Ovarian Development and Parity Determination in *Culiseta melanura* (Diptera: Culicidae)

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ABSTRACT A laboratory colony of *Culiseta melanura* (Coquillett) was used to follow ovarian development from emergence to oviposition and to validate the accuracy of using follicular dilatations to age grade females. We observed no change in the size of the primary follicles in unfed females from emergence to 3 d of age. Sugar feeding stimulated follicular growth and produced the following 3 types of ovarioles: (1) large primary follicles that eventually developed into functional ovarioles; (2) lesser numbers of small primary follicles that developed small amounts of yolk after blood feeding but degenerated in the latter stages of development; and (3) small primary follicles that did not develop yolk after blood feeding, exhibited accelerated growth, and became rogue ovarioles with multiple false dilatations. The yolk of the small primary follicles that degenerated after blood feeding was resorbed during stage IV of egg development, and the degenerating follicles resembled gonotrophic dilatations in the latter stages of the cycle. This process produced gravid females with some secondary follicles that appeared to possess a gonotrophic dilatation. Other authors have termed these artifacts false or agonotrophic dilatations. Degenerating ovarioles bearing these artifacts were used to determine physiological age in blood fed and gravid *Cs. melanura*. Nulliparous females in the latter stages of gonotrophic development have single false dilatations on degenerating ovarioles, 1-parous females have 2 false dilatations on degenerating ovarioles. In unfed, nulliparous females, false dilatations can be distinguished from true dilatations because they are attached to secondary follicles that are much smaller than the primary functional follicles that fill most of the ovary. In blood fed and gravid females, follicles that support false dilatations always lack yolk. Rogue ovarioles are unreliable indicators of physiological age and should not be used for diagnostic purposes.

KEY WORDS *Culiseta melanura*, age grading, false dilatation, rogue ovariole

EASTERN EQUINE ENCEPHALOMYELITIS virus (EEE) is perpetuated along the coast of North America in a cycle that involves *Culiseta melanura* (Coquillett) as the primary enzootic vector and passerine birds as the amplification hosts (Morris 1988, Scott and Weaver 1989, Crans et al. 1994). *Aedes sollicitans* (Walker) is considered the primary vector to humans in coastal areas of New Jersey (Crans et al. 1986). Equine cases have been linked to *Coquillettidia perturbans* (Walker) in inland areas of the state (Crans and Schulze 1986). *Cs. melanura* is used as the primary indicator of virus activity for encephalitis surveillance and control (Crans and McCuiston 1993). Specimens are collected weekly at established study sites and tested for EEE. When virus is detected in *Cs. melanura*, the epizootic and epidemic vector species are targeted for control. *Cs. melanura* is associated with a number of threatened and endangered species and is not controlled in either the larval or adult stage. Controlling mammalian feeding species prevents human and equine cases but has no effect on the maintenance phase of the cycle.

Duration of the gonotrophic cycle, frequency of multiple blood meals and longevity of the vector are important parameters in the epidemiology of every insect-borne disease. In many families of Diptera, the number of gonotrophic cycles completed by wild-caught females may be assessed by counting the number of dilatations on the ovarian follicles (Detinova 1962, Lewis 1970, Mullens and Schmidtmann 1982, Reisen et al. 1986). Although, 1 dilatation per ovarian cycle is the general rule, some mosquito species form false dilatations, making age grading of populations unreliable (Yajima 1970, Oda et al. 1978, Nayar and Knight 1981). This problem can be minimized by studying ovarian development and dilatation formation in known nulliparous, one-parous, or multiparous females under controlled laboratory conditions and identifying common artifacts encountered with a given species (Fox and Brust 1996).

Longevity of the maintenance vector and the number of gonotrophic cycles completed by host-seeking females is critical for a complete understanding of EEE epidemiology. We recently developed a thermal heat summation model to predict duration of the gonotrophic cycle of *Cs. melanura* in nature (Mahmood and

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Table 1. Rate of egg development in functional and degenerating ovarioles of nulliparous *Cs. melanura* females at 25°C

Treatment	Days after treatment	Follicle length μm ± SD		Follicle width μm ± SD		Yolk μm ± SD	
		Functional	Degenerating	Functional	Degenerating	Functional	Degenerating
Unfed	Emergence	29.6 ± 3.5g		28.3 ± 2.9g		0.0 ± 0.0f	
	3	27.8 ± 2.4g		28.1 ± 2.4g		0.0 ± 0.0f	
Sugar fed	6	55.7 ± 5.4f	47.2 ± 5.8d	55.3 ± 5.1f	46.3 ± 5.8d	0.0 ± 0.0f	0.0 ± 0.0d
Blood fed	0.5	119.4 ± 23.5e	103.5 ± 8.8c	97.9 ± 12.4e	87.0 ± 8.9b	45.6 ± 17.3e	36.0 ± 9.7c
	1	185.8 ± 27.0d	125.8 ± 24.3b	129.6 ± 14.9d	92.8 ± 14.3b	114.6 ± 29.0d	62.4 ± 23.3b
	2	202.4 ± 30.5c	142.8 ± 24.3a	142.9 ± 16.4c	102.8 ± 7.4a	128.7 ± 37.1c	73.0 ± 12.7a
	3	297.2 ± 36.2b	127.3 ± 53.9b	186.6 ± 15.2b	69.3 ± 17.5c	239.6 ± 41.0b	0.0 ± 0.0d
	4	655.2 ± 19.6a	NM	153.8 ± 6.6a	NM	654.6 ± 19.5a	NM

Values (mean ± SD) in columns followed by different letters were significantly different when tested by Duncan new multiple range test (Sokal and Rohlf 1981). NM = not measured but <25 μm total length.

Crans 1997a), but no information is available on the reliability of using follicular dilatations for age grading purposes. Wild caught *Cs. melanura* feed to repletion >95% of the time (Mahmood and Crans 1997b), making age grading meaningful for natural populations. In the current study, we followed ovarian development from emergence to oviposition using a laboratory colony of *Cs. melanura* to observe developmental stages of the egg and validate the accuracy of using follicular dilatations to age grade females.

Materials and Methods

Strain. We used a colony of *Cs. melanura* in the F_{13}–F_{20} generation that was originally established from field stock collected from southern New Jersey (Mahmood and Crans 1994a). The colony was maintained in an insectary at 24 ± 2°C under a photoperiod of 16:8 (L:D) h, with 1.5 h of dusk and 1 h of dawn. Adults were provided with a 10% sucrose solution changed weekly. To initiate the gonotrophic cycle, females were offered a restrained northern bobwhite quail (*Colinus virginianus*) and allowed to blood feed overnight.

Rate of Egg Development. We determined the rate of egg development in newly emerged, 3-d-old unfed, 6-d-old sugar fed, and 6- to 9-d-old sugar fed females 0.5, 1, 2, 3, and 4 d after a full blood meal. Engorged females were isolated in 3.8-liter paper cages constructed from ice cream cartons. The ovaries from 5–7 females each were removed at 0, 1, 2, 3, and 4 d postblood feeding using the methods described by Reisen et al. (1986). We separated individual follicles within each ovary by the method of Hitchcock (1968). The length and width of each ovarian follicle was measured using an ocular micrometer on a phase contrast microscope by the methods of Mahmood et al. (1991). The space occupied by yolk was measured separately and used to assign stages of follicular development according to the scheme developed by Christophers (1911) as modified by Mer (1936). The 5 largest (functional) and 5 smallest (degenerating) ovarian follicles were measured for each female.

Detection of Follicular Dilatations. To determine the accuracy of using dilatations for age grading purposes, we fed 100 newly emerged females with 10% sucrose solution. This series was not offered blood, and a subsample was dissected on days 1 and 6 to examine pedicels from unfed specimens for false dilatations. Similarly, 200 newly emerged adults were fed 10% sucrose solution daily and offered blood on the 9th night to initiate oogenesis. The blood fed females were isolated and provided with 10% sucrose solution for 4 d. Surviving specimens were placed in individual vials with deionized water and a sucrose source and were checked daily for oviposition. These females were dissected 0, 18, 24, and 48 h after oviposition and examined for follicular dilatations on both functional and degenerating follicles by the methods described by Detinova (1962). One-parous females were fed blood and dissected at the same intervals to observe if the pedicel reforms after oviposition. The accuracy of using dilatations to correctly identify parity was validated with blind tests performed on cohorts of known 2-wk-old nulliparous specimens as well as parous specimens that had oviposited from 1 to 14 d earlier.

Results

The ovarian follicles of newly emerged *Cs. melanura* females did not increase significantly in size for the first 3 d after emergence (Table 1). Significant increase in length ($F = 2{,}078.48$; df = 7, 202; $P < 0.001$) and width ($F = 278.08$; df = 7, 202; $P < 0.001$) took place before oviposition after ingestion of sugar and a blood meal. Ingestion of blood produced a significant increase in yolk deposition ($F = 2{,}000.05$; df = 7, 202; $P < 0.001$).

Stage N. The primary follicles of the newly emerged (<1 h) and 3-d-old unfed females were at stage N of ovarian development. Stage N follicles were separated from their germaria with 8 differentiated monomorphic cells (Fig. 1 a and b). The primary follicles of 0–3-d old females were nearly round, ranging from 25 to 33 μm in length and 25 to 31 μm in width.

Stage I. Sugar feeding stimulated a differential growth response that produced 3 types of ovarioles: (1) large primary follicles that eventually developed into functional ovarioles (LF1 in Fig. 1c); (2) lesser numbers of small primary follicles that developed small amounts of yolk after blood feeding, but degenerated in the latter stages of development (SF1 in Fig. 1 c and d); and (3) small primary follicles that did not develop yolk after blood feeding underwent uncon-

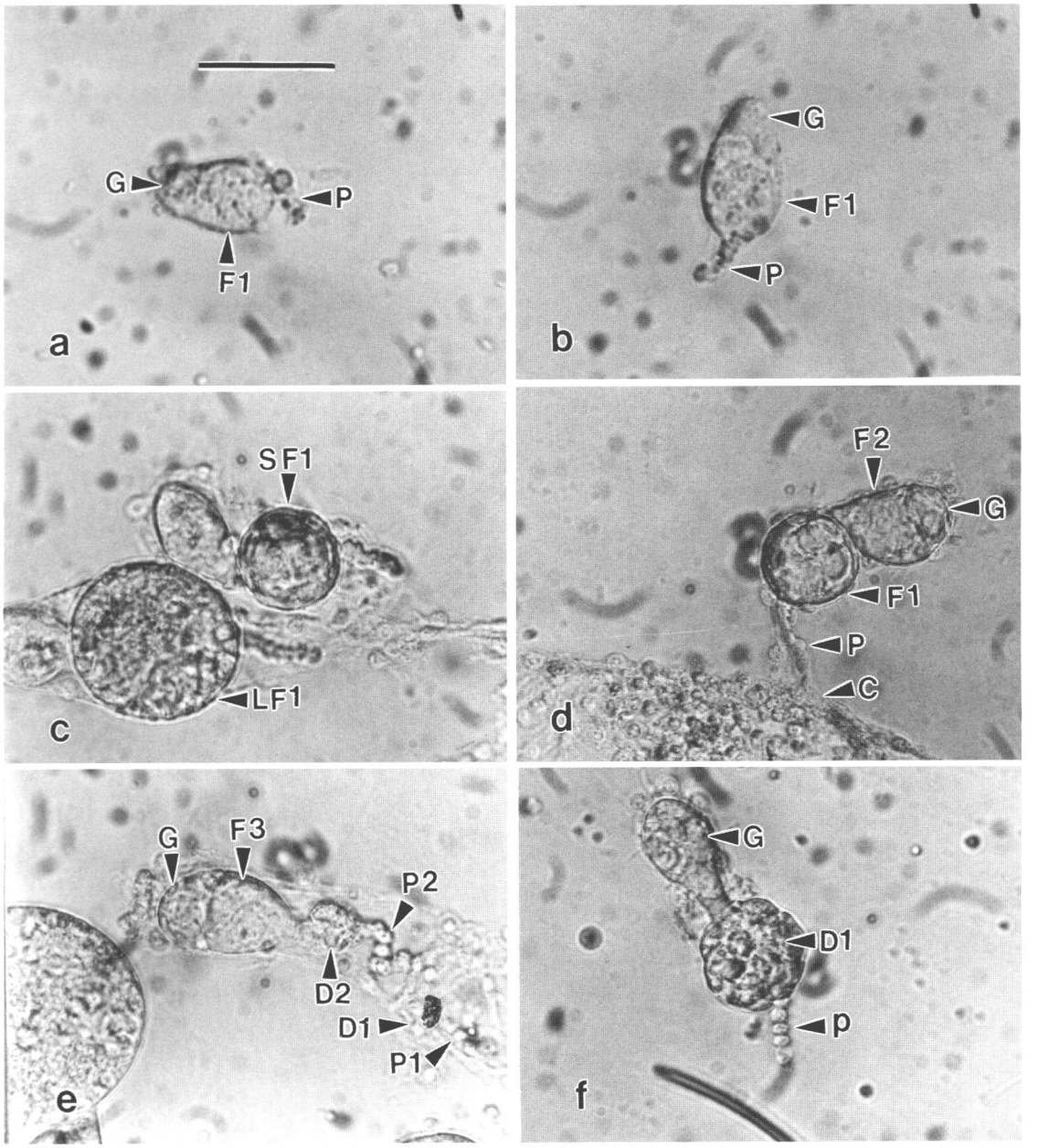

Fig. 1. Ovarian follicular development in newly emerged to 6-d-old sugar fed nulliparous females. (a) Ovariole of a newly emerged female; (b) ovariole of an unfed, 3-d-old female; (c) functional primary versus degenerating primary follicle of a 6-d-old sugar fed female; (d) degenerating primary follicle of a 6-d-old sugar fed female; (e) rogue ovariole exhibiting accelerated growth and degeneration of the primary and secondary functional follicles; and (f) degenerating primary functional follicle of a 6-d-old sugar fed female exhibiting asynchronous cellular division and an internal mass of undifferentiated cells. C = Calyx; D1, D2 = false dilatations formed by degeneration of the primary and secondary follicles; F1, F2, and F3 = primary, secondary and tertiary functional follicles; G = germarium; LF1 = large primary functional follicle; SF1 = small primary follicle; P1, P2 = primary and secondary pedicles. Bar = 50 μm for all.

trolled cellular division and became rogue ovarioles with multiple false dilatations (Fig. 1e).

Functional follicles in stage I of ovarian development were separated fully from their germaria and had 7 nurse cells with an oocyte that lacked yolk granules (LF1 in Fig. 1c). Degenerating follicles were separated from their germaria but lacked nurse cells and oocytes. Some of the degenerating follicles exhibited asynchronous cellular division at this point in their development and produced an internal mass of undifferentiated cells (D1 in Fig. 1e). Other small follicles degenerated during stage N and produced ova-

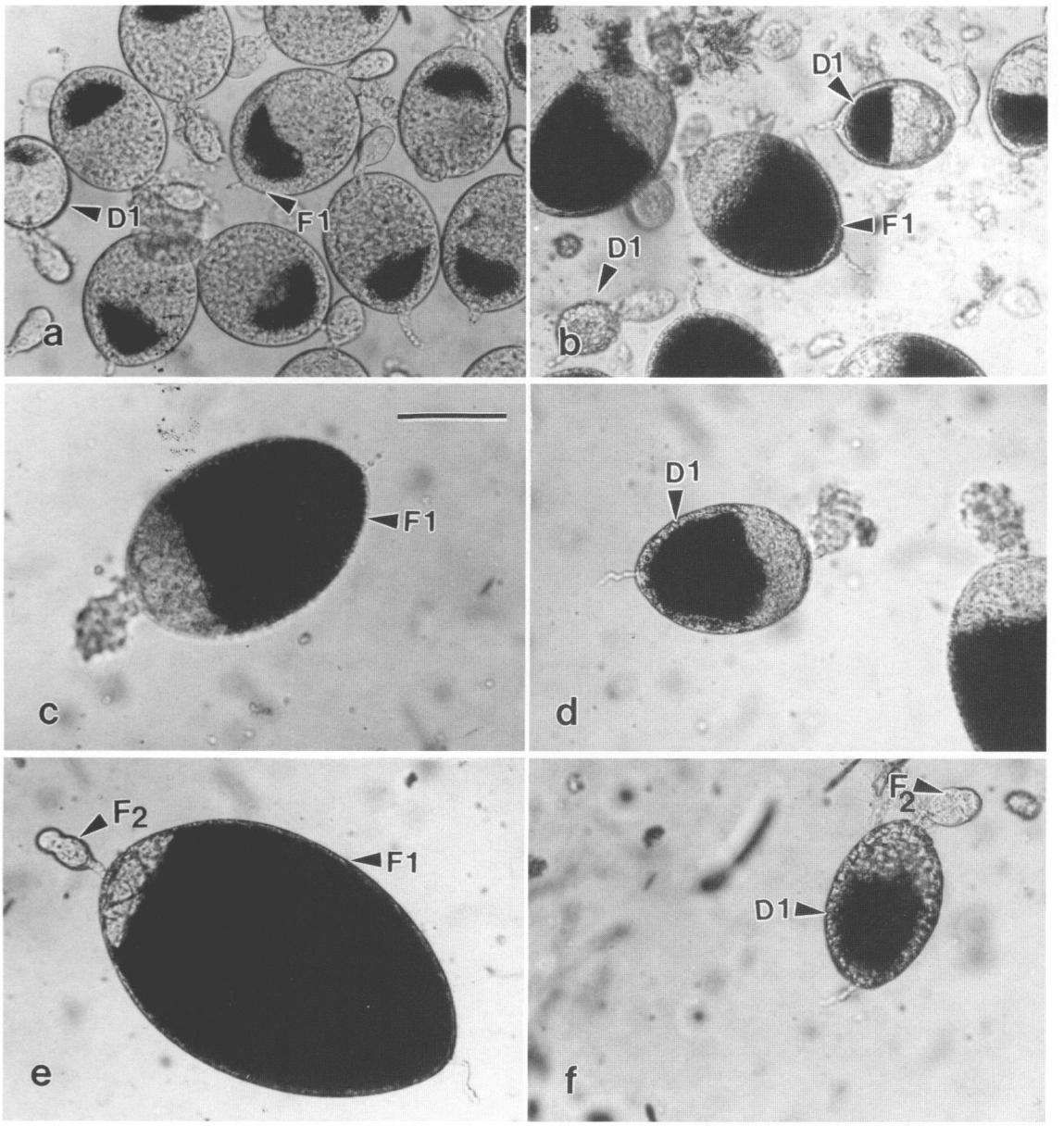

Fig. 2. Stages of ovarian follicular development in 6- to 9-d-old females at various times after a blood meal. (a) Primary follicles in late stage II, 18 h after blood feeding; (b) primary follicles in early stage III, 48 h after blood feeding; (c) functional primary follicle in late stage III, 72 h after blood feeding; (d) degenerating primary follicle from same female72 h after blood feeding; (e) functional primary follicle in early stage IV, 78 h after blood feeding; (f) degenerating primary follicle from same female 78 h after blood feeding. F1, F2 = primary and secondary follicle; D1 = degenerating primary follicle. Bar = 100 μm for all.

rioles with degenerated primary and secondary follicles (D1 and D2 in Fig. 1e). Reisen et al. (1989) and Fox and Brust (1996) noticed similar degeneration in *Cs. inornata* and termed them rogue ovarioles. The degenerating follicles of rogue ovarioles resembled dilatations but were attached to undeveloped tertiary follicles rather than fully developed secondary follicles (F3 in Fig. 1e). The pedicels adjoining primary and secondary follicles in nulliparous females had 6–9 distinct cells that gave the pedicel the appearance of a string of beads (P in Fig. 1e).

Stage II. The time intervals used in these experiments did not permit the observation of stage I-II (Mer 1936) that lasted <6 h. in blood fed *Cs. melanura*. By 12 h after blood feeding, all follicles had entered stage II. Table 1 shows that at 0.5 d, functional as well as

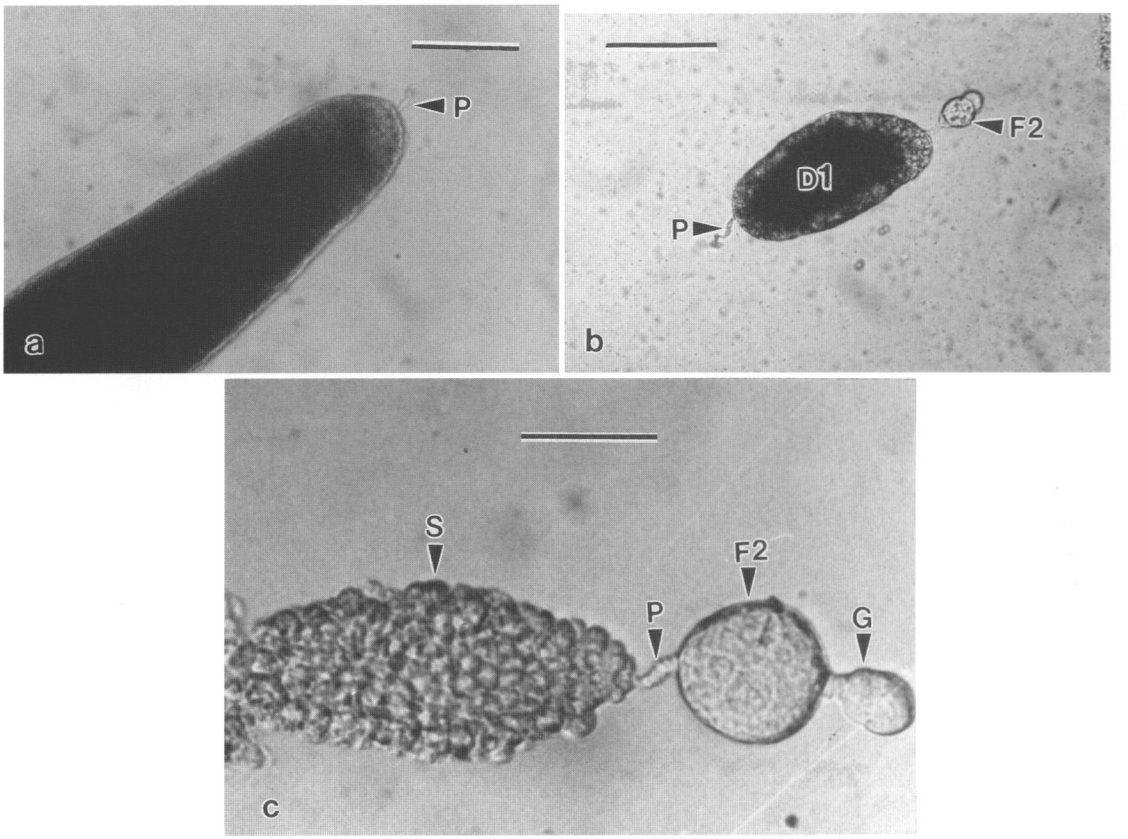

Fig. 3. Stages of ovarian development in females 4–5 d after a bloodmeal. (a) Basal portion of primary follicle in late stage IV, (b) degenerating follicle from same female showing reduction in size as degeneration occurs, (c) ovarian follicle from a 1-parous female within 1 h of oviposition showing the flaccid sac previously occupied by the stage V egg. F2 = secondary functional follicle; G = germarium; *P* = pedicle; S = flaccid sac. Bars = 50 μm.

degenerating follicles exhibited a significant increase in both length and width ($P < 0.001$). Functional follicles became distinctly oval in shape as because of yolk accumulation. Although size increased significantly in all follicles during stage II, less yolk was deposited in follicles in the process of degeneration (D1 in Fig. 2 a and b) (Table 1). One day after blood feeding, functional follicles had 7 nurse cells that were much smaller than the oocyte. At this stage of development, the oocyte nucleus was no longer visible because of yolk accumulation (F1 in Fig. 2a).

Stage III. Follicular development proceeded to stage III 2–3 d after blood feeding. In functional follicles, the oocyte occupied ≈½ of the follicle in early stage III, and the secondary follicles were not yet separated from their germaria (Fig. 2b). By late stage III, the oocyte occupied ¾ of the follicle and developing secondary follicles were separated distinctly from their germaria (Fig. 2c). Degenerating primary follicles increased significantly in both length ($F = 53.47$; df = 4, 95; $P < 0.001$) and width, ($F = 94.15$; df = 4, 95; $P < 0.001$) for the first 2 d after blood feeding (Table 1), but steadily decreased in size during days 3 and 4 (F1 versus D1 in Fig. 2 a and b, d and f). On the 3rd d after blood feeding, yolk loss in degenerating follicles resulted in "wrinkled bodies" filled with a mass of yellow granules that were larger than gonotrophic dilatations and served as precursors of false dilatations.

Stage IV. Follicles entered early stage IV of development 3–5 d after blood feeding and became distinctly oblong in shape. At this stage, most of the follicle was occupied by the oocyte, and the nurse cells were pushed to the proximal end (Fig. 2e). Well formed secondary follicles at stage N were attached to both functional and degenerating stage IV functional follicles ("wrinkled bodies") (F2 in Fig. 2 e and f). Secondary follicles lacked yolk granules and were separated completely from their germaria. On the 4th d, degenerating follicles were distinctly smaller than functional stage IV follicles and became distorted in shape (Fig. 3 a versus b). On the 5th d after blood feeding, degenerating follicles were wrinkled, greatly reduced in size, and resembled gonotrophic dilatations normally found in parous specimens (Fig. 3c). However, these false dilatations were attached to functional secondary follicles that were still at stage N of development rather than stage II follicles that would normally occur in parous host-seeking specimens at this stage of gonotrophic development.

Table 2. Comparison of dilatation formation on functional and degenerating ovarioles of female *Cs. melanura* before and after the 1st oviposition

Interval	No. females examined	Functional ovarioles: No. of dilatations on functional follicles			No. females with degenerating ovarioles	Degenerating ovarioles: No. of dilatations on degenerated follicles		
		0	1	2		0	1	2
		n (%)	*n* (%)	*n*		*n* (%)	*n* (%)	*n* (%)
Nulliparous								
Time after emergence								
24 h[a]	10	10 (100)	0 (0)	0	0	0 (0)	0 (0)	0 (0)
6 d[b]	20	20 (100)	0 (0)	0	2	18 (90)	1 (5)	1 (5)
1-parous								
Time after oviposition								
0 h	6	0 (0)	6 (100)[c]	0	6	0 (0)	0 (0)	6 (100)
12–18 h	17	0 (0)	17 (100)[d]	0	10	0 (0)	0 (0)	10 (58.8)
24 h	18	0 (0)	17 (97.4)	1	11	0 (0)	1 (5.6)	10 (55.5)
48 h	9	0 (0)	9 (100)	0	6	0 (0)	1 (11.1)	5 (55.5)

[a] Degenerating ovarioles not yet formed.
[b] 40% of these females possessed some degenerating ovarioles.
[c] All in sac stage.
[d] All >½ contracted.

Stage V. Stage V of ovarian development occurred 4–5 d after a blood meal. In this stage, the follicle was distinctly elongate and possessed a clearly visible micropyle. In this stage of development, nulliparous specimens contained large numbers of stage V eggs. The degenerating follicles distinctly resembled dilatations, but were still connected to secondary follicles in stage N of ovarian development. Detinova (1962) found similar artifacts in *Anopheles maculipennis* Meigen and termed them diagnostic follicles in parous females.

Dilatation Formation. We examined 30 nulliparous females, 20 of which had taken a sugar meal, and found no evidence of dilatation formation on the primary follicles in any of the specimens (Table 2). Some of the ovarioles of sugar fed females possessed degenerating follicles that had progressed to the point where they resembled dilatations. As a result, some of the ovarioles in nulliparous specimens had secondary or tertiary follicles (with 1 or 2 dilatations) as early as 6 d after emergence. Eight (40%) of the sugar fed females in this sample had noticeable degenerating follicles, 1 (5%) had a single dilatation, and 1 had 2 dilatations (the rogue ovariole in Fig. 1f).

All of the 1-parous females examined within 1 h of oviposition had functional ovarioles with dilatations in the flaccid sac stage (S in Fig. 3d). Degenerating ovarioles in these specimens had 2 dilatations (D1 and D2 in Fig. 4a) that were attached to tertiary follicles (F3 in Fig. 4a). Dilatations on the degenerating ovarioles of 1 parous females therefore represented the primary and secondary follicles on ovarioles that had not matured. All of the 1-parous females that oviposited 12–18 h earlier had ½ contracted sacs on most of the secondary follicles of functional ovarioles.

None of the 1-parous females examined 12–18 h after oviposition had a single dilatation on any of their degenerating ovarioles, but 59% had degenerating ovarioles that bore 2 dilatations (Table 2) (Fig. 4a). The pedicels of functional follicles had undifferentiated cells that gave the pedicel the appearance of a woven cord (P1 in Fig. 4b). The primary as well as the secondary pedicels of degenerating ovarioles possessed distinct cells that gave the pedicel the appearance of a string of beads (P3 in Fig. 4a).

Most functional ovarioles of 1-parous females had 1 fully contracted dilatation 24 h after oviposition (D1 in Fig. 4c). A single female had 2 clear dilatations on what appeared to be a functional ovariole (Table 2). After 48 h, 100% of the females we examined had 1 fully contracted dilatation on all functional ovarioles (D1 in Fig. 4d). A single female in this sample had 1 dilatation on a degenerated ovariole and 5 (55.5%) had degenerated ovarioles with 2 dilatations.

In total, 11 nulliparous and 11 parous females were included in a blind test to determine the accuracy of the dilatation technique. All of the nulliparous females were identified correctly even though 1 had a number of degenerated ovarioles bearing false dilatations. One of the parous females was classified as nulliparous. The remainder were classified accurately as 1-parous. Four (36%) of the parous females had degenerating ovarioles, 3 (27%) had degenerating ovarioles with 2 dilatations. The dilatations on degenerating ovarioles were recognized as false dilatations in this blind test because they were attached to secondary follicles that were much smaller than the others.

Discussion

Ovarian follicular growth in newly emerged female mosquitoes is affected by the release of juvenile hormone from the corpora allata. Juvenile hormone stimulates the primary follicles of newly emerged females to grow in size to a previtellogenic resting stage (Lea 1963, Gwadz and Spielman 1973). Meola and Readio (1988) measured resting stage follicles in sugar fed *Aedes aegypti* (L.) and showed that they did not in-

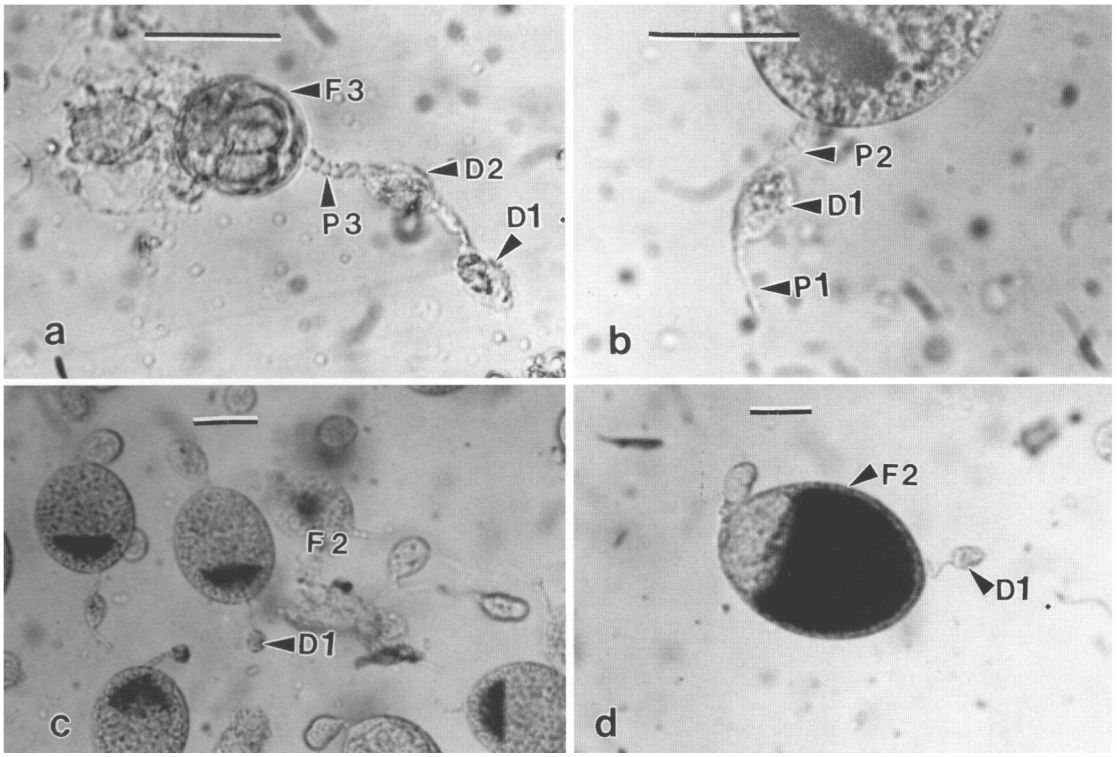

Fig. 4. Ovarian follicles in parous females. (a) Two false dilatations on a degenerating follicle of a 1-parous gravid female, (b) 1-true gonotrophic dilatation in 1-parous female with fresh blood meal, (c) secondary functional follicles of a 1-parous blood fed female, (d) secondary functional follicle of a 1-parous blood fed female with 1 true gonotrophic dilatation. D1, D2 = primary and secondary dilatations. F2, F3 = secondary and tertiary functional follicles, P1, P2, and P3 = primary, secondary and tertiary pedicles. Bar = 50 μm.

crease in size after reaching 50–75 μm in length. The resting stage follicles of sugar fed *Cs. melanura* females also stopped their development at stage I and did not grow beyond 55 μm in length. The disproportionate growth of small primary follicles (SF_1) in *Cs. melanura* after sugar feeding may have resulted from inadequate juvenile hormone stimulation.

Detinova (1962) described abortive oogenesis in the ovarian follicles of *An. maculipennis* and suggested that degeneration of follicles only occurred in blood fed females. Oda et al. (1978) reported follicular degeneration in gonoactive, unfed, nulliparous *Cx. tritaeniorhynchus* Giles in the laboratory, but felt the phenomenon was probably rare in the field. Rosay (1969) noted resorption of nonfunctional follicles in sugar fed *Culex pipiens quinquefasciatus* Say, *Culex tarsalis* Coquillett, and *Cs. inornata* Williston, but did not describe retarded growth in follicles that had developed to the point where they possessed oocytes and nurse cells. We found that retarded growth and degeneration of primary follicles were common in sugar fed *Cs. melanura*. As described by Detinova (1962), functional follicles with retarded growth can accumulate yolk granules up to late stage III after blood feeding and still degenerate to produce false dilatations.

Detinova (1962) noted that the agonotrophic dilatations that formed on degenerating follicles were detected easily during the latter stages of gonotrophic development and designated them as diagnostic follicles for physiological age grading of parous females. We found that degenerating follicles have considerable value determining parity in blood fed and gravid *Cs. melanura* where true dilatations are difficult to find because of the fragility of the pedicel during late stages of follicular development. Resting box populations of *Cs. melanura* contain large numbers of blood fed and gravid specimens (Mahmood and Crans 1994b). We feel that false dilatations can be used to accurately assess physiological age in blood fed and gravid *Cs. melanura* from resting populations (Fig 5). Nulliparous females with primary follicles at stage V have single false dilatations on degenerated ovarioles, whereas 1-parous, gravid females have 2 false dilations on each of the degenerating ovarioles. Degenerating ovarioles (the diagnostic ovarioles of Detinova) are detected easily because in gravid and subgravid females follicles that support the false dilatations always lack yolk granules. Parity status of unfed females may be confirmed by drying the 2nd ovary in a drop of distilled water and examining the tracheolar skeins. Rogue ovarioles and mace ovarioles (Sokolova 1994) were relatively common in *Cs. melanura* but were unreliable indicators of physiological age and should not be used for diagnostic purposes.

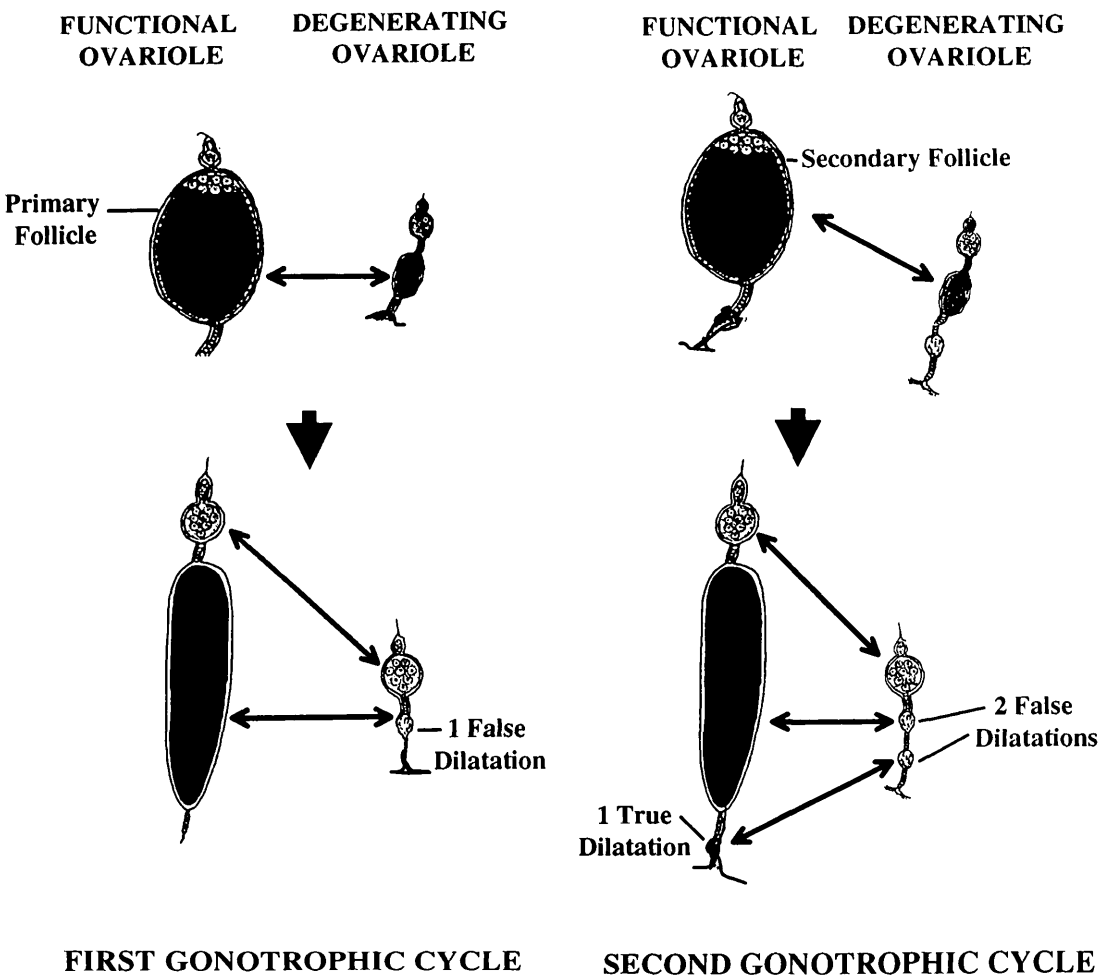

Fig. 5. Comparison of functional and degenerating ovarioles in *Cs. melanura* during the 1st and 2nd gonotrophic cycle.

Acknowledgments

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