

# COMPARATIVE STUDY OF THE CELL CYCLES IN THE LARVAL BRAIN TISSUE OF TWO SPECIES OF MOSQUITOES, *ORTHOPODOMYIA SIGNIFERA* (COQUILLET) AND *Aedes TRISERIATUS* (SAY)<sup>1</sup>

JESSE COCKE,<sup>2</sup> JONATHAN DODD<sup>3</sup> AND JAMES D. LONG

Cells of proliferating tissues pass through a series of chemical and morphological changes which are referred to collectively as the cell cycle (Howard and Pelc, 1951). The total cell cycle ( $T_c$ ) is divided sequentially into four phases: (a) a post-mitotic phase ( $G_1$ ), a synthesis phase (S), a post-synthetic phase ( $G_2$ ) and mitosis (M). Breland (1959) demonstrated a simple technique for preparing chromosome squashes using mosquito larval brain tissues and this technique has been used in a number of cytogenetic studies (e.g.,

Baker and Aslamkhan, 1969; Craig and Hickey, 1967; and Laven, 1969). With the exception of the study by Mukherjee and Rees (1969), however, no attention has been given the nature of the cell cycle in the brain tissue of these animals. They confirmed a definite cell cycle time of 10 hrs. in the dividing larval brain cells of the mosquito, *Aedes dorsalis* (Meigen) by using tritiated thymidine ( $H^3TdR$ ) autoradiography. Mitosis,  $G_1$ , S and  $G_2$  durations were found to be  $1\frac{1}{4}$  hr., 7 hrs., 1 hr., and  $\frac{3}{4}$  hr., respectively.

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<sup>1</sup> From the Biology Department, Sam Houston State University, Huntsville, Texas 77340. Prepared as part of a thesis in partial fulfillment of the requirements for the Master of Arts degree.

<sup>2</sup> Present address: 209 Redbud, Bryan, Texas 77801.

<sup>3</sup> Present address: 3125 9th Street, Lot 25, Hillside Trailer Park, Wichita Falls, Texas 76301.

The primary objective of this study was to examine the cyclic nature of the dividing larval brain tissues of two species of mosquitoes (*O. signifera* and *A. triseriatus*) by determining total cell cycle and subsection times for each species studied. A second purpose was to compare and contrast the cell cycles and subsection times

of the dividing larval brain tissues of these two species.

**MATERIALS AND METHODS.** Larvae of the two species of mosquitoes studied occur naturally in water found in tree holes. Specimens were collected in the Huntsville, Texas area, brought to the laboratory and sorted as to species and size. Plastic culture dishes (25 cm. diameter x 9 cm. deep) containing 300 ml. distilled water plus 50 ml. concentrated stump water were used to culture larvae. Finely ground Fleischmann's yeast and Purina Dog Chow were added daily to the culture media for feeding until specimens became late instar larvae.

The procedure for pulse labeling (Thrasher, 1966) involves exposing culture tissue cells or entire organisms to  $H^3TdR$  solutions for only 30 min., then removing the  $H^3TdR$  solution immediately. The bottom of a 14 cm. plastic culture dish was removed and a section of nylon hosiery stretched and glued across the opening formed to facilitate transporting a large number of larvae through the solutions used in this procedure. This straining device fitted snugly within 15 cm. dishes in which larvae were sequentially labeled and washed.

Six hundred larvae were selected from culture at random and placed into the transport device. To remove debris, specimens were washed in several distilled water baths. The strainer containing the larvae was transferred into 125 ml.  $H^3TdR$  solution (New England Nuclear, specific activity 2.0 curies/mM at a final concentration of 2.0  $\mu\text{C}/\text{ml.}$ ) for exactly 30 min. (Thrasher, 1966), moved through five rinses of distilled water to remove excess labeled thymidine and ultimately held in a solution of larval food and excess thymidine triphosphate (Nutritional Biochemical) until sacrificed.

Room temperature was maintained at  $27 \pm 1^\circ\text{C}$  during larval incubation and sacrifice. Groups of 20 larvae randomly chosen from the incubation dish were placed in modified Carnoy's fixative at intervals for 20 hrs. after the pulse label was

applied (*O. signifera* 0.5, 1.0, 2.0, 3.0 . . . 20 hrs; *A. triseriatus* 0.5, 1.5, 2.0, 3.0, 4.0 . . . 20 hrs). Specimens were removed individually from the fixative and placed on microscope slides on the stage of a dissecting microscope. The superenteric ganglia were dissected from each head (Breland, 1959), debris and excess fluids removed, a drop of 50 percent acetic acid added and a 22 mm square coverslip placed on the brain tissue. Each brain was then squashed. Coverslips were removed after freezing the squash preparations in liquid nitrogen and the slides dried for approximately 3 min. on the slide warmer at  $47^\circ\text{C}$ . When dry they were stained with 0.5 percent aceto-orcein for approximately 4 min. (French, Baker and Kitzmiller, 1962). Stained slides were placed on end upon 4 x 4 in. gauze sponges (Johnson and Johnson) to drain excess stain, dipped into 70 percent alcohol, immersed in three successive baths of distilled water and allowed to air dry. Preparations from each sacrifice time were placed into properly labeled boxes and stored in desiccators until coated with Kodak NTB-3 photographic emulsion. Slides were held for approximately one month at  $4^\circ\text{C}$  in a refrigerator.

The autoradiograms were then developed in full strength Kodak D-19 developer for 4 min., stopped in indicator stop bath for 30 sec., fixed in Kodak fixer for 5 min., washed for 45 min. in running tap water and allowed to drain dry. Each brain squash was examined using the compound microscope at 970X and the numbers of labeled and unlabeled metaphases were recorded. A metaphase figure with at least two silver grains was considered labeled.

**RESULTS.** Frequencies of labeled metaphases in the brain tissues of *O. signifera* at various times after  $H^3TdR$  pulse labeling are shown in Figure 1. At 0.5 hr. after pulse labeling 55 percent of the metaphases observed were labeled. The frequency increased sharply to 98 percent at the first hour and remained high until the 11th hr. where a drop to a 55 percent level

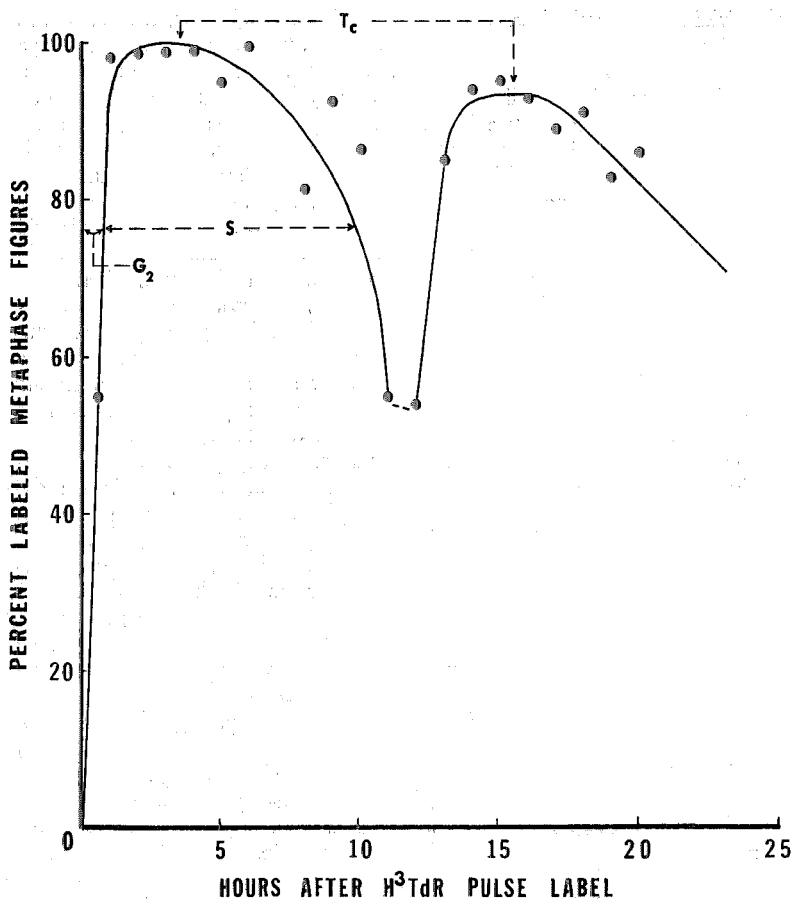


FIG. 1.—Percent labeled metaphase figures as a function of time after  $H^3TdR$  pulse labeling of *O. signifera*.

was observed. By the 15th hr. 95 percent of the metaphase figures were again labeled. The labeling decreased to only 86 percent in the 20th hr.

The 77.5 percent point used for interpretation of these data represents a mean of the high and low labeling frequencies obtained. Thrasher (1966) obtained values near zero after the first wave of labeled metaphases and therefore used the 50 percent point for estimation of the subsection times. In this study the lowest labeling percentage after a similar wave

was only 55 percent, so the 77.5 percent point was accepted for estimation of cell cycle subsections times.  $G_2$  was estimated from the 77.5 percent on the ascending limb of the first wave of labeled metaphases to be 30 min. A total cell cycle time of 11.75 hrs. was estimated from the midpoint of the first wave of labeled metaphases to the midpoint of the second. A 9 hr S duration was approximated from the 77.5 percent on the ascending limb of the first wave to the same level on the descending limb of this wave.

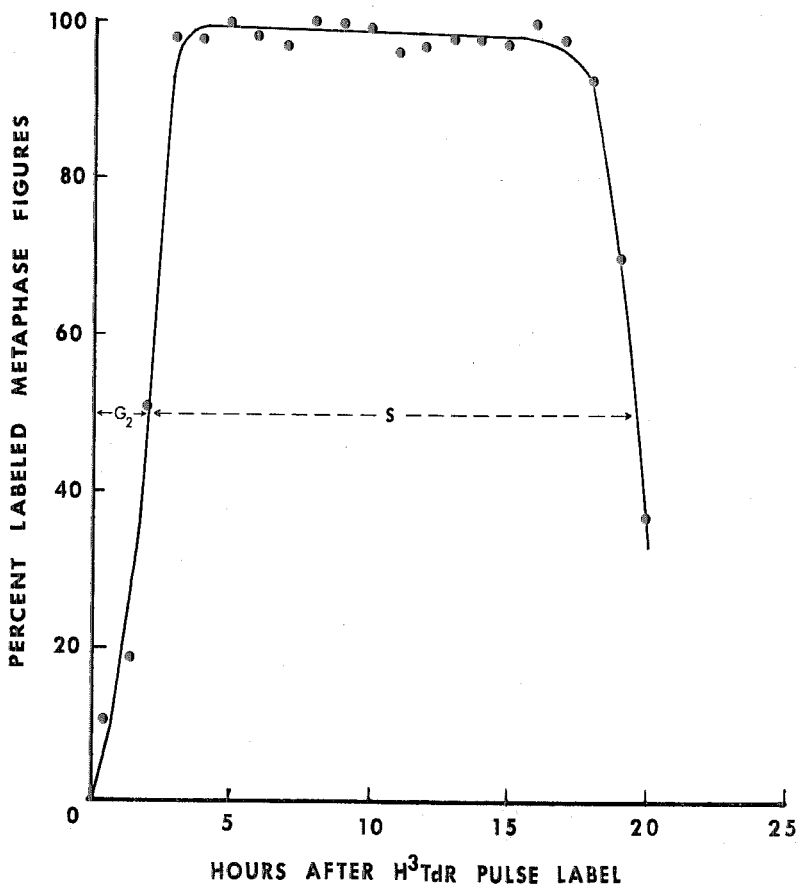


FIG. 2.—Percent labeled metaphase figures in the brain tissue of *A. triseriatus* as a function of time after  $H^3TdR$  pulse label.

A mitotic index (M.I.) was determined for the brain tissue at each sacrifice time after pulse label by determining the number of mitotic figures per 1000 nuclei observed in the population. A mean mitotic index of 0.038 for all 21 time periods (21,000 cells) was calculated. The durations of M and  $G_1$  were calculated using the relationship shown by Thrasher (1966). Table I gives a summary of the durations of the subsection times and total cell cycle time of the brain tissue of *O. signifera*.

The frequencies of labeled metaphase figures in the brain tissue of *A. triseriatus*

at various sacrifice times after  $H^3TdR$  pulse labeling are shown in Figure 2.

TABLE I.—Estimated durations of cell cycle and its subsections in *O. signifera*

Period	Hours	Minutes
$G_1$	1	48
S	9	0
$G_2$	0	30
M	0	27
$T_c$	11	45

Mitotic Index = 0.038.

The frequency did not approach 100 percent until the 3rd hr. after pulse labeling, indicating a considerably longer  $G_2$  period than the proliferating brain cells of *O. signifera*. This high labeling frequency remained until the 18th hr. where a drop to 93 percent was observed. The 19th and 20th hrs. after pulse labeling showed 70 percent and 37 percent labeling frequencies, respectively. Only a single wave of labeled metaphases was observed. Since a labeling frequency below 50 percent was observed after this wave, the duration of  $G_2$  and S were estimated at the 50 percent point. The duration of the  $G_2$  period was approximately 2 hrs. and that of the S period 17.5 hrs. A total cell cycle time ( $T_c$ ),  $G_1$  and M could not be approximated, because only a single wave of labeled metaphases was observed. However  $T_c$  appears to be longer than 20 hrs. A mitotic index was not needed for calculations.

The curve representing the cell cycle in the brain tissue of *O. signifera* deviated slightly from the theoretical curve proposed by Thrasher (1966). Although a cyclic pattern was obtained, the frequency of labeled metaphases after the first wave dropped to only 55 percent suggesting a heterogenous population of cells.

Heterogenous populations of male and female larvae whose specific ages were unknown were used in this study. There was no practical way to quickly identify the larval instars or sex of large numbers of larvae studied. Also, sex cannot be determined on the basis of chromosome morphology. Specimens used in this investigation were assumed to be 3rd or 4th instar, yet were selected on the basis of size alone. Due to this method of selection neither larval sex nor specific age was known. Fluctuations in the mitotic index from 0-9 percent in the larval brain tissue examined indicate a high degree of variation in mitotic activity among larvae.

The independent influence of sex on the cell cycle has been demonstrated by Jan and Boyes (1970). They were unable to establish a definite cyclic pattern in the

dividing brain cells of pooled male and female *Musca domestica* larvae. The sex chromosomes are heterochromatic and somatically unpaired in *M. domestica* so a differential count of labeled metaphase figures was made on XX and XY cells. Considerable deviation in labeling frequency was found in male and female metaphases at the same time intervals after the pulse label was applied. *O. signifera* and *A. triseriatus* both show three pairs of somatically paired chromosomes (2 long pairs of similar length, 1 short). Sex chromosomes have not been identified; consequently, a differential sex count could not be made with these species. Male and female larvae of the same age may have differential rates of development due to differences in timing of hormonal secretion. The molting hormone ecdysone has been shown to activate (Crouse, 1968) or inhibit DNA synthesis (Danieli and Rodino, 1967; Rodman, 1967, 1968). This secretion could produce partial synchrony within some dividing tissues in the brain and cause results deviating from those theoretically expected. Use of 3rd and 4th instar larvae treated as a single pooled population could have produced variations due to differences in hormonal levels among individuals of different ages.

Mosquito larval brain tissue is possibly not in a steady state of mitotic division. Due to hormonal variations, cells in the dividing compartment(s) (Thrasher, 1966), may be in partial synchrony at different times throughout larval development. More than a single dividing compartment in each larval brain may be present with its own characteristic cell cycle. Christophers (1960) has described several sizes of nuclei in the three regions of the larval brain of *Aedes aegypti* (L.). Jan and Boyes (1970) have also shown that several sizes of nuclei are present in each larval brain of the house fly, *Musca domestica*. Labeled nuclei of different sizes were observed in the larval brain tissues of both *O. signifera* and *A. triseriatus*. The cytoplasm of the brain cells was

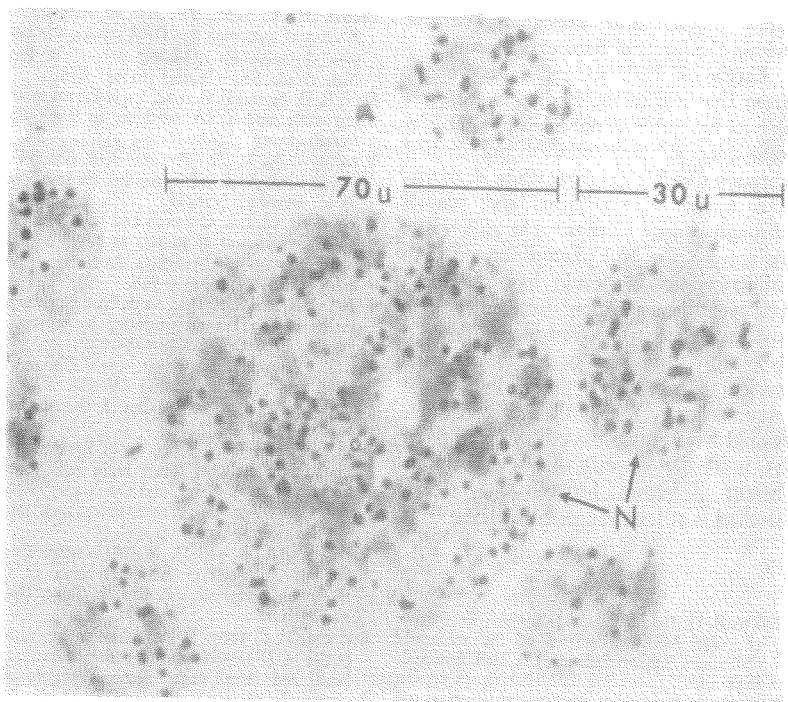


FIG. 3.—Nuclei (N) in *O. signifera* brain tissue showing heavy labeling. x 4000.

disrupted by squashing, consequently only the squashed nuclear diameters could be measured. Large labeled nuclei were found to be 70–75  $\mu$  in diameter whereas most nuclei observed ranged from 20–30  $\mu$  in diameter. No large chromosomes of commensurate size were observed, yet heavy labeling in these nuclei indicate considerable DNA synthesis (Fig. 3). Nuclei of different sizes point to the possibility of more than a single dividing compartment (Thrasher, 1966) within each larval brain, adding to the heterogeneity of the population.

The effects of various concentrations of  $H^3TdR$  on cell cycles in mosquito brain tissues have not been studied. Greulich, Cameron, and Thrasher (1961) demonstrated that thymidine stimulates mitotic activity in mouse duodenal epithelium.

Wimber and Quastler (1963) have shown that 4  $\mu C/ml$   $H^3TdR$  increases the  $G_2$  phase of *Tradescantia* root tip cells. The effects of 2  $\mu C/ml$   $H^3TdR$  solution on the dividing brain tissues of the two species of mosquitoes studied could be different, thus producing other than theoretically expected results proposed by Thrasher (1966).

**SUMMARY AND CONCLUSIONS.** The pulse labeling, autoradiographic, and brain squash procedures employed in this study served as a useful technique in detecting differences in cell cycle times of *O. signifera* and *A. triseriatus*. The data obtained for *O. signifera* indicate a total cell cycle time of 11 hrs. and 45 min., whereas a total time for *A. triseriatus* could only be approximated to be greater than 20 hours.

These differences are difficult to interpret within the limits of these experiments.

Larvae of both species were brought from the field to the laboratory for rearing 48 hrs. prior to pulse labeling. *O. signifera* larvae were collected and studied through October 24, 1970, while *A. triseriatus* larvae were collected and studied through March 7, 1971. The differences in temperature and photoperiod prior to laboratory rearing could have produced cell cycle time variations between the two species. The change from a natural to a laboratory diet also may have induced variation in cell cycle times. It does not appear, however, that these factors alone would have caused the cell cycles to differ as markedly as was observed. An inherent difference between the species studied is at least suggested by these data.

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