (fall, winter) within years (2010-2011 and 2011-2012) and three ovarian metrics compared (follicle stage, primary follicle length and the ratio of primary to secondary follicles; Table 2). Mean follicle stage significantly varied between species and sampling seasons (interaction, $F = 6.39$, $df = 2$, 1878 , $P = 0.002$) and between species and sampling years ($F = 48.91$; $df = 3$, 1974 ; $P < 0.001$). Only in urban *Cx. pipiens* females was the mean follicle stage significantly different between sampling years ($F = 27.88$; $df = 1$, 96 ; $P < 0.001$), with a larger mean follicle stage observed during 2011-2012 than 2010-2011 (Table 2). During both winters, *Cx. tarsalis* females arrested follicular development at stage I (Fig. 3) and maintained this stage until late winter, when a significant increase ($F = 39.38$; $df = 1$, 949 ; $P < 0.001$) occurred after the winter solstice (Fig. 2). A similar pattern was observed in *Cx. stigmatosoma*, although no significant difference ($F = 0.20$; $df = 1$, 49 ; $P = 0.655$) was observed, as few females were collected during late winter in 2011-2012 and none were collected in 2010-2011. During both winters, the majority of rural and suburban *Cx.*

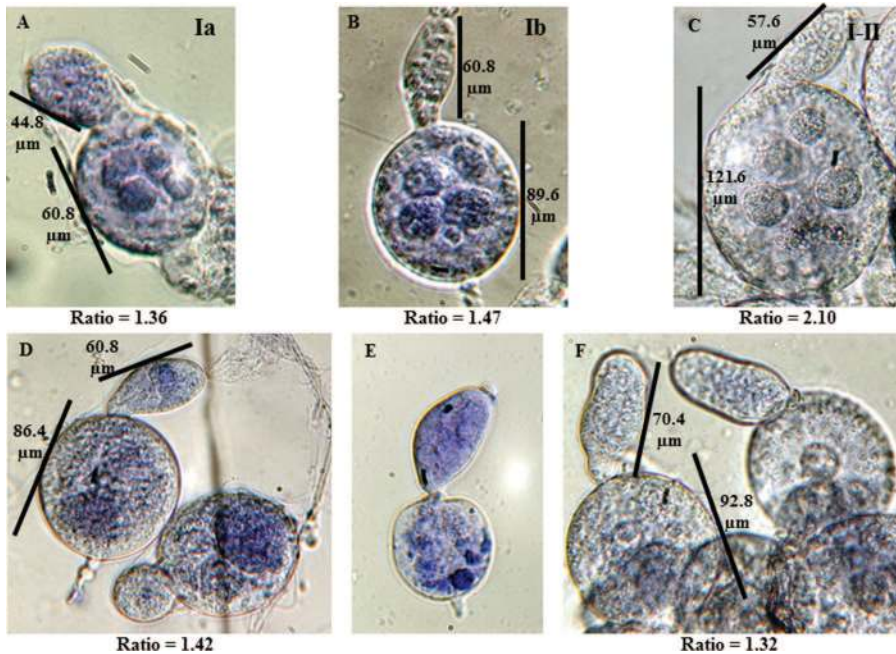


Fig. 3. Nulliparous ovarioles from diapausing stage Ia–Ib (A and B) and host-seeking arrested stage I–II (C) *Cx. tarsalis* females, and from gonotrophically active anautogenous *Cx. pipiens* complex females (D–F). Follicles D–F are in various stages of degeneration; totally degenerated follicles such as E were not measured. (Online figure in color.)

pipiens females did not arrest follicular development at diapause stage I, but proceeded to \geq stage I–II. However, there was a significant increase ($F = 88.15$; $df = 1, 877$; $P < 0.001$) in the mean follicle stage during late winter, depicting the presence of some ephemeral diapausing forms during October–December ($\approx 40\%$ [89/222 and 169/428]). For *Cx. tarsalis* and *Cx. stigmatosoma*, there were no significant differences in the mean follicle stage between species and sampling seasons (Tukey's HSD; $P > 0.05$), but the mean follicle stage was significantly larger in rural and suburban *Cx. pipiens* females than that in *Cx. tarsalis* and *Cx. stigmatosoma* (Tukey's HSD; $P < 0.001$). During October–December, the mean follicle stage in urban *Cx. pipiens* females was significantly larger than that in all other species (Tukey's HSD; $P < 0.001$), with follicles developed to $>$ stage I–II, indicating most females were autogenous (80% [49/61] and 84% [31/37]).

For mean primary follicle length, there was a significant interaction between species and sampling seasons ($F = 57.98$; $df = 2, 1801$; $P < 0.001$), but no interaction between species and sampling years. For *Cx. tarsalis* ($F = 557.69$; $df = 1, 938$; $P < 0.001$), rural and suburban *Cx. pipiens* ($F = 67.49$; $df = 1, 813$; $P < 0.001$), and *Cx. stigmatosoma* ($F = 6.34$; $df = 1, 47$; $P = 0.015$), the mean primary follicle length was significantly smaller during October–December than January–February (Table 2). No significant difference in the length of the primary follicles was observed between *Cx. tarsalis* and *Cx. stigmatosoma* during 2010–2011 (Tukey's HSD; $P > 0.05$), although follicles were significantly smaller in *Cx. stigmatosoma* during 2011–2012 (Tukey's HSD; $P < 0.001$). From October to

December, the mean primary follicle length was significantly larger in rural and suburban *Cx. pipiens* than in *Cx. tarsalis* (Tukey's HSD; $P = 0.002$) and *Cx. stigmatosoma* (Tukey's HSD; $P < 0.001$). However, during January–February, *Cx. tarsalis* had significantly larger primary follicles than rural and suburban *Cx. pipiens* (Tukey's HSD; $P < 0.001$), because primary follicles in *Cx. pipiens* females during late winter appeared to have degenerated and were shrunken and granular (Fig. 3).

For the mean follicular ratio, there was significant interaction between *Culex* species and sampling seasons ($F = 47.17$; $df = 2, 1801$; $P < 0.001$), but no interaction between species and sampling years. For *Cx. tarsalis* ($F = 324.37$; $df = 1, 938$; $P < 0.001$) and rural and suburban *Cx. pipiens* ($F = 13.97$; $df = 1, 813$; $P < 0.001$), the mean primary follicle length was significantly smaller in October–December than January–February (Table 2), but no significant difference was observed in *Cx. stigmatosoma* ($F = 0.97$; $df = 1, 47$; $P = 0.330$). For *Cx. tarsalis* and *Cx. stigmatosoma*, there was no significant difference in the mean follicular ratio during October–December (Tukey's HSD; $P > 0.05$), but ratios were significantly larger in *Cx. tarsalis* during January–February (Tukey's HSD; $P < 0.001$). Similar to mean primary follicle lengths, ratios were significantly larger in rural and suburban *Cx. pipiens* than *Cx. tarsalis* (Tukey's HSD; $P < 0.001$) and *Cx. stigmatosoma* (Tukey's HSD; $P = 0.002$) during October–December, but significantly smaller than *Cx. tarsalis* during January–February ($P < 0.001$). This resulted from shrunken primary follicles and elon-

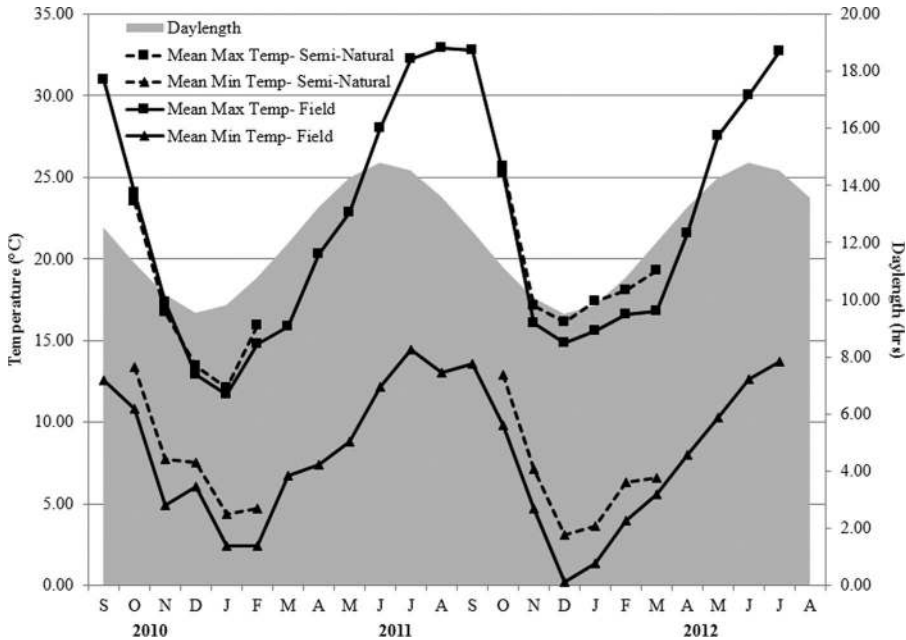


Fig. 4. Monthly mean air temperatures (max and min) for field and seminatural studies (within mosquito house) in the Sacramento Valley, CA, during 2010–2012. Air temperatures for seminatural studies were collected from October to February in 2010–2011 and October to March in 2011–2012. Also shown is daylength in hours.

gated secondary follicles (Fig. 3) in *Cx. pipiens* during late winter.

Diapause Termination. More than 50% of *Cx. tarsalis* females had terminated diapause when collected on 2 February 2011 (1.1°C minimum, 16.2°C maximum, 10.3 h daylength) and 8 February 2012 (5.7°C minimum, 17.9°C maximum, 10.5 h daylength). Mean monthly values for maximum and minimum air temperatures and daylength hours are shown in Fig. 4. During 2010–2011, the median termination of *Cx. tarsalis* diapause on 2 February 2011 corresponded to an accumulation of 66.8 DD, whereas during 2011–2012, emergence on 8 February 2012 corresponded to 128.2 DD. The average accumulation of DDs combining both sampling periods was 97.52 ± 30.7 DD. Because few *Cx. stigmatosoma* females were collected during late winter, diapause termination could not be evaluated. Anautogenous *Cx. pipiens* females did not enter diapause in the Sacramento Valley, but the proportion of blood-fed and gravid females increased in accordance with diapause termination in *Cx. tarsalis*. The number of blood-fed and gravid *Cx. pipiens* complex females increased from 7% (1/14) on 29 December to 36% (5/14) on 2 February during 2010–2011 and from 3% (1/35) on 28 December to 42% (10/24) on 8 February during 2011–2012.

Seminatural Studies. As in field studies, stages of follicular development, the primary follicle length, and the primary to secondary follicular ratio were evaluated for cohorts with known emergence dates of each species (Table 3; Fig. 5). *Cx. tarsalis* and *Cx. stigmatosoma* cohorts emerging under seminatural conditions in the mosquito house during mid-late Oc-

tober entered reproductive diapause. In contrast, rural and suburban *Cx. pipiens* cohorts from the Sacramento Valley progressed follicular development to host-seeking arrest (stage II) or had degenerated follicles when dissected; urban and autogenous *Cx. pipiens* complex females developed follicles to stage V. Unexpectedly, *Cx. pipiens* females from Shasta County (≈ 223 km north of Sacramento) did not uniformly arrest vitellogenesis at stage I.

Diapause Induction. During 2010, the first *Cx. tarsalis* cohort emerged on 4 October (9.8°C minimum, 21.0°C maximum, 11.4 h daylength) and 58% (7/12) of these females progressed vitellinogenesis to host-seeking arrest stage I–II by the end of October. Because the majority of these females did not enter diapause, even though they emerged after the autumnal equinox, they were removed from subsequent analyses. The remaining *Cx. tarsalis* cohorts emerged on or after 2 November (daylength ≤ 10.3 h) and 100% of these maintained reproductive diapause until termination after the winter solstice (Fig. 5). During 2011, the first *Cx. tarsalis* cohort did not emerge until 27 October (daylength, 10.5 h) and >90% of these females entered diapause compared with 100% of those emerging after 1 November. No *Cx. stigmatosoma* females were kept in the mosquito house during 2010–2011; however, a few were evaluated during 2011–2012. All *Cx. stigmatosoma* females emerged between 27 October and 1 November and 100% of females entered and remained in diapause until the winter solstice.

Ovarian Morphometrics. For mean follicle stage, there was a significant interaction between species

Table 3. Reproductive status of *Cx. tarsalis*, *Cx. stigmatosoma*, *Cx. pipiens* complex females, and *Cx. pipiens* from Shasta County reared in a mosquito house during the overwintering period in the Sacramento Valley, CA, from 2010 to 2012

Species	2010–2011													
	<i>Cx. tarsalis</i>				Rural and suburban <i>Cx. pipiens</i>				Urban <i>Cx. pipiens</i>				Shasta ^c <i>Cx. pipiens</i>	
	Oct.–Dec.	Jan.–Mar.	Jan.–Feb.	Oct.–Dec.	Jan.–Mar.	Jan.–Feb.	Oct.–Dec.	Jan.–Mar.	Jan.–Mar.	Oct.–Dec.	Jan.–Mar.	Jan.–Feb.	Oct.–Dec.	Jan.–Feb.
No. evaluated ^a	30	16	50	30	16	337	337	561	45	73	150	181	73	113
Mean follicle stage (95% CI)	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.28 (1.09–1.47)	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1.92 (1.82–2.02)	1.92 (1.16–1.71)	2.12 (2.05–2.18)	3.9 (3.64–4.18)	4.82 (4.65–5.00)	1.82 (1.76–1.88)	2.02 (1.98–2.05)	1.53 (1.42–1.65)	1.71 (1.60–1.81)
No. evaluated ^b	30	16	49	30	16	301	15	432	—	—	150	180	73	105
Mean 1 ^o follicle length, μm (95% CI)	57.0 (54.0–60.0)	50.8 (44.8–56.7)	81.1 (75.8–86.3)	57.0 (54.0–60.0)	50.8 (44.8–56.7)	73.2 (70.6–75.9)	65.3 (54.0–76.6)	82.0 (80.2–83.8)	—	—	80.8 (77.6–83.9)	97.4 (94.5–100)	67.1 (64.3–69.8)	84.5 (81.4–87.7)
Mean 1 ^o /2 ^o follicle ratio (95% CI)	1.23 (1.21–1.24)	1.15 (1.07–1.23)	1.50 (1.42–1.58)	1.23 (1.21–1.24)	1.15 (1.07–1.23)	1.34 (1.30–1.37)	1.23 (1.09–1.37)	1.45 (1.42–1.48)	—	—	1.48 (1.43–1.53)	1.61 (1.57–1.65)	1.20 (1.16–1.24)	1.41 (1.36–1.47)

^a The mean follicle stage was evaluated for all dissected females.

^b Autogenous females and those with degenerated follicles were not measured.

^c *Cx. pipiens* mosquitoes were reared from egg rafts collected in Shasta County, CA.

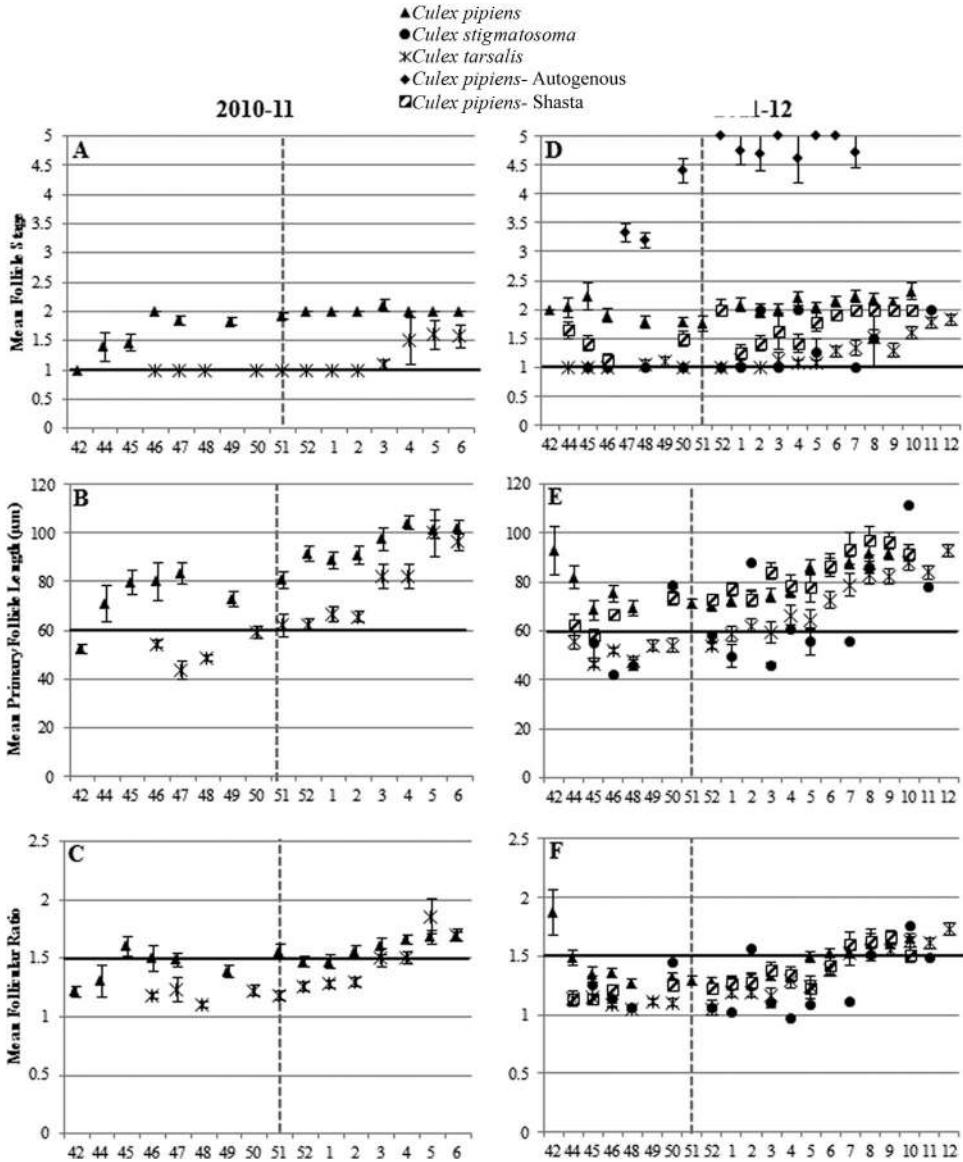


Fig. 5. Temporal changes in the mean follicle stage (A and D), mean primary follicle length (B and E), and mean follicular ratio (C and F) among field-collected *Culex* females reared and held under seminatural conditions in the mosquito house. Represented on the x-axis are week numbers for 2010–2011 and 2011–2012. Dark horizontal lines denote diapause thresholds, and dashed vertical lines denote the occurrence of the winter solstice.

and sampling seasons ($F = 7.48$; $df = 4, 1959$; $P < 0.001$), but no interaction between species (*Cx. tarsalis* and rural and suburban *Cx. pipiens* only) and sampling years. The mean follicle stage of all *Cx. tarsalis* ($F = 53.49$; $df = 1, 401$; $P < 0.001$), rural and suburban *Cx. pipiens* ($F = 20.02$; $df = 1, 1226$; $P < 0.001$), *Cx. pipiens* from Shasta County ($F = 4.60$; $df = 1, 184$; $P = 0.033$), *Cx. stigmatosoma* ($F = 11.67$; $df = 1, 30$; $P = 0.002$), and urban *Cx. pipiens* ($F = 35.10$; $df = 1, 116$; $P < 0.001$) dissected during October–December was significantly smaller than those dissected during January–March (Table 3). For *Cx. tarsalis* and *Cx. stigmatosoma*, there were no significant

differences in the mean follicle stage between species and seasons (Tukey's HSD; $P > 0.05$). For rural and suburban *Cx. pipiens*, the mean follicle stage during October–December was significantly larger than for *Cx. pipiens* from Shasta County (Tukey's HSD; $P = 0.001$), *Cx. tarsalis* (Tukey's HSD; $P < 0.001$), and *Cx. stigmatosoma* (Tukey's HSD; $P < 0.001$). The mean follicle stage in *Cx. pipiens* from Shasta County during October–December was also significantly larger than that in *Cx. tarsalis* (Tukey's HSD; $P < 0.001$) and *Cx. stigmatosoma* (Tukey's HSD; $P = 0.001$). A similar pattern was seen during January–March; however, there was no significant difference between *Cx. stig-*

matosoma (Tukey's HSD; $P > 0.05$) and *Cx. pipiens* from Shasta County (Tukey's HSD; $P > 0.05$). As in field studies, the majority of rural and suburban *Cx. pipiens* females reached \geq stage I–II during early winter (82% [123/150] in 2010 and 71% [240/337] in 2011). However, the significant increase in the mean follicle stage during late winter was related to the presence of a low number of diapausing forms during October–December (18% [27/150] in 2010 and 29% [97/337] in 2011). During 2011–2012, the mean follicle stage of urban *Cx. pipiens* females was significantly larger than that in all other species (Tukey's HSD; $P < 0.001$). Females remained autogenous throughout the winter period and 5–10 surviving females were allowed to oviposit in March. These eggs hatched and progeny, when reared under natural conditions, were all autogenous after 7 d postemergence ($n = 10$). Autogeny also was observed in rural and suburban *Cx. pipiens* cohorts (<1% [1/331] in 2010 and 9% [77/898] in 2011) and in one female from Shasta County (0.5% [1/186]). *Cx. pipiens* from Shasta County also exhibited a mixed response to diapause induction cues, with the majority of females reaching host-seeking arrest during early winter (53% [39/73]), although significantly more females arrested development at stage I than did those from the Sacramento Valley ($\chi^2 = 11.44$; $df = 1$; $P = 0.001$).

For mean primary follicle length, there were significant interactions between species and sampling seasons ($F = 11.88$; $df = 3$, 1663; $P < 0.001$) and between species (*Cx. tarsalis* and rural and suburban *Cx. pipiens* only) and sampling years ($F = 5.63$; $df = 1$, 1458; $P = 0.018$). For *Cx. tarsalis* ($F = 242.56$; $df = 1$, 396; $P < 0.001$), rural and suburban *Cx. pipiens* ($F = 78.31$; $df = 1$, 1060; $P < 0.001$), *Cx. pipiens* from Shasta County ($F = 62.38$; $df = 1$, 176; $P < 0.001$), and *Cx. stigmatosoma* ($F = 6.18$; $df = 1$, 29; $P = 0.019$), the mean primary follicle length was significantly smaller during October–December than January–February (Table 3). The mean primary follicle length was significantly larger during 2010–2011 than 2011–2012 in *Cx. tarsalis* ($F = 8.93$; 1, 396; $P = 0.003$) and rural and suburban *Cx. pipiens* females ($F = 77.06$; $df = 1$, 1060; $P < 0.001$). For *Cx. tarsalis* and *Cx. stigmatosoma*, there was no significant difference in the mean follicle length during October–December (Tukey's HSD; $P > 0.05$), but mean lengths were significantly smaller in *Cx. stigmatosoma* during January–February (Tukey's HSD; $P = 0.026$). For rural and suburban *Cx. pipiens* females, the mean primary follicle length during October–December was significantly larger than those in *Cx. tarsalis* (Tukey's HSD; $P < 0.001$), *Cx. stigmatosoma* (Tukey's HSD; $P < 0.001$), and *Cx. pipiens* from Shasta County (Tukey's HSD; $P = 0.004$), but only significantly larger than *Cx. tarsalis* (Tukey's HSD; $P < 0.001$) and *Cx. stigmatosoma* (Tukey's HSD; $P < 0.001$) during January–February.

For the mean follicular ratio, there was significant interaction between species and sampling seasons ($F = 13.39$; $df = 3$, 1663; $P < 0.001$), but no significant interaction between species (*Cx. tarsalis* and rural and suburban *Cx. pipiens* only) and sampling years. The

mean follicular ratio was significantly smaller during October–December than during January–February for *Cx. tarsalis* ($F = 170.02$; $df = 1$, 396; $P < 0.001$), rural and suburban *Cx. pipiens* females ($F = 38.75$; $df = 1$, 1060; $P < 0.001$), and *Cx. pipiens* from Shasta County ($F = 36.25$; $df = 1$, 176; $P < 0.001$), but not significantly different for *Cx. stigmatosoma* ($F = 1.19$; $df = 1$, 29; $P = 0.284$) (Table 3). For *Cx. tarsalis* and *Cx. stigmatosoma*, there was no significant difference in the mean follicular ratio during October–December (Tukey's HSD; $P > 0.05$), but ratios were significantly smaller in *Cx. stigmatosoma* during January–February (Tukey's HSD; $P = 0.003$). For rural and suburban *Cx. pipiens* females, the mean follicular ratio during October–December was significantly larger than those in *Cx. tarsalis* (Tukey's HSD; $P < 0.001$), *Cx. stigmatosoma* (Tukey's HSD; $P = 0.016$), and *Cx. pipiens* from Shasta County (Tukey's HSD; $P < 0.001$), but only significantly larger than *Cx. stigmatosoma* (Tukey's HSD; $P = 0.006$) during January–February.

Diapause Termination. More than 50% of *Cx. tarsalis* females held under seminatural conditions terminated diapause by 2 February 2011 (5.1°C minimum, 16.3°C maximum, 10.3 h daylength) and 22 February 2012 (7.7°C minimum, 24.0°C maximum, 11.1 h daylength). During 2010–2011, the median termination of diapause by *Cx. tarsalis* females on 2 February 2011 corresponded to an accumulation of 83.6 DD, whereas during 2011–2012, median termination occurred on 22 February 2012, 3 wk later, after 242.1 DD, average = 162.85 ± 79.3 DD. Termination did not commence in *Cx. stigmatosoma* until after the winter solstice, with the first female in host-seeking arrest dissected on 11 January 2012. All *Cx. stigmatosoma* females reached host-seeking arrest by 7 March 2012. As the majority of *Cx. pipiens* females from the Sacramento Valley did not enter a reproductive diapause during either winter, diapause termination dates could not be determined.

Environmental Chamber Studies. When dissected 21 d postemergence, <20% of *Cx. pipiens* complex progeny from the Sacramento Valley were in diapause after being held under experimental midwinter conditions in a bioenvironmental chamber (Fig. 6). Overall, most *Cx. pipiens* complex females had follicles at \geq stage I–II or degenerated. After 21 d postemergence, 87% (26/30) of F_1 progeny reared from autogenous *f. molestus* females collected from a manhole in Old Sacramento had ovarian follicles progressed autogenously to stages III–V. Autogeny was also observed infrequently in the progeny of females collected from aboveground locations, with 3% (1/29) and 19% (11/59) of females autogenous from Woodland and Elk Grove, respectively. *Cx. pipiens* from Shasta County also were reared under diapause conditions as a *f. pipiens* diapause control, but unexpectedly, 83% (5/6) did not enter diapause and were in host-seeking arrest 21 d postemergence, indicating this population was not pure *f. pipiens*. *Cx. tarsalis* progeny were reared concurrently and after 21 d postemergence, 100% ($n = 11$) remained in diapause.

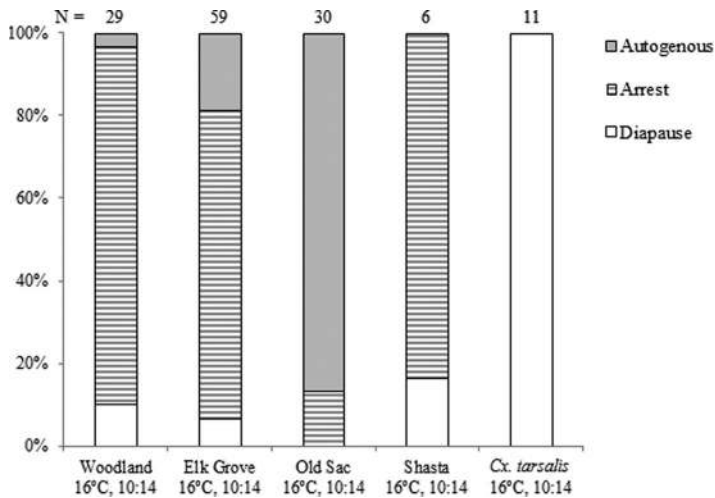


Fig. 6. Percentage of *Cx. pipiens* complex populations and *Cx. tarsalis* females that were diapausing, in host-seeking arrest, and autogenous after being held for 21 d postemergence under mid-winter experimental conditions in a bioenvironmental chamber (10:14 [L:D] h, 16°C). *Culex pipiens* from Shasta County, CA, were included as a diapause control. Old Sac, Old Sacramento.

Discussion

Cx. tarsalis, members of the *Cx. pipiens* complex, and *Cx. stigmatosoma* were collected from resting sites in the Sacramento Valley of California during the winters of 2010–2011 and 2011–2012. Members of the *Cx. pipiens* complex were collected most frequently, followed by *Cx. tarsalis* and *Cx. stigmatosoma*. In accordance with previous studies, most *Cx. tarsalis* females exposed as larvae to autumnal conditions entered reproductive diapause and arrested primary follicles at stage I, with primary to secondary follicular ratios <1.5 (Reisen et al. 1986a,b, 1995, 2010). Although our sample sizes were low, *Cx. stigmatosoma* also entered and maintained reproductive diapause during winter, agreeing with observations in Oregon (Skultab and Eldridge 1985). In comparison, anautogenous *Cx. pipiens* females from the Sacramento Valley failed to enter diapause and instead arrested ovarian development at prehost-seeking stage II during winter. Degenerating granular follicles were observed in the majority of females, indicative of quiescence, where hormonal secretion brings the eggs to the host-seeking arrest stage and then allows degeneration if a bloodmeal is not taken (Spielman 1974). Autogenous populations of *f. molestus* were found primarily in manholes and catch basins in urban Sacramento. These females remained reproductively active during winter, with autogenous, blood-fed, gravid, and parous females collected from October to January. Similar to observations in Massachusetts (Spielman 1971), autogenous populations declined during late winter, although elimination by mosquito control and catch basin maintenance efforts could not be excluded. In addition, a few autogenous females were collected resting aboveground in rural and suburban locations during winter, similar to findings in aboveground populations in southern Europe (Gomes et al. 2009) and central California (Strickman and Fonseca 2012), which were composed of both

forms of *Cx. pipiens* (*pipiens* and *molestus*). Although summer populations of *Cx. tarsalis* in the Sacramento Valley frequently express autogeny (Spadoni 1974, Spadoni et al. 1974), autogenous females were not observed during fall and winter in the current study. Previously, diapause-inducing conditions during autumn were found to suppress the phenotypic expression of autogeny in genetically autogenous females (Reisen 1986).

Diapause Induction. During both winters, diapause was induced in >50% of *Cx. tarsalis* and *Cx. stigmatosoma* females by mid-to-late October, whereas those emerging in early October were a mix of diapausing and nondiapausing forms. In southern California, Reisen et al. (1995) found that immature *Cx. tarsalis* females experiencing warm mean water temperatures (>20°C) and short photoperiods (12:12 [L:D] h) during early autumn exhibited a mixed response, with some females entering diapause and others remaining gonotrophically active. Conversely, females that emerged during November through mid-December, when water temperatures dropped to <18°C and photoperiod had decreased to <10.3 h, consistently entered and maintained diapause. Spielman and Wong (1973b) found a similar pattern in *Cx. pipiens*, with only half of the population entering diapause when exposed to warm water temperatures (>22°C). Although the average of minimum and maximum temperatures in the Sacramento Valley for October was ≈18°C during both winters, the mean maximum temperature was ≈24°C and may have prevented diapause induction in early emerging cohorts. Conversely, 80–90% of *Cx. tarsalis* females were in diapause by November, when the average temperature was ≈11°C and mean maximum temperatures remained <18°C.

Culex females of all species and forms continually repopulated resting sites after removal sampling, and blood-fed, gravid, parous, and nondiapausing females

were collected throughout winter. These findings agree with patterns reported previously for southern California (Bellamy and Reeves 1963, Kliever et al. 1969, Reisen et al. 1986a,c), and indicate that in the Sacramento Valley, overwintering females also remain vagile. Because females were found to be fructose-positive throughout winter (Reisen et al. 1986c), it appeared that they also remained metabolically active. These results suggested that mild winter temperatures in the Sacramento Valley may allow nondiapausing females horizontally infected with WNV during autumn to survive the short California winter period. Conversely, studies conducted in colder climates found that diapausing *Culex* females remained sedentary at collection sites throughout winter and did not take bloodmeals (Mitchell 1979, Farajollahi 2005). Interestingly, parous females comprised a small portion of overwintering populations, regardless of latitude and temperature (Nelson 1964, Kliever et al. 1969, Mitchell 1979, Andreadis et al. 2010). However, average temperatures in the Sacramento Valley from November to March were below the thermal minimum for WNV replication, indicating that infected females may not become infectious until spring. This could explain why the single WNV-positive female collected on 25 January 2012 contained little WNV RNA ($C_t = 37.2$).

Follicular Observations. In the Sacramento Valley of California, *Cx. tarsalis* and *Cx. stigmatosoma* females had small primary follicles and follicular ratios <1.5 during October–December. The mean monthly primary follicle length was $<63 \mu\text{m}$ from October to December and increased to $63\text{--}90 \mu\text{m}$, in accordance with diapause termination after the winter solstice. Follicular ratios from October to December were ≤ 1.2 , but were between 1.4 and 1.6 during late winter in *Cx. tarsalis*. These findings agreed with previous studies that demonstrated ovarian diapause in *Culex* occurred when primary follicle size was $<60 \mu\text{m}$ and the primary to secondary follicular ratio was <1.5 , whereas females in host-seeking arrest have larger ($>70 \mu\text{m}$) primary follicles with ratios >1.5 (Spielman 1974, Eldridge and Bailey 1979, Reisen et al. 1986a, Eldridge 1987, Vinogradova 2000, Farajollahi 2005). However, these criteria were not reliable in distinguishing diapausing from nondiapausing *Cx. pipiens* complex females from the Sacramento Valley. During late winter, primary follicles degenerated to $<60 \mu\text{m}$, whereas secondary follicles became precocious and differentiated, yielding ratios that remained <1.5 (Fig. 2). Other studies also found disagreement in the metrics used to define ovarian diapause in *Culex* mosquitoes in this region (Reisen et al. 1986a,c, 2010). In these and the current study, follicular ratios >1.5 and large primary follicles with no visible yolk were observed in diapausing *Cx. tarsalis* females. Taken together, these findings suggest that stages of follicular development based on yolk deposition and differentiation may be the most reliable criteria used in determining reproductive status, because it delineated the hormonal, physiological, and morphological changes in the ovarian follicles. However, the primary follicle length and the primary to sec-

ondary follicular ratio should be used to validate these presumptive observations.

Follicular morphology in nulliparous anautogenous *Cx. pipiens* complex females from the Sacramento Valley was similar to those previously described among overwintering *Cx. quinquefasciatus* in southern California (Reisen et al. 1986a). *Cx. quinquefasciatus* females exhibited degenerative dilatations during winter, indicative of quiescence, whereas degenerated follicles were rarely observed among overwintering *Cx. tarsalis*. In addition, degenerate follicles containing granular material and measuring $<50 \mu\text{m}$ have been previously reported in *Cx. pipiens* (Spielman and Wong 1973a,b). These degenerate follicles were observed even when females were held continuously (up to 30 d) under short-day conditions (8 h of light). In a study by Spielman (1974), topical applications of juvenile hormone caused the primary follicles to degenerate and the secondary follicle to begin development. Small primary and large secondary follicles were observed repeatedly among *Cx. pipiens* complex females during late winter in the current study, suggesting continued secretion of juvenile hormone from the corpora allata.

Genetic Characterization of *Cx. pipiens* Populations. To assist in interpreting our phenotypic observations, the genetic structure of *Cx. pipiens* complex populations from our study areas in the Sacramento Valley was characterized by microsatellite analysis and was found to consist of a complex admixture of *pipiens*–*quinquefasciatus*, *f. pipiens*–*f. molestus*, and *f. pipiens*–*f. molestus*–*quinquefasciatus* hybrids; only a small portion from populations in Davis and Elk Grove were composed of genetically pure *pipiens f. pipiens* (Kothera et al. 2013). These winter findings agreed with Spielman and Wong (1973b), who demonstrated that only pure anautogenous *Cx. pipiens* entered reproductive diapause. Under both diapausing and nondiapausing conditions, autogenous mosquitoes remained gonotrophically active and developed mature eggs, whereas hybrid individuals remained reproductively active and expressed autogeny or had ovaries that progressed to the host-seeking arrest stage and then degenerated if a bloodmeal was not taken. It seems that extensive genetic introgression coupled with mild temperatures in the northern Central Valley of California have permitted variation in the overwintering strategies among *Cx. pipiens* complex populations, allowing parous, quiescent, diapausing, gonotrophically active, and autogenous females to persist during winter.

Temperature Effects. Warm fall temperatures may have caused a significant portion of prehibernating *Cx. pipiens* complex females to terminate diapause and persist in a state of host-seeking arrest when winter conditions were amenable for their survival. Eldridge and Bailey (1979) found that even when female *Cx. pipiens* were reared and maintained under diapause conditions, if warmed to 25°C for 3–5 d while retaining a winter photoperiod, the ovarian follicles developed to the host-seeking arrest stage and females took bloodmeals. Whereas in female *Cx. tarsalis*, warming alone (at 25°C) for up to 8 d did not terminate diapause in a significant portion of the population

(Mitchell 1981). Only when held under long photophase and warm temperature did female *Cx. tarsalis* terminate diapause. Therefore, the interaction of temperature on diapause potential in *Cx. pipiens* complex females from the Sacramento Valley was evaluated in a bioenvironmental chamber set to midwinter conditions. In agreement with field and mosquito house populations, anautogenous females remained at reproductive arrest \geq stage II (Kawai 1969) 21 d postemergence, even under short photophase (10:14 [L:D] h) and cool temperature (16°C). Surprisingly, the majority of *Cx. pipiens* females from northern Shasta County also progressed to reproductive arrest rather than remain at diapause under midwinter conditions. Although these findings were unexpected, genetic analysis found that *Cx. pipiens* populations from Shasta County also were highly introgressed, with 35% assigned to *f. pipiens*, <5% to *f. molestus*, <2% to a group of genetically similar individuals of hybrid origin, and the remainder admixed among clusters (Kothera et al. 2013). Autogenous members of the *Cx. pipiens* complex did not enter a reproductive diapause under any environmental conditions. Genotyping of specimens identified up to 70% of individuals from manholes and a catch basin in urban Sacramento as *f. molestus* (Kothera et al. 2013). In addition, autogenous females were collected resting aboveground in Woodland, Davis, Elk Grove, and Shasta County, and these findings also were supported by genetic data that identified between 4 and 18% of individuals from these locations as *f. molestus* (Kothera et al. 2013).

Diapause Termination. Diapause termination was staggered under both field and seminatural conditions, with a small proportion of *Cx. tarsalis* females terminating immediately after the winter solstice, and then increasing steadily until late February. Concurrent with diapause termination, as determined by ovarian morphometrics, was an increase in the proportion of blood-fed female *Cx. tarsalis* and *Cx. pipiens* complex collected resting in nature. A previous study done in the Central Valley of California also found that diapause termination was asynchronous in field populations, with females first becoming reproductively active by January (Reisen et al. 2010). Overall, diapause termination in *Cx. tarsalis* after the winter solstice seemed to be limited by temperature, with an average of 97.52 ± 30.7 DD and 162.85 ± 79.3 DD necessary for diapause termination in at least 50% of field and seminatural populations, respectively. During 2010–2011, termination occurred on the same date in both field and seminatural mosquito house populations, whereas termination was delayed by 2 wk in seminatural populations during 2011–2012. Consistently, more heat accrual was required for termination in females held under seminatural conditions than observed in field populations. Because California *Culex* are mobile and use various microenvironments throughout winter, heat accrual and thus diapause termination in field populations may vary, especially if females egress from resting sites and sugar feed during late afternoon (Reisen et al. 1986c), thereby exposing them to warm ambient temperatures. Plasticity in the diapause ter-

mination response within the same overwintering populations may allow *Culex* mosquitoes to use favorable weather conditions, while protecting a portion of the population against disastrous changes in weather (Reisen et al. 1986a, 2010). This would seem especially advantageous for species at northern latitudes, where freezing temperatures can occur from late winter into spring, as seen in *Cx. pipiens* from New Jersey, where diapause termination is delayed until April–May (Farajollahi 2005). Conversely, in the southeastern deserts of California, mild temperatures coupled with high metabolic rates in mosquitoes do not allow diapause persistence through late winter, and females terminated diapause on or immediately after the winter solstice (Nelson 1971, Reisen et al. 1995). In the more northern Kern County, where temperatures are $\approx 5^\circ\text{C}$ cooler than Coachella Valley, termination is delayed several weeks until after the winter solstice (Bellamy and Reeves 1963). Taken together, these findings suggest that the cessation of diapause is attributed to temperature accrual after the winter solstice, which terminates hormonal constraints on *Culex* ovarian development.

Virus Persistence. Overall, multiple overwintering strategies used by populations of *Culex* mosquitoes from the Sacramento Valley may enable virus persistence by multiple mechanisms, as horizontally and vertically infected females may persist during winter and initiate transmission when temperatures are above the minimum for virus replication. Vertical transmission of WNV was demonstrated for *Culex* females collected during fall in California (Nelms et al. 2013) and by detection of RNA in a pool of *Cx. tarsalis* males collected during November. In addition, WNV RNA was detected in one blood-fed *Cx. tarsalis* female collected in January. It is unclear whether this female was infected vertically during fall and had renewed blood feeding after diapause termination or was horizontally infected by feeding on an infected host. Our high Ct values indicated that a limited amount of virus may have been present, which probably precluded isolation of infectious virus. In agreement, previous studies also failed to isolate infectious virus from winter mosquitoes positive for WNV RNA (Nasci et al. 2001, Bugbee and Forte 2004, Farajollahi et al. 2005). Reisen et al. (2006a) found that nondiapausing *Cx. tarsalis* females incubated in the laboratory at 10°C after an infectious bloodmeal failed to become infectious. In addition, females imbibing an infectious bloodmeal at room temperature and maintained at 18°C for 1 wk before transfer to 10°C for 21–42 d became infected, but infectious virus was not isolated. Overall, these findings provide possible evidence for local overwintering of WNV in diapausing, nondiapausing, or both *Cx. tarsalis*.

Although average temperatures in the Sacramento Valley were not consistently above the 14°C threshold (Reisen et al. 2006a) required for WNV replication until April, mean maximum temperatures during January were $\approx 15^\circ\text{C}$ and the first recent positive (Ct score <30) dead bird of the 2012 season, a House Finch, was collected on 18 January in Sacramento

(Arbovirus Surveillance Bulletin #2, CA Department of Health Services). These data indicated that some infected *Culex* females may be able to transmit WNV immediately after diapause termination during late winter. Therefore, DD models forecasting the termination of *Culex* female diapause or arrest also may signify the onset of the virus transmission season and inform the timing of vector control measures.

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