Fate of the Blood Meal in Force-Fed, Diapausing *Culex pipiens* (Diptera: Culicidae)

CARL J. MITCHELL[1] AND HANS BRIEGEL[2]

J. Med. Entomol. 26(4): 332–341 (1989)

ABSTRACT Diapausing *Culex pipiens* L. do not display host-seeking behavior and can be induced to take blood only by being placed in contact with or in proximity to a host for prolonged periods. Such "force-fed" females do not use the blood for lipogenesis, and only some of them use the blood to initiate vitellogenesis. Diapausing *Cx. pipiens* that are induced to feed eject an average of 4.2–4.6 μl of blood during overnight feeding periods compared with an average of 0.1 μl for nondiapausing controls. The reduced avidity of diapausing females for blood, even under optimum conditions, and the ejection by fed females of blood volumes in excess of volumes usually retained indicate that such females are not physiologically programmed for taking and retaining blood. Data for uric acid and hematin excretion and bloodmeal volumes retained by diapausing females are positively correlated with diapause termination and yolk deposition. The occurrence of gonotrophic dissociation need not be invoked to explain the failure of some diapausing females to initiate vitellogenesis following a blood meal. Instead, this is explained by retention of small quantities of blood followed by incomplete digestion and is the expected result of a dose-dependent phenomenon determined by threshold blood volumes. Our data support the concept that the overwintering strategy of *Cx. pipiens* is limited to gonotrophic concordance in which overwintering females in nature do not take blood or develop eggs until diapause is terminated.

KEY WORDS Insecta, *Culex pipiens*, physiology, diapause

DEBUCK & SWELLENGREBEL (1934) coined the term "enforced feeding" to describe a phenomenon in diapausing *Anopheles messeae* Falleroni where "... the same mosquitoes consistently refusing to bite a person's bare arm introduced into their cage of 2.4 cub. ft. (0.068 m^3) ... feed all, or nearly all, when they are enclosed within a jar of less than 0.2 cub. ft. (0.006 m^3) although they are put to the inconvenience to feed through gauze." Since then, it has been shown that different sensory receptors are involved in host seeking and in blood feeding (McIver 1982). Host-seeking behavior is absent in diapausing *Culex tarsalis* Coquillett and *Cx. pipiens* L. (Mitchell 1981, 1983; Bowen et al. 1988), but if the host-seeking step in the sequence of events that leads to feeding is bypassed, some diapausing females may feed that otherwise would not do so (Mitchell 1981, 1983). Even most gravid *Cx. pipiens* will take a blood meal when they are confined near a host but not when they have to locate the host at a distance (Meola & Readio 1987). These abnormal "enforced feeding" phenomena may be explainable in evolutionary terms. The lack of blood feeding by diapausing and gravid females has survival value; therefore, because blood feeding in nature invariably is preceded by host seeking, selection pressure for the control of blood feeding may reside at the level of the host-seeking response (Mitchell 1981, Bowen et al. 1988). Consequently, if the host-seeking step is omitted by placing females in proximity or in contact with a host, sensory responses concerned with gorging may be activated in individuals that otherwise are unprepared physiologically for taking, retaining, or digesting blood.

Previous studies on gonotrophic dissociation and winter survival of blood-fed *Cx. pipiens* support the concept that enforced feeding is necessary to induce diapausing females to take a blood meal. Eldridge (1968) used a small cage, made from a 0.0005-m^3 glass jar (1 pint), designed to increase mosquito contact with the host. The bottom of each jar was flooded with water, and mosquitoes were confined for 16 h with the leg of a chicken inserted through a hole in the top cover. This technique, and others that relied on confining large numbers of mosquitoes with hosts in small cages, was used by Bailey et al. (1982) to obtain blood-fed, diapausing *Cx. pipiens* for their study on winter survival.

Diapause in *Culex* mosquitoes is characterized by ovarian diapause, reduced blood avidity, hypertrophy of the fat body, and relative inactivity (Eldridge 1987). Ovarian diapause is defined as a condition in which the ovarian follicles of the female mosquito do not develop past Stage N of Kawai (1969), or a follicle/germarium ratio of about 1.5:1 (Spielman & Wong 1973, Eldridge 1987).

The fact that some force-fed, diapausing *Cx. pipiens* may fail to initiate vitellogenesis is undisputed. Some investigators have maintained that the blood taken by such females is used for increasing

[1] Division of Vector-Borne Viral Diseases, Center for Infectious Diseases, Centers for Disease Control, Public Health Service, U.S. Department of Health and Human Services, P.O. Box 2087, Fort Collins, Colo. 80522.

[2] Department of Zoology, University of Zurich, Zurich CH-8057, Switzerland.

lipid reserves for winter survival instead of for ovarian development (Eldridge 1968, 1981; Eldridge & Bailey 1979). However, the data upon which this claim is based (Eldridge 1968) are inconclusive because the females tested for lipids had been fed 5% sucrose before and after blood feeding. Reexamination of this question showed that diapausing *Cx. pipiens* cannot increase their lipid reserves by taking a single blood meal; in fact, such reserves decrease significantly in blood-fed females kept on a water diet for 6 d after feeding (Mitchell & Briegel 1989). Therefore, a question arises about the fate of the blood meal in diapausing *Cx. pipiens* that do not use the blood for either vitellogenesis or lipogenesis.

We have investigated this question quantitatively by determining the volumes of blood ejected during overnight feeding periods, volumes retained, and the total amounts of uric acid and hematin excreted following a blood meal. Uric acid totals, hematin totals, and bloodmeal volumes were correlated with the initiation of vitellogenesis and yolk deposition. Uric acid is a catabolite of protein digestion, and, among nondiapausing mosquitoes, quantities excreted are positively correlated with dietary protein intake (Briegel 1986). In contrast, hematin is an undigested waste product that never enters the hemocoel, and quantities voided are directly proportional to the amount of hemoglobin in the blood meal (Briegel 1986). Briegel (1986) differentiates between excretion and defecation when referring to removal of uric acid and hematin, respectively. To be concise, both functions are referred to as excretion in this report.

Materials and Methods

The strain of *Cx. pipiens* used was from Fort Collins, Colorado. It was colonized in 1981 and enters diapause uniformly when exposed to short day lengths and cool temperatures (Wilton & Smith 1985, Bowen et al. 1988, Mitchell & Briegel 1989). The stock colony was maintained at 27°C, 85% RH, and a photoperiod of 14:10 (L:D). Larvae were reared on a high-protein diet (Lea 1964). Adults were fed 10% sucrose ad lib. and allowed to feed overnight on a chicken once each week.

Diapausing females were reared from the egg or first instar at 22°C and 9:15 (L:D). Diapause induction under these conditions is close to 100% (Bowen et al. 1988). Larvae were fed the same diet as the stock colony, but the feeding period was extended from 8 to 11 d because of slowed development at the cooler temperature. Adults were fed 1 or 10% sucrose ad lib. from eclosion until offered a blood meal at 7–11 d of age. These concentrations of sucrose were shown to yield adult females with minimal and maximal amounts of lipids, respectively (Mitchell & Briegel 1989). Ovaries were dissected in saline, and follicles with attached germaria were measured at 100× under a compound microscope. A follicle/germarium (F:G) ratio equal to or less than 1.5:1 (ovary class 1) was used as the criterion for ovarian diapause (Spielman & Wong 1973). Follicle lengths of such females typically were less than 50 μm (this study, Bowen et al. 1988). Two additional ovarian classes based on yolk length were arbitrarily selected for females that terminated diapause following blood feeding. These were females with a F:G ratio greater than 1.5:1 after blood digestion and with yolk lengths less than 100 μm (ovary class 2) or equal to or greater than 100 μm (ovary class 3). Yolk lengths of nondiapausing controls also were measured. To confirm the status of experimental cohorts, five follicles and germaria from five females were measured from each brood of diapausing mosquitoes and colony controls on the day of each blood-feeding trial.

Diapausing females were induced to take blood by being placed in groups of 50 in 250-ml (0.00025 m^3) plastic cups covered with netting upon which a small chick was restrained overnight (1630–0800 hours) at 22°C, 9:15 (L:D), with lights off at 1700 hours. Colony females used as controls, and females in which diapause had been terminated by exposure to 27°C and 14:10 (L:D) for 1 wk, were fed in the same manner under the same conditions. Some diapausing and nondiapausing females were given measured volumes of heparinized chick blood by enema (Briegel & Lea 1975).

Following the overnight feeding period of 15.5 h, the number of blood-fed females per cup was determined by sorting obviously blood-fed individuals and dissecting the remainder for occult blood in the digestive tract. The volume of blood ejected into each cup during the overnight feeding period was determined as described below. Blood-fed females were placed in individual tubes (10 by 75 mm) and restrained in the lower portion by hollow glass inserts with cotton plugs in the bottom. Water was added to the cotton plugs for consumption and to increase humidity. Mosquitoes were transferred to clean tubes at intervals, and excreta were stored for analysis. Mosquitoes were dissected for ovarian classification at the end of bloodmeal digestion, usually 3–6 d after feeding depending on incubation temperature. Any gut contents present at this time were added to the excreta to be tested for uric acid and hematin.

Blood-fed diapausing and nondiapausing colony controls were incubated at 9:15 (L:D) and temperatures of 18, 22, and 27°C, and a daily 18–25°C cycle with the low and high temperatures corresponding to the times the lights were turned on (0800 hours) and off (1700 hours). Some blood-fed colony controls also were incubated at 14:10 (L:D) and 27°C. The 18°C temperature was chosen because it has been reported that ". . . any bloodmeals taken (by diapausing *Cx. pipiens*) result in fat body development and not ovarian development" when incubated at 18°C or below during the time the blood meal is being digested (Eldridge & Bailey 1979).

Sample sizes varied according to feeding success

Table 1. Ejection of blood by *Cx. pipiens* during a 15.5-h overnight feeding period at 22°C

Diet and status before blood meal	Fat body hypertrophy[a]	No. feeding trials[b]	No. ♀♀ tested	% Fed	Avg μl blood ejected per ♀ per trial	
					$\bar{x}$	SEM
1% Sucrose						
Diapausing	No	13	626	33.7	4.6[c]	0.19
10% Sucrose						
Diapausing	Yes	45	2,175	37.5	4.2	0.06
Nondiapausing	No	8	352	88.6	0.1	0.01
Diapause broken	Yes	2	101	81.2	0.2	0.02

[a] See Mitchell & Briegel 1989.
[b] Usually 50 females tested per trial.
[c] Based on 426 females tested in nine trials; volume ejected not determined for others.

and the objectives of experiments. All specimens of excreta were tested for total hematin, and associated females were dissected for ovarian classification after blood digestion was completed. Bloodmeal volumes were then derived from total hematin values and hemoglobin titers of associated chicks. Because of time constraints, only some of the samples tested for hematin were tested for uric acid.

Assays. To determine hemoglobin titers in the hosts, blood samples were drawn from each chick by jugular venipuncture at the end of each feeding period, and duplicate samples of 3, 6, and 9 μl were mixed with 1 ml of Drabkin's reagent and compared spectrophotometrically with a human hemoglobin standard (Sigma 525-18, Sigma Diagnostics, St. Louis) (Briegel et al. 1979). Ejecta from mosquitoes collected in each plastic cup during overnight feeding periods were dissolved in 10 ml of Drabkin's reagent. Three 1-ml aliquots from each cup were centrifuged for 1 min at 14,000 rpm. Hemoglobin concentrations in the three samples were determined spectrophotometrically at 540 nm and compared with those of individual chicks to estimate the volume of blood in each cup.

Excreta in the tubes were dissolved in 1 ml each of 1% lithium carbonate and used for hematin and uric acid determinations (Briegel 1980). The stoichiometric molar relationship between bloodmeal hemoglobin and fecal hematin (Briegel 1986) made it possible to retrospectively estimate the volume of blood each female contained when it was placed in a tube following the overnight feeding period.

Data were analyzed for statistical significance by using χ^2, probit analysis, analysis of covariance, and Student's t test, as appropriate. In the text, arithmetic means are routinely followed by standard errors of the means.

Results

The percentage of different cohorts of *Cx. pipiens* that took a blood meal and the quantities of blood they ejected during the overnight feeding period are shown in Table 1. The proportion of diapausing females that took blood was not significantly ($P > 0.05$) affected by the concentration of sucrose in their diet before they were offered a blood meal. However, the proportion of diapausing females that took blood was significantly lower ($P < 0.001$) than that of nondiapausing controls and females that had terminated diapause before feeding. Diapausing females ejected copious quantities of blood during the overnight feeding period. The average volumes ejected per female were 42–46 times greater, and significantly higher ($P < 0.001$), than the average volume ejected by nondiapausing controls. The average volume of blood ejected by females that had terminated diapause was twice that of nondiapausing controls but was 21–23 times less ($P < 0.001$) that of diapausing females.

Overnight access to a host in a 0.00025-m^3 cage under crowded conditions resulted in average feeding rates of only 33.7–37.5% among diapausing *Cx. pipiens* (Table 1). The reduced avidity of diapausing females for blood, and our desire to avoid interrupting the dark phase of the photoperiod, made it impractical to obtain individually fed females that had fed to repletion and from which ejecta were collected simultaneously. This precluded determining the proportion of the blood meal ejected by individual females soon after feeding. Therefore, the average volume of blood ejected per female during the 15.5-h overnight feeding period was determined for females that were fed in groups. To obtain additional information, and to determine the volume of blood ejected in relation to bloodmeal size, groups of 10 diapausing females that had been maintained on 10% sucrose were each given measured volumes of heparinized chick blood by enema. Quantities ejected during the next 24 h at 18°C and 9:15 (L:D) were determined for 49 females and expressed as percentages of original volumes. An average of 62% (±7.2) was ejected, and the range was from 51% for the 7-μl meals to 90% for the 1-μl meals. Experiments designed to determine the blood volume necessary to initiate vitellogenesis in diapausing females by using the enema technique failed because of this premature expulsion phenomenon.

Baseline data on uric acid and hematin excretion patterns were obtained for 22 nondiapausing col-

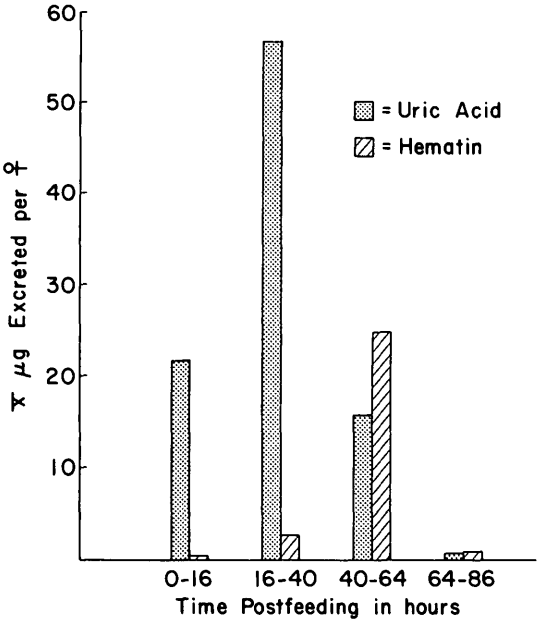

Fig. 1. Temporal pattern of uric acid and hematin excretion by 22 nondiapausing *Cx. pipiens* incubated at 27°C and 14:10 (L:D).

ony controls that were placed in tubes within 15 min of engorgement and incubated for 86 h at 27°C and 14:10 (L:D) (Fig. 1). Excreta were collected during the 16 h after feeding (roughly equivalent to the 15.5-h overnight feeding period used in most experiments) and daily thereafter. The average amount of uric acid excreted per female was 94.6 ± 5.1 μg, and the average amount of hematin excreted was 28.8 ± 1.0 μg. The average bloodmeal volume was 9.2 ± 0.3 μl. Most of the uric acid (59.9%) was excreted between 16 and 40 h; however, a significant proportion (22.7%) was excreted during the 16 h after feeding. A relatively small proportion of hematin (1.4%) was excreted during the first 16 h, and most (85.8%) was excreted between 40 and 64 h after feeding.

Temporal patterns of uric acid and hematin excretion were determined for diapausing *Cx. pipiens* incubated at each of the four temperature regimens and 9:15 (L:D) during the time of blood digestion. Digestion proceeded more rapidly as the incubation temperature increased and was essentially completed by day 3 at 27°C and by day 6 at 18°C.

The total amounts of uric acid excreted by 453 females fed 10% sucrose and 70 females fed 1% sucrose before blood feeding are shown in Table 2. Considering only females in the 10%-sucrose cohort that were diapausing when they took a blood meal, average uric acid totals were significantly lower ($P \leq 0.01$) for females that remained in diapause (ovary class 1) compared with those that terminated diapause and deposited at least 100 μm of yolk (ovary class 3) at each temperature tested except 27°C. Other statistically significant differences in stepwise comparisons of average uric acid totals among the females in the 10%-sucrose cohort that were diapausing when they took a blood meal follow. At 18°C, females that remained in diapause (ovary class 1) excreted significantly less ($P < 0.001$) uric acid than females that developed class 2 ovaries. At 18–25°C, females that developed class 2 ovaries excreted significantly less uric acid ($P < 0.001$) than females that developed class 3 ovaries.

The total amounts of hematin excreted by 578 females fed 10% sucrose and 70 females fed 1% sucrose before blood feeding are shown in Table 3. Among the females in the 10%-sucrose cohort that were diapausing when they took a blood meal, average hematin totals were significantly lower ($P < 0.05$) for females that remained in diapause (ovary class 1) at each temperature, in comparison to females that developed class 3 ovaries. Other statistically significant comparisons among the females in the 10%-sucrose cohort that were diapausing when they took a blood meal are as follows: At 18°C and at 18–25°C, females that remained in diapause (ovary class 1) excreted significantly less ($P < 0.05$) hematin than females that developed class 2 ovaries. Also, at 22 and 27°C, females that developed class 2 ovaries excreted significantly less ($P < 0.05$) hematin than females that developed class 3 ovaries.

All nondiapausing females used as controls in the experiments summarized in Tables 2 and 3 became gravid after taking a blood meal. The average amounts of uric acid and hematin excreted by these females were at least twice as great as the average amounts excreted by females in the 10%-sucrose cohort that were diapausing when they took a blood meal and subsequently terminated diapause (ovary classes 2 and 3). The differences are highly significant ($P < 0.001$) at each temperature where paired comparisons were made.

Among mosquitoes that were diapausing when fed, only 8.6% ($n = 70$) of females in the 1%-sucrose cohort initiated vitellogenesis following a blood meal compared with 40.1% ($n = 439$) of females in the 10%-sucrose cohort (Table 3); this difference is highly significant ($P < 0.001$). These cohorts also differed in the amounts of uric acid and hematin excreted by females incubated at the same temperature and with the same ovarian classification. Females in the 1%-sucrose cohort that remained in diapause following a blood meal (ovary class 1) excreted significantly more ($P < 0.05$) uric acid than did females in the 10%-sucrose cohort that remained in diapause (Table 2). A significant difference ($P < 0.05$) in average uric acid totals also was observed among females in 1%- and 10%-sucrose cohorts that terminated diapause and developed class 3 ovaries at 18–25°C. Paired comparisons of average hematin totals between females in the 1%- and 10%-sucrose cohorts that were diapausing when they took a blood meal and had the same ovarian classification following digestion of the

Table 2. Average μg of uric acid excreted by 523 *Cx. pipiens* fed 1% or 10% sucrose before blood feeding and grouped by ovarian classification following blood digestion at different temperatures and 9:15 (L:D)

Incubation temp, °C		1% Sucrose diet			10% Sucrose diet			
		Diapausing when fed blood			Diapausing when fed blood			Nondiapausing when fed blood
		1[a]	2	3	1	2	3	[b]
18	*n*	28	1	0	55	14	19	47
	$\bar{x}$	43.3	41.9	—	15.3	33.6	43.7	108.1
	SEM	5.7	—	—	2.2	3.2	8.0	7.0
18–25	*n*	20	0	4	104	12	35	29
	$\bar{x}$	23.8	—	87.2	12.7	19.1	52.6	105.8
	SEM	4.6	—	15.2	1.6	4.3	6.0	7.4
22	*n*	16	0	1	44	15	27	Not done
	$\bar{x}$	24.9	—	131.9	11.9	18.4	26.2	—
	SEM	3.5	—	—	1.8	3.6	3.3	—
27	*n*		Not done		15	11	15	11
	$\bar{x}$	—	—	—	20.5	21.8	30.8	60.6
	SEM	—	—	—	2.7	4.9	5.4	6.9

[a] Ovary classes: 1, remained in diapause; 2, diapause terminated, yolk <100 μm; 3, diapause terminated, yolk ≥100 μm.
[b] All nondiapausing controls deposited >100 μm of yolk.

blood at the same temperature, show the following statistically significant differences. The females in the 1%-sucrose cohort with class 1 ovaries at 18°C and with class 3 ovaries at 18–25°C excreted significantly more ($P < 0.05$) hematin than did their counterparts in the 10%-sucrose cohort (Table 3). With one exception, females in the 1%-sucrose cohort that initiated vitellogenesis took larger blood meals than those that did not.

The volume of blood ejected during the 24 h immediately following the 15.5-h overnight feeding period was determined for 447 of the 578 females in the 10%-sucrose cohort included in Table 3. The top portion of Fig. 2 shows the average volumes of blood retained at the end of the overnight feeding period, and the bottom portion shows the percentages of those volumes ejected during the 24 h immediately following removal of the blood source (i.e., chicks). Because all mosquitoes were fed under identical conditions, and because subsequent incubation temperatures could not have influenced the data for average blood volumes retained as shown in the top portion of Fig. 2, these data were combined for analysis. Among mosquitoes that were diapausing when fed, those that remained in diapause retained significantly smaller ($P \leq 0.01$) blood volumes (average 2.3 ± 0.1 μl) at the end of the overnight feeding period than females that subsequently terminated diapause. Similarly, each of the remaining stepwise comparisons is significantly ($P \leq 0.001$) different; i.e., the average blood volume retained by females with

Table 3. Average μg of hematin excreted by 648 *Cx. pipiens* fed 1% or 10% sucrose before blood feeding and grouped by ovarian classification following blood digestion at different temperatures and 9:15 (L:D)

Incubation temp, °C		1% Sucrose diet			10% Sucrose diet			
		Diapausing when fed blood			Diapausing when fed blood			Nondiapausing when fed blood
		1[a]	2	3	1	2	3	[b]
18	*n*	28	1	0	55	14	19	47
	$\bar{x}$	10.5	8.2	—	6.6	12.0	14.0	27.9
	SEM	7.1	—	—	4.4	3.1	6.9	10.6
18–25	*n*	20	0	4	104	13	36	71
	$\bar{x}$	8.4	—	29.7	7.2	10.8	13.6	27.6
	SEM	8.1	—	14.2	5.3	4.7	7.5	8.8
22	*n*	16	0	1	89	27	41	10
	$\bar{x}$	6.3	—	18.5	7.4	9.0	13.5	34.3
	SEM	5.5	—	—	4.6	4.9	6.2	5.7
27	*n*		Not done		15	11	15	11
	$\bar{x}$	—	—	—	6.3	6.7	12.3	24.4
	SEM	—	—	—	2.5	4.2	9.0	7.0

[a] Ovary classes: 1, remained in diapause; 2, diapause terminated, yolk <100 μm; 3, diapause terminated, yolk ≥100 μm.
[b] All nondiapausing controls deposited >100 μm of yolk.

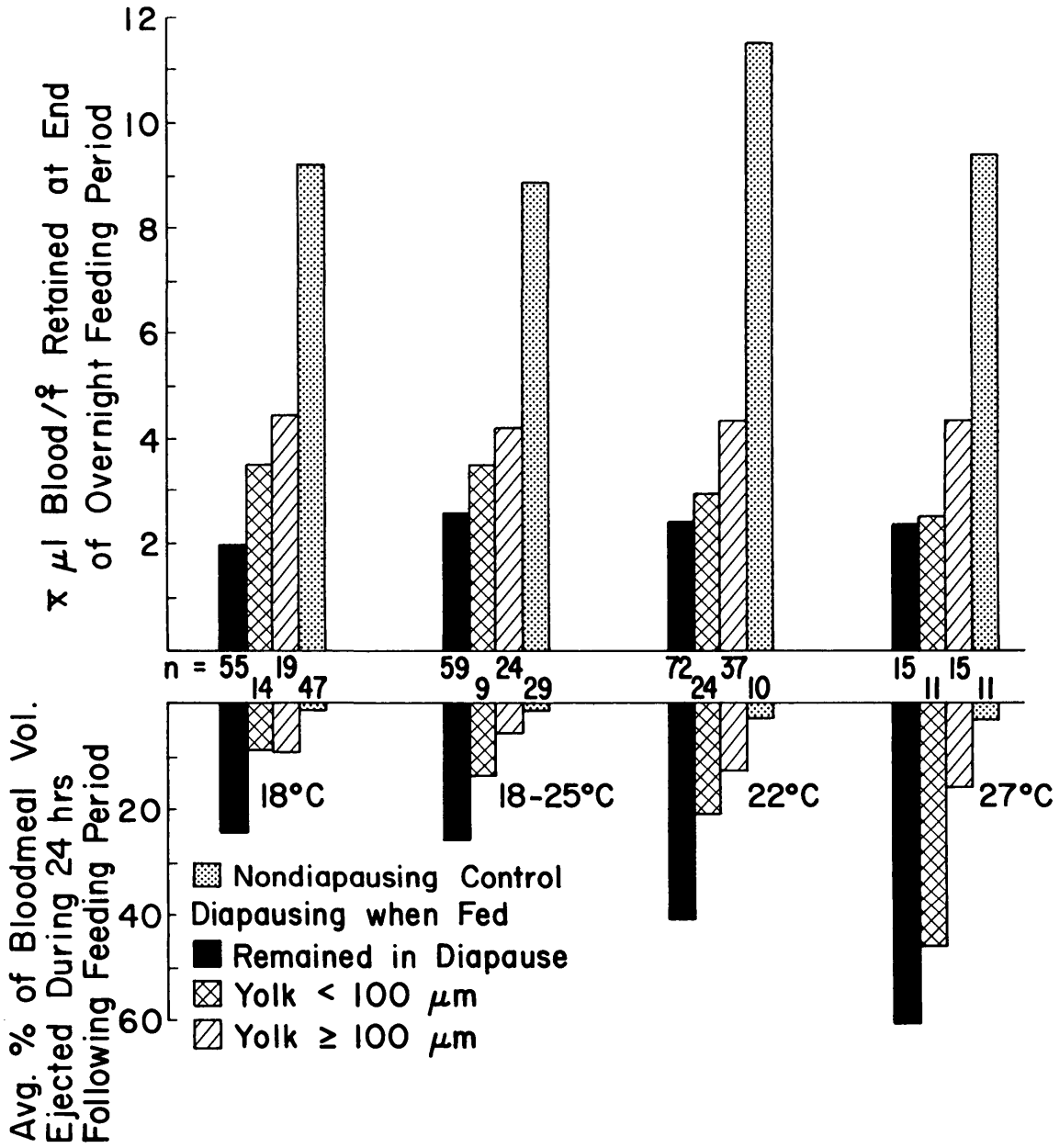

Fig. 2. Average blood volumes retained by diapausing and nondiapausing *Cx. pipiens* at the end of the overnight feeding period in relation to vitellogenesis and percentages of those volumes ejected during the 24 h following the feeding period.

class 2 ovaries (average 3.1 ± 0.2 μl) at the end of the overnight feeding period is significantly less than that of females with class 3 ovaries (average 4.3 ± 0.2 μl), and the latter is significantly less than that of the nondiapausing controls (average 9.3 ± 0.3 μl).

With regard to ejection of blood during the 24 h following the 15.5-h overnight feeding period, each cohort that remained in diapause ejected significantly greater ($P < 0.05$) percentages of the blood volume than did any other cohort incubated at the same temperature. In addition, females that terminated diapause after feeding ejected significantly greater ($P < 0.05$) percentages of their blood volumes during this 24-h period than did nondiapausing controls, except at 27°C. At the latter temperature, females that terminated diapause and developed class 2 ovaries ejected a significantly greater ($P < 0.001$) percentage of their blood volumes than nondiapausing controls; however, fe-

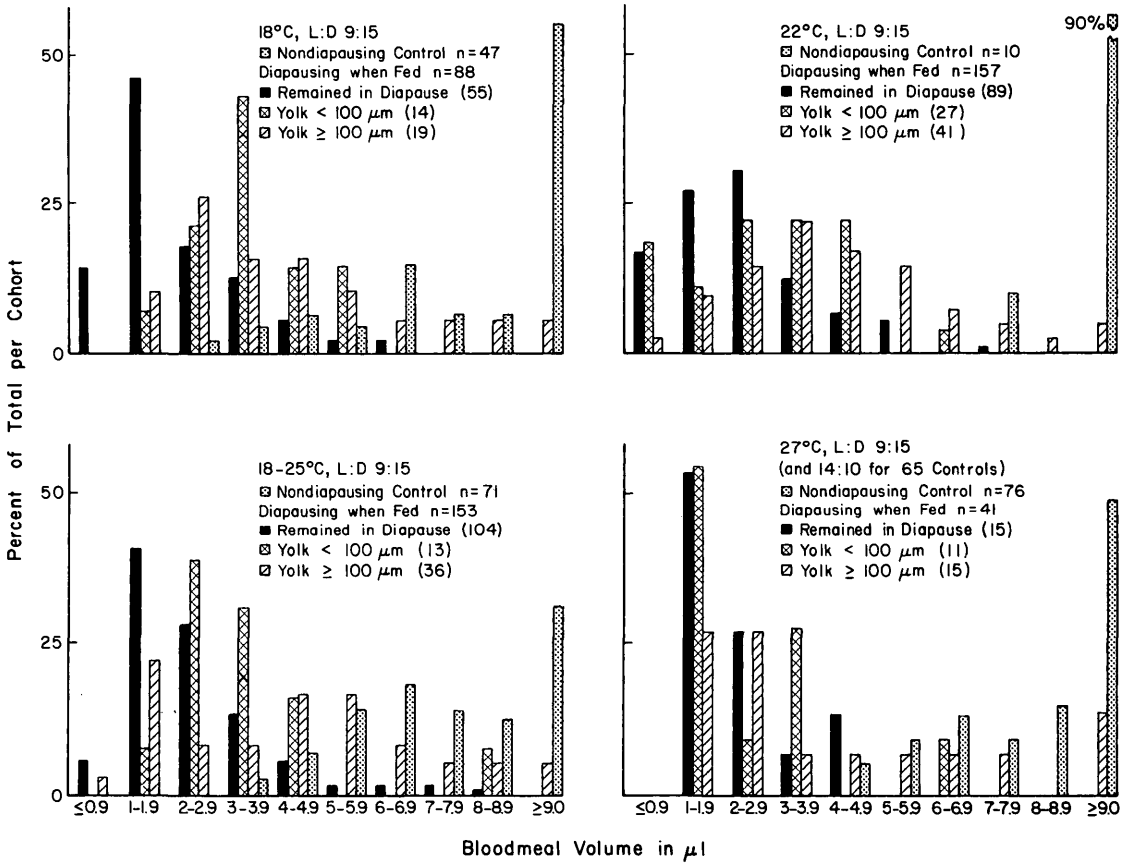

Fig. 3. Relationship between blood volumes retained at the end of the overnight feeding period and the initiation of vitellogenesis in diapausing and nondiapausing *Cx. pipiens*.

males with class 3 ovaries did not differ significantly in this respect from the controls.

The relationship between blood volumes retained at the end of the overnight feeding period and the initiation of vitellogenesis by 439 females maintained on 10% sucrose before blood feeding and that were diapausing when they took blood is shown in Fig. 3. Similar data also are presented for 204 nondiapausing controls, all of which became gravid. At 27°C, 65 of the 76 controls were incubated at 14:10 instead of 9:15 (L:D); however, this would not have affected bloodmeal volume because all females were fed under identical conditions before being incubated at the different regimens. The average blood volumes retained by the nondiapausing controls at the end of the overnight feeding period were 9.2 ± 0.5 μl at 18°C, 8.0 ± 0.3 μl at 18–25°C, 11.5 ± 0.6 μl at 22°C, and 9.1 ± 0.3 μl at 27°C. With a single exception, each of these averages is at least twice as high as that of any cohort that was diapausing when fed and incubated at the same temperature. The exception is for females that developed class 3 ovaries at 18–25°C (average 4.4 ± 0.3 μl). In each case, the blood volumes of nondiapausing controls were significantly higher ($P < 0.01$) than those of females that were diapausing when fed and incubated at the same temperature.

The 50% endpoints of blood volumes required to initiate vitellogenesis among females that were diapausing when fed and incubated at various temperatures follow: 4.8 μl (95% C.I. 4.0–5.7) at 18–25°C, 4.0 μl (3.1–5.1) at 18°C, 3.6 μl (2.8–4.3) at 22°C, and 1.6 μl (0.1–3.0) at 27°C. Stepwise comparisons show the following statistically significant differences. The 50% endpoint at 18–25°C is significantly greater than the endpoints at 22°C ($P < 0.05$) and 27°C ($P < 0.01$). The 18 and 22°C endpoints are significantly greater ($P < 0.05$) than the 27°C endpoint. Endpoints for blood volumes required to initiate vitellogenesis in orally-fed nondiapausing controls and females maintained on 1% sucrose before blood feeding could not be determined because the former all became gravid and only 6 of 70 of the latter did so.

An attempt was made to determine the 50% endpoint for the blood volume required for vitellogenesis in nondiapausing controls by giving measured volumes (0.5, 1, 1.5, 2, and 3 μl) of heparinized chick blood (126.2 μg of protein/μl) by enema and incubating the mosquitoes at 27°C and 14:10 (L:D). In this experiment, the criterion for vitel-

logenesis was yolk lengths of at least 100 μm in two or more follicles at 72 h after enema. Each of 12 females that received 2 and 3 μl, respectively, and 10 of 13 (77%) that received 1.5 μl, met this criterion. Because 8 of 13 (62%) females given 1 μl and 5 of 10 (50%) given 0.5 μl also met the criterion, the 50% endpoint must be in the vicinity of 0.5 μl, and the amount of blood protein required must be about 63 μg.

Uric acid output is an indication of the efficiency of protein digestion (Briegel 1986); therefore, we determined whether females that remained in diapause after taking a blood meal differed in this respect from females that terminated diapause after feeding. The analysis is restricted to 366 females maintained on 10% sucrose before blood feeding that were diapausing when they took blood and for which we have total uric acid output (Table 2) and bloodmeal volumes (data not shown). There is a high correlation ($P < 0.01$) between total uric acid and bloodmeal volume for females that remained in diapause ($r = 0.71$) and for females that terminated diapause ($r = 0.47$) after feeding. This also is true for these cohorts analyzed separately at each temperature except for females that terminated diapause at 18–25°C. Further, when analysis of covariance was used to adjust for bloodmeal volume, the average uric acid totals for females that remained in diapause were significantly lower than those of females that terminated diapause at 18–25°C ($P < 0.001$) and at 22°C ($P < 0.05$) after feeding. No significant difference was noted for the groups incubated at 18 and 27°C. Also, the ratio of uric acid output to bloodmeal volume was significantly greater ($P < 0.001$) among nondiapausing controls than among females in the 10%-sucrose cohort that remained in diapause (ovary class 1) and that terminated diapause (ovary classes 2 and 3) after taking a blood meal.

Discussion

Diapausing females maintained on 1% sucrose and nondiapausing controls maintained on 10% sucrose before blood feeding had small fat bodies. In contrast, diapausing females maintained on 10% sucrose before blood feeding, and females in which diapause had been terminated by exposure to a long photoperiod and warm temperature, had hypertrophied fat bodies (Mitchell & Briegel 1989). Therefore, it is possible to examine the effects of diapause and fat body size in *C. pipiens* on avidity for blood and on bloodmeal retention. Both lean and fat diapausing females had a reduced avidity for blood, and those that did feed ejected copious quantities of blood during the overnight feeding period (Table 1). This suggests that diapausing *Cx. pipiens*, regardless of fat body size, are not physiologically programmed for taking or retaining blood. In contrast, lean nondiapausing controls, and fat females that had terminated diapause after being exposed to 27°C and 14:10 (L:D) for 1 wk, fed readily and ejected relatively small quantities of undigested blood during the overnight feeding period.

Hudson (1979) noted that diapausing *Culiseta inornata* (Williston) collected from the field took small meals, ejected them prematurely, and did now show any tryptic activity. Our results differ somewhat in that some tryptic activity must have occurred in those *Cx. pipiens* that terminated diapause and initiated vitellogenesis after taking blood. However, our results support Hudson's (1979) conclusion that such females normally do not take blood in nature.

Briegel (1969) established a nitrogen balance for blood-fed *Culex pipiens quinquefasciatus* (Say) and showed that the intake of dietary nitrogen is in excess of reproductive requirements. In *Aedes aegypti* (L.), excretory processes occur in two waves. A peak in uric acid excretion occurs the day after the blood meal, and a peak in hematin excretion occurs the following day (Briegel 1986). The temporal pattern of uric acid and hematin excretion by nondiapausing *Cx. pipiens* is similar (Fig. 1). Because a significant proportion (22.7%) of total uric acid was excreted during the 16 h after feeding at 27°C, uric acid lost during overnight feeding periods probably resulted in an underestimation of total uric acid for some cohorts in some experiments. This is especially so for nondiapausing controls because most of them would be expected to have fed during the early part of the period.

Collectively, the data for uric acid excretion (Table 2), hematin excretion (Table 3), and bloodmeal volumes (Fig. 2 and 3) show clear and unequivocal trends. Among cohorts fed 10% sucrose before being offered a blood meal, nondiapausing controls retained more than twice as much blood as diapausing females at the end of the overnight feeding period. The controls also excreted more than twice as much uric acid and hematin as any cohort that was diapausing when fed and incubated under the same conditions. Further, with only one exception, the differences between females that remained in diapause after taking blood and those that terminated diapause and developed class 3 ovaries are statistically significant with respect to blood volume retained at the end of the overnight feeding period, total uric acid output, and total hematin output. At each incubation temperature, females that remained in diapause retained significantly less blood and excreted significantly less uric acid and hematin that did females that developed class 3 ovaries. The single exception for statistical significance is for uric acid totals at 27°C. The occurrence of gonotrophic dissociation need not be invoked to explain the failure of some diapausing females to initiate vitellogenesis following a blood meal. Instead, this is readily explained by retention of small quantities of blood followed, in some cases, by incomplete digestion. This is the expected result of a dose-dependent phenomenon in which a threshold blood volume must not only be ingested but retained and digested before vitellogenesis can be initiated.

Females that remained in diapause after taking blood not only retained significantly smaller blood volumes at the end of the overnight feeding period but also ejected significantly greater percentages of those volumes during the next 24 h in comparison with cohorts that terminated diapause and nondiapausing controls (Fig. 2). Diapausing females given known blood volumes by enema showed a similar trend. There was an inverse relationship between original volumes and the proportion ejected during the 24 h following the enemas. The tendency of diapausing females with small blood meals to eject disproportionate amounts further reduces the potential value of such meals; however, it is these individuals that would be classified as having undergone gonotrophic dissociation using the criteria of most previous studies.

At first glance, a regression of blood volumes on yolk lengths would seem to be the method of choice for analyzing the effect of blood volume on vitellogenesis; however, this is not a valid approach. Although a small blood meal may result in only a few eggs, these eggs may have yolk lengths comparable with those resulting from a large meal because of resorption of developing oöcytes and developmental arrest of oöcytes in an early state of vitellogenesis (Lea et al. 1978, Clements & Boocock 1984). Therefore, correlations between blood volume and vitellogenesis are based on ovarian classes instead of yolk lengths of individual females. Because the number of developing follicles was not counted, it is impossible to say whether quantitative differences existed between class 3 ovaries of females that terminated diapause and those of nondiapausing controls. To answer this question, future investigators may wish to determine total nitrogen in one ovary from each female and dissect the other to determine ovarian class.

Among mosquitoes that were diapausing when they took blood, significantly more females that had been maintained on 10% sucrose before blood feeding initiated vitellogenesis (40.1%) than did females that had been maintained on 1% sucrose before blood feeding (8.6%). These data, combined with those for uric acid and hematin outputs for cohorts within the same ovarian class, suggest that the threshold blood volume required to initiate vitellogenesis is higher among females with limited lipid reserves (i.e., females fed 1% sucrose). We have shown that diapausing *Cx. pipiens* fed 1% sucrose do not use blood for synthesizing lipids (Mitchell & Briegel 1989). Because most do not use the blood for initiating vitellogenesis, and because uric acid totals suggest that at least some of the blood is being digested, we conclude that females with limited energy reserves may be able to use the digested portion of the blood for immediate energy needs. Nonetheless, a single blood meal provides very little sustenance to females with limited reserves because only 50% of such females can survive for 20 d in simulated hibernation (Mitchell & Briegel 1989). The physiological mechanism causing some diapausing *Cx. pipiens* to terminate diapause and initiate vitellogenesis after taking blood while others do not is unknown, but it is clearly related to the volume of blood retained and the efficiency of blood digestion.

It is important to emphasize that in the experiments with diapausing *Cx. pipiens*, ejection of undigested blood did not automatically cease when females were transferred to tubes at the end of the 15.5-h overnight feeding period. The temporal excretion pattern of hematin among nondiapausing controls shows that only a small proportion of the total hematin is excreted during the 40 h after feeding (Fig. 1), a period equivalent to the 15.5-h overnight feeding period plus the first 24 h (day 1) of excreta collection in all experiments involving blood-fed, diapausing *Cx. pipiens*. Our tests could not distinguish between hemoglobin in undigested blood and hematin from digested blood. Therefore, in light of the temporal excretion pattern of hematin by nondiapausing controls, it is probable that most of the "hematin" detected in excreta of diapausing females during the 24 h immediately following the overnight feeding period, and unknown proportions thereafter, represents undigested blood that did not provide any nourishment.

Incomplete digestion of the blood meal by diapausing *Cx. pipiens* also is reflected by significantly lower ratios of uric acid output in relation to blood volume compared with nondiapausing controls. This may explain the seemingly high blood volumes required to initiate vitellogenesis in diapausing females. Endocrine functions may become slow or inactive in diapausing females; this could result in reduced tryptic activity. Trypsin was not detected in the midguts of blood-fed, diapausing *Cs. inornata* (Hudson 1979), and deactivation of the endocrine system in *Ae. aegypti* is known to suppress the full expression of midgut trypsin (Graf 1986).

Swellengrebel (1929) used the term "dissociation gono-trophique" to describe a condition in which the repeated taking of blood meals by *Anopheles atroparvus* Van Thiel during the autumn and winter failed to result in egg production. This led to speculation that other mosquito species, including *Cx. pipiens*, might occasionally take a blood meal before entering hibernation and digest the blood without initiating vitellogenesis (Eldridge 1968, Eldridge & Bailey 1979). There are, however, important differences in the overwintering strategies of *An. atroparvus* and *Cx. pipiens*. The former spends the winter in warm houses and animal shelters, takes multiple blood meals during the winter, does not store large quantities of fat, and presumably uses the blood for short-term energy needs (Swellengrebel 1929, Washino 1977). In contrast, *Cx. pipiens* overwinters in cool, moist, hibernacula well-protected from external temperature variations; rarely, if ever, takes a blood meal during the winter; and synthesizes large quantities of lipids before entering hibernation principally, if not exclusively, from plant juices rich in carbohydrates (Mitchell 1983, Jaenson 1987, Mitchell & Briegel 1989).

Our results support evidence indicating that the overwintering strategy of *Cx. pipiens* closely resembles that of *An. messeae*. The latter species develops hypertrophied fat bodies before entering hibernation, and diapausing females can be induced to take blood only by being placed in proximity to a host (DeBuck & Swellengrebel 1934). In nature, overwintering females do not take blood or develop eggs. Swellengrebel (1929) called this phenomenon "gonotrophic concordance" and considered it to be the mark of true hibernation.

Acknowledgment

We thank S. Zaba for maintaining the stock mosquito colony and R. Haigis for assistance with the chemical analyses. We appreciate the contributions made by the Swiss National Science Foundation (No. 3.149.85 to H.B.) and the support given by the "Georges and Antoine Claraz-Schenkung, Zürich," as well as the "Stiftung für wissenschaftliche Forschung an der Universität Zürich." We are especially grateful to Mary F. Bowen, Northern Illinois University, for her critical reading of the manuscript.

References Cited

Bailey, C. L., M. E. Faran, T. P. Gargan & D. E. Hayes. 1982. Winter survival of blood-fed and non blood-fed *Culex pipiens* L. Am. J. Trop. Med. Hyg. 31: 1054–1061.

Bowen, M. F., E. E. Davis & D. A. Haggart. 1988. A behavioral and sensory analysis of host-seeking behavior in the diapausing mosquito *Culex pipiens*. J. Insect Physiol. 34: 805–813.

Briegel, H. 1969. Untersuchungen zum aminosauren- und proteinstoffwechsel wahrend der autogenen und anautogenen eireifung von *Culex pipiens*. J. Insect Physiol. 15: 1137–1166.

1980. Determination of uric acid and hematin in a single sample of excreta from blood-fed insects. Experientia 36: 1428.

1986. Protein catabolism and nitrogen partitioning during oogenesis in the mosquito *Aedes aegypti*. J. Insect Physiol. 32: 455–462.

Briegel, H. & A. O. Lea. 1975. Relationship between protein and proteolytic activity in the midgut of mosquitoes. J. Insect Physiol. 21: 1597–1604.

Briegel, H., A. O. Lea & M. J. Klowden. 1979. Hemoglobinometry as a method for measuring bloodmeal sizes of mosquitoes (Diptera: Culicidae). J. Med. Entomol. 15: 235–238.

Clements, A. N. & M. R. Boocock. 1984. Ovarian development in mosquitoes: stages of growth and arrest and follicular resorption. Physiol. Entomol. 9: 1–8.

de Buck, A. & N. H. Swellengrebel. 1934. Behavior of Dutch *Anopheles atroparvus* and *messeae* in winter under artificial conditions. Rev. Malariol. (Rome) 13: 404–416.

Eldridge, B. F. 1968. The effect of temperature and photoperiod on blood-feeding and ovarian development in mosquitoes of the *Culex pipiens* complex. Am. J. Trop. Med. Hyg. 17: 133–140.

1981. Vector maintenance of pathogens in adverse environments (with special reference to mosquito maintenance of arboviruses), pp. 143–157. *In* J. J. McKelvey, B. F. Eldridge & K. Maramorosch [eds.], Vectors of disease agents. Praeger, New York.

1987. Diapause and related phenomena in *Culex* mosquitoes: their relation to arbovirus disease ecology. Curr. Top. Vector Res. 4: 1–28.

Eldridge, B. F. & C. L. Bailey. 1979. Experimental hibernation studies in *Culex pipiens* (Diptera: Culicidae): reactivation of ovarian development and blood-feeding in prehibernating females. J. Med. Entomol. 15: 462–467.

Graf, R. 1986. Mosquito trypsin—purification, in vivo synthesis and physiology in *Aedes aegypti*. Ph.D. dissertation, University of Zurich, Zurich, Switzerland.

Hudson, J. E. 1979. Follicle development, blood feeding, digestion and egg maturation in diapausing mosquitoes, *Culiseta inornata*. Entomol. Exp. Appl. 25: 136–145.

Jaenson, T. G. T. 1987. Overwintering of *Culex* mosquitoes in Sweden and their potential as reservoirs of human pathogens. Med. Vet. Entomol. 1: 151–156.

Kawai, S. 1969. Studies on the follicular development and feeding activity of the females of *Culex tritaeniorhynchus* with special reference to those of autumn. Trop. Med. (Nagasaki) 11: 145–169.

Lea, A. O. 1964. Studies on the dietary and endocrine regulation of autogenous reproduction in *Aedes taeniorhynchus* (Wied.). J. Med. Entomol. 1: 40–44.

Lea, A. O., H. Briegel & H. M. Lea. 1978. Arrest, resorption, or maturation of oocytes in *Aedes aegypti*: dependence on the quantity of blood and the interval between blood meals. Physiol. Entomol. 3: 309–316.

McIver, S. B. 1982. Sensilla of mosquitoes (Diptera: Culicidae). J. Med. Entomol. 19: 489–535.

Meola, R. & J. Readio. 1987. Juvenile hormone regulation of the second biting cycle in *Culex pipiens*. J. Insect Physiol. 33: 751–754.

Mitchell, C. J. 1981. Diapause termination, gonoactivity, and differentiation of host-seeking behavior and blood-feeding behavior in hibernating *Culex tarsalis* (Diptera: Culicidae). J. Med. Entomol. 18: 386–394.

1983. Differentiation of host-seeking behavior from blood-feeding behavior in overwintering *Culex pipiens* (Diptera: Culicidae) and observations on gonotrophic dissociation. J. Med. Entomol. 20: 157–163.

Mitchell, C. J. & H. Briegel. 1989. Inability of diapausing *Culex pipiens* to use blood for producing lipid reserves for overwinter survival. J. Med. Entomol. 26: 318–326.

Spielman, A. & J. Wong. 1973. Studies on autogeny in natural populations of *Culex pipiens*. III. Midsummer preparation for hibernation in autogenous populations. J. Med. Entomol. 10: 319–324.

Swellengrebel, N. H. 1929. La dissociation des fonctions sexuelles et nutritives: (dissociation gono-trophique) d'*Anopheles maculipennis* comme cause du paludisme dans les Pays-Bas et ses rapports avec "l'infectión domiciliaire." Ann. Inst. Pasteur (Paris) 43: 1370–1389.

Washino, R. K. 1977. The physiological ecology of gonotrophic dissociation and related phenomena in mosquitoes. J. Med. Entomol. 13: 381–388.

Wilton, D. P. & G. C. Smith. 1985. Ovarian diapause in three geographic strains of *Culex pipiens* (Diptera: Culicidae). J. Med. Entomol. 22: 524–528.

Received for publication 21 September 1988; accepted 19 October 1988.