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by

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ABSTRACT

A method of dissection followed by staining was initiated to allow physiological age-grading based on ovarian development in order to more completely describe the physiological development of adult female stable flies [*Stomoxys calcitrans* (L.)]. The gonotrophic developmental continuum from a non-differentiated cell in teneral females to mature eggs at the time of oviposition was first arbitrarily divided into six stages by using distinct landmarks within the developing oocyte. Then, nulliparous, uniparous, and biparous+ females were differentiated on the basis of the presence or absence of stained follicular relics in the ovariole sheaths. The combination of the two techniques made it possible to assign dissected adult female stable flies to one of 12 age-classes.

A Technique for Physiologically Age-grading Female Stable Flies, *Stomoxys calcitrans* (L.)

Philip J. Scholl[1]

INTRODUCTION

A technique to describe the physiological aging process of adult female stable flies [*Stomoxys calcitrans* (L.)] was required to complete the total developmental description initiated by Berry *et al.* (1977) and Kunz *et al.* (1977). This bulletin describes a technique for placing individual female stable flies into 12 arbitrary stages based on gonotrophic development. The technique should serve as a basis for future experimental work that will relate physiological age to calendar age and the degree to which environmental factors such as temperature, light, and feeding behavior affect this relationship.

Techniques used to describe gonotrophic development in Diptera require two separate steps. For the first, one must describe oogenesis as a developmental continuum of individual oocytes from non-differentiated cells in teneral females to mature eggs at the end of the first complete gonotrophic cycle. For the second, the first complete gonotrophic cycle must be distinguished from subsequent oviposi-tions.

The most commonly used system for arbitrarily dividing the continuum into distinct stages was proposed by Christophers (1911) and later modified by Mer (1936) for anopholine mosquitoes. Thus, in the early work with *S. calcitrans* by various Russian investigators (Derbenieva-Ukhova 1942, Kuzina 1942, Kuzina 1950, and Detinova 1962), this eight-stage classification system was used to describe the stages in the first gonotrophic cycle; and the system is still used to describe ovarian development in dipterous insects (Cupp and Collins 1979, Moobola and Cupp 1978).

Numerous authors have described follicular development of the polytrophic ovaries of Diptera. King *et al.* (1956) described oogenesis in *Drosophila melanogaster* Meigan as having 14 stages in the first cycle. Adams and Mulla (1967) divided oogenesis into 10 stages for *Hippelates collusor* (Townsend), and this system was later used to describe development in the house fly, *Musca domestica* L. (Adams 1974); the screwworm fly, *Cochliomyia hominovorax* (Coquerel) (Adams and Reinecke 1979); the stable fly, *S. calcitrans* (Buschman and Patterson 1980); and the Australian blow fly, *Lucilia cuprina* (Wiedemann)

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(Beattie and Cheney 1979). Also, the first ovarian cycle has been described as being a seven-stage process for the house fly, *Musca domestica vicina* Macquart (Sakurai 1973); as a six-stage process for the blow fly, *L. cuprina* (Vogt *et al.* 1974) and the house fly (Trepte 1979); and as a five-stage process for the eye gnat, *Hippelates pusio* Loew (Schwartz 1965).

The second step, as noted, is accurate classification of female physiological development to age-grade populations. For this, the researcher must differentiate the first egg-laying cycle from those that follow. Detinova (1962) summarized several techniques for age-grading dipteran insects, especially the Culicidae. Also, descriptions of methods for age-grading muscid flies (including *S. calcitrans*) were summarized by Detinova (1962) and Anderson (1964). In brief, flies that have not yet oviposited (nulliparous) can be distinguished from those that have (parous) by using the accumulation of "yellow bodies" (follicular relics) that remain in the ovariole pedicels and lateral oviducts as first described by Kuzina (1942). With subsequent ovipositions, and therefore increased deposition of such relics, the yellow bodies become more pronounced. Anderson (1964) noted that the major difficulty in distinguishing nulliparous from parous flies arose with those flies that were gravid uniparous (one previous oviposition) because the relics were too dispersed within the ovariole sheath to be easily distinguishable. Nevertheless, several authors have reported success in distinguishing different gonotrophic cycles in such cyclorraphous Diptera as the face fly, *Musca autumnalis* DeGeer (Miller and Treece 1968); *L. cuprina* (Clift and McDonald 1973, Vogt *et al.* 1974); the Australian bush fly, *Musca vetustissima* Walker (Tyndale-Biscoe and Hughes 1968); *M. domestica* (Smith 1968); and a black fly, *Simulium ochraceum* Walker (Cupp and Collins 1979).

The investigation reported here was an effort to find a method to distinguish between, and arbitrarily number, stages in the follicular development of female *S. calcitrans,* and to simplify the system so it would require minimum training and equipment. Additionally, a technique was desired that would make it possible for any investigator to easily distinguish between nulliparous, uniparous, and biparous+ (two or more ovipositions) female flies.

MATERIALS AND METHODS

Adult stable flies were obtained from the colony at the U.S.D.A. Livestock Entomology Laboratory facility in Lincoln, NE. These flies were from a parent colony started from flies collected in Lancaster Co., NE in the summer of 1979, currently in the 20th generation.

The rearing technique for the colony has evolved with changing availability of materials. Eggs are collected on moistened paper towels folded so one end is submerged in a 473-ml wide-mouth jar of water and secured with a rubber band. The other end of the towel is loosely

folded over to produce a dark "tunnel", apparently the preferred oviposition site for this species. Eggs are washed off the towel into a beaker, and 1 ml (≃800 eggs) is pipetted into wells dug into rearing medium (2 parts vermiculite, 1 part wheat bran meal, 0.25 parts ground fish meal and 1.25 parts tap water). Each day after the third day, the medium is stirred and remoistened until the vermiculite particles glisten (Jones 1966), a process that is continued until the third-instar larvae begin to pupate. Pupae are spooned from the surface of the medium, floated in a large tub of water, collected on a sieve, and placed on paper towels to dry. Then they are placed in 30cm² screened cages until emergence. Once a day adult flies are fed sodium citrated (1 tablespoon/gal) fresh bovine blood from soaked sanitary napkins placed on the top of each cage.

The purpose of the initial trials was to become familiar with the physiological changes in the female reproductive tract that corresponded with their ovipositional history. Therefore, individual records of female fertilization and oviposition were made by using single-pair rearing cages (Figure 1). These consisted of a 472 ml (1 pt) paper carton with a 4 cm-diameter hole in the bottom and the center cardboard removed from the lid, so only a ring was left. Additional materials required included two tops from 9 cm plastic petri dishes, a 13 cm-diameter piece of bridal veil, a 25 ml Erlenmeyer flask, a 10 cm strip of absorbent cotton that served as a wick, a figure "8" 10 × 5 cm piece of black cotton muslin, a No. 8 rubber band, a straight pin, and a small strip of cotton gauze. The black cotton piece had one end secured with the rubber band so one end was always in contact with and moistened by the protruding cotton wick. The other end was folded over and pinned loosely to produce the tunnel. The bridal veil was secured on the top of the cage by the cardboard ring, the blood-soaked cotton gauze strip was placed on top, and the entire cage was covered by the second petri dish lid. Water was injected daily into the cotton wick with a large syringe until it was saturated.

Subsequently, the developmental sequence of individual oocytes from female cohorts was photographed. Teneral flies were allowed to emerge for 1 hr in a 30 cm² screened cage and placed in a diurnal growth chamber at 25°C with a light regime of 12L:12D. In the first study, three cohort females were removed every 12 hours, anesthetized with CO_2, dissected in Yeager's insect saline solution, and examined microscopically with a phase contrast microscope. Representative ovarioles were photographed with ASA 160 tungsten film. In the second study, three female cohorts were processed in this way every six hours.

The dissected ovaries were stained as described by Vogt *et al.* (1974) with neutral red stain for 2-5 seconds at a dilution of 1 gm per 100 ml of saline solution. This stain was preferable to other stains tested because it accentuated the follicular relics of parous flies and

enhanced photography of individual follicles. After staining, the ovaries were rinsed in Yeager's insect saline solution and teased apart.

Spermathecae were also removed and squashed under a coverslip for subsequent determination of fertilization.

RESULTS

Fertilization

Determination of fertilization by dissection was rapid and definitive. Spermatozoa can be seen through phase contrast (Figure 2) as they quickly leave the crushed spermathecae of a fertilized female. However, flies should be dissected soon after death for accurate determination of fertilization because spermatozoa degenerate quickly and are difficult to discern in flies that have been dead for several hours or that have been frozen or preserved. It is best to dissect flies immediately or to keep them alive in a refrigerator when fertilization is to be determined.

Developmental Stages

The reproductive system of the adult female stable fly is normally described as being of the meroistic type (each ovariole contains both oocytes and nurse cells) and of the polytrophic subtype (the nurse cells accompany each oocyte as it develops) (Bonhag 1958). Since ovarian development is thus a continual process, division into separate and distinct developmental stages is, at best, arbitrary and tentative. The correspondence between the system devised as a result of the present studies and seven previous systems is summarized in Table 1. It obviously is most similar to those systems having five or six stages per cycle. Systems based primarily on histological techniques are, of course, more accurate, but the proposed system is not inaccurate and can more easily be used for field classification of stages and by personnel with minimum equipment.

The criteria used in placing cells into each of the proposed six stages for the first ovarian cycle are identified in Table 2. Occasional

Table 1. Comparison and correspondence between proposed stage designations and previous stage designations describing gonotrophic development in Diptera.

Proposed system	Christophers (1911)	King *et al.* (1956)	Schwartz (1965)	Adams & Mulla (1967)	Vogt *et al.* (1974)	Trepte (1979)	Sakurai (1973)
0	N, NO	1, 2, 3, 4	--	1	0	1	1
1	I	5, 6	1	2, 3	I	2	2
2	IIa, IIb	7, 8	2	4, 5	II	3	3
3	III	9, 10, 11	3	6, 7	III	4	4, 5
4	IV	12, 13	4	8, 9	IV	5	6
5	V	14	5	10	V	6	7

Table 2. Summary of ovariole stage descriptions for gonotrophic development in adult female *Stomoxys calcitrans*.

Primary oocyte stage desig-nation	Fig-ures	Physical characteristics of stage	Length to width ratio (range)	Percent of egg occupied by oocyte & yolk	Sequent follicular development (ND=not distinguishable) 2°	3°	4°	5°	Fig.
0	3-4	*Follicle not separated from germarium*; at end of stage 0 cell begins to organize and to constrict into figure 8 shape	Not separated	0	ND	ND	ND	ND	17
1	5-6	*Follicle separated from germarium at beginning of stage 1 and clearly spherical*; at end of stage 1, *oocyte visible* without yolk and with nucleus unstained.	1.0-1.4	0-20	0	ND	ND	ND	18
2	7-9	For duration of stage 2 *nucleus stains dark red*; yolk deposition clearly visible; follicle begins to elongate, becoming ovoid.	1-4-1.75	20-35	1	0	ND	ND	19
3	10-12	*Oocyte nucleus obscured by yolk*; period of greatest growth with *yolk occupying 3/4 of egg at end of stage*; follicle "football shaped" at beginning of stage to distinctly elongated at end of stage 3.	1.75-3.7	35-75	2	1	0	ND	20
4	13-14	*Disintegrating nurse cells occupy less than 1/4 of total egg, "red cap" at end of stage*; egg nearly full size and extremely fragile, bursting during dissection.	3.7-4.0	75-95 (including "red cap")	2-3	1-2	0	ND	21
5	15-16, 23	*Chorion hardened with hatching pleat*; egg at maximum size and maturity; disintegrated nurse cells reduced to small cluster; egg evacuated from membrane easily during dissection.	4.0	100	3	2	1	0	22

arbitrary decisions had to be made about the placement of some stages since development is a continuum. The portions of the stage descriptions that are perhaps the most easily discernable, are italicized.

Representative photographic examples of each stage are shown in Figures 3-16. Scale lines on the plates represent 100 um. Figures 17-22 depict examples of the sequential follicular development for each stage. Figure 23 shows the full display at time of oviposition.

The increase in ovariole size as growth progresses in each ovary is depicted both by the scale lines on the photograph of individual follicles and by the photograph of whole ovaries (Figure 24) where all six stages are shown to illustrate relative size.

Follicular Relics

The identification of the parous state of individual females has been more difficult than assigning them to a developmental stage, as Anderson (1964) noted, because the follicular relics of uniparous females are widely dispersed within the follicular membrane and there are too few that can be readily distinguished. The ovaries of older females which have oviposited several times are readily recognizable by the large "yellow bodies" that can be seen without staining (Figure 25). Figures 26-28 show the difference between nulliparous, uniparous, and biparous+ females, especially for the unstained ovary. Yellow bodies cannot be seen in either nulliparous or uniparous females without staining; they become increasingly more visible with two or more ovipositions. The standard procedure, therefore, was to stain only one ovary of each pair and to make the determination of parous state based on the following criteria:

	stained ovary	**unstained ovary**
nulliparous	no relics	no relics
uniparous	relics (red)	no relics
biparous+	relics (red)	relics (yellow)

Immediately following oviposition, follicular remnants are still finely diffused within the ovariole membrane. These, when stained, have the appearance of long ribbon-like strands (Figure 29). These eventually coalesce to form button-like forms as in Figure 28. This sequence can be used to temporally place parous females relative to their oviposition, at least within 24 hours at 25°C. The example shown (Figure 29) was obtained when a female in the process of oviposition was dissected. Most of the ripe eggs from the stained ovary had been voided, and mature eggs can still be seen in both ovaries.

Follicular relics that have already coalesced are shown under higher magnification in Figures 30-31. The first was obtained from a female which had oviposited approximately twice; the latter was obtained from a female which had oviposited more than four times.

From photographs and observations of the various stages, it appears that at the time of normal oviposition, the secondary follicle (2° in photographs) is at stage 3. Therefore, in the proposed rating system there are 12 possible physiological age categories to which female flies can be assigned with assurance: nulliparous 1-6, uniparous 3-5, and biparous+ 3-5. To date, the author has not been able to use this staining procedure to distinguish between 2, 3, 4, or more ovipositions as is possible with *Lucilia cuprina* (Vogt *et al.* 1974). This is not a great disadvantage, however, with an estimated 75% daily survival rate (LaBrecque *et al.* 1975), only a very small proportion of adult females (<5%) should theoretically live long enough in nature to be included in the category of biparous +, approximately 12 days at 25°C.

Abnormalities

On rare occasions abnormal ovaries were found during routine dissections which did not follow the course of events as described above. Probably the most common abnormality was the occasional finding that one of the ovaries had not developed past stage 2 though the other had developed normally. This condition was rarely observed in field-collected females.

Abnormal ovarian development can be produced in the laboratory by preventing mating. The female, whose follicle is shown in Figure 32, was isolated from males at emergence. Instead of "normal" oviposition and development of subsequent follicles on approximately day 7, the figure illustrates the condition of the ovarioles from a female 10 days from emergence. The developing secondary follicles had become distorted because the primary follicles had not vacated the ovariole sheath to allow subsequent normal development and enlargement. The tolerable limits of such overcrowding of ovarioles due to lack of mating have not been determined, but preliminary trials indicate that females could develop and retain full components of eggs without oviposition for up to 16 days.

Figures 33-35 depict follicles in various stages of degeneration that resulted from unknown causes. Abnormalities of this type may be laboratory artifacts since such observations have not been detected in field-collected flies. In one case (Figure 33), the primary and secondary follicles have degenerated but still are recognizable; in Figure 34, it is difficult to determine the stage of the remains prior to disintegration. Figure 35 was obtained from a female that uniformly reabsorbed only the secondary follicle in each ovariole. Further experimentation may help in understanding the causes for such abnormal development.

SUMMARY

A technique to determine the physiological age of female stable flies, *Stomoxys calcitrans,* is described in this bulletin. It was devised

because of the need for a quick and accurate method of classifying adult female populations according to physiological age. As understanding of the relationship between environmental conditions and the physiological and calendar age of the insects increases, so too will the ability to use the proposed system more precisely in population sampling and life table modeling. Preliminary trials have indicated that the technique may also be useful in determining gonotrophic development, parity, and age structure of other Diptera as well as insects in other orders.

Key to Abbreviations used in Figs. 3-29

1°	Primary follicle	FR	Follicular relics
2°	Secondary follicle		("Yellow bodies")
3°	Tertiary follicle	GE	Germarium
4°	Quaternary follicle	HP	Hatching pleat
5°	Quintan follicle	NC	Nurse cells
DNC	Degenerating nurse cells	OCN	Oocyte nucleus
FM	Follicular membrane	YO	Yolk

Scale lines on each figure represent 100 um between tips of arrows

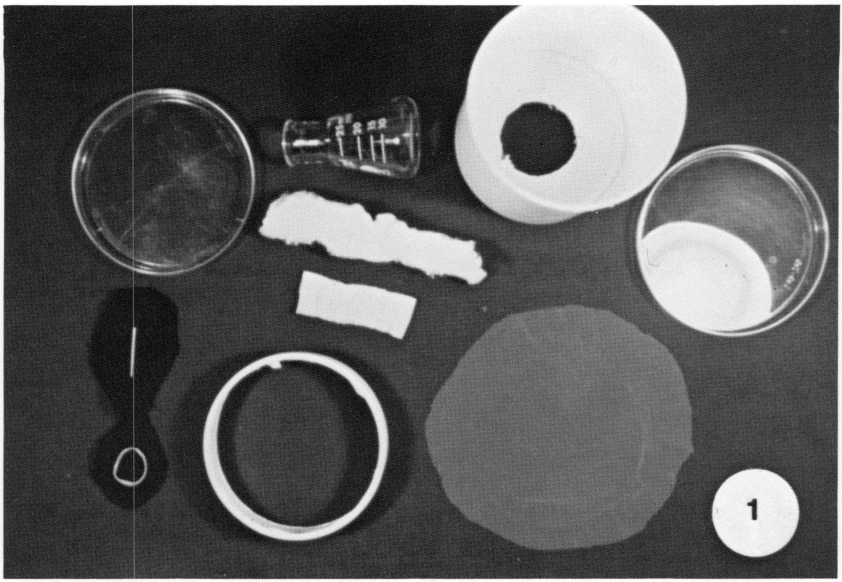

Figure 1. Break away of component parts of individual rearing cage.

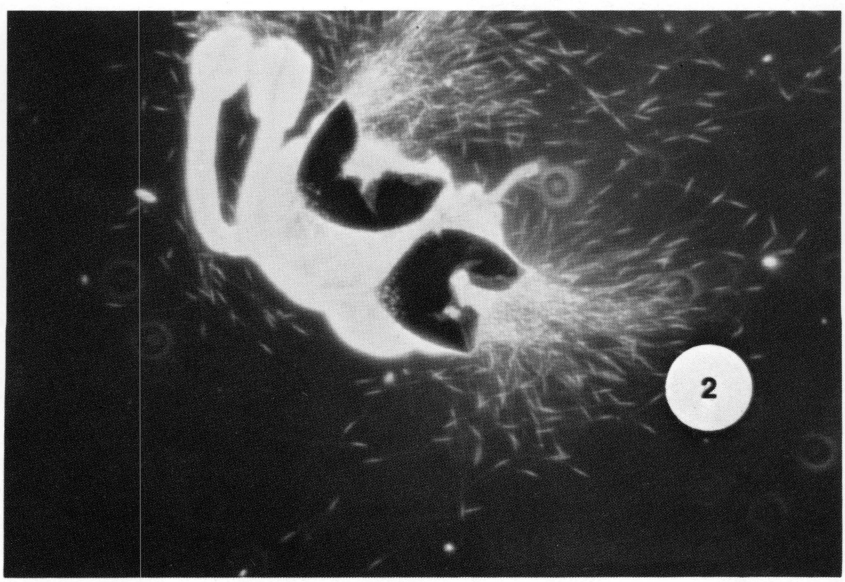

Figure 2. Crushed spermathecae from fertilized *S. calcitrans* female.

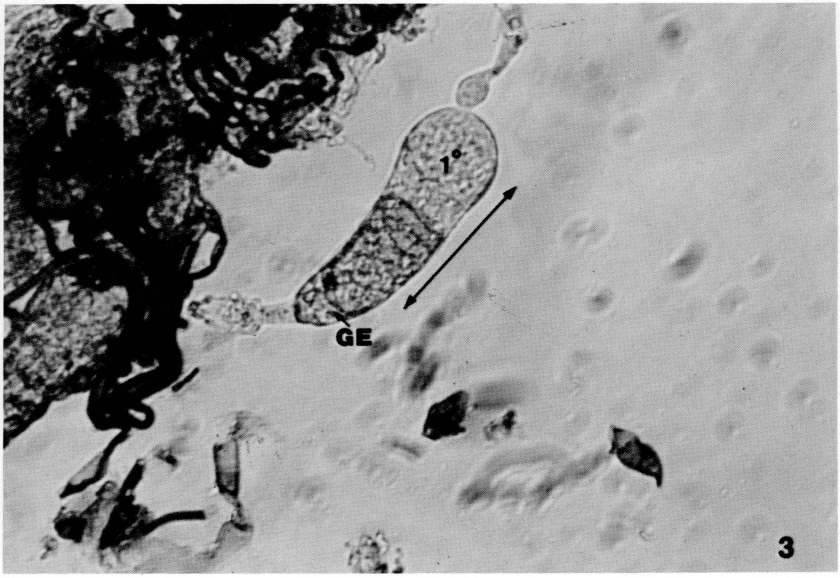

Figure 3. Primary oocyte, early stage 0.

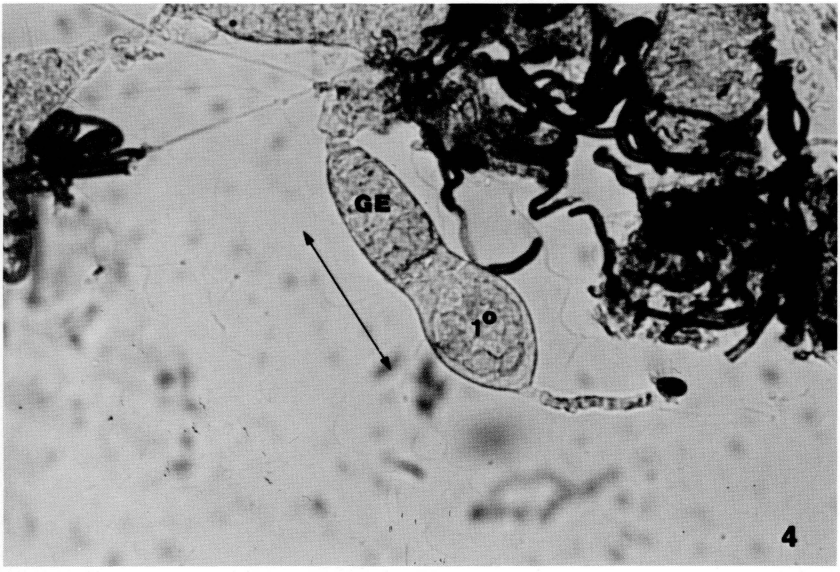

Figure 4. Primary oocyte, late stage 0.

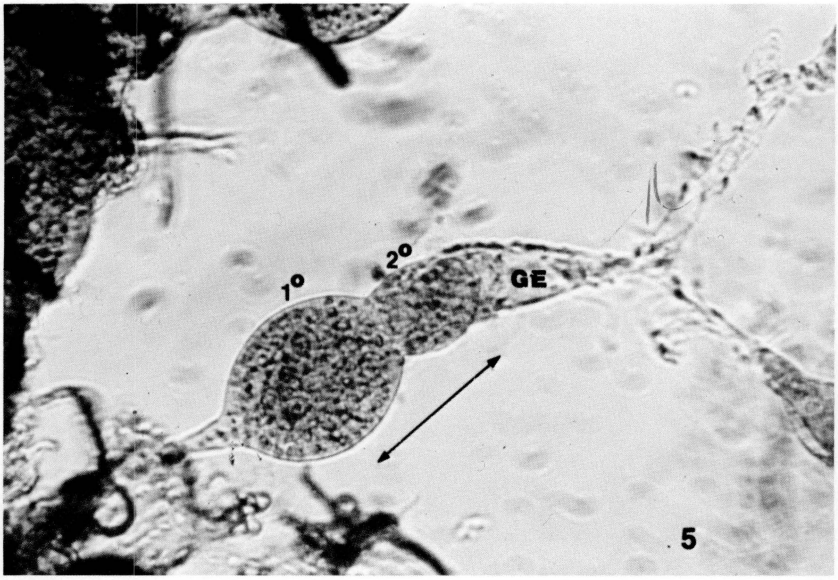

Figure 5. Primary oocyte, early stage 1.

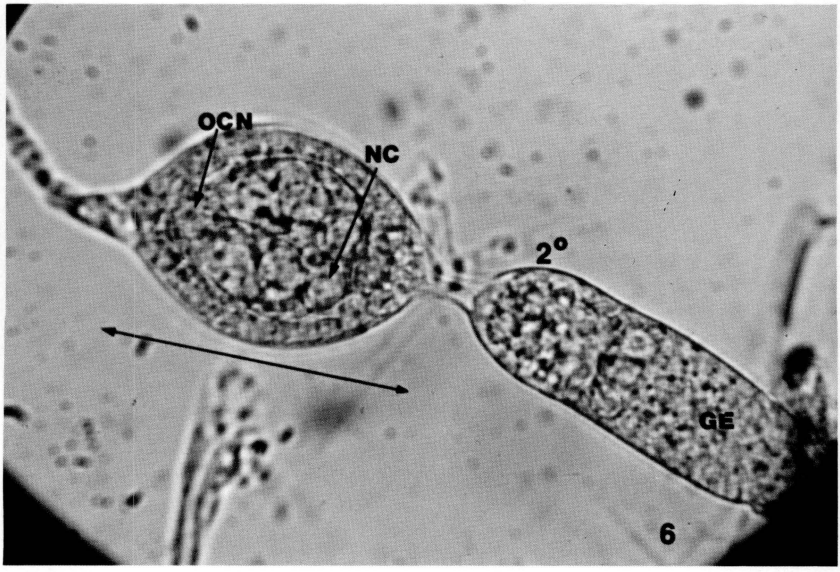

Figure 6. Primary oocyte, late stage 1.

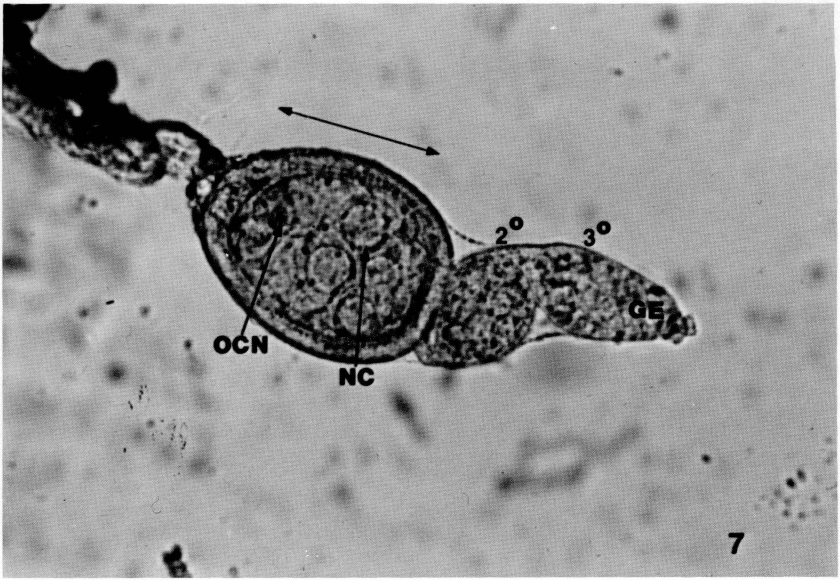

Figure 7. Primary oocyte, early stage 2.

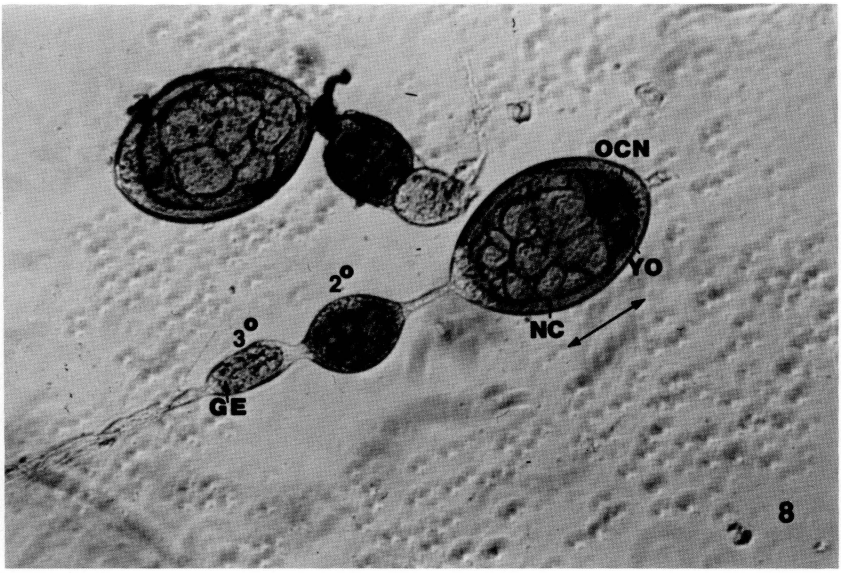

Figure 8. Primary oocyte, stage 2.

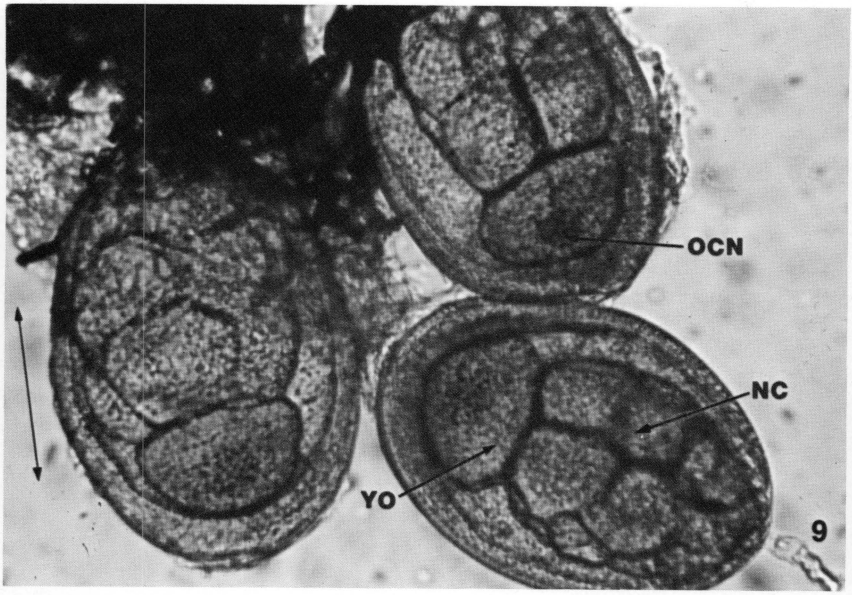

Figure 9. Primary oocyte, late stage 2.

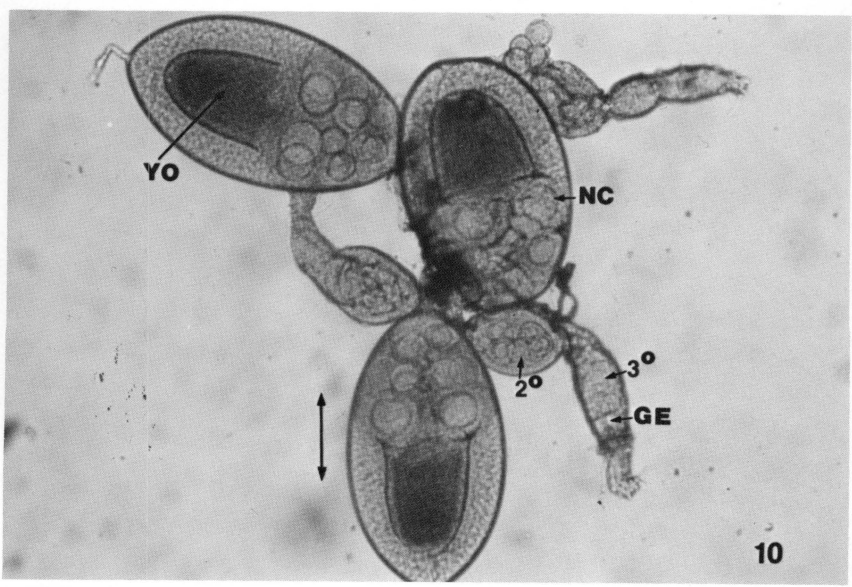

Figure 10. Primary oocyte, early stage 3.

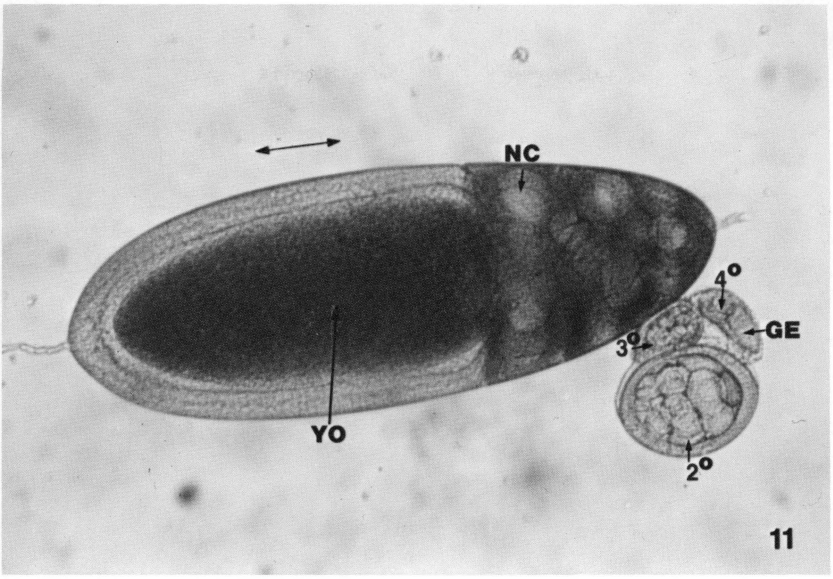

Figure 11. Primary oocyte, stage 3.

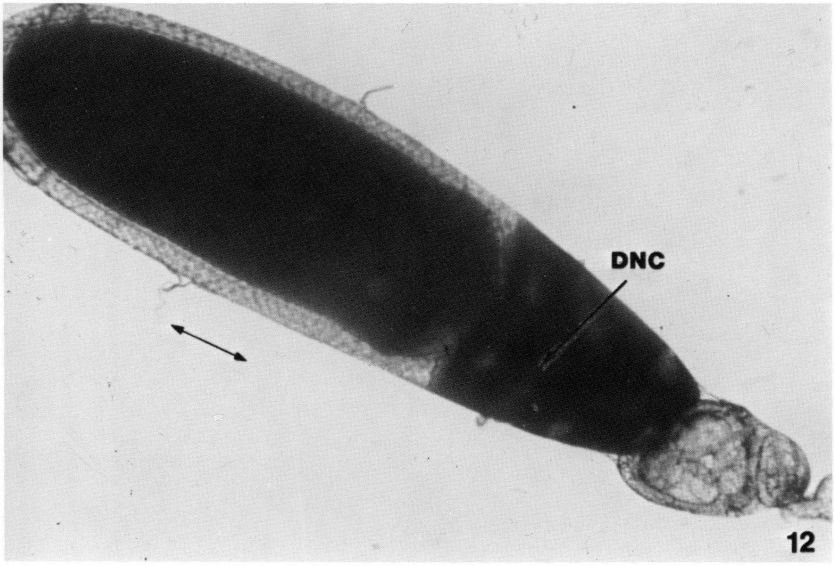

Figure 12. Primary oocyte, late stage 3.

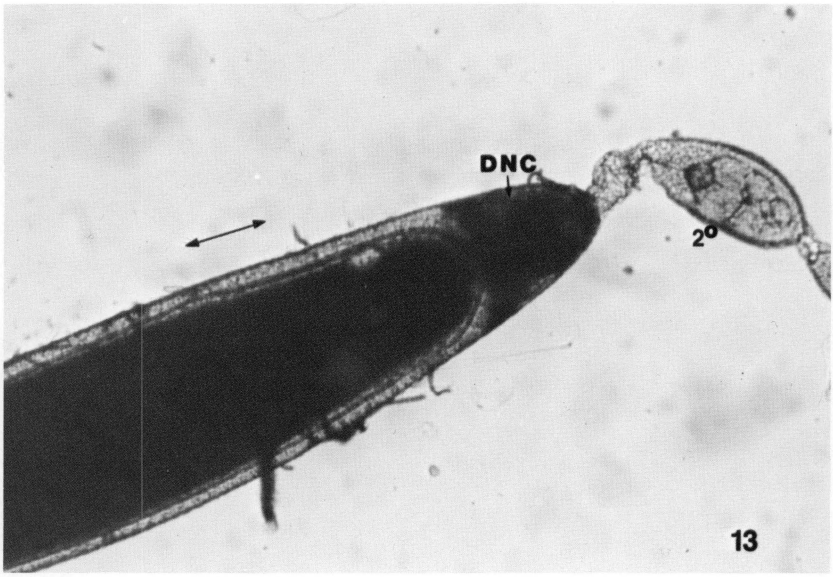

Figure 13. Primary oocyte, early stage 4.

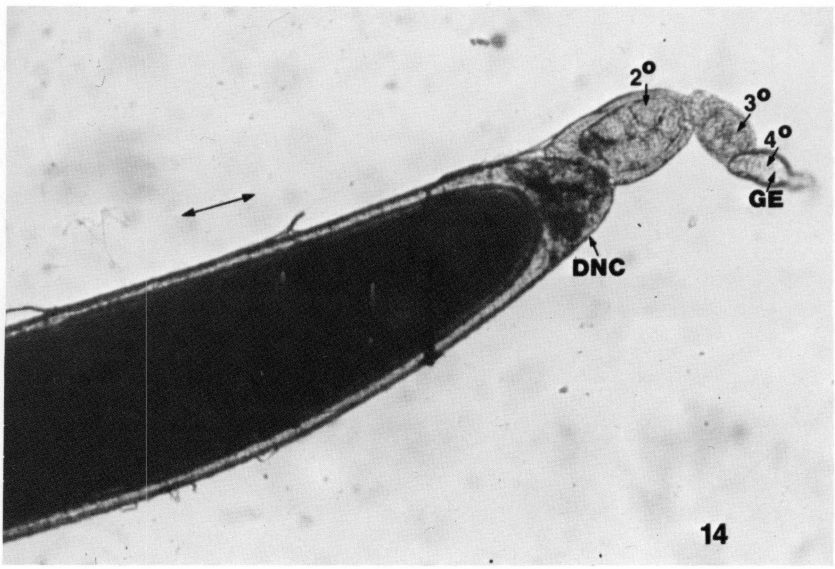

Figure 14. Proximal end of late stage 4 depicting "red cap" (final stage of degenerated nurse cells).

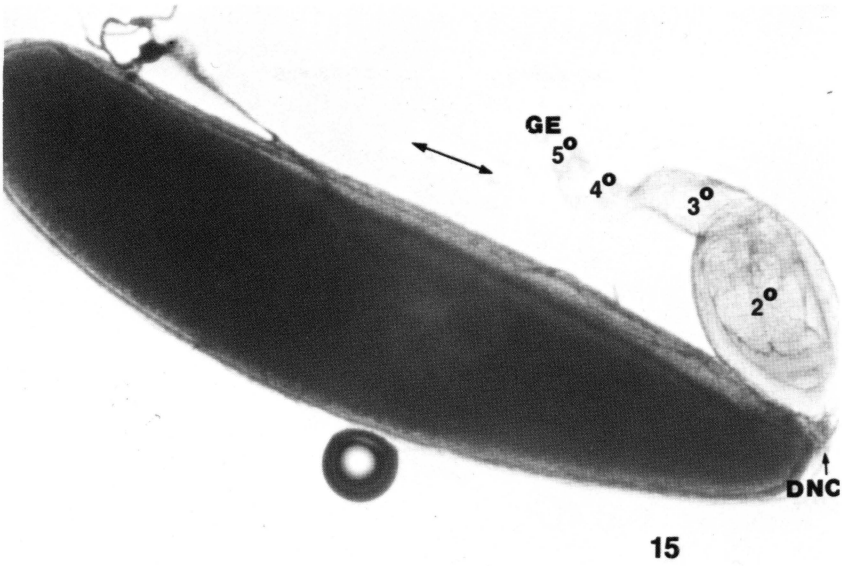

Figure 15. Primary oocyte, stage 5.

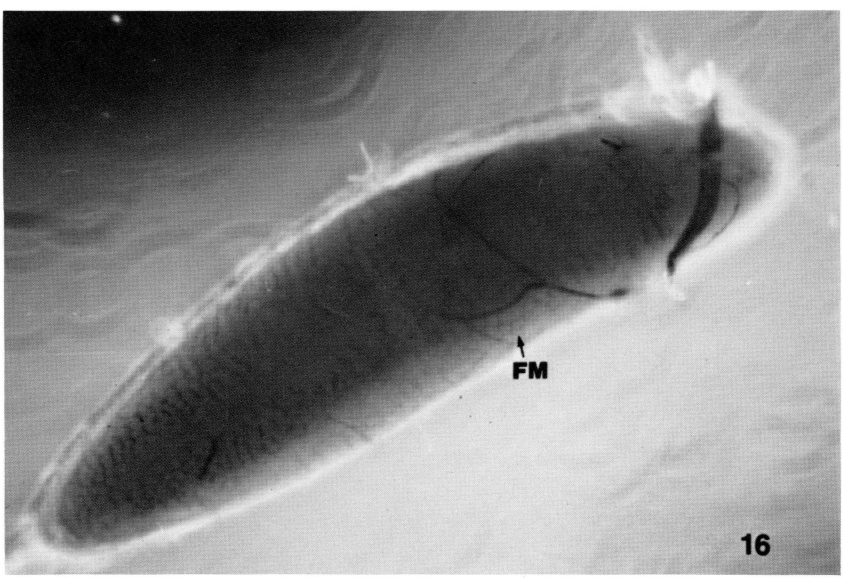

Figure 16. Primary oocyte, stage 5 enclosed in stained follicular membrane.

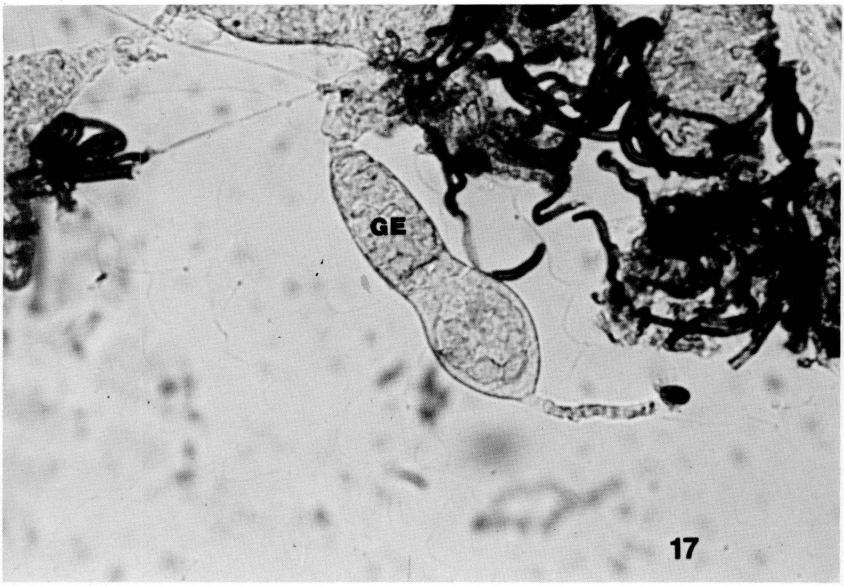

Figure 17. Sequent follicular development when primary follicle is at stage 0.

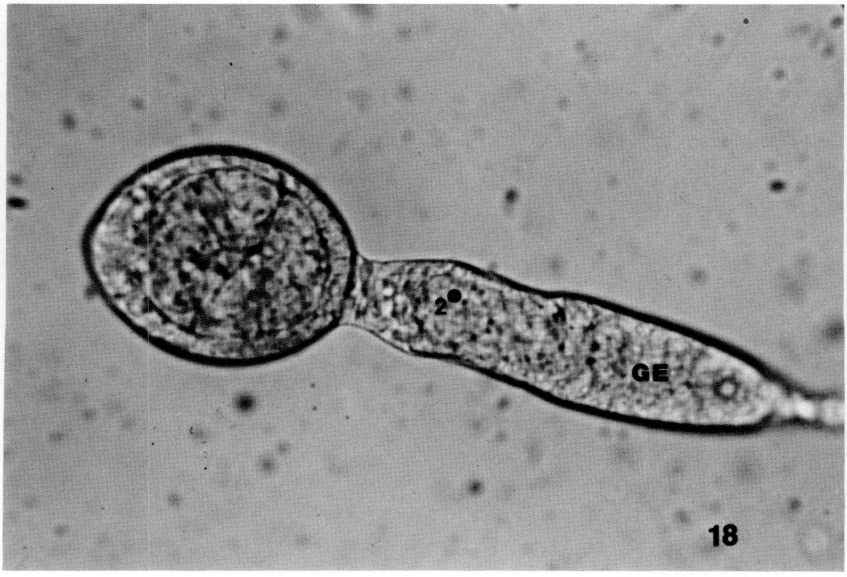

Figure 18. Sequent follicular development when primary follicle is at stage 1.

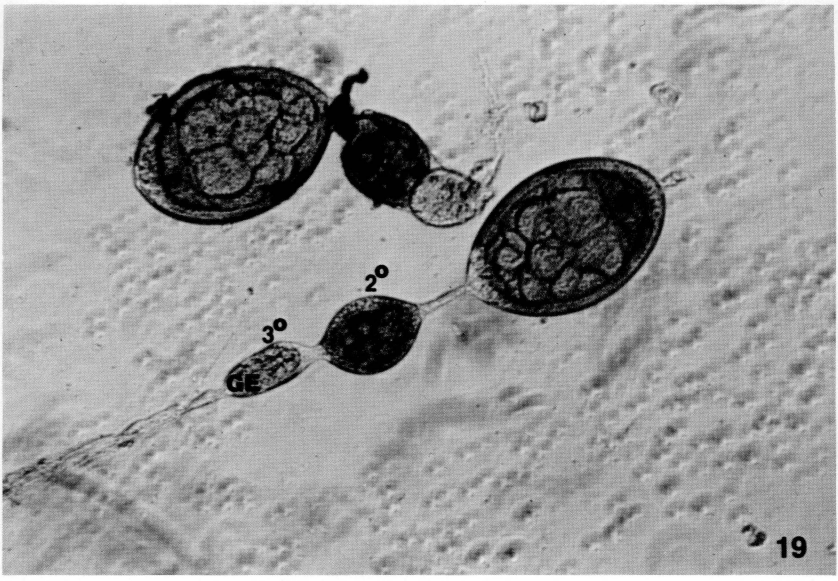

Figure 19. Sequent follicular development when primary follicle is at stage 2.

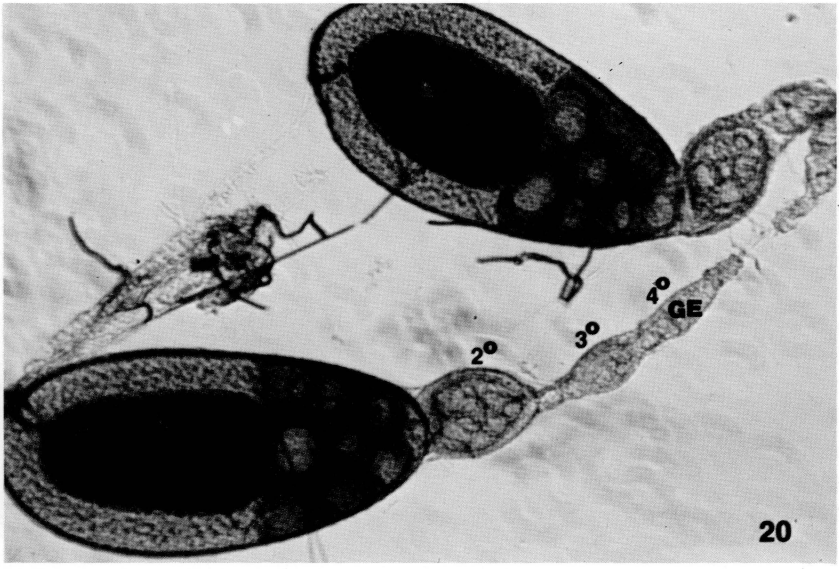

Figure 20. Sequent follicular development when primary follicle is at stage 3.

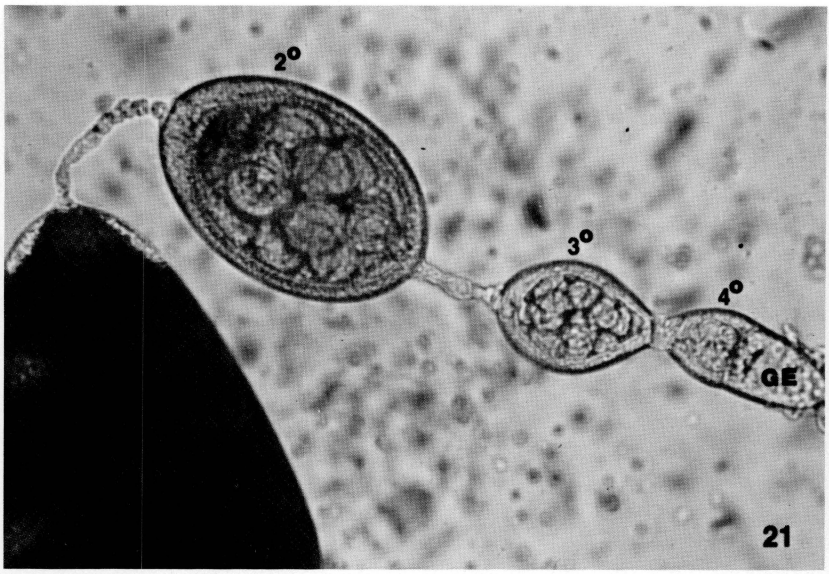

Figure 21. Sequent follicular development when primary follicle is at stage 4.

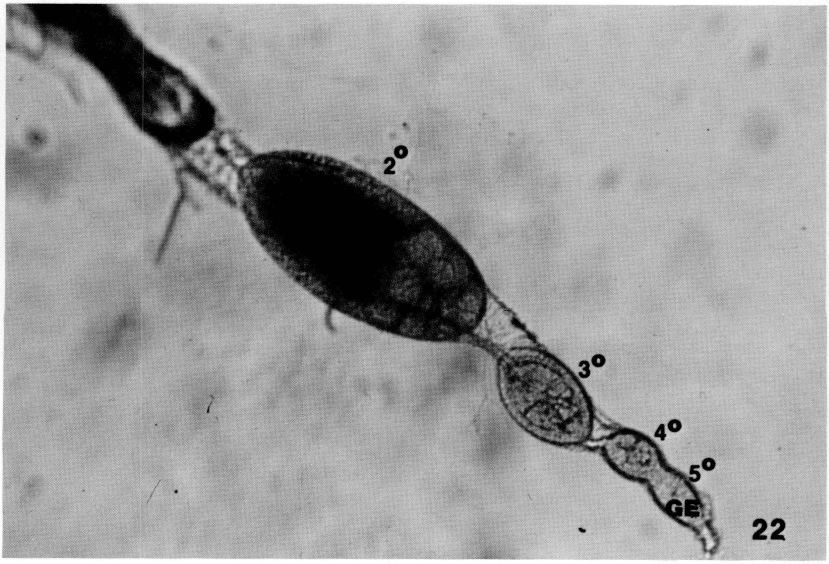

Figure 22. Sequent follicular development when primary follicle is at stage 5.

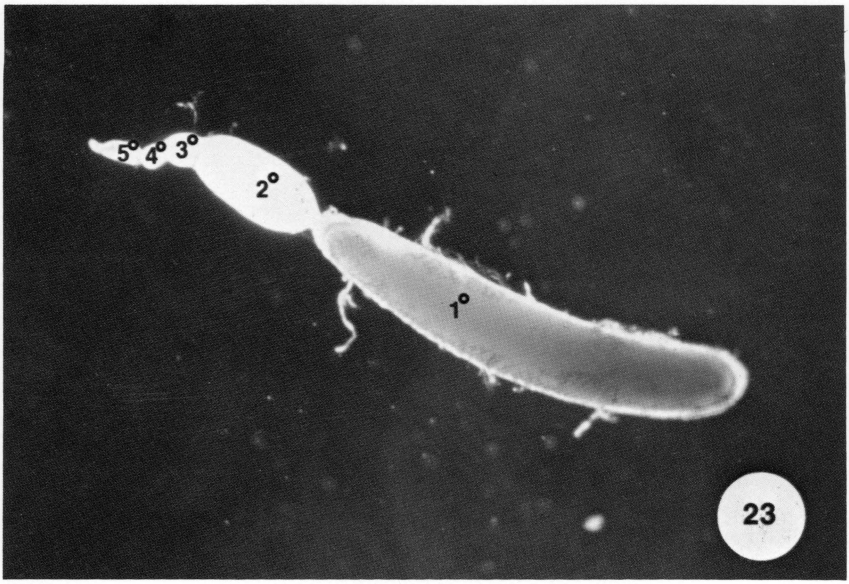

Figure 23. Full display of primary and subsequent follicles, 1-5, at time of oviposition.

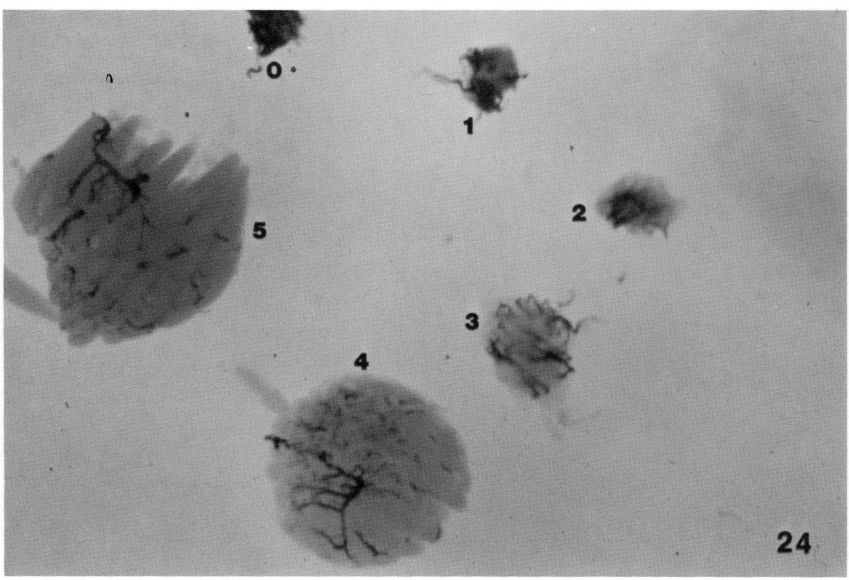

Figure 24. Size increase of whole ovaries from stages 0 to 5.

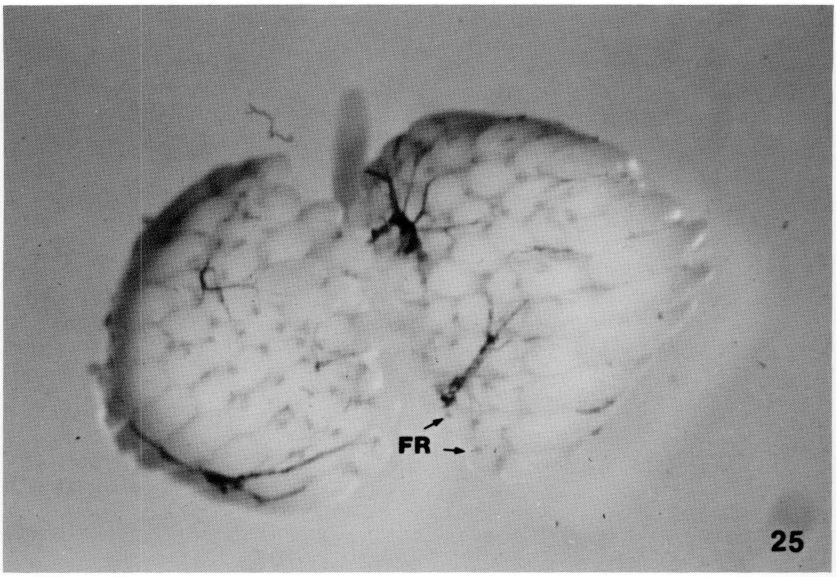

Figure 25. Unstained whole ovaries from multiparous female with "yellow bodies" visible.

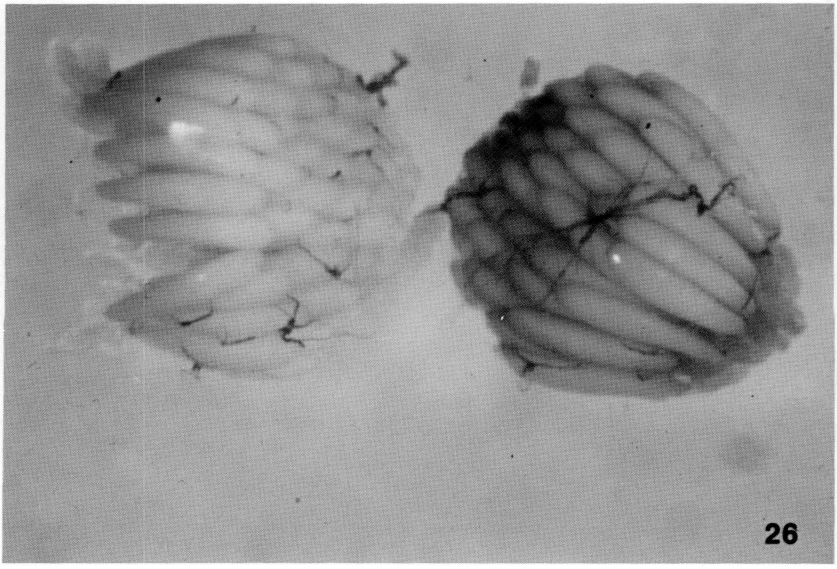

Figure 26. Stained and unstained ovaries from nulliparous female.

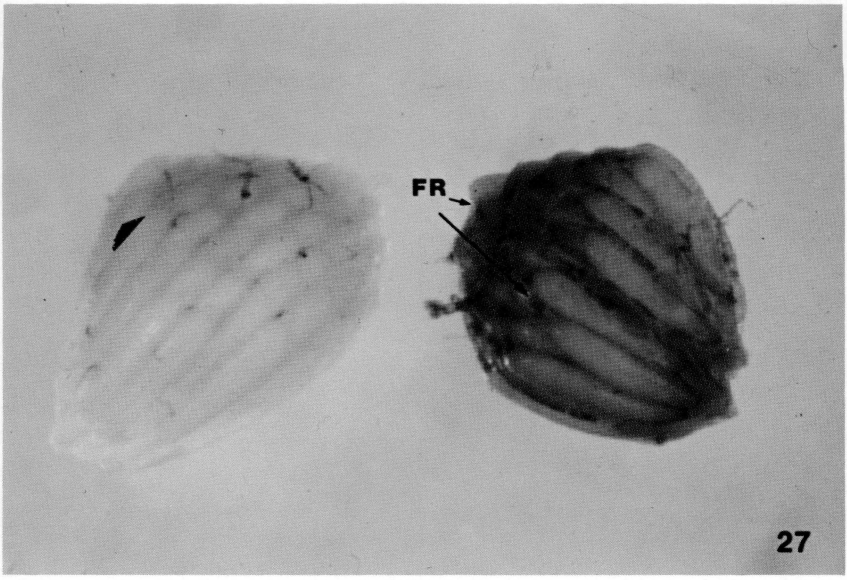

Figure 27. Stained and unstained ovaries from uniparous female.

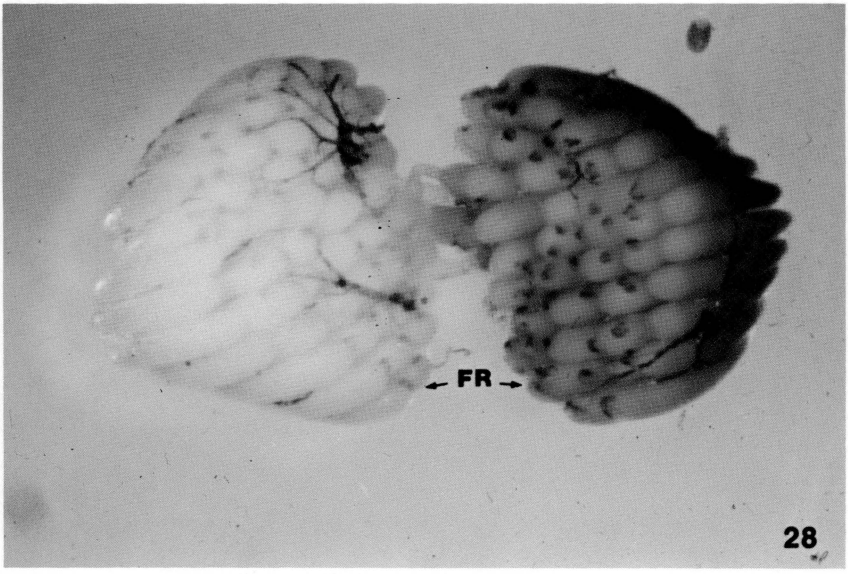

Figure 28. Stained and unstained ovaries from biparous+ female.

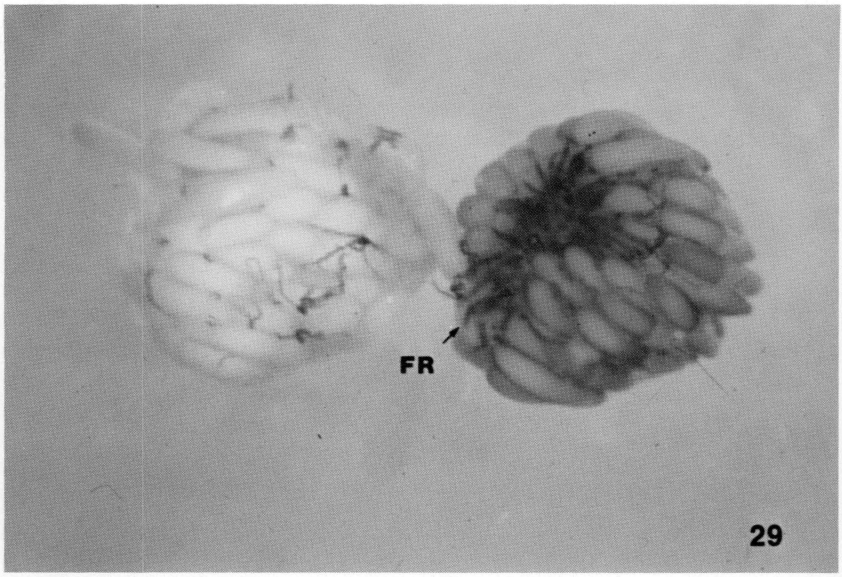

Figure 29. Stained and unstained ovaries from uniparous female which has recently oviposited.

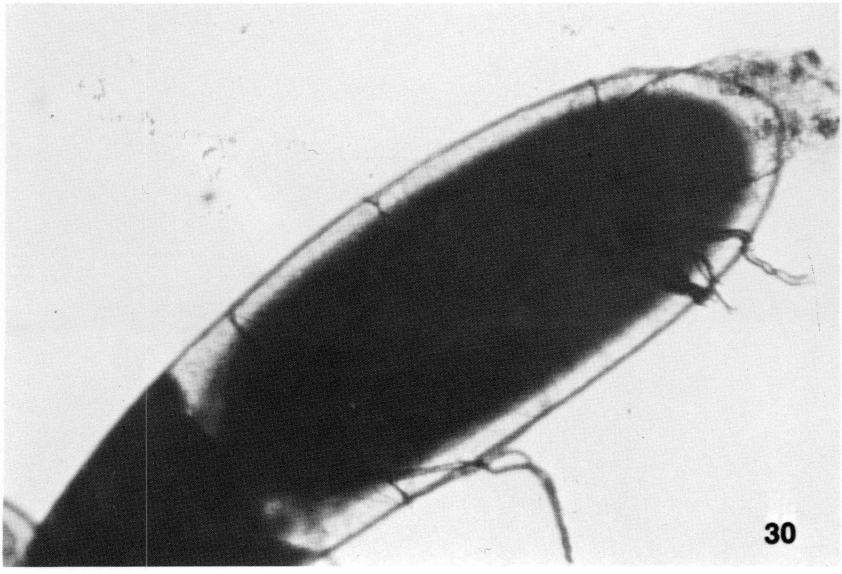

Figure 30. Close-up of follicular relics in ovariole sheath from female that had oviposited at least 2 times.

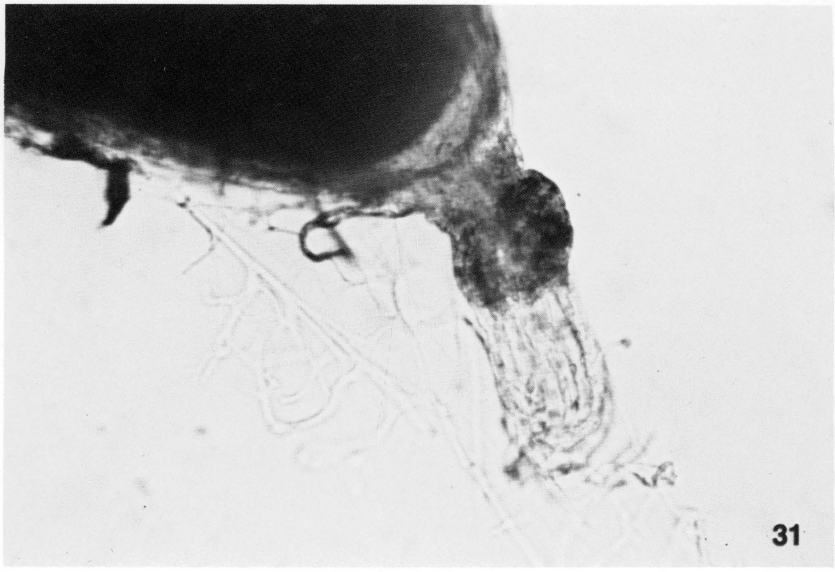

Figure 31. Close-up of follicular relics in ovariole sheath from female that had oviposited at least 4 times.

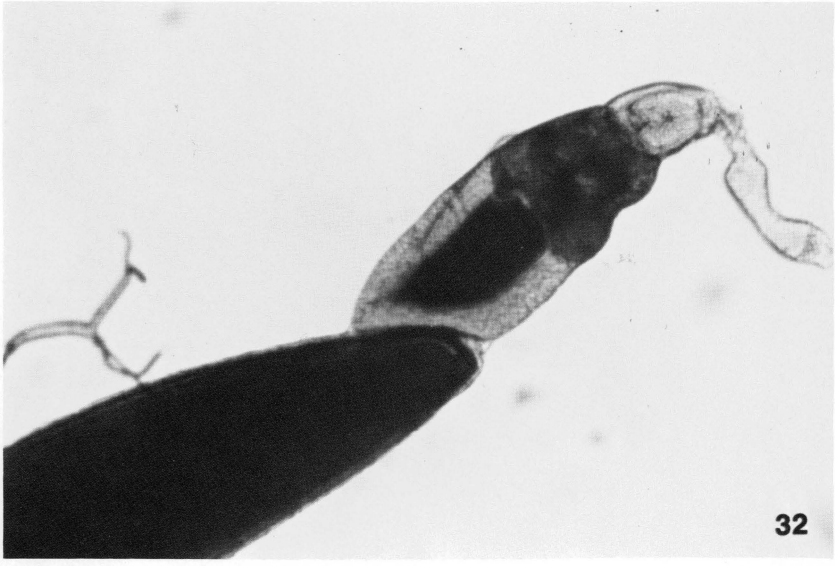

Figure 32. Distortion of secondary follicle due to crowding of ovariole in unmated female.

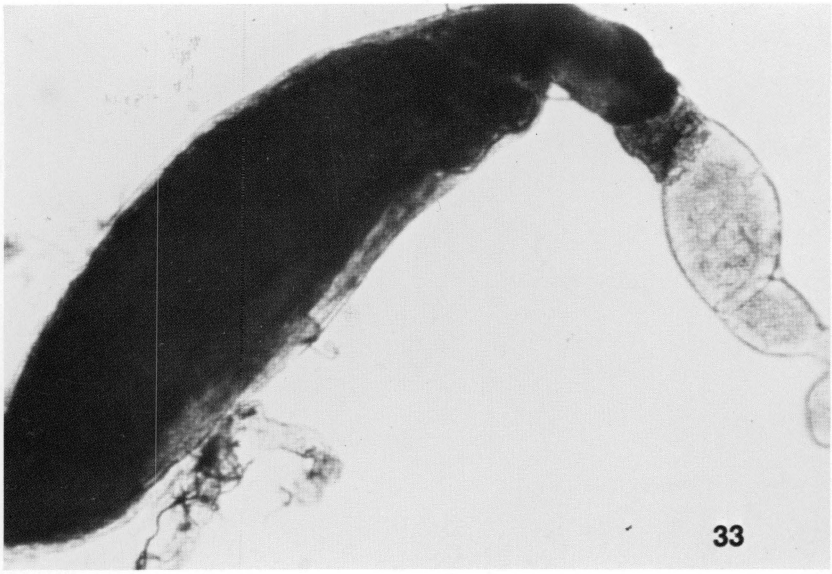

Figure 33. Degeneration of primary follicle, unknown cause(s).

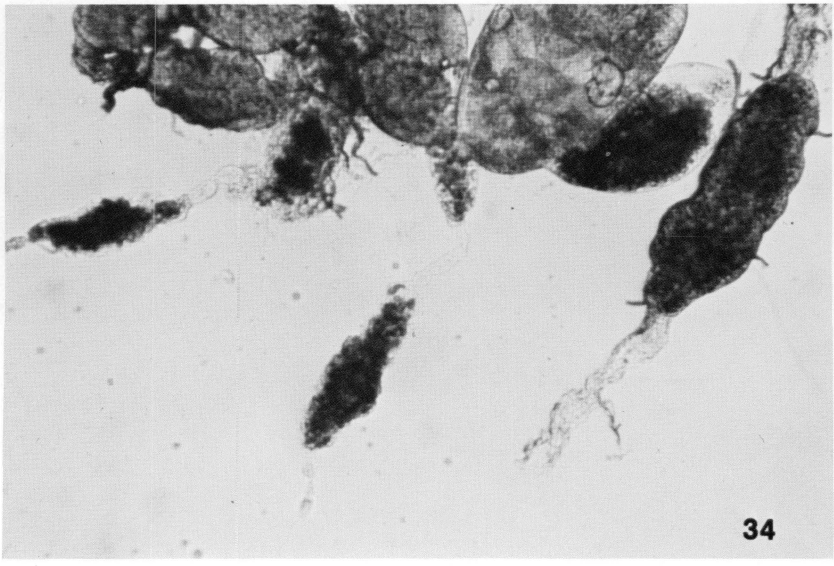

Figure 34. Degeneration of all primary and secondary oocytes, unknown cause(s).

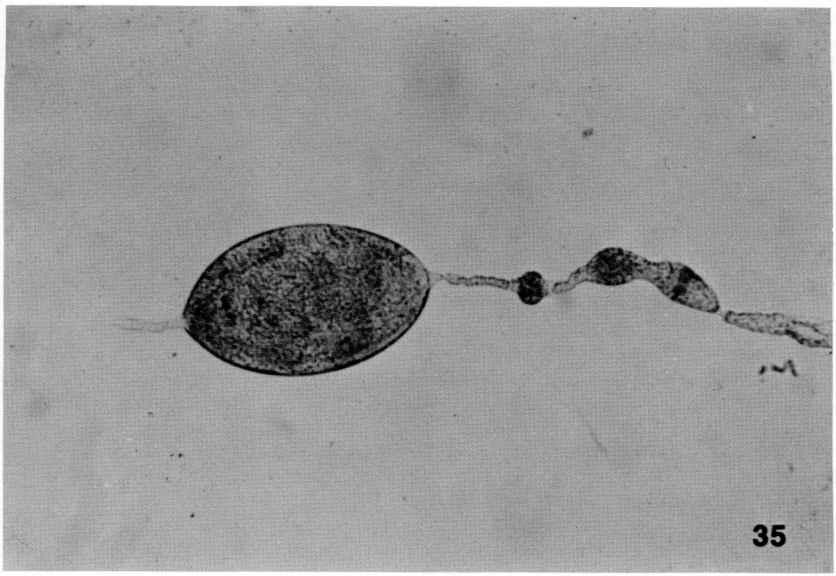

Figure 35. Degeneration of secondary follicle only, unknown cause(s).

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