DETERMINATION OF CHRONOLOGICAL AGE IN *CULEX PIPIENS* S.L.

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ABSTRACT. Daily survival rate is a major determinant of the vectorial capacity of mosquitoes, but current methods are difficult or lack the accuracy needed for epidemiologic models. We used daily growth bands on the thoracic apodemes to estimate age and survival of *Culex pipiens* s.l. from Memphis, Tennessee. Coded specimens of known age were used to evaluate the procedure's accuracy. Field-collected specimens of unknown age were also examined. We were unable to consistently determine the age of the coded, cage-reared specimens. Growth bands in cage-reared specimens were less well-defined than those in field-collected material. In its present form, the method is impractical for ecological or epidemiologic studies.

INTRODUCTION

The age structure of the vector population is a crucial consideration in understanding the dynamics of vector-borne disease. In most models of vector-borne disease, vectorial capacity—the average number of infective bites delivered by the vectors feeding on one host in one day (Fine 1981)—is exponentially proportional to the survival rate, *s* (Macdonald 1952, Garrett-Jones 1964). The survival rate is, therefore, a crucial factor in arbovirus epidemics (Miller et al. 1973, Smith 1975) but, for several reasons, it is seldom measured. Current methods may be overly expensive or labor-intensive (e.g., mark-release-recapture) or crucial assumptions of the method may be violated (e.g., stable age distribution and the use of parous rate data). Certain essential parameters may not be known (e.g., dispersal out of the study area) or may be highly irregular (e.g., time between successive ovipositions) (Service 1976). Furthermore, typical estimates of survival lack the precision needed to predict changes in vectorial capacity, and there is little incentive or funding to mount the massive studies that could provide more accurate estimates.

Many techniques have been used to estimate survival rates of mosquitoes and other insects of public health importance (Detinova 1968, Gillies 1974, Service 1976, Neville 1983). Most of these assess the "physiologic" rather than the calendar age of the individual, usually the number of ovarian cycles completed by a female. In addition to being time-consuming and requiring great manual dexterity, physiologic aging techniques may give misleading results for a number of species (Rosay 1969, Bellamy and Corbet 1974, Nayar and Knight 1981). The various problems with ovarian dissection techniques have prompted a search for simpler, more reliable methods, particularly in the area of actual calendar age measurement.

Neville (1963) showed that the endocuticle in adult insects is laid down in the form of daily growth layers, and this discovery resulted in extensive studies on the physiology of cuticle deposition (Neville 1983). Daily growth bands on the thoracic apodemes have been found to be reliable indicators of chronological age in *Anopheles* (Schlein and Gratz 1972, 1973; Schlein 1979). We report on attempts to apply the methods of Schlein and Gratz to the *Culex pipiens* complex.

MATERIALS AND METHODS

Specimens of *Cx. pipiens* s.l. from Memphis, Tennessee, were obtained in two ways. First, specimens of unknown age were collected by truck trap, air-dried, and shipped to the Division of Vector-Borne Viral Diseases, Centers for Disease Control, Fort Collins, Colorado (referred to as "truck trap" specimens). Second, *Cx. pipiens* larvae were collected and reared to adulthood in Memphis, Tennessee. Adults were held in an outdoor cage (approximately 0.06 m^3) for 1 to 14 days and fed on water and either sucrose or raisins. Specimens of known age were killed, air-dried, given a code number, and sent to Fort Collins for age determination (referred to as "cage-reared" specimens).

The methods of Schlein (1979) were used to prepare the specimens. Dried mosquitoes were immersed in hot potassium hydroxide (7%) for several minutes and then dissected under a stereo microscope at about 10-30X. Fragments of muscle and other tissues were removed, and the appropriate structures were transferred to a suitable container for staining. For work with

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individual specimens, we used 96-well, disposable serological plates (Micro-Titer U-well).[4] The small well size reduced reagent volumes and shortened the time needed to search for the tiny apodemes. Substage lighting (which can be used with the clear plastic plates) is very useful in steps where specimens are in dark staining solutions such as potassium permanganate. The complete staining procedure is shown in Table 1.

Table 1. Staining procedure for thoracic apodemes of mosquitoes (modified from Schlein 1979).

1. Fix in saturated picric acid, 10 min.
2. Rinse in water, 2 × 5 min.
3. Oxidize in 1% potassium permanganate, 10 min.[a]
4. Rinse in water, 2 × 5 min.
5. Place in mordant (fresh 1% ammonia alum), 10 min.[a]
6. Rinse in water, 2 × 20 min.
7. Stain in ripe hematoxylin (Heidenhain's), 0.2% in 70% ethanol for up to 1 min (checked under microscope, 100×, to avoid overstaining).
8. Rinse in water, briefly.
9. Dehydrate in absolute ethanol.
10. Clear in xylene.
11. Mount in Canada balsam (or equivalent medium).

[a] Times were varied to find optimal combinations for *Cx. pipiens* (see text).

Because Schlein (1979) found that different *Anopheles* species required different lengths of time in the various chemicals to produce optimal results, we conduct preliminary tests to determine the staining time parameters for *Cx. pipiens* Linnaeus. Truck trap specimens were used for this experiment. Approximately 100 picric acid-fixed, washed metathoraxes were randomly distributed among 16 wells of a 96-well microtiter plate that had been divided into 16 blocks of 6 wells. Oxidation, rinsing, and mordant treatment steps were carried out in the 6 wells assigned to each block. Times were 5, 10, 15 and 20 min for both oxidant and mordant, giving 16 combinations.

Specimens were individually stained in hematoxylin with microscopic observation (100–250X) to control staining intensity (Schlein and Gratz 1973). Specimens were then dehydrated and mounted in Permount[4] (Fisher Scientific).

Preparations were examined by transmitted light, phase contrast, and polarized light microscopy. Each specimen was ranked on an arbitrary scale of 0 to 4 for clarity of the growth bands and correspondence between the number of bands on the metathoracic phragma (Tph) and furca (Fu) (Fig. 1A). Specimens less than 2 days old or those without one or more structures were discarded from the analysis.

Cage-reared, coded *Cx. pipiens* were stained by the procedure outlined in Table 1. For each specimen, the number of lines on the metathoracic phragma and furca (Fig. 1B, 1C) were counted. In addition, the growth bands on the intersegmental ridge (Fig. 1D) of the metathorax (Owen 1977) were counted. Based on the evidence from all structures, an estimate was made of the probable age of the specimen. After all specimens had been examined and their ages estimated, a list of the actual ages was obtained for comparison.

RESULTS AND DISCUSSION

Of the 110 thoraxes used, only 72 were 2 days old or older and had all of the apodemes intact after the dissection and staining. Even with this small sample, the relation between oxidant and mordant times was reasonably clear. Equal times in oxidant and mordant gave the best preparations with our material (Table 2), and staining times of 10 min or more were required. We chose 10 min in each solution to reduce the time needed to process the material (Table 1). In addition, there were differences in the average scores obtained from the three types of microscopy. Normal light microscopy was the least efficient at resolving the bands; phase contrast (suggested by Y. Schlein, personal communication) was intermediate; and polarized light microscopy was the most efficient. Of the three metathoracic structures studied, the furcal (Fig. 1B) bands were the most distinct and the phragmal (Fig. 1C) lines least distinct. The bands of the intersegmental ridge were of varying clarity and tended to be paired. Banding of the intersegmental ridge has not been reported previously.

Figure 2 shows the age distribution of the truck trap specimens. In addition to the 72 specimens 2 days or older. there were 13 specimens less than 2 days old for which a calendar age could be assigned with reasonable confidence (total = 85). Most specimens were 3 to 4 days old (55%). The smaller percentage (32%) of young (0 to 2 days) females suggests that either a) young females were not flying in the truck path during the trapping period (the collection route of the truck trap was some distance from the nearest major breeding site), b) there was a drop in emergence several days prior to the collection, or, c) there was a defect

[4] Use of trade names or commercial sources is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

in the age determination procedure that caused young individuals to be assigned to an older age group.

The relationship between estimated and true calendar age of 123 coded, cage-reared females of known age is shown in Fig. 3. Three major problems were noted. First, the age of 1- to 4-day-old mosquitoes was greatly overestimated, while that of specimens more than 7 to 8 days old was underestimated. The latter result

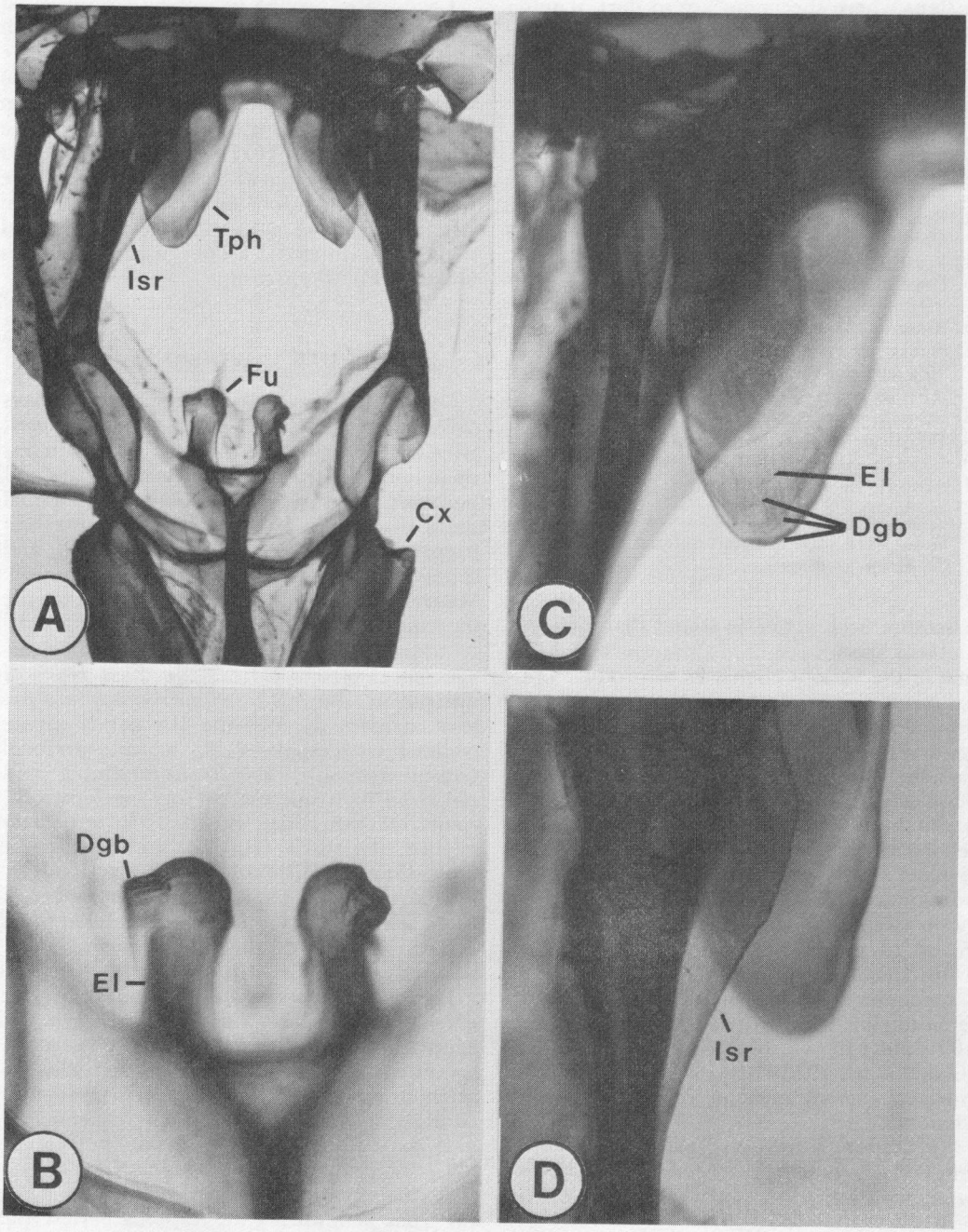

Fig. 1. Daily growth bands in thoracic apodemes of *Culex pipiens*. A—Metathorax showing coxa (Cx), furca (Fu), intersegmental ridge (Isr), and phragma (Tph); B—Metathoracic furca showing eclosion line (El) and daily growth bands (Dgb); C—Second phragma showing three weak growth bands; D—Intersegmental ridge showing five growth bands.

Table 2. Average clarity scores (subjective rating scale of 0–4) for growth bands on thoracic apodemes of *Culex pipiens* s.l. treated with different combinations of oxidant (potassium permanganate) and mordant (ammonia alum), n = 72.

Time in mordant (min)	Time in oxidant (min)			
	5	10	15	20
5	1.0 (2)[a]	1.2 (6)	1.9 (4)	1.6 (5)
10	0.7 (3)	2.4 (5)	2.0 (7)	2.0 (5)
15	1.7 (5)	1.6 (5)	2.2 (4)	1.9 (5)
20	1.9 (5)	1.8 (4)	2.0 (4)	2.3 (3)

[a] Number examined in parentheses.

was expected since the bands in older individuals are narrower and more close-packed. However, we did not expect to overestimate ages of the young specimens. The presence of extra growth bands in the young specimens could be due to problems in the technique or to counting of the "secondary" lines or striations (Schlein and Gratz 1972) as primary growth lines. Second, within each age group, there was excessive spread in estimated age (e.g., the range of estimates for 2-day-old specimens was 6 days and for other groups was 3 to 4 days). Third, growth lines in many of the coded "unknown" specimens were more difficult to resolve than those in specimens from the truck trap. Schlein

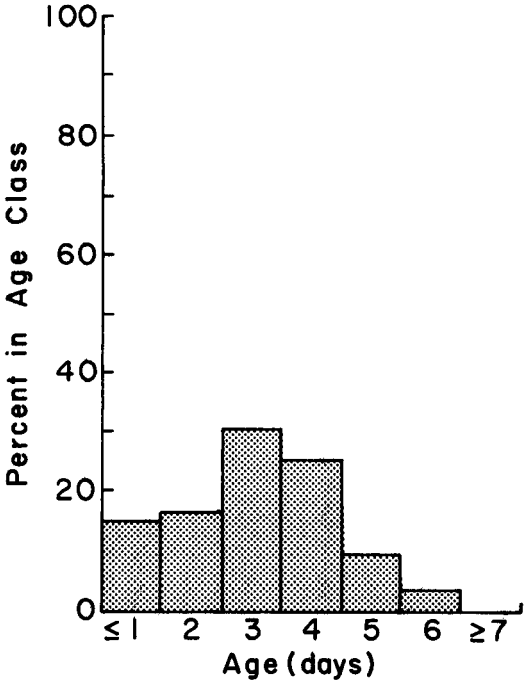

Fig. 2. Estimated age distribution of *Culex pipiens* s.l. collected by truck trap in Memphis, Tennessee. n = 85.

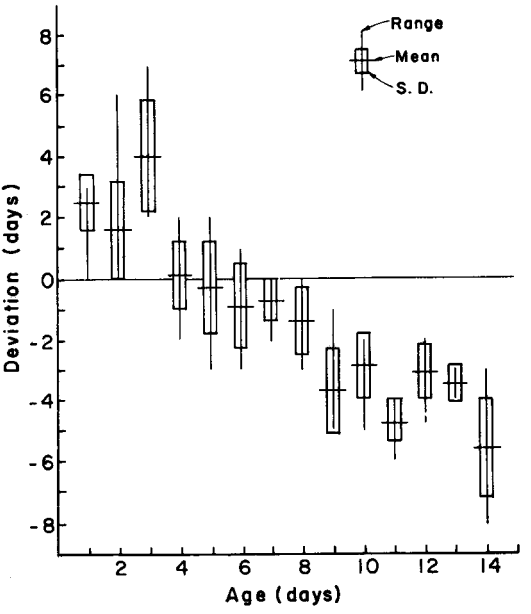

Fig. 3. Deviation of estimated age from actual age in *Culex pipiens* s.l. from Memphis, Tennessee. Age estimates based on daily growth bands on thoracic apodemes. n = 123.

and Gratz (1973) found that mosquitoes reared in the laboratory or at fluctuating "room temperature" had less distinct bands than did field-collected specimens. Such an effect might have contributed to some of the variability described above since these specimens were held in a cage within a screened porch. However, we felt the staining procedure itself contributed the major portion of the within-group variation.

It seems likely that many of the foregoing problems in the apodeme technique as described by Schlein and Gratz (1972, 1973) and Schlein (1979) could be eliminated or reduced with more experience. However, at present, the problems of lack of accuracy and the time-consuming staining procedure prevent the method from being used as a tool for ecologic and epidemiologic studies. We are currently investigating other methods of visualizing the daily bands, such as differential interference (Nomarski) microscopy (Ellison and Hampton 1982, Johnston and Ellison 1982). These seem to offer more promise, both by increasing resolution of growth bands and by eliminating the need for staining.

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