NOTES ON THE OVERWINTERING OF *CULEX TRITAENIORHYNCHUS* GILES IN JAPAN [1]

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Culex tritaeniorhynchus has been extensively studied in its relation to Japanese encephalitis virus (Mitamura *et al.*, 1938; Petrischeva and Shubladse, 1940; Hammon *et al.*, 1949; Sasa and Sabin, 1950; and others). Taxonomically *C. tritaeniorhynchus* is assigned to the typical subgenus *Culex*, of which all known members overwinter in the adult stage in temperate regions. Sasa (1949) and La Casse and Yamaguti (1950) assumed that *C. tritaeniorhynchus* should conform to the same overwintering pattern and speculated that this species overwinters as adults in small numbers. Precise information on the overwintering habits of this species is of significance in relation to determining whether the virus of Japanese encephalitis overwinters in the arthropod vector as appears to be the case with western equine encephalitis virus in *Culex tarsalis* (Blackmore *et al.*, 1956 and Reeves *et al.*, 1958). Studies on this phase have been pursued by this laboratory for the past eight years, although searches for the overwintering stage of the mosquito have been essentially negative (Anon. 1949–1956).

In the fall of 1957 it was decided to continue the search for overwintering adults primarily because of the observation that this species develops hibernation characteristics when the daylight period becomes less than 12.5 hours and that adult females appear before immature stages and males in the spring. Nakata (1954) observed a single specimen of *C. tritaeniorhynchus* in the laboratory from October 1953 until March 1954. The temperature data presented by Nakata indicate that *C. tritaeniorhynchus* could hibernate under laboratory conditions.

PROCEDURE. Field work commenced with concentration on adult collections from habitats previously unreported as well as from known classical mosquito hibernation habitats. The primary study area was Shinhama in Chiba Perfecture, Honshu, Japan. This area had been previously surveyed for mosquito larvae and adults during the spring and numerous virus isolations had been made during the fall of 1957 from *C. tritaeniorhynchus* by the Department of Virus and Rickettsial Diseases of this organization. Immature stages of *Culex vishnui*, indistinguishable from *C. tritaeniorhynchus* in the adult stage, have not been collected from this area.

Routine dissections of overwintering *C. pipiens* were made to determine the status of hibernation characteristics as have been described by various authors (Swellengrebel, 1929; MacGregor, 1931 and Buxton, 1935). Observations were made on abdominal fat body development, ovarian development, insemination, and evidence of recent engorgement in hibernating mosquitoes to establish criteria for determining whether a *Culex* specimen collected in winter was unequivocally a hibernating adult.

Adult hibernating female mosquitoes were dissected in 0.6 percent saline solution under a dissecting microscope and then the dissected parts were examined under a compound microscope. Sudan IV stain was added to the material on the slide to aid in fat determination. Spermatozoa were readily seen when present by adding slight pressure to the cover slip thus crushing the spermathecae. Subjective categories for fat development were: stage 1 (++++), abdomen with extensive fat body causing distention; stage 2 (++), fat bodies on dissection and abdomen not distended; stage 3 (+), definite fat body development usually found in anterior third of abdomen and traces around sper-

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mathecae; stage 4 (—), few or no fat cells present in abdomen. Subjective categories for ovarian development were: stage 1 (+++), mature eggs; stage 2 (++), yolk (ovum) filling over half the follicle; stage 3 (+), yolk (ovum) filling half the follicle or less; and stage 4 (—), little or no yolk present in follicle. Stages 3 and 4 are comparable to Christopher's (1936) stages 2 and 1 respectively.

RESULTS. The percentages of hibernating characteristics as revealed by dissection of 712 adult *Culex pipiens* collected from October through December 1957 are shown in Table 1. It was observed that all the population of *C. pipiens* did not acquire overwintering characteristics at the same time. Egg rafts of this species were still being collected in October when part of the population had already begun to hibernate. Of approximately 9,000 hibernating *C. pipiens* collected in November through February, none showed evidence of a blood meal. The first spring-collected *C. pipiens* that showed evidence of a blood meal were collected during the second week of March 1958. Because of these data on *C. pipiens* the following combination of characteristics of a winter-collected adult female mosquito is considered to be unequivocal evidence of hibernation and overwintering: (1) presence of fat-body development in the abdomen; (2) lack of ovarian development; (3) presence of spermatozoa in the spermathecae; and (4) lack of recent engorgement, if in the state of true hibernation. Detailed data on hibernation characteristics are presented in the annual professional report of the 406th Medical General Laboratory of 1957 (Anon. 1957).

TABLE 1.—Percentages of hibernation characteristics as revealed by dissections of *Culex pipiens* collected from October through December 1957

Month	Number of specimens	Fat body present			Inseminated	Not engorged	Without ovarian (—) development
		+++	++	+			
October	110	73.6	14.4	03.6	92.7	99.3	99.3
November	333	95.0	00.3	00.7	95.0	99.5	99.5
December	269	96.3	02.6	00.0	98.7	100.0	98.7
Totals	712	91.6	03.4	0.85	96.0	99.8	99.7

During this study, adult *C. tritaeniorhynchus* were collected in the Kanto Plain area every month during winter of 1957–1958, except March. The data on hibernating *C. tritaeniorhynchus* females collected in nature from 18 September through 21 February 1958 are shown in Table 2. Table 2 does not include specimens which were collected in October and were not dissected. All specimens possessed hibernation characteristics when dissected. Although the number of specimens collected was not large, it was established that *C. tritaeniorhynchus* does overwinter as hibernating adults in the Kanto Plain area.

The main hibernating habitats of *C. tritaeniorhynchus* adults were in brush and wood piles of the common pine, *Pinus densiflora* Sieb. et Zucc. Specimens were also collected from bundled bamboo, *Pseudossa japonica* Makino, wood piles composed of *Quercus acuta* Thumb. and *Zelkova serrata* Makino, and a cave. Undoubtedly *C. tritaeniorhynchus* can be collected in other habitats in winter and it is believed that bamboo ground litter should be given particular attention in future surveys. Adults collected from brush piles were often found to be lethargic and did not readily fly when disturbed.

Temperature and humidity data were obtained from a self-recording hygrometer and thermograph apparatus placed 20 inches from the edge and surface of a pine brush pile. The winter temperature ranged from 14° to —1.0° C., but seldom reached 0° C. in winter. It is probable that the centers of large brush piles never freeze.

TABLE 2.—Collection data and results of dissections of hibernating *C. tritaeniorhynchus* females collected in nature from 18 September 1957 through 21 February 1958

	Date	Recent engorgement	Ovarian development	Abdominal fat body	Insemination	Habitat	Location
1	18 Sept. '57	—	—	+	+	pig-baited Magoon trap	Sagiyama
2	18 Sept. '57	—	—	++	+	pig-baited Magoon trap	Sagiyama
3	22 Sept. '57	—	—	+++	+	pig-baited Magoon trap	Shinhama
4	26 Sept. '57	—	—	?	+	pig-baited Magoon trap	Shinhama
5	12 Nov. '57	—	—	+++	+	woodpile *Pinus densiflora*	Kiso near Tokiwa
6	16 Nov. '57	—	—	+++	+	bamboo thicket *Pseudossa japonica*	Shinhama
7	3 Dec. '57	—	—	+++	+	bundled bamboo *Pseudossa japonica*	Shinhama
8	3 Dec. '57	—	—	++	+	brush pile *Pinus densiflora*	Shinhama
9	3 Dec. '57	—	—	++	+	brush pile *Pinus densiflora*	Shinhama
10	18 Dec. '57	—	—	++	+	cut bamboo *Pseudossa japonica*	Shinhama
11	19 Dec. '57	—	—	++	+	woodpile *Zelkova serrata* and *Pinus densiflora*	Onuma near Zama
12	7 Jan. '58	—	—	++	+	brush pile *Pinus densiflora*	Shinhama
13	13 Jan. '58	—	—	+	+	brush pile *Pinus densiflora*	Shinhama
14	14 Jan. '58	—	not checked	not checked	+	brush pile *Pinus densiflora*	Shinhama
15	18 Feb. '58	—	—	+	+	cave	Yokosuka
*16	21 Feb. '58	—	+++	—	+	woodpile *Quercus acuta*	Yokosuka

* This specimen was placed in the insectary on the 21 February 1958. It engorged on rabbit blood on the 26 February and died on 6 March without oviposition. Dissections were performed on the 6 March 1958.

The relative humidity ranged from 76 to 91 percent.

From a total of 11,622 hibernating mosquitoes of all species, collected from all habitats during winter, 4,255 specimens were collected from brush and wood piles.

Russian workers have reported (literature unavailable) that adult females of *C. tritaeniorhynchus* may overwinter in moss, cut grain fields and shrubbery. No specimens have been collected from similar habitats in the Kanto area.

During this study, *Culex vorax* and *Culex rubensis* were collected in winter in Kanagawa Prefecture. This laboratory has no previous record of these two species being collected during winter months.

A few *Culex hayashii* males were collected each month during the winter.

Barr, *vide* Wallis *et al.* (1958), states that after taking a blood meal it seems improbable that *Culex* mosquitoes can undergo the physiological adaptation process (gonadotrophic-dissociation) which is necessary to convert egg production to hibernation. Pilot studies on hibernation characteristics conducted at this laboratory show that it is physiologically possible for colonized *C. tritaeniorhynchus* to develop hibernation characteristics with or without a blood meal when subjected to less than optimal light period.

C. tritaeniorhynchus can be made to develop characteristics, such as fat body development, on a diet of sugar water when subjected to less than an optimum light schedule. If this occurs in nature, it would be unlikely that this species would be an overwintering host for virus, unless transovarian transmission is involved. Perhaps there is not a true gonotrophic dissociation in *C. tritaeniorhynchus* as stated by Newson and Blakeslee (1957) but just the lack of a blood meal stimulus for ovarian development. It may be possible to determine whether a hibernating *Culex* mosquito takes a blood meal before hibernating by determining nitrogen levels in experimentally fed mosquitoes over different time intervals and in mosquitoes collected in nature throughout the year, and particularly in the winter.

Other observations on hibernating characteristics were that ovaries of laboratory reared *C. tritaeniorhynchus*, fed only on sugar water, occasionally develop to the stage in which the ovum occupies half of the follicle. When a group of *C. tritaeniorhynchus* females are given a single blood meal, fresh apples, and subjected to less than optimal light period, part of the group will develop overwintering characteristics such as gonadotrophic dissociation. This was accomplished experimentally by placing caged and recently engorged mosquitoes in a location where they did not receive the benefit of a light control box that extended the day light period to 15 hours.

SUMMARY. During the winter of 1957–1958, it was determined that *C. tritaeniorhynchus* overwinters as adults in Japan. The main habitats in which specimens were collected were brush and wood piles of the common pine, *Pinus densiflora*. Specimens have also been collected from wood piles of *Zelkova serrata* and *Quercus acuta*, bundled bamboo, and from a cave. Data from pilot laboratory studies indicate that there is a possibility that *C. tritaeniorhynchus* does not require a blood meal to overwinter. If this be the case in nature, it would be unlikely that this species would be an overwintering host for virus unless transovarian transmission is involved.

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References

(ANON.) 1949–1956. Professional reports, 406th Medical General Laboratory, APO 343, San Francisco, California.

(ANON.) 1957. Professional report, 406th Medical General Laboratory, APO 343, San Francisco, California.

BELLAMY, R. E., REEVES, W. C., and SCRIVANI, R. P. 1958. Relationship of mosquito vectors to winter survival of encephalitis virus. II. Under experimental conditions. Amer. J. Hyg. 67:90–100.

BLACKMORE, J. S., and WINN, J. F. 1956. A winter isolation of western equine encephalitis virus from hibernating *Culex tarsalis* Coquillett. Soc. Expt. Biol. and Med. Proc. 91:146–148.

BULLOCK, H. R. 1957. Bionomics of *Culex tritaeniorhynchus* Giles. Field Studies. The Armed Services Research and Development Report on Insect and Rodent Control. October-December. Number XXXIX pp. 11–12.

BULLOCK, H. R., MURDOCH, W. P., *et al.* 1958. Overwintering of *Culex tritaeniorhynchus* Giles Kanto Plain. Proceedings of the 10th Annual Meeting of Japan Society of Sanitary Zoology. p. 19.

BUXTON, P. A. 1935. Composition during hibernation, *Culex* (Diptera). Parasit. 27:263–265.

CHRISTOPHER, S. R., STINTON, J. A., and COVELL, G. 1936. How to do a malaria survey. Hlth. Bull. Malar. Inst. India, No. 14 (4th ed.), iv and 147.

HAMMON, W. McD., REES, D. M., COCALA, J., and MEIKLEJOHN, GORDON. 1949. Experimental transmissions of Japanese B encephalitis virus by *Culex tritaeniorhynchus* and *Culex pipiens* var. *pallens*, suspected natural vectors. Amer. J. Hyg. 50:46–50.

HAMMON, W. McD., TIGERTT, W. D., SATHER, G., and SCHENKER, H. 1949. Isolation of Japanese B encephalitis virus from naturally infected *Culex tritaeniorhynchus* collected in Japan. Amer. J. Hyg. 50:51–56.

HURLBUT, H. S. 1950. Transmission of Japanese B encephalitis by mosquitoes after experimental hibernation. Amer. J. Hyg. 51:265–268.

LACASSE, W. J., and YAMAGUTI, S. 1950. Mosquito fauna of Japan and Korea. U.S. Army, Office of the Surgeon, Hq., 8th Army, APO 343, pp. viii + 268 + 2 appendices of 7 and 268 pp.

MACGREGOR, M. E. 1931. The nutrition of adult mosquitoes. Trans. Roy. Soc. Trop. Med. and Hyg. 24:465.

MITAMURA, T., KITAOKA, M., MORI, K., and OKUBO, K. 1938. Isolation of the virus of Japanese epidemic encephalitis from mosquitoes caught in nature. Reports to the Ninth Meeting of the Committee on Encephalitis. Tokyo Iji Shishi 62:820–824.

NAKATA, GOICHI. 1954. Observations on wintering habits of mosquitoes about Kyoto, Japan. Jap. J. of Sanit. Zool. 5:60–61.

NEWSON, H. D. and BLAKESLEE, T. E. 1956. A study of *Culex tritaeniorhynchus* oviposition activity during 1955. Mosq. News 16:224–228.

NEWSON, H. D., and BLAKESLEE, T. E. 1957. Observations of a laboratory colony of the mosquito *Culex tritaeniorhynchus* Giles. Mosq. News 17: 308–311.

PETRISCHEVA, P. A., and SHUBLADSE, A. K. 1940. The vectors of autumn encephalitis in the maritime district. Arkhiv. Biol. Nauk. 59:72–77.

REEVES, W. C., BELLAMY, R. E. and SCRIVANI, R. P. 1958. Relationships of mosquito vectors to winter survival of encephalitis viruses. I. Under natural conditions. Amer. J. Hyg. 67:78–89.

SASA, MANABU. 1949. Zoophilism, hibernation and appearance of mosquitoes of Japan. Jap. Med. J. 2:99–107.

SASA, MANABU, and SABIN, ALBERT B. 1950. Ecological studies on the mosquitoes of Okayama in relation to the epidemiology of Japanese B encephalitis. Amer. J. Hyg. 50:21–35.

SWELLENGREBEL, N. H. 1929. Sexual and nutritional factors, *Anopheles* (Diptera). Ann. Inst. Pasteur. 43:1370–1380.

WALLIS, R. C., TAYLOR, R. M., MCCOLLUM, R. W., and RIORDAN, J. T. 1958. Study of hibernating mosquitoes in eastern equine encephalomyelitis epidemic areas in Connecticut. Mosq. News 18:1–4.