Grodhaus, G. 1963. Chironomid midges as a nuisance. II. The nature of the nuisance and remarks on its control. Calif. Vector Views. 10:27–37.

Mulla, M. S. 1974. Chironomids in residential-recreational lakes. An emerging nuisance problem—measures for control. Entomol. Tidskr. 95(Suppl):172–6.

Mulla, M. S. and A. M. Khasawinah. 1969. Laboratory and field evaluation of larvicides against chironomid midges. J. Econ. Entomol. 62:37–41.

Mulla, M. S., H. A. Navvab-Gojrati and H. A. Darwazeh. 1978a. Biological activity and longevity of synthetic pyrethroids against mosquitoes and some nontarget insects. Mosq. News 38:90–6.

Mulla, M. S., H. A. Navvab-Gojrati and H. A. Darwazeh. 1978b. Toxicity of synthetic pyrethroids to 4 species of freshwater fishes Environ. Entomol. 7:428–30.

Pelsue, F. W. and G. C. McFarland. 1971. Laboratory and field studies of a new chironomid species in the Southeast Mosquito Abatement District. Proc. and Pap. Calif. Mosq. Control Assoc. 39:74–9.

THE RELATION OF FEMALE POLYGAMY TO GONOTROPHIC ACTIVITY IN THE *ROCK* STRAIN OF *AEDES AEGYPTI*[1]

ROGER W. WILLIAMS AND AGNES BERGER

Division of Tropical Medicine and Division of Biostatistics, School of Public Health, College of Physicians and Surgeons, Columbia University, New York, N.Y. 10032

ABSTRACT. It is a widely held concept that the pheromone matrone acts to prevent a second insemination during the entire life of fully inseminated females of *Aedes aegypti*. We have demonstrated that females of the ROCK strain, inseminated at 4 days post emergence, which become chronologically old (28 days) but remain physiologically young by being denied blood, cannot be inseminated a second time. In contrast, some fully inseminated females which through the ingestion of human blood progressed through a number of gonotrophic cycles, becoming physiologically older, were inseminated a second time. Within groups of 100, second insemination occurred in 6% following the 4th cycle, 22% after the 5th, 38% after the 6th and 48% after the 7th.

INTRODUCTION

Craig (1967) demonstrated that when male accessory glands were implanted, or extracts of these glands were injected, into virgin females of *Aedes aegypti* (L.) these females remained refractory to insemination for life. The substance responsible is the pheromone matrone. It has become accepted that, once fully inseminated, a female of this species cannot again be inseminated during her lifetime. Gwadz and Craig (1970) noted that incomplete insemination resulted from copulation with partially depleted males or from interruption of copulation and that females involved in such procedures remain receptive to a second insemination.

Williams et al. (1978) found that when females of the ROCK (Rockefeller) strain of *Ae. aegypti* were injected with matrone its activity was associated with the dilution and not related to the amount of that dilution injected, i.e. the results in preventing a second insemination were the same for 1, 2, or 4 ul of a given dilution. It would, therefore, appear that the effect

[1] This project was supported in part by BSRG grant SO7-RR-05449 awarded by the Biomedical Research Support Program, Division of Research Resources, National Institutes of Health.

of naturally induced matrone, through a successful mating, would be greatly influenced by the dilution factor of the components of the seminal material. At the time of an initial insemination only a fraction of the accessory gland substance, diluted with seminal components, is transferred from the male to the bursa of the female.

The definition of "insemination," as used in this discussion, will be the successful deposition of seminal material, including spermatozoa, by a male mosquito into the female bursa. Williams and Hagan (1977) showed that naturally induced matrone prohibited the introduction of sperm and other seminal material into the bursa of the ROCK strain of female *Ae. aegypti* 3 days after initial insemination. These investigators also called attention to the possibility of strain differences within the species *Ae. aegypti* regarding the effectiveness of matrone, since their results with the ROCK strain differed from those of Spielman et al. (1967), and Jones and Makhukar (1976), who worked with different strains.

Does the natural deposition of a diluted fraction of the total amount of matrone, placed into a female bursa at the time of a complete insemination, protect that female for life from a second insemination as does the implantation of whole male accessory glands or the injection of full strength matrone? If not, for how long is this protection evident? Is matrone activity affected by chronological age or related to gonotrophic activity and physiological age? To attempt to answer these questions, experiments were conducted with the ROCK strain of *Ae. aegypti* as described below.

MATERIALS AND METHODS

Ae. aegypti were reared and maintained in an environmental chamber at a temperature of 27° C. at 75–80% RH. Liver powder was used for the larval food. For exposure to males, females were placed in mosquito cages, 25 per cage. These cages were made from 2.785 liter (1 gallon) ice-cream cartons, each with a sleeve and a netting under the lid rim. The proper number (see below) of 4 day post emergent virgin males were transferred to these cages for mating. Following exposure to males, the females were removed and dissected for examination for insemination, or placed by group (see below) into larger metal cages. Canned, washed apple slices, freshly supplied every 2–3 days, were made available to all such mosquitoes. Those females which were to ingest blood were offered a human hand and arm, through the sleeve of the metal cage, every 3–4 days. On each occasion, those females which did not ingest blood when first offered were given a second opportunity later the same day. Any that still refused blood were removed from the experiments, as were those that died during the course of the experiments. An oviposition beaker containing some water and lined with filter paper was placed in each cage following blood ingestion.

Two experiments were conducted (Experiment I and Experiment II), the second as a consequence of the results of the first. Williams et al. (1978) found that at 4 days post emergence just as many virgin females became inseminated by bouncing a cage (to stimulate flight) of 25 females with 100 males for 10 min as when a similar number of females were exposed to 50 males for 24 hr without bouncing. Gwadz and Craig (1968, Table 1) found that a like number of females became inseminated when exposed for the first time at 28 days post emergence to a 2/1 ratio of males for 24 hr as is known to occur among chronologically young females at 4 days post emergence. In Experiment I the bouncing procedure was followed while in Experiment II the latter procedure involving 17 hr of cohabitation with males was used. The 17 hr was selected in preference to 24 hr since it was thought that perhaps, among females which might become inseminated for the second time, the bursae could return nearly enough to the normal deflated appearance in 24 hr to make them difficult

Table 1. (Experiment I). Observed insemination rates in experimental groups* of *Aedes aegypti* (females exposed to a 4:1 ratio of males and bounced for 10 minutes).

		First Exposure to Males			Second Exposure to Males			
			Inseminated Only On This Exposure			Inseminated		
Group*	No. Dissected	Days Post-Emergence	No.	(%)	Days Post-Emergence	No.	(%)	No. of Blood Meals
A	108	28	60	(55.6)	—	—	—	0
B	114	4	100	(87.7)	14	14	(12.3)	0
C	106	4	94	(88.7)	28	12	(11.3)	0
D	109	4	98	(89.9)	14	11	(10.1)	2
E	114	4	105	(92.1)	21	9	(7.9)	5
Aver. of B-E	110.75	—	—	—	—	11.5	(10.38)	—
F	125	4	86	(68.8)	28	39	(31.2)	7
G	103	28	80	(77.7)	—	—	—	7

* See section on Materials and Methods for description of the groups.

to read. Females which ingested blood were exposed for a second time to males 4 days after the last feeding to allow for the completion of the gonotrophic cycle. The day of exposure to males was always counted post emergence. For some mosquitoes the day of exposure was actually 1 day later than stated for the group, i.e. a group described as exposed on day 4 contained some females that were 5 days post emergence. The males utilized were always 4–5 days post emergent virgins. The descriptions of the experimental groups in Experiment I were as follows:

GROUP A. Virgin females exposed to males for the first and only time on day 28 post emergence, were not offered blood and, therefore, deposited no eggs. The selection of 28 days was made since McDonald (1977) found that in his study area some of a natural population of *Ae. aegypti* survived this length of time during the season of his investigation.

GROUP B. Virgin females were exposed to males on days 4 and 14, were not offered blood and, therefore, deposited no eggs.

GROUP C. Virgin females were exposed to males on days 4 and 28, were not offered blood and, therefore, deposited no eggs.

GROUP D. Virgin females were exposed to males on days 4 and 14, ingested blood following the first exposure to males and again on day 9, and as a group deposited 2 batches of eggs.

GROUP E. Virgin females were exposed to males on days 4 and 21, ingested blood 5 times and as a group deposited 5 batches of eggs.

GROUP F. Virgin females were exposed to males on days 4 and 28, ingested blood 7 times and as a group deposited 7 batches of eggs.

GROUP G. Virgin females were exposed to males on day 28 only, ingested blood 7 times and deposited scatterings of infertile eggs.

The sample of females studied in each group was selected at random. If the bursa could not be seen or was not found in a mosquito which had been twice exposed to males (which occurred about 10% of the time, Williams and Hagan, 1977) that mosquito was not counted and another female selected, as a result a bursa was seen from every mosquito counted. The number in each sample was determined by the time available for dissection, although 100 was set as a minimum for each group. In Experiment II the dissections were limited to 100, irrespective of time, and the procedures for groups A, C, F, and G in Experiment I were repeated. Group C was repeated twice and Group F was repeated through

4,5, and 6 gonotrophic cycles with dissections occurring at a somewhat earlier chronological age among those groups given fewer blood meals (F_3,F_2,F_1, Table 2). A group X was included to make sure that the bursae could be properly read after 17 hr of cohabitation with males.

To establish whether insemination had taken place, on a particular exposure to males, females were dissected in saline and the bursa of each examined microscopically for seminal material following the exposure.

In previous experiments female mosquitoes which were inseminated following a second exposure to males in 3 days or less after the first exposure were known to fall into 2 categories; those that were not inseminated on the first exposure (about 5%, Williams and Hagan 1977, Williams et al. 1978) and those that were insufficiently inseminated on the first exposure to prevent a second insemination (About 7%, Gwadz and Craig 1970). All other females could be expected to be fully inseminated (except for rare individuals which might not be inseminated on either exposure to males). Our own results for the total of these 2 categories are given in Table I and discussed below (Results and Discussion). If, following the second exposure to males, some in our groups became inseminated in numbers and percentages significantly greater than that expected in these 2 categories, then matrone had failed to protect these previously fully inseminated females from a second insemination.

RESULTS AND DISCUSSION

The observed insemination rates among females in Experiment 1 are presented in Table 1. In groups A and G, where females were exposed to males for the first and only time at 28 days post emergence (chronologically old), 55.6% and 77.7% became inseminated, but as reported by Williams and Hagan (1977) when the first and only exposure to males was on day 4 (chronologically young) the insemination rate was 109/114 or 95.6%. Since group A was similar to the latter group in every respect, the significantly lower insemination rate in group A ($P<<.005$) must be attributed to the advanced chronological age. Group G differed from A in having ingested blood 7 times and developed and deposited some infertile eggs. This group had a 22% increase in insemination rate over group A, indicating that blood ingestion diminished the effect of chronological age on the proportion inseminated, although there still existed a statistically significant difference between 95.6%, the insemination rate of chronologically young mosquitoes, and the 77.7% rate of the chronologically old, blood fed group G($P<<.005$).

Insemination rates from first exposure to males on day 4 in groups B–E were essentially the same despite the fact that, by the time of dissection, the groups had been screened according to different criteria, as previously described.

Williams and Hagan (1977) found in 2 groups of 100 females exposed to males only at 4 days post emergence that 4.4% and 6.7% were not inseminated and Williams et al. (1978) reported that in a similar group of 300 females 4.9% were not inseminated. The weighted average of non-inseminated females in these 3 groups of 500 mosquitoes is 5.16%. In groups B–E (Table 1) the weighted average per cent inseminated on the second exposure to males was 10.38. Approximately 5.16% of these could be considered to have been inseminated on this occasion because they were not inseminated on the first exposure to males. The insemination of the remainder, 5.22%, may be attributed to interruption of copulation or to copulating with depleted males on the first exposure.

The difference between 55.6% inseminated in group A, on the first exposure to males on day 28, and about 6% in group C (11.3%–5.16% or 11%–5%) on the second exposure on day 28 shows the significant inhibitory effect of matrone over and above the influence of chronological age,

Table 2. (Experiment II). Observed insemination rates in experimental groups* of *Aedes aegypti* (females exposed to a 2:1 ratio of males for 17 hours).

		First Exposure to Males		Second Exposure to Males			
Group*	No. Dissected	Days Post-Emergence	No. & % Inseminated Only On This Exposure	Days Post-Emergence	No. & % Inseminated	No. of Blood Meals	Comments
X	100	4	97	—	—	—	Bursae readable at 17 hours
A	100	28	97	—	—	—	
C	100	4	91	28	6	0	3 not inseminated on either exposure
C	100	4	90	28	10	0	
F_3	100	4	83	19	17	4	17% − 11% = 6% of fully inseminated ♀♀ inseminated a second time
F_2	100	4	67	23	33	5	33% − 11% = 22% of fully inseminated ♀♀ inseminated a second time
F_1	100	4	51	25	49	6	49% − 11% = 38% of fully inseminated ♀♀ inseminated a second time
F	100	4	41	28	59	7	59% − 11% = 48% of fully inseminated ♀♀ inseminated a second time
G	100	28	95	—	—	7	

* See section on Materials and Methods for dsecription of the groups.

in the absence of blood ingestion ($P<<.005$).

While no significant differences were found in the rates of second insemination between groups B–E, group F results differed. In this group 31.2% were inseminated on the 2nd exposure to males, significantly more than in group C ($P<<.005$), suggesting a possible lack of matrone action and/or in the effect of aging, in about 20% (31.2%–10.38% or 31%–11%) of females previously fully inseminated.

It was apparent while bouncing cages of chronologically old females that this stimulus did not encourage flight to the degree that it did among chronologically young mosquitoes. This inaction is apparently a consequence of chronological age, and no doubt accounts for the lower insemination rates at exposure to males at 28 days. To ascertain if we could confirm the results found in group F, of Experiment I, and determine if these results were due to the age factor or to the lack of inhibition of matrone, we conducted Experiment II which eliminated bouncing of cages in favor of 17 hours of cohabitation with a 2:1 ratio of males. The results are presented in Table 2.

The results obtained in group A and G confirm those of Gwatz and Craig (1968) indicating that as many chronologically old females (28 days) will become inseminated, as do young one (4 days), when cohabiting with males for 17–24 hr, which is in marked contrast to the results following the bouncing of cages of chronologically old females for 10 min (Experiment I). Since the age factor was thus eliminated in Experiment II the effect of blood meals on the insemination of aged females at first exposure to males does not become apparent as in Experiment I. In Experiment II the effect of physiological age as a result of successive gonotrophic cycles on second insemination rates becomes much more apparent in the F series. This series indicated that about 6% (17%–11%) of some fully inseminated females became inseminated a second time following the 4th gonotrophic cycle. The per cent inseminated a second time did not become significant until after the 5th cycle when 22% (33%–11%) were receptive ($P<<.005$), followed by 38% (49%–11%) after the 6th, and 48% (59%–11%) after the 7th cycle.

Although these percentages should not be considered as absolute they do indicate a definitive direction in favor of second insemination associated with increased physiological age and confirm the findings of group F in Experiment I. The difference in results between Experiments I and II must be attributed largely to the manner of exposure of the females to males. As in Experiment I, chronologically old mosquitoes, which did not experience successive gonotrophic cycles, group C, remained refractory to a second insemination. We do not know whether sperm from a second insemination, occurring days after the initial complete insemination, in females of the ROCK strain, find their way to the spermathecae to impregnate these females. However, insemination is necessary before impregnation is possible, and the fact that any seminal material was found in the bursae following second insemination attempts was contrary to the findings when these attempts were made 3 days after the first (Williams and Hagan 1977). Thus it has been demonstrated that, under laboratory conditions, matrone is not entirely active in preventing a second insemination during the lifetime of many females of our ROCK strain of *Ae. aegypti* which live long enough to complete 4 or more gonotrophic cycles. The concept that fully inseminated females of this species are all refractory to subsequent insemination during their lifetimes would appear to be incorrect. Further study should be made utilizing strains with genetic markers for the second insemination attempts, or radiotracer methology as described by Young and Downe (1979), to ascertain if fully inseminated females, inseminated a second time, become impregnated with sperm from the second insemination.

If loss of refractiveness to a second in-

semination should be associated only with blood ingestion and not with complete gonotrophic cycles, then it may be possible for some females in nature to become susceptible to a second insemination within 2 weeks of emergence since certain strains are known to ingest blood more than once per gonotrophic cycle (McClelland and Conway 1971, Pant and Yasuni 1973, Do Si Hien 1976). There are, no doubt, natural strains of *Ae. aegypti* which normally complete 6 gonotrophic cycles within a time span of several days less than the 25 utilized in Experiment II. Should the refractiveness to a second insemination be lost in natural populations and should such females become impregnated, then these facts may be of some significance with respect to mosquito control, particularly in genetically altered or sterile male releases. Some females inseminated by such males could become receptive to normal males later in life and might deposit some viable eggs from which normal mosquitoes could develop. Such a phenomenon could explain, in part at least, why some natural populations of *Ae. aegypti,* thought to be ecologically isolated, increased more rapidly than expected after cessation of releases of altered males, as noted by McDonald et al. (1977).

Jones and Wheeler (1965) and Spielman et al. (1967) give a full description of the bursa and its contents following initial insemination in chronologically young females. The bursal wall increases 5 to 10 times in thickness, there are numerous granules from the ejaculate and within 3 to 5 min many large clear globules or spherules appear. Sperm can best be seen just under the thickened wall. Within a day or two the bursa loses most of its volume, the globules have disappeared and the wall becomes thin again. Spielman et al. (1967) also noted that the bursae of females inseminated a second time usually did not develop a thick wall. Spielman et al (1969), while studying the bursae of virgin females, noted that by 40 hr post emergence a clear fluid was present in the lumen and that when the fluid came into contact with packets of coarse granular material from the male accessary glands the packets were transformed into transparent "spherules." This rarely occurred in bursae from non virgin females. We found that in females inseminated when chronologically old and in those inseminated a second time that the bursal morphology and content, as seen within a few minutes following insemination, appeared not unlike the description given for the bursae of young females seen 24 hr after insemination. The walls of such bursae were frequently entirely thin or thickened only in scattered areas. Because globules were usually absent, or relatively few in number, bursae lacked full distention. Since granules were usually not utilized for globular formation they were frequently so numerous as to make sperm detection difficult, although sperm could be seen in some. Globules were more common among females inseminated the first time at 28 days post emergence than among females inseminated a second time and, when seen, appeared to have lost some of their characteristic hyaline appearance. The fact that the bursal wall remained thin, or mostly so, both on the occasions of a second insemination and on first insemination occurring late in life, could be explained by postulating that the thickening of the bursal wall is caused by a substance in the lumen, or in the wall, of the bursa when it combines with seminal material; this substance is usually depleted upon the first insemination, is never fully replenished and deteriorates with chronological age. Likewise, as demonstrated by Spielman et al. (1969), there is, following the first insemination, a depletion of the substance in the female bursa which, when combined with the male accessory gland material, produces globules. This substance, different from that which helps produce a thick bursal wall, seems also not to be replenished and likewise deteriorates with chronological age, although at a slower rate.

The usual number of spermathecae is 3 in *Ae. aegypti.* In dissecting thousands of

females we have seen 4 on a number of occasions and during these experiments we dissected 1 female which had 5, 3 containing sperm.

References Cited

Craig, G. B., Jr. 1967. Mosquitoes. Female monogamy induced by male accessory substance. Science, Wash. 156:1499–1501.

Do Si Hien. 1976. Biology of *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse). IV. The feeding females. Acta Parasit. Polonica 24:27–35.

Gwadz, R. W. and G. B. Craig, Jr. 1968. Sexual receptivity in female *Aedes aegypti*. Mosq. News 28:586–593.

Gwadz, R. W. and G. B. Craig, Jr. 1970. Female polygamy due to inadequate semen transfer in *Aedes aegypti*. Mosq. News 30:355–360.

Jones, J. C. and B. V. Makhukar. 1976. The potency of male accessory gland material in the mosquito (*Aedes aegypti*). Experientia 32:451–453.

Jones, J. C. and R. E. Wheeler. 1965. Studies on spermathecal filling in *Aedes aegypti* (Linnaeus). I. Description. Biol. Bull., Woods Hole 129:134–150.

McClelland, G. A. H. and G. R. Conway. 1971. Frequency of blood feeding in the mosquito *Aedes aegypti*. Nature, Lond. 232:485–486.

McDonald, P. T. 1977. Population characteristics of domestic *Aedes aegypti* (Diptera: Culicidae) in villages on the Kenya coast. I. Adult survivorship and population size. J. Med. Ent. 14:42–48.

McDonald, P. T., W. Hauserman and N. Lorimer. 1977. Sterility introduced by release of genetically altered males to a domestic population of *Aedes aegypti* at the Kenya coast. Am. J. Trop. Med. and Hyg. 26:553–561.

Pant, C. P. and M. Yasuno. 1973. Field studies on the gonotrophic cycle of *Aedes aegypti* in Bangkok, Thailand. J. Med. Ent. 10:219–223.

Spielman, A., Sr. M. G. Leahy and V. Skaff. 1967. Seminal loss in repeatedly mated female *Aedes aegypti*. Biol. Bull., Woods Hole 132:404–412.

Spielman, A., Sr. M. G. Leahy and V. Skaff. 1969. Failure of effective insemination of young female *Aedes aegypti* mosquitoes J. Insect Physiol. 15:1471–1479.

Williams, R. W. and N. K. B. Hagan. 1977. Efficiency of a single insemination in preventing a second in the ROCK strain of the mosquito. *Aedes aegypti*. J. Insect Physiol. 23:1205–1207.

Williams, R. W., N. K. B. Hagan, A. Berger and D. D. Despommier. 1978. An improved assay technique for matrone, a mosquito pheromone, and its application in ultrafiltration experiments. J. Insect Physiol. 24:127–132, and Erratum, Ibid. 24:457.

Young, A. D. M. and A. E. R. Downe. 1979. Quantitative assessment with radiotracers of sperm transfer by male *Aedes aegypti* (Diptera: Culicidae). J. Med. Entomol. 15:259–264.