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Immuno-Fluorescent Analysis of Follicular Growth and Development in Whole Ovaries of the Indianmeal Moth

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ABSTRACT The differentiation and growth of ovaries was analyzed using immuno-fluorescence microscopy and then correlated with the changes in the external morphology of female pupae during metamorphosis of the Indianmeal moth, *Plodia interpunctella* (Hübner). Fourteen developmental points coincident with a daily change in the light:dark cycle were chosen for observation to describe the progress of cuticular, ovarian, and follicular development during metamorphosis. Follicular structure was examined in whole mounts of ovaries using an immuno-fluorescent labelling technique. The growth of oocytes and nurse cell cap in terminal follicles was measured throughout ovarian development. A rapid increase in the relative size of the nurse cells began during the fourth scotophase and continued until the beginning of the sixth scotophase. Following the sixth scotophase, the relative size of the nurse cells decreased until they disintegrated prior to choriogenesis. Oocytes began to grow rapidly during the fifth scotophase, coincident with the initiation of vitellogenesis, and continued to grow until choriogenesis was initiated just after adult eclosion. The rate of follicular growth was related to the position of the follicle in the ovariole; the closer to the terminal position, the greater the rate of growth. Thus, at adult eclosion, each ovariole contained a linear array of follicles in progressive stages of development with the terminal follicles ready to begin choriogenesis.

Females of the Indianmeal moth, *Plodia interpunctella* (Hübner), complete the majority of egg maturation during metamorphosis and emerge as adults having terminal oocytes that can be fertilized within a few hours after eclosion. The paired ovaries in newly eclosed females have a gross morphology similar to the mature polytrophic ovaries in other lepidopterans (Norris, '32; Telfer, '75. King and Büning, '85; Griffith and Lai-Fook, '86). Each ovary is composed of four ovarioles containing a linear array of follicles in progressive stages of development with the terminal follicles closest to the oviduct being the most advanced (Norris, '32). Following adult eclosion, the majority of the follicles complete maturation sequentially during the first 32 hr and join the terminal oocytes in readiness for fertilization.

Considerable information has been gathered on the regulation and physiology of adult differentiation, vitellogenesis, and egg maturation of ovaries in insects (Engelmann, '79; Hagedorn and Kunkel, '79; King and Büning, '85; Bownes, '86). In moths of the families Bombycidae, Pyralidae, and Saturniidae, adult ovarian differentiation and maturation of the terminal follicles occurs during metamorphosis. In *Bombyx mori,* the initiation of follicular differentiation and growth has been correlated with the early pupal ecdysteroid peak (Hanaoka and Ohnishi, '74; Tsuchida et al., '87). Isolation of pupal abdomens prior to the first pupal ecdysteroid peak blocks follicular differentiation in both *B. mori* (Sakurai and Hasegawa, '69; Chatiani and Ohnishi, '76; Ohnishi, '87; Tsuchida et al., '87) and *Hyalophora cecropia* (Williams, '52); injection of ecdysteroids into isolated abdomens of these moths stimulates follicular differentiation and growth. After the initiation of adult differentiation, the follicles become or-

ganized and develop to maturity in the vitellarium. Follicular morphology has been described for other lepidopterans, i.e., *H. cecropia* (King and Aggarwal, '65), *Ephestia kuehniella* (Cruickshank, '71; Guelin and Durand, '80; Torres, '81), *B. mori* (Yamauchi et al., '81; Yamauchi and Yoshitake, '84), and *Calpodes* (Griffith and Lai-Fook, '86), where each follicle consists of an oocyte connected to seven nurse cells via intercellular canals and a single layer of follicular cells, which surrounds the oocyte.

Although there is information known about the physiology of ovarian development and egg maturation during early adult development and late vitellogenesis, limited information is available about the control and progress of ovarian developmental changes during pupal and pharate adult development. As components of an analysis of egg production in *Plodia interpunctella,* the yolk proteins have been characterized (Shirk et al., '84; Bean et al., '88) and the regulation of yolk protein synthesis by ecdysteroids has been described (Shirk and Brookes, '87; Shirk, et al., '90). Previously, the temporal sequence of adult development under conditions of continuous light was described for *P. interpunctella* (Smithwick and Brady, '71), and general schemes for follicular development were described for other lepidopterans (King and Aggarwal, '65; Cruickshank, '71; Torres, '81; Yamauchi and Yoshitake, '84). However, these descriptions do not offer an adequate correlation of the temporal sequence of metamorphosis with ovarian growth so that a precise understanding of the physiology regulating metamorphosis and egg maturation in *P. interpunctella* can be attained. This study provides a temporal description of ovarian development beginning before follicular differentiation and continuing to choriogenesis and correlates these events with changes in the external morphology during pupal and pharate adult development.

MATERIALS AND METHODS

Animal preparations

The *Plodia interpunctella* colony was reared according to Silhacek and Miller ('72) in a 16 hr light:8 hr dark cycle at 30°C and 70% relative humidity. The initiation of adult development and eclosion in *P. interpunctella* was found to be under photoperiodic control (Zimowska et al., unpublished). Females that pupated just prior to the beginning of a scotophase were found to develop synchronously and eclosed after 136 (±2) hr. Those that pupated after scotophase were divided into two groups: one that required a little more than 5 day to eclose and a second that was delayed about 1 day. To obtain pupae that would develop synchronously, newly molted white pupae (±½ hr) were collected just prior to the beginning of the scotophase to obtain synchronous cohorts and were aged to specific times. Developmental stages were identified on the criteria of time from pupation and external morphological characters (Table 1). Cohorts of a developmental stage were used to prepare tissues for that time point. Insect age is expressed in hours from the time of pupation.

Immunohistochemistry

Cells in the ovary were stained using as a primary antiserum a monospecific polyclonal rabbit antiserum to a 14 kDa polypeptide (embryonic protein = EP) that was isolated from embryos of *Plodia interpunctella* (Shirk, unpublished). The antiserum to EP was chosen to stain the ovaries because the accumulation of this antigen was restricted primarily to germline cells of both ovaries and testes of this species. Based on a lack of antigenic cross-reactivity, EP was unrelated to the previously identified yolk polypeptides (YPs) of this species. Specifically, monospecific antisera for each of the YPs did not cross-react with EP and vice versa. In addition, the cellular compartmentalization of EP was different than that of the YPs because EP was localized within the cytoplasm of the oocyte and was not present in the yolk granules as are the YPs.

Immunofluorescent staining of EP in whole mounts of ovaries, which provided a unique visualization of structural changes during development, was performed essentially as described by Davis ('87). Ovaries were dissected from pupae in Weevers' saline ('66). Whole ovaries were fixed for 12 hr at 4°C in 4% (W/V) depolymerized paraformaldahyde in 0.1 M phosphate buffer (pH 7.4). The ovaries were washed 5 times for 1 hr in 0.1 M phosphate (pH 7.4), 1% azide, and 1% Triton-X 100 (PBAT) at 24°C, and placed in PBAT at 4°C overnight. The ovarian tissues were blocked with 2% nonreactive goat serum in PBAT at 24°C and then incubated with monospecific rabbit anti-EP serum diluted 1:250 in PBAT at 24°C overnight. The ovaries were washed in PBAT 6 times for 1 hr each and then incubated with anti-rabbit goat IgG conjugated with fluorescein isothio-

TABLE 1. Correlation of externally observable morphological characters with the growth and development of ovaries in pupae and pharate adults of P. interpunctella

Pupal age (hours)	Pupal stage	Observable structures	Description	Ovarian stage	Relative size of ovariole mean (SD)
0–½	P1	White pupa	Cuticle soft and translucent, no apparent tanning	A	2 (0.1)
9	P2	Tan pupa with clear eyes	Cuticle sclerotized and tanned, eyes unpigmented (fat body dissociated)	A	3 (0.1)
24	P3	Tan pupa with eyes ¼ pigmented	Pigment spots appear in ¼ facets of the ventro-anterior edge of the eyes	B	4 (0.4)
33	P4	Tan pupa with eyes ⅓ pigmented	Pigment spots appear in ⅓ facets of the ventro-anterior edge of the eyes	B	5 (0.2)
48	P5	Tan pupa with ¾ pigmented	Pigment spots appear in ¾ facets of the eyes	B	7 (0.3)
57	P6	Tan pupa with eyes ⅝	Pigment spots appear in ⅝ facets of the eyes	B&C	8 (0.6)
72	P7	Tan pupa with dark brown eyes	All facets of eyes are dark brown	C	9 (0.0)
81	P8	Tan pupa with black-brown eyes	Eyes are black-brown (fat body reassociating)	C	12 (0.6)
96	P9	Black eyes, Tarsal claws −	Eyes are black; tarsal claws have not begun to melanize	C	20 (0.8)
100	P10	Tarsal claws +	Light melanization of tarsal claws	C&D	24 (0.9)
105	P11	Tarsal claws brown		C&D	25 (0.4)
120	P12	Wings red-brown	Integument of the wings shows a red-brown pattern	C&D	51 (3.4)
124	P13	Abdomen yellow, wings dark brown		D	57 (3.9)
129	P14	Brown pupa		D	77 (10.3)
136	I	Newly eclosed adult		D	100 (12.1)

cyanate diluted 1:40 in PBAT containing 2% nonreactive goat serum at 4°C overnight. The ovaries were washed in PBAT 5 times for 1 hr each at 24°C. The ovarian preparations were dehydrated in a graded series to 75% ethanol, and then cleared in a graded series to 80% glycerine diluted with 50 mM carbonate buffer (pH 9.4). Whole mounts of ovaries in 80% glycerine were examined and photographed with an Olympus BHS microscope equipped with a BH2-RFL reflected light fluorescence attachment with a blue 490 nm excitation filter, a blue 455 nm supplementary exciter filter, and a G-520 barrier filter.

Feulgen staining

The ovaries were stained by a Feulgen reaction essentially as described by Humason ('79). Ovaries were dissected from pupae in Weevers' saline, and adhering tissues were removed. The dissected ovaries were hydrolyzed in 1 N HCl for 15 min at 60°C and then stained with Schiff's reagent for 30 min at 24°C. After rinsing in sulfurous acid, the ovaries were dehydrated in a graded series of ethanol, transferred to acetone, and finally transferred to xylene. The stained ovaries were mounted in Permount on microscope slides for viewing.

Measurement of follicular and oocyte size

The sizes of the ovarioles, follicles, nurse cells (as measured by the area of the nurse cell cap), and oocytes were measured as planar areas from photomicrographs of whole mounts of ovaries. Area measurements for the designated structures were made from same-magnification photomicrographs or fluorescent photomicrographs of the ovaries using a Reel Digitizer (Jandel Scientific) and analyzed with Sigma-Scan software (Jandel Scientific). The means and standard deviations were computed and plotted using Fig.P software (Biosoft). The three-dimensional graphs were generated using Plotit software (Scientific Programming Enterprises). The term "relative size" of the designated structures is expressed as a percentage of the mean area either of ovarioles or of prechoriogenic oocytes or follicles from ovaries of 2-hr-old newly eclosed adults. Prechoriogenic follicles are identified here as follicles depositing vitellin membrane, also defined as follicles in

terminal growth phase (Telfer and Anderson, '68).

RESULTS

Staining characteristics of the EP antiserum

As seen in fluorescent micrographs of whole mounts of stage B ovaries from P4 and P7 pupae, the EP antiserum staining was localized in the cytoplasm of the cystocytes (Fig. 1A) and in the nuclei of the ovarian sheath cells (Fig. 1). In paraffin sections of vitellogenic follicles from ovaries of newly emerged adult females, EP antiserum staining was found in the cytoplasm of oocytes and nurse cells, and in the nuclei of nurse cells (Fig. 2) and was observed in these cells in all stages of developing follicles. The EP antiserum staining was absent from the follicular epithelium cells at all stages of pupal and pharate adult development.

General description of ovarian growth and correlation with pupal development

In the designated culture conditions, females that molted into pupae just prior to scotophase required 136 (±2) hr to complete metamorphosis and eclose as adults (Table 1). Follicular differentiation was initiated shortly after pupation, with the majority of vitellogenesis in terminal follicles being completed by eclosion. To mark the progress of development during the pupal and pharate adult stages, 14 time points corresponding to 1) the daily beginning of each scotophase, 2) the daily one-hour after the end of each scotophase, 3) the beginning of vitellogenesis, and 4) the beginning of nurse cell regression were chosen for observation. For the externally observable morphological structures, the first nine time points were associated with changes in growth and pigmentation of the compound eyes, while the remaining five time points were associated with changes in the melanization and pigmentation of integumental features of the pharate adult (Table 1). Initiation of adult metamorphosis was observed between 12 and 24 hr based on the appearance of apolysis; thus, all stages from P3 to adult eclosion were considered pharate adults. The sequence of follicular differentiation and follicular growth was then correlated with the changes in the externally observable morphological structures at the 14 time points to provide an accurate temporal sequence for ovarian development during metamorphosis (Table 1; Fig. 3).

The development of the ovaries was divided into 5 stages (A–E) based on the progress of differentiation, nurse cell growth, and oocyte maturation (Table 1; Fig. 3). The fat body began to dissociate in newly molted P1 pupae. Beginning with Stage A, each of the paired ovaries consisted of 4 germaria that were continuous with the tubular ovarioles, and the ovarioles were connected to the

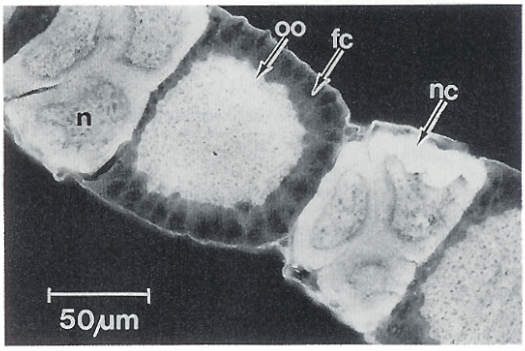

Fig. 2. Fluorescent micrograph of a cross section of early vitellogenic follicles from the ovary of a newly eclosed adult female. fc, follicle cells; n, nucleus of nurse cell; nc, nurse cell; oo, oocyte.

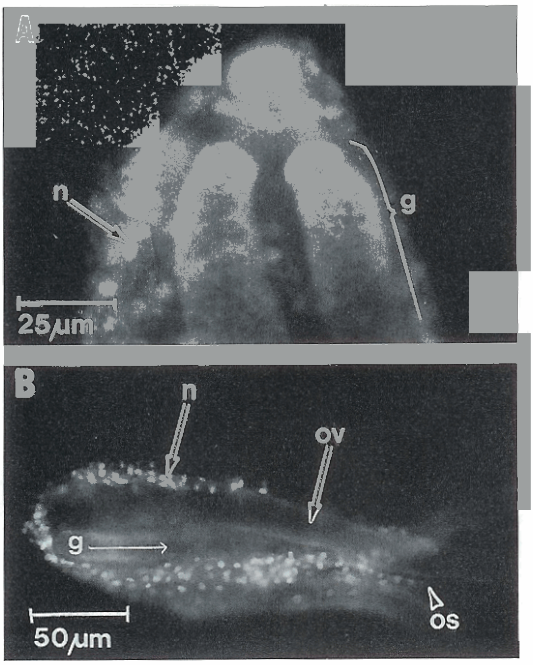

Fig. 1. Fluorescent micrographs of the apical portion of the ovaries from P4 pharate adults (A) and P7 pharate adults (B). g, germarium; n, nucleus of an ovariole sheath mesodermal tissue cell; os, ovariole sheath; ov, ovariole.

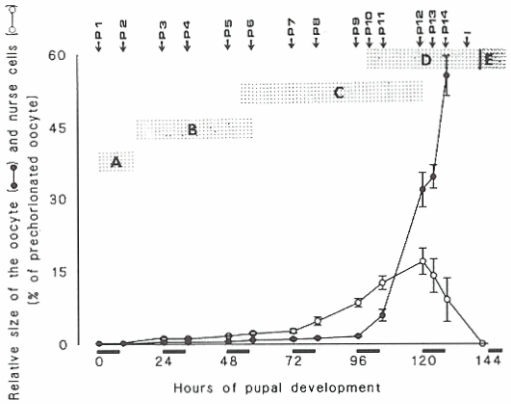

Fig. 3. The increase in size of oocytes and nurse cells of terminal follicles in ovaries of *Plodia interpunctella* during metamorphosis. The relative size of the oocytes and nurse cell caps was determined from the fluorescent micrographs. The mean relative size with standard deviation bars are shown for oocytes and nurse cell caps from the terminal 3–5 follicles of ovarioles from 4–6 females for each time point. Horizontal bars below the X-axis denote the occurrence of scotophase. The designations P1 to P14 and I are as described in Table 1, and letters A to E represent ovarian stages as described in text. The stippled bars represent the duration of ovarian stages

calyxes (Fig. 4A). In Stage A ovaries from P1 pupae, only cystocytes were observed; no differentiated follicles were found in the ovarioles. Thus, Stage A ovaries were at a point of development just prior to the initiation of adult development (Figs. 4A, 5 P1). Between 12 and 24 hr after pupation, the ovaries began to differentiate (Stage B), and organized follicles that contained discernible oocytes, nurse cells, and follicular epithelium cells were present (Figs. 4B, 5 P3). The initiation of endopolyploidization of the nurse cells in terminal follicles, i.e., those follicles most distal (posterior) from the germarium in the ovarioles, marked the beginning of Stage C (Fig. 5 P6). Once endopolyploidization was initiated, the nurse cells also began a rapid increase in relative size (Fig. 5 P6–P10). Stage D ovaries had vitellogenic terminal follicles, and Stage E ovaries, found in newly eclosed adults, had postvitellogenic terminal follicles.

Ovarian growth during the first 80 hr was slow. During this time period, the ovarioles increased in relative size from 2% of the size of the ovarioles of newly eclosed adults in P1 pupae to 12% of the relative size of adult ovarioles in P8 pharate adults (Table 1). Vitellogenesis was initiated in P10 pharate adults between 96 and 100 hr, just following the reassociation of cells comprising the adult fat body. With the onset of vitellogenesis, the ovaries, especially the oocytes, began to increase rapidly in size and continued to increase in size until 24 hr after adult eclosion.

The relative size increase of oocytes and nurse cells in the terminal follicles during metamorphosis was correlated with the externally observable morphological structures and with the stages of ovarian development (Fig. 3). The relative size of the nurse cells of the terminal follicles increased slowly until the 4th scotophase when they began a period of rapid growth. The relative size of the nurse cells reached a maximum at the beginning of the 6th scotophase, and then the cells began to regress. Within a few hours after eclosion, the nurse cells of the terminal follicles had transferred their cytoplasm into their interconnected oocyte and disintegrated (data not shown). The terminal oocytes, and therefore follicles, began to increase rapidly in relative size after the initiation of vitellogenesis during the 5th scotophase and continued their growth until they became prechoriogenic in newly eclosed adults (Fig. 3).

Ovarian developmental stages

Stage A—cystocytes

In newly molted P1 female pupae, each of the paired ovaries consisted of four germaria on the anterior ends of the ovarioles and were surrounded by a sheath of mesodermal tissue. The germaria contained cystocytes, identified in thin sections by the presence of synaptonemal complexes and cytoplasmic bridges (data not shown) but no differentiated follicles (Fig. 4A).

Stage B—follicular differentiation

Between 12 and 24 hr after pupation, follicles had begun to differentiate from the dividing cystocytes in the ovarioles, and by 36 hr defined oocytes, nurse cells, and follicular epithelium cells were recognizable (Figs. 4B, 5 P3). The formation of prefollicles (nurse cell-oocyte clusters without a surrounding layer of follicular epithelium cells) resulted from incomplete divisions of cystocytes. As a result of three nuclear divisions with incomplete cytokinesis, a single cystocyte gave rise to seven nurse cells and a single oocyte that were interconnected by intercellular channels (data not shown). The fate of individual nurse cells was not followed through follicular maturation. However, a differential

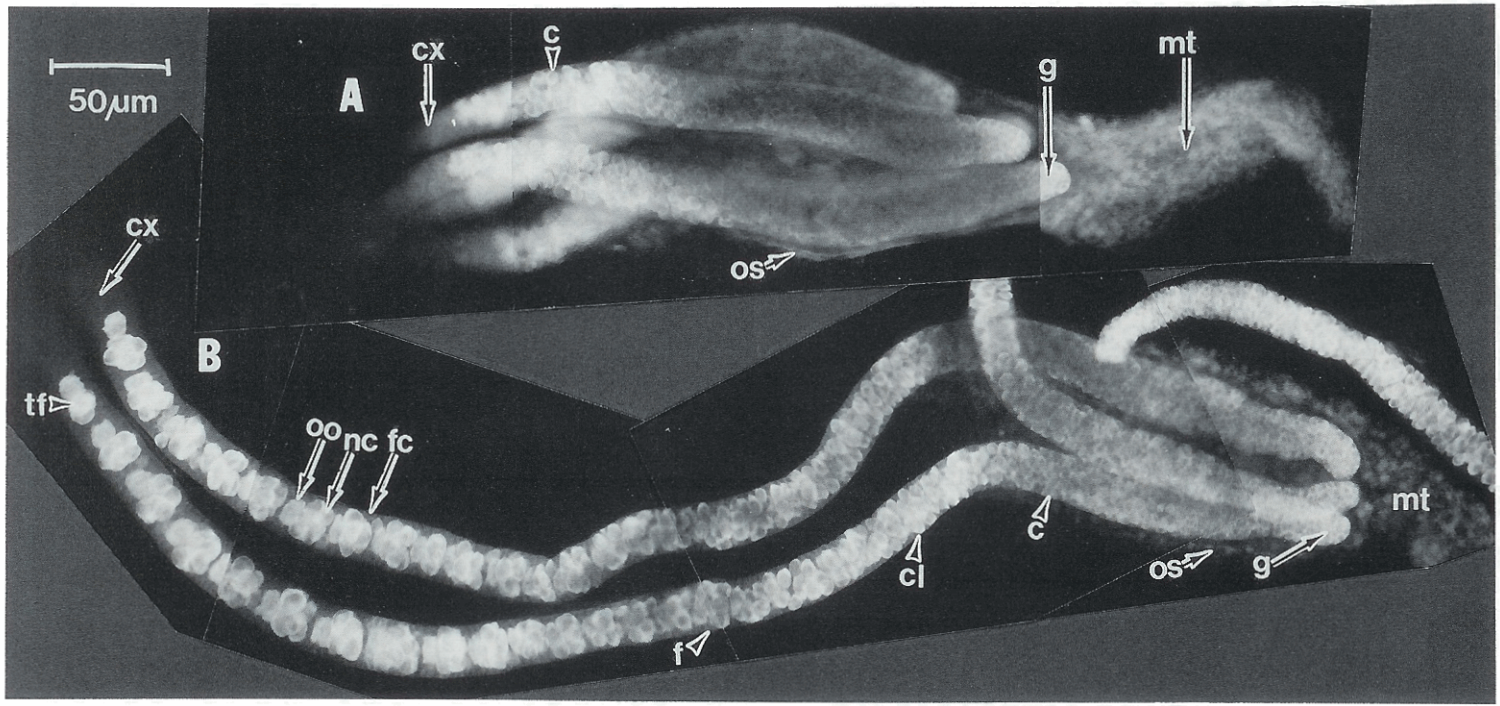

Fig. 4. Fluorescent micrographs of ovaries from a (**A**) P1 pupa prior to follicular differentiation and from (**B**) a P3 pharate adult after the differentiation of the terminal follicles. c, cystocytes; cl, oocyte-nurse cell clusters; cx, calyx; f, differentiating follicles; fc, follicular epithelium cells; g, germarium; mt, mesodermal tissue; nc, nurse cells; oo, oocyte; os, ovariole sheath; tf, terminal follicle.

growth rate for the nurse cells was indicated by the observation that the terminal follicles in newly eclosed females contained six large nurse cells and one that was one tenth of the size of the other six nurse cells (data not shown). Once the prefollicles were formed, they became arranged in a linear array in the ovarioles with the terminal oocytes being the most developed and the most distal from the germarium (Fig. 4B). In ovaries from P3 pharate adults, 9 (±1.5) prefollicles per ovariole were present, and cystocytes giving rise to new prefollicles were observed (Fig. 4B).

The rate of follicular differentiation and the total number of follicles per ovariole increased rapidly until the end of the third scotophase (56 hr). For example, in P4 pharate adults there were 12.8 (±1.3) follicles per ovariole, and the rate of formation during this period (P3 to P4) was 0.4 follicles per hr per ovariole. At the beginning of the third scotophase, Stage P5, 21 (±1.7) follicles per ovariole had differentiated at a rate of 0.55 follicles per hr per ovariole (P4 to P5). However, during the third scotophase, the rate of follicular differentiation increased to 0.75 follicles per hr per ovariole, so that by 56 hr, P6 pharate adults contained 27 (±5.3) follicles per ovariole (P5 to P6). Following the third scotophase, the rate of follicular differentiation fell to about 0.2 follicles per hr per ovariole and remained at this level until the beginning of vitellogenesis.

The end of Stage B was arbitrarily set with P6 pharate adults, which contained 27 ± 5.3 follicles per ovariole, a developmental point when 50% of the follicles had formed. At maturity, the maximum number of follicles per ovariole ranged from 45 to 65 per ovariole in ovaries from 6 hr adult females.

Stage C—endopolyploidization and growth of nurse cells

In addition to the rapid rate of follicular differentiation, the amount of Feulgen staining material in the nuclei of the nurse cells in the terminal follicles increased during the third scotophase (compare staining of nurse cell nuclei in insets in Fig. 5 P5–P6). Similar increases in Feulgen staining material in nurse cell nuclei of other insects has been shown to be associated with endopolyploidization (Mulligan and Rasch, '85). During the fourth scotophase, the size of the nurse cells in the terminal follicles increased rapidly, reaching a maximum in P12 pharate adults at the beginning of the sixth scotophase (Fig. 3). The size of the nurse cells in the terminal follicles started to decrease during the sixth scotophase. The nurse cells could still be observed in the terminal follicles of virgin females until the initiation of vitellin membrane synthesis and the beginning of the terminal growth phase (Telfer and Anderson, '68) approximately 6 hr after adult eclosion. However, if the females mated within 15–30 min after eclosion, the regression of the nurse cells and the termination of vitellegenesis in the terminal follicles could begin within 1 hr after adult eclosion.

Stage D—vitellogenesis

Yolk accumulation was first observed in the terminal oocytes of each ovariole of P9 pharate adults during the fifth scotophase, between 96 and 100 hr. Once yolk uptake began, the relative size of the terminal oocytes increased rapidly (Fig. 3). This increase in the relative size of the oocytes and follicles was correlated with their position within the ovarioles for various developmental points during vitellogenesis beginning with P9 pharate adults. The rate of increase in the relative sizes of oocytes and follicles was dependent upon both the position in the ovariole and the developmental point during vitellogenesis (Figs. 6, 7). The rate of increase in relative size for a positional group of oocytes or follicles was calculated as the change in relative size between the two developmental points divided by the time elapsed between the developmental points. The rate of relative size increase was the same for all oocytes and all follicles through P11 pharate adults. However, after this time the relative size increases for oocytes more proximal to the germarium was slower than for the more terminal oocytes and follicles. Between P11 (Figs. 6A, 7A) and P12 (Figs. 6A, 7A), the terminal 15 oocytes and follicles maintained a similar rate of increase in relative size, while the oocytes and follicles more proximal to the germarium were less developed. Between P12 and P14 (Figs. 6A, 7A), the terminal 10 oocytes and follicles maintained a similar rate of increase in relative size while again those more proximal were less developed. Finally, between P14 and adult eclosion, the five terminal oocytes and follicles continued to increase rapidly to the maximal follicular size while the remainder of the oocytes and follicles lagged behind.

The relationships between the rates of growth for the follicles in the various positions in the ovarioles was most clearly visual-

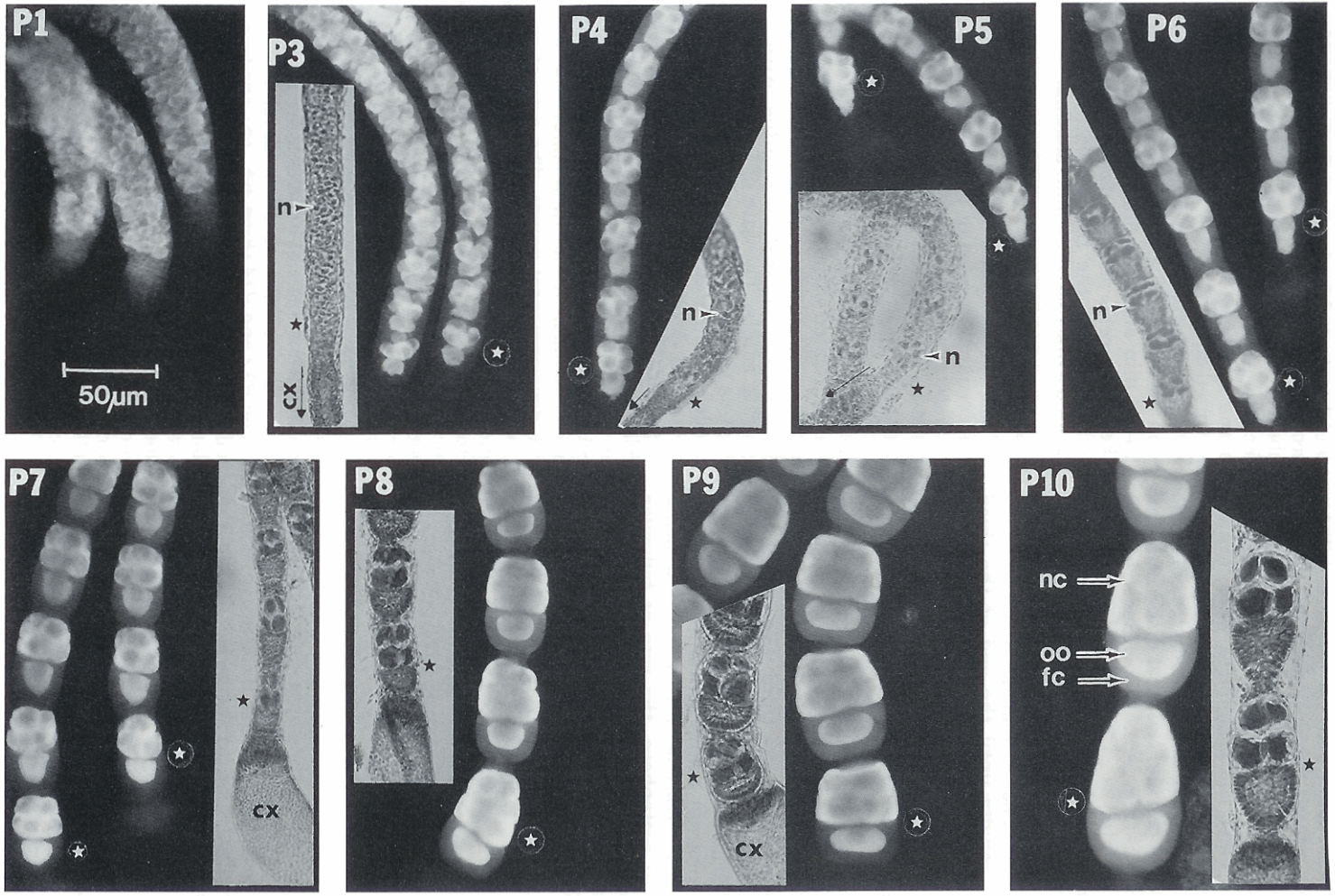

Figure 5

ized by examining the relative size grid lines of the 3D surface plots for the increases in relative size (Figs. 6B, 7B). The slope of the relative size grid lines for the terminal group of five oocytes and follicles became much steeper after 120 hr than for the second group of five oocytes and follicles, while before 120 hr the slopes for the increase in relative size were nearly the same. A similar observation was noted for the relative size grid lines between the second 5 group and the third 5 group as well as for the third 5 group and the fourth 5 group of oocytes and follicles. This pattern of oocyte maturation resulted in a linear array of developing follicles within the ovarioles.

Stage E—post-vitellogenesis

The terminal five follicles reached maximum size in adults 2 to 6 hr after eclosion when the oocytes had completed vitellogenesis and the follicle cells had initiated vitellin membrane synthesis (prechoriogenic follicles). The termination of vitellogenesis and the concomitant transformation of the follicular epithelium to prechoriogenic activity were correlated with the collapse of the nurse cell cap (Fig. 8). The last vitellogenic follicle retained a reduced space in the nurse cell cap (Fig. 8, follicle a), while the adjacent distal follicle, which was prechoriogenic, showed no remaining nurse cell cap (Fig. 8, follicle b).

The termination of vitellogenesis was observed in whole mounts of ovarioles from 2-hr-old adult females by staining either with EP antiserum immunofluorescence (Fig. 9) or with trypan blue (data not shown). The follicles a and b in Figure 8 were observed with fluorescence microscopy after staining with EP antiserum. The intercellular spaces between the follicular epithelium cells in follicle a were heavily stained with the EP antiserum, showing that the follicle was still patent and capable of yolk protein uptake (Fig. 9A). However, in the adjacent follicle b, the interfollicular cell spaces had closed, signalling the end of vitellogenesis and the beginning of the terminal growth phase. As a control, non-reactive serum did not result in the staining of the interfollicular cell spaces of vitellogenic oocytes (Fig. 9B). The closing of the interfollicular cell spaces was also observed when ovarioles were stained with trypan blue according to Telfer and Anderson ('68). The loss of EP staining and the lack of trypan blue uptake by a follicle were correlated with the same stage of follicular development, i.e., when the nurse cell cap had disintegrated (data not shown). Following disintegration of the nurse cell cap and the closing of the interfollicular cell spaces, the follicles entered the terminal growth phase, which indicated the beginning of vitellin membrane synthesis. To determine the number of follicles with incomplete vitellin membrane synthesis, the trypan blue stained ovarioles were immersed in 0.9 M sucrose. Three follicles distal to the last follicle showing trypan blue uptake were crenated due to water loss. Follicles more distal than the three crenated follicles were insensitive to the hyperosmotic solution indicating that the vitellin membrane was complete (Telfer and Anderson, '68).

Fig. 5. Growth of terminal follicles in ovaries from the various stages of previtellogenic females. Each panel shows a fluorescent micrograph of the ovary with an inset showing a Feulgen stained micrograph of the same stage ovary. Panel designations indicate the corresponding stage of pupal development as described in Table 1. cx, calyx; fc, follicular epithelium cells; n, nuclei of nurse cells; nc, nurse cell caps; oo, oocyte. Asterisk designates the position of the terminal follicle. Arrows in panel P3, P4, and P5 extend from the beginning of the calyx.

DISCUSSION

The morphology and development of ovaries and follicles in *Plodia interpunctella* are similar to that reported for other moths (King and Aggarwall, '65; Cruickshank, '71; Torres, '81; Yamauchi and Yoshitake, '84; King and Büning, '85). However, the temporal sequence of development is different for each species: i.e., *Bombyx mori* and *Hyalophora cecropia* complete choriogenesis of most oocytes before adult eclosion, whereas *P. interpunctella* completes choriogenesis during the first day following adult eclosion. While the previous studies have been mostly concerned with the description of ovaries that contain follicles in all stages of development, the work presented here describes various stages of ovarian development. By describing a temporal sequence for the externally observable morphological structures at defined points in the light:dark cycle throughout pupal and pharate adult development, this study has provided a profile of markers useful in the early as well as the later stages of metamorphosis and established the course of development in reference to circadian rhythms. These two features provide considerable refinement of the developmental profile described by Smithwick and Brady ('71).

Two aspects of the technical approach contributed significantly to the assessment of ovarian growth reported in this study. First,

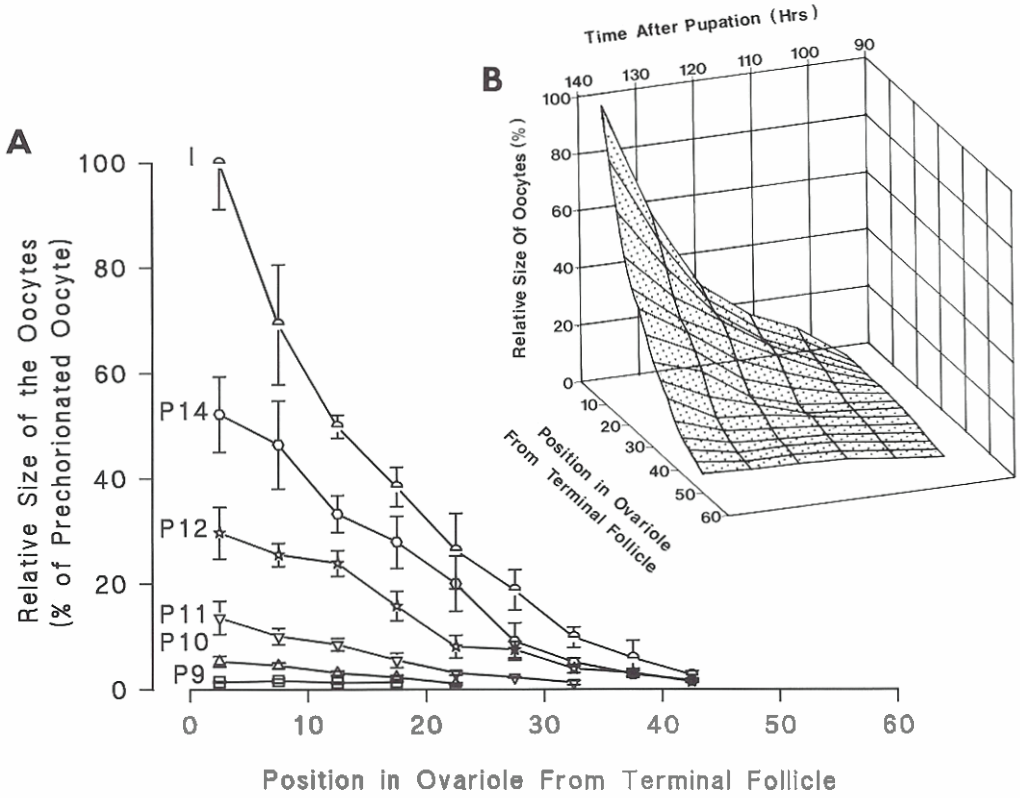

Fig. 6. Increasing size of oocytes from various positions within ovarioles during the stages of vitellogenesis. **A:** Profiles of the relative sizes of oocytes in the ovarioles at selected time points during vitellogenesis. The relative size of the oocytes was determined from micrographs of Feulgen stained preparations. The relative size of the oocytes is presented as the percent of the planar area of terminal prechorionated oocytes in newly eclosed adult females. Each point represents the mean and SD of five sequential oocytes from one female. Line designations indicate the corresponding stage of pupal development as described in Table 1. **B:** Three-dimensional surface plot of the relative oocyte size during vitellogenesis. The stippled area represents the upper surface of the data plot.

the use of the EP antiserum as a means of selectively staining the ovarian preparations proved especially useful in the analysis of the development of previtellogenic ovaries. The use of the EP antiserum provided us with the ability to observe for the first time germline cells in whole mounts of the tissue without interference from structures of the overlying follicular epithelium cells. This technique provided a clear assessment of the growth of the nurse cell-oocyte complex through all of the stages of adult ovarian development. In addition, the use of EP antiserum to stain the whole mounts provided an accurate determination of the point at which a follicle began synthesizing the vitellin membrane (see Fig. 9). The intact follicles showed the same sensitivity to staining with EP antiserum as for staining with trypan blue. Thus, the transition of a follicle to terminal growth phase could be determined accurately using only the EP antiserum immuno-fluorescent staining. This technique also produced consistent tissue effects, i.e., minimal tissue shrinkage and distortion, so that reliable size measurements could be made from different preparations used for making comparisons between the various developmental stages.

The second aspect was the use of relative size calculated from photomicrographs of nurse cell caps, oocytes, and follicles as a measure to describe ovarian growth quantitatively, which avoided some of the difficulties associated with computing volumes. Because the shapes of the follicles, the nurse cell capsules, and the oocytes in the fixed prepara-

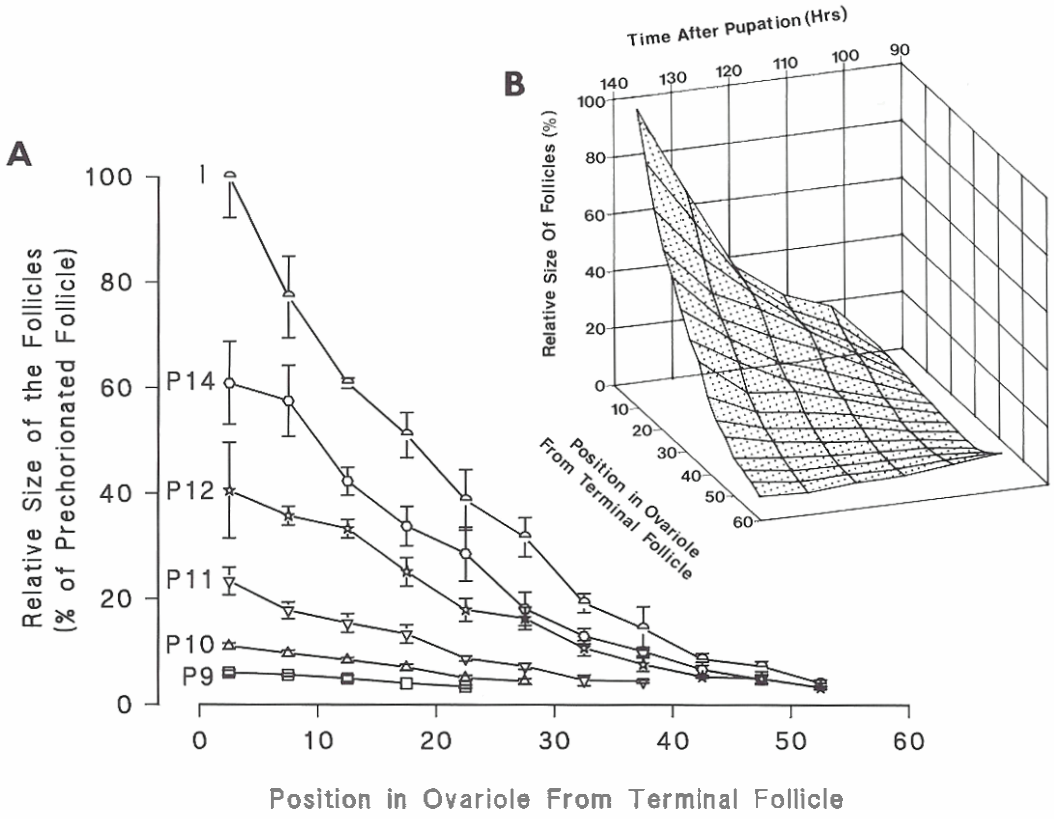

Fig. 7. Increasing size of follicles from various positions within ovarioles during the stages of vitellogenesis. **A:** Profiles of the relative sizes of follicles in the ovarioles of selected time points during vitellogenesis. The relative size of the follicles was determined from micrographs of Feulgen stained preparations. The relative size of the follicles is presented as the percent of the planar area of the terminal prechorionated follicles in newly eclosed adult females. Each point represents the mean and SD of five sequential follicles from one female. Line designations indicate the corresponding stage of pupal development as described in Table 1. **B:** Three-dimensional surface plot of the relative follicle size during vitellogenesis. The stippled area represents the upper surface of the data plot.

tions were not regular spheres, the determination of volume from our data as previously described (Torres, '81) led to a greater variability in the computed values than did the use of relative size. Therefore, the use of relative size was employed to decrease variability.

Follicular differentiation in *Plodia interpunctella* began between 12 and 24 hr after pupation. The ovarioles of P3 pharate adults contained a mean of nine prefollicles each consisting of an oocyte and seven nurse cells. The oocyte was oriented posterior to the nurse cells within the ovariole. The same number of nurse cells per follicle has been reported for other lepidopterans (Telfer, '75; King and Büning, '85). Although Norris ('32) previously reported that there were only five nurse cells per follicle, the presence of the other two nurse cells was probably not observed because of the large differential in the size of the nurse cells. A size differential between the nurse cells occurs as the result of asynchronous endomitotic cycles of the nurse cells following segregation from the oocyte (Telfer, '75).

Following the initiation of follicular differentiation, there were four major events in the development of terminal follicles that were coincident with scotophases, i.e., the initiation of nurse cell endopolyploidization, the rapid increase in the relative size of the nurse cells, the initiation of vitellogenesis, and the decrease of nurse cell cap size. Only the last event in the maturation of the terminal follicles, which is the onset of choriogene-

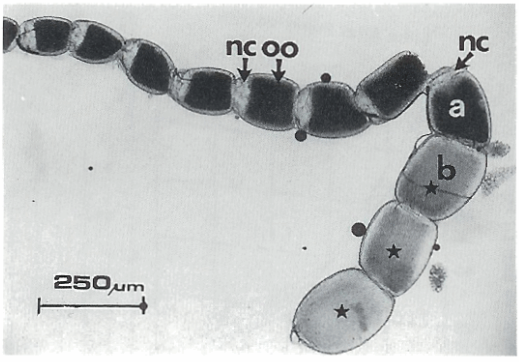

Fig. 8. Light micrograph of a whole fixed ovariole from a 6-hr-old virgin adult female. The micrograph was taken using transmitted light. The ovariole was fixed and stained for immuno-fluorescence and is shown using fluorescence microscopy in Figure 9. The follicles a and those more proximal to the germarium were vitellogenic. Follicles marked with a * were post-vitellogenic. nc, nurse cell cap; oo, oocyte.

sis, began during a light phase occurring after adult eclosion. The coincidence of the four earlier events during scotophases suggests that development of the terminal follicles, which is controlled by regulatory mechanisms that are influenced by photoperiod. However, a further assessment of the influence of photoperiod on the initiation of these follicular growth parameters must be made before this can be concluded.

Based on calculations from data in Figure 3, the growth profiles for nurse cells and oocytes of terminal follicles also showed that the rates of growth increased at specific times. Prior to the fourth scotophase (P1 to P7) the rate of increase in relative size of the nurse cells was less than 0.06% per hr of the size of prechoriogenic oocyte. After the beginning of the fourth scotophase (P7 to P12), the rate of increase in the relative size of the nurse cells increased four-fold to 0.25% (±0.03) per hr. Similarly, the rate of oocyte growth was less than 0.03% per hr of the size of prechoriogenic oocytes prior to the fifth scotophase (P1 to P9). However, following the initiation of vitellogenesis during the fifth scotophase, the rate of increase in oocyte relative size increased from 0.47% per hr between P9 to P11 (96 to 105 hr) to 2.63% per hr between P12 to P14 (120 to 129 hr) to 8.9% per hr between P14 (129 hr) and eclosion (136 hr).

The rate of follicular growth during pharate adult development was dependent on the position of the follicle within the ovarioles. Zimowska et al. (unpublished) observed that approximately 15 oocytes became vitellogenic in the 4 hr between P9 and P10 pharate adults. From the data presented here, the growth rates of the terminal 15 oocytes and follicles remained equal through P12 (Figs. 6, 7). However, at each subsequent developmental point, the rate of growth for the terminal five oocytes and follicles increased rapidly whereas the development of the more anterior oocytes and follicles was at a slower rate. The existence of differential growth rates for the various groups of oocytes and follicles indicates that their growth rates are regulated along the length of the ovariole, and as a consequence the ovarioles of newly emerged adult females contain a linear array of oocytes in progressive stages of development.

The growth profiles of ovaries during pharate adult development suggest the presence of a regulatory mechanism that either promotes an increase in the growth rate of the terminal follicles or retards the growth of follicles more proximal to the germarium or both. Even though the general regulation of follicular growth and vitellogenesis was shown to be dependent on the decline of ecdysteroid titers (Shirk and Brookes, '87; Shirk et al., '90), this cannot account for the differential growth of individual follicles. In *Hyalophora cecropia,* the differential growth between the terminal follicles and the more anterior follicles was suggested to be the result of declining vitellogenin titers in the hemolymph that reduced availability of vitellogenins to the maturing oocytes during the later stages of pharate adult development (Telfer and Rutberg, '60). However, this does not appear to be the case in *Plodia interpunctella* because the hemolymph titers of the vitellogenins remain high throughout the entire period of vitellogenesis (Shirk et al., unpublished).

The regulated growth of individual follicles during pharate adult development may be the result of interfollicular communication. Dominance of one follicle over another could be established through a gradient communicated via ionic coupling (Woodruff, '79; Woodruff and Telfer, '90). The presence of any regulatory mechanism controlling follicular development has not been elucidated and must be further assessed before completely understanding ovarian development during metamorphosis.

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In memory of Christa L. Hoyt, whose dedication and warmth enriched the lives of all

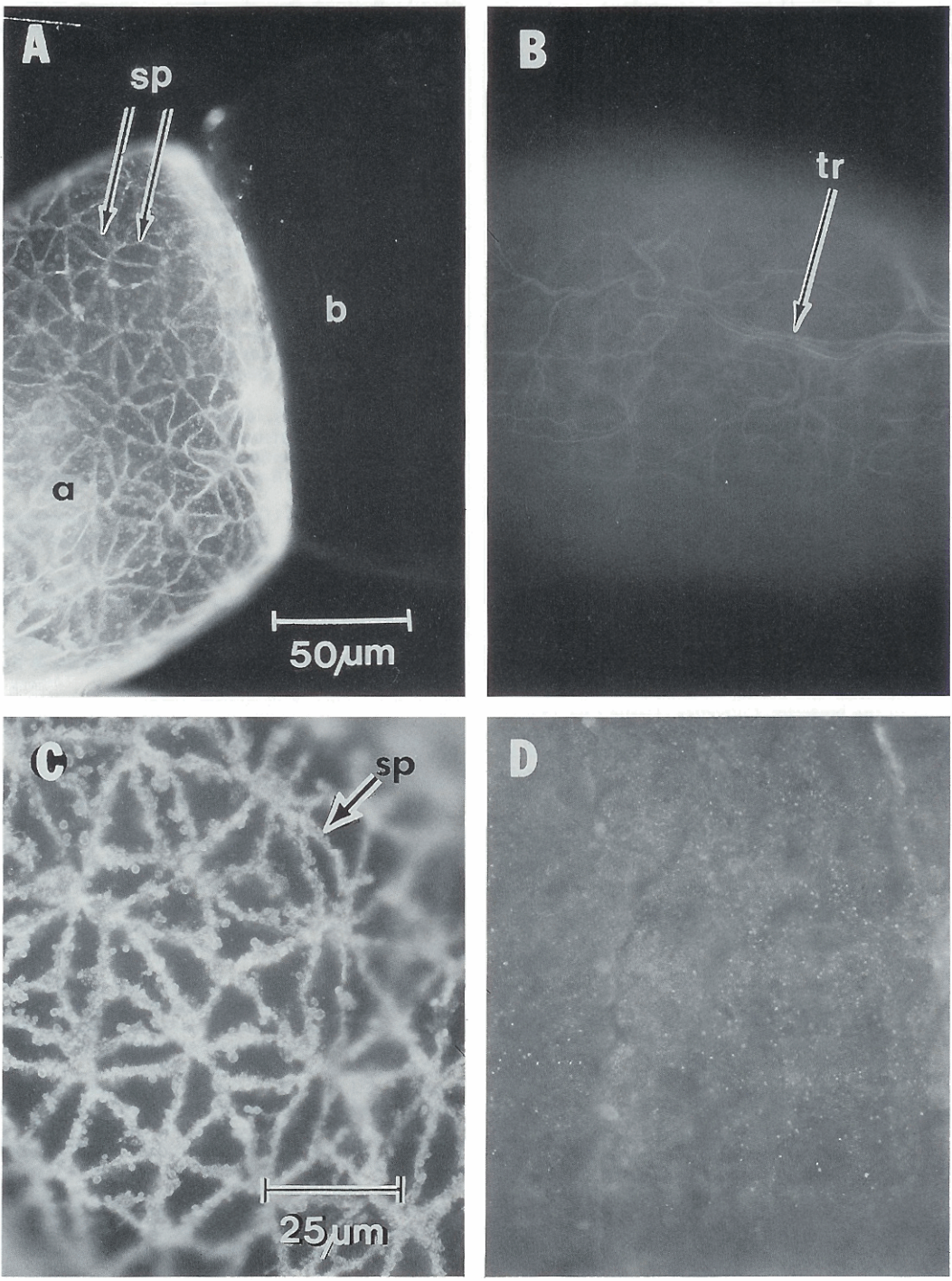

Fig. 9 Fluorescent micrographs showing the presence of EP antiserum staining in the interfollicular cell spaces of vitellogenic follicles (Aa,C), but not in post-vitellogenic follicles (Ab,D). B shows background fluorescence of a vitellogenic follicle with nonreactive serum. The magnification bars in A and C are the same for B and D, respectively. sp, interfollicular cell spaces; tr, trachea.

who worked on this project and whose tragic death has cut short a future of immense potential. We wish to thank Drs. E. Beckemeyer, K. Iatrou, and W. Telfer for their comments. We thank Christa Hoyt, Curtis Murphy, and Karen Ogren for technical assistance. This work was supported in part by a BARD grant (US-1122-86R) to D.L.S., E.S., and P.D.S.

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