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ABSTRACT. The overwintering ecology of *Culiseta melanura* was studied in a seasonally flooded evergreen forest swamp in south central Connecticut in an effort to clarify which larval stages successfully overwinter in the northeastern USA, and to determine the degree to which larval development and/or mortality occur during the winter months. A total of 8,626 immature *Cs. melanura* were collected weekly for analysis from subterranean crypts and cavities located under the roots of trees from December 13, 2011 to May 31, 2012. Despite the formation of ice on the surface water at the entrance holes to the crypts, water temperatures within the cavities remained above freezing (average = 1.8°C) throughout the coldest winter months of January and February. A heterogeneous population of 2nd, 3rd, and 4th instars were recovered throughout the winter and early spring in the same relative proportions (30%, 30%, 40%, respectively), with no significant change in their comparative abundance during this period, providing unequivocal evidence that all 3 instars successfully overwinter in the region. Findings further demonstrate that larvae undergo no development during the winter and do not appear to be impacted by any measurable mortality. The cessation of larval diapause and a resumption of development was observed in mid-April and was coincident with a gradual increase in water temperature within the crypts to 9°C, in agreement with a previously calculated developmental thermal minimum of 8.5°C for *Cs. melanura.* This resulted in a protracted period of pupation that encompassed a minimum of 5 wk, followed by a staggered emergence of adults and an overlap of the residual overwintering population with larvae of the 1st summer generation.

KEY WORDS *Culiseta melanura*, larval development, overwintering, diapause, eastern equine encephalitis

INTRODUCTION

Culiseta (*Climacura*) *melanura* (Coquillett) is a widespread mosquito whose distribution includes most of the eastern half of the USA and portions of southeastern Canada (Wood et al. 1979, Darsie and Ward 2005). The ecology of the species is of considerable interest because it is the primary enzootic vector of eastern equine encephalitis virus (EEEV). Eastern equine encephalitis virus has recently exhibited a sustained resurgence of activity within established foci in the northeastern USA, and unprecedented northward expansion into new regions where it had been historically rare or previously unknown, including northern New England (CDC 2006, Gibney et al. 2011, Mutebi et al. 2011) and the Canadian Maritimes (Lindsey, personal communication). The underlying conditions responsible for this resurgence are unknown.

Culiseta melanura predominates in densely wooded freshwater swamps and sphagnum bogs (Joseph and Bickley 1969, Morris et al. 1976). Larvae develop in subterranean "crypts" beneath mats of sphagnum and in deep shaded cavities under the roots of trees (Siverly and Schoof 1962, Muul et al. 1975, Pierson and Morris 1982), where water temperatures remain below 20°C most of the summer (Mahmood and Crans 1998). The habitat is comparatively stable and generally contains cool acidic water throughout most of the year. Larval development is exceptionally slow, extending over a period of 2–3 months, and in the northeastern USA, there are typically 1 overwintering and 2–3 summer generations a year (Morris et al. 1976, Andreadis 2002, Mahmood 2002).

The species is unusual in that it overwinters in the larval stage during a period of quiescence induced by low temperature rather than short photoperiod (Maloney and Wallis 1976). However, because of the cryptic nature of the habitat and freezing temperatures, larvae are exceedingly difficult to find during the winter months (Hayes 1961, Joseph and Bickley 1969). As a result, little definitive published information exists on the overwintering biology of *Cs. melanura*, and in particular, which stage(s) successfully overwinter. Early limited observations in freshwater swamp habitats in the Mid-Atlantic and northeastern USA have yielded conflicting results. Joseph and Bickley (1969) found 1st–4th instars throughout the winter months in the Pocomoke Swamp in the Maryland Eastern Shore, but were unable to determine if overwintering larvae were in true diapause or if development progressed slowly from January through March because of low water temperatures that ranged from 3.9°C to 12.8°C. Muul et al. (1975), working in the same swamp habitat, did not examine larval populations during the winter months, but found a preponderance of overwintering 4th instars in April and May prior to pupation and adult emergence. Burbutis and Lake (1956), in contrast

to Joseph and Bickley (1969), recovered primarily 4th-stage larvae and just a few 2nd and 3rd instars during the winter months from a sphagnum bog habitat in northern New Jersey, thus inferring overwintering by mainly 4th instars. Similar observations were noted in central New York State by Morris et al. (1976), who found 1st–4th instars in September and October, but 4th instars only in November, leading them to conclude that *Cs. melanura* overwintered as a 4th instar in this more northerly region. Wallis (1962) also concluded that in Connecticut, *Cs. melanura* persisted throughout the winter as 4th instars in a period of suspended growth, but provided no quantitative data or details.

In December 2011, we initiated a study to examine the overwintering ecology of *Cs. melanura* more precisely in an effort to clarify which stages successfully overwinter in this region and determine the degree to which larval development and/or mortality may occur during the winter months. The results of this investigation are reported here.

MATERIALS AND METHODS

The study was conducted in a seasonally flooded needle-leaved evergreen forest swamp (Metzler and Barrett 2006) with a history of high *Cs. melanura* populations located in south central Connecticut (41°23′09″N, 72°29′39″W). The swamp is dominated by Atlantic white cedar (*Chamaecyparis thyoides*), red maple (*Acer rurum*), yellow birch (*Betula alleghaniensis*), and eastern hemlock (*Tsuga canadensis*) with a well-developed understory of mountain laurel (*Kalmia latifolia*) and an extensive *Thudidium delicatulum* and *Sphagnum* spp. moss ground cover (Fig. 1A). Sampling was conducted once a week from mid-December through May. Immature stages were collected with a hand-held dipper from a minimum of 25 water-containing "crypts" or cavities that were formed by uprooted trees and arching roots of living trees that typically extended up to a meter in length and 20 cm in depth under the root mass (Fig. 1B). Water temperatures were recorded on each collection date from the same cavity. Water samples containing field-collected mosquitoes were transferred to the laboratory, where all individuals were identified to species and enumerated (Andreadis et al. 2005). Larval stages for *Cs. melanura* were determined by the same individual (JJS) based on visual inspection of head capsule width, overall body size, and morphological features gleaned from rearing observations and measurements with our established laboratory colony (Mahmood and Crans 1994). Field-collected pupae were isolated and identified as adults following emergence. The comparative proportion of each stage was computed, and weekly collection data from each crypt were combined for analysis. Data on the proportions of each instar were subject to regression analysis (Systat 2008) from mid-December through mid-April (18 wk) to ascertain possible development and/or mortality during the winter months. Similar analysis was conducted from mid-April through the end of May (7 wk) in an effort to document the initiation of renewed development in the spring. The emergence of adult populations was additionally monitored with the 1st detection of pupae in early May by the deployment of 6 CO_2-baited Centers for Disease Control and Prevention miniature light traps and an equal number of nestable fiber pot resting boxes that were placed in the immediate vicinity of the larval collection sites on May 10 (Komar et al. 1995).

Fig. 1. (A) Seasonally flooded evergreen forest swamp habitat for *Culiseta melanura* where study was conducted in south central Connecticut. (B) A typical *Cs. melanura* larval habitat. A moss-covered water-containing subterranean cavity under the base of tree roots.

RESULTS

A total of 8,626 immature *Cs. melanura* were collected for analysis during the 25-wk sampling period, December 13, 2011–May 31, 2012 (Fig. 2). A thin layer of ice formed on the surface water surrounding the crypts from late December through early February; however, water temperatures

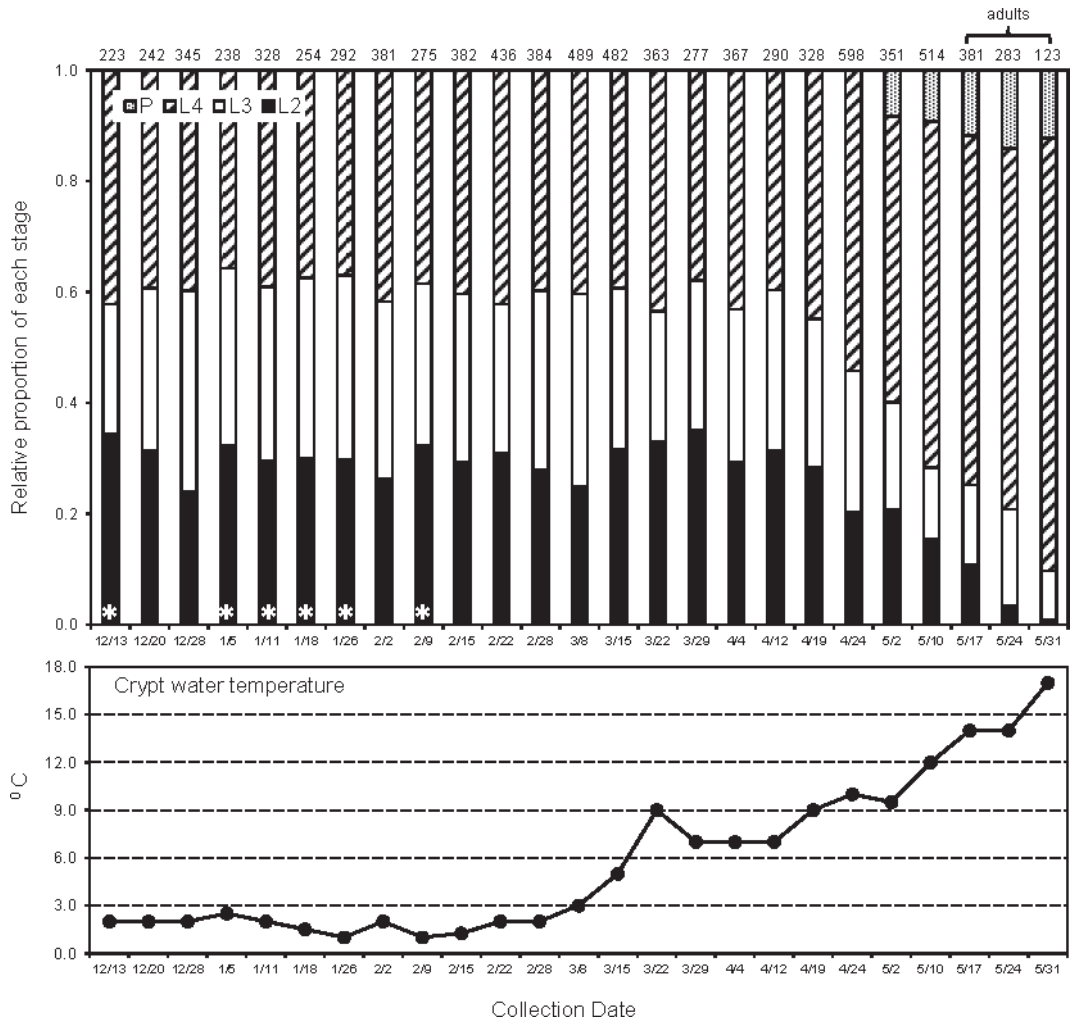

Fig. 2. Weekly water temperatures and comparative abundance of immature stages of *Culiseta melanura* collected from subterranean crypts in an evergreen forest swamp habitat in south central Connecticut, December 13, 2011–May 31, 2012. P, pupae; L4, 4th instars; L3, 3rd instars; L2, 2nd instars; *, presence of 1st instars. Numbers on top of each weekly column indicate number of immature *Cs. melanura* collected in sample. The detection of emerging adults collected in traps placed in the immediate vicinity of the swamp is also indicated for each respective date.

within the cavities were remarkably consistent during this period (mean ± SD = 1.8 ± 0.5°C; range = 1.0°C–2.5°C), and remained above freezing throughout the entire winter (Fig. 2).

Second, 3rd, and 4th instars of *Cs. melanura* were found within the crypts with each collection (Fig. 2). A few (n = 1–3) 1st instars were detected on 6 occasions only: December 13, January 5, 11, 18, and 26, and February 9. From mid-December through mid-April, the relative proportions (mean ± SE%) of 2nd (30.0 ± 0.7%, n = 1,807), 3rd (30.0 ± 0.8%, n = 1,821), and 4th (39.8 ± 0.5%, n = 2,420) instars collected from the crypts were nearly identical and remained virtually unchanged (Fig. 2). An analysis of the comparative abundance of each larval instar over this 18-wk period (December 13–April 12) as determined by linear regression revealed no significant changes in proportion of 2nd, 3rd, or 4th instars (Fig. 3). Thereafter, beginning on April 19 and continuing through the end of May, steady significant declines were observed in the proportion of 2nd and 3rd instars (Fig. 3). This was coincident with a significant measured increase in the proportion of 4th instars, and the appearance of increasing numbers of pupae that were 1st detected on May 2. Associated with this renewed development of larvae was an increase in water temperature within the crypt from 9°C to 17°C.

The 1st emerging adults were collected on May 17. Numbers of each sex collected were as follows: May 17 = 8 males, 19 females; May 24

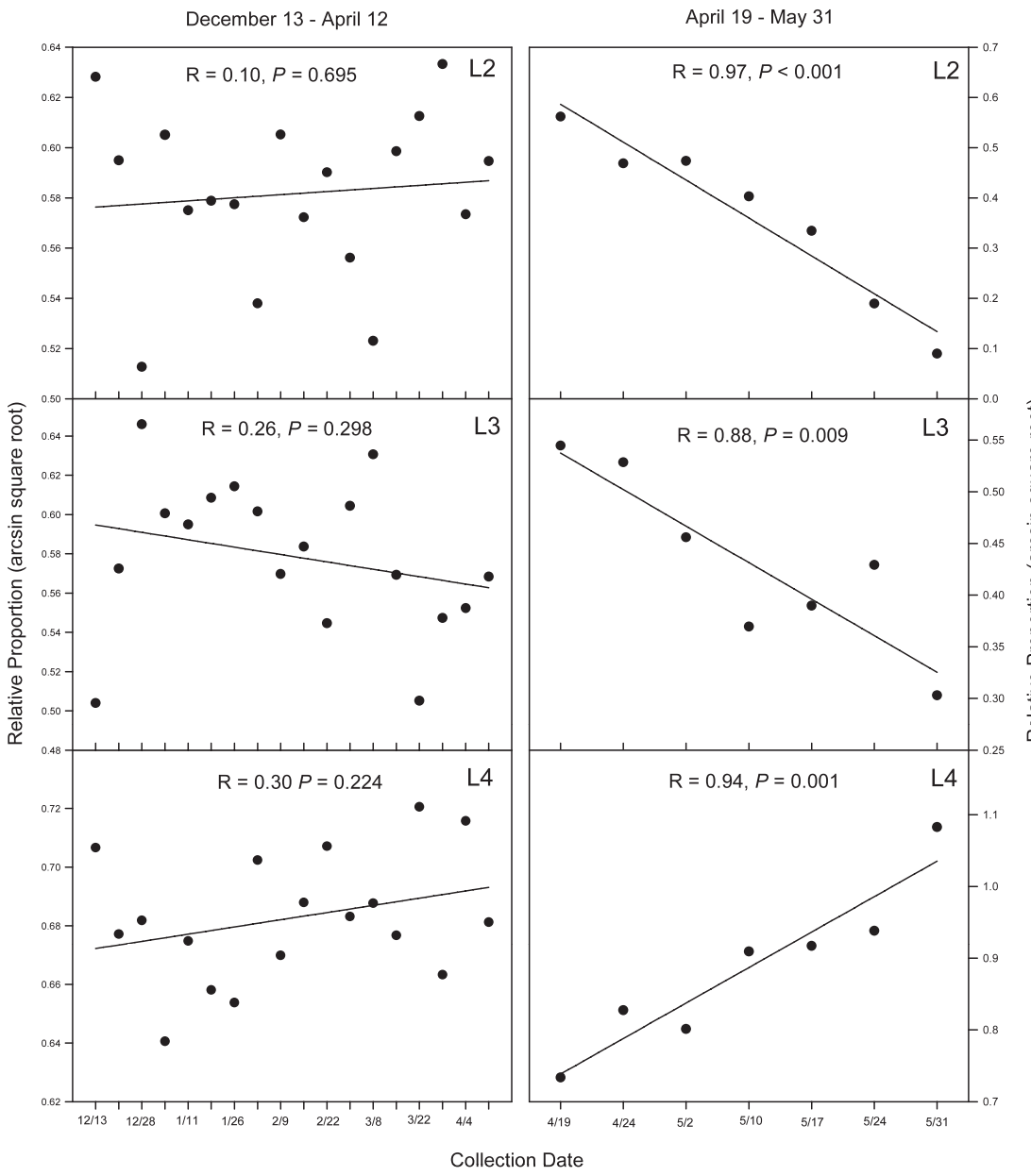

Fig. 3. Results of regression analysis showing no significant changes in the relative proportions of overwintering 2nd (L2), 3rd (L3), and 4th (L4) instars of *Culiseta melanura* collected from subterranean crypts from December 13, 2011 through April 12, 2012, and significant declines in 2nd and 3rd instars and coincident with significant increases in 4th instars from April 19 to May 31, 2012.

= 79 males, 253 females; May 31 = 86 males, 320 females. First instars of the summer generation were detected 2 wk later on May 31 (n = 17), indicating the initiation of egg laying.

DISCUSSION

With this investigation, we provide unequivocal evidence that in freshwater swamp habitats in this region of the northeastern USA, a heterogeneous population of 2nd–4th instars of *Cs. melanura* successfully overwinter in subterranean crypts beneath the roots of trees where water temperatures remain above freezing throughout the winter months. The recovery of large numbers of all 3 instar stages in the same relative proportions throughout the winter, with no discernible change in their comparative abundance,

further lead us to conclude that larvae undergo no development during this period and are not severely impacted by any measurable mortality. These observations concur with those of Joseph and Bickley (1969), who found 1st–4th instars throughout the winter months in freshwater swamp habitats located in coastal Maryland. However, they contrast sharply with reports from similar sites in northern New Jersey (Burbutis and Lake 1956) and central New York (Morris et al. 1976), where 4th instars were the primary or only stage recovered during the late fall and winter months, leading these investigators to conclude that *Cs. melanura* persisted throughout the winter primarily as a 4th instar in these more northerly regions. Although our investigations do not support this view, we cannot rule out the possibility that variations could be due to local differences in habitat conditions.

Despite the formation of ice on the surface water at the entrance holes to the crypts, water temperatures within the cavities remained slightly above freezing (average = 1.8°C) during the coldest winter months of January and February. The winter of 2011–12 was notably warmer than normal (deviations from norm: December = 3.9°C, January = 4.0°C, February = 4.3°C, March = 5.3°C) (http://www.erh.noaa.gov/box/dailystns.shtml), and thus these comparatively warmer temperatures may have enhanced larval survival, especially among early instars. However, larvae have a marked resistance to cold temperatures (Mahmood and Crans 1998) and laboratory studies (Maloney and Wallis 1976) have shown all 4 instars can survive in a state of arrested development at least 2 months at 4°C with little mortality, provided they are cooled gradually, in agreement with our findings and conclusions. Maloney and Wallis (1976) have also demonstrated that 4th instars can survive over 7 months at 4°C, and upon rewarming, pupate within 3 wk and emerge as adults in 3–4 days. Even under conditions where water temperatures within the crypts result in more sustained freezing, *Cs. melanura* larvae are known to avoid freezing by burrowing into mud up to a depth of 15 cm where temperatures do not go below 1.4°C, and then return to open water as spring approaches (Hayes 1961), which is entirely consistent with our observations in the field.

The declines in the number and comparative proportions of 2nd and 3rd instars of *Cs. melanura* and corresponding increase in the proportion of 4th instars observed in mid-April and May were interpreted as cessation of larval diapause and a resumption of development. This was coincident with a gradual increase in water temperature within the crypts to 9°C, and a corresponding photoperiod of 13 h 32 min on April 19. Our observations on the renewal of larval development when water temperatures attained 9°C seemed to validate the laboratory findings of Mahmood and Crans (1998), who, using a heat summation model, calculated a developmental thermal minimum of 8.5°C for *Cs. melanura.* This minimum developmental temperature was further substantiated by the lack of any discernible development within the larval population during late March and early April when water temperatures of 7°C were consistently recorded in the crypts for 3 straight weeks.

Our final observation of significance was the presence of a diverse population of 2nd–4th instars in the spring with the termination of larval diapause. This resulted in a protracted period of pupation that encompassed a minimum of 5 wk, followed by a staggered emergence of adults, and an overlap of the residual overwintering population with larvae of the 1st summer generation. These observations support a degree-day model of Mahmood (2002), which predicts that temperature-driven development of 1st–4th overwintering instars of *Cs. melanura* creates a cascade of adult emergence peaks among the 1st generation in the spring and early summer based on the degree days required for completion of each stadium. It has been suggested (Joseph and Bickley 1969) that adult emergence over an extended period may enhance survival and reproduction of this species by ensuring that a certain percentage of adults are present in the population at all times. It has also been suggested (Mahmood 2002) that EEEV amplification within a region is directly related to the size, survival, and age structure of overwintering larval population. How these phenomena impact the ecology of EEEV in the region by increasing the likelihood of adult blood feeding on potentially viremic migratory and resident nesting birds, and how warming winter temperatures associated with climate variability may impact the maintenance and amplification of this virus, are intriguing questions that remain to be explored.

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