ANOPHELES CULICIFACIES GILES: SOME RELATIONSHIPS AMONG OVIPOSITION, REFEEDING AND SURVIVORSHIP

WILLIAM K. REISEN AND FARIDA MAHMOOD

Pakistan Medical Research Center, University of Maryland School of Medicine, 6, Birdwood Road, Lahore, Pakistan.

ABSTRACT. A series of laboratory experiments was performed to help explain why few unfed, parous *An. culicifacies* females are routinely collected in nature. *An. culicifacies* was observed to have a pregravid phase requiring a 2nd blood meal to initiate oögenesis beyond the resting ovarian Stage IIb, with about half of a newly emerged cohort taking their initial blood meal on the 1st and 2nd nights after emergence. Oviposition activity in the insectary was essentially bimodal with peaks occurring after dusk and during dawn. Females refed the night of 1st oviposition, although those not offered a blood meal survived until the following evening without sugar or water. These data were used to calculate the proportion of unfed females expected to be collected in afternoon resting samples during summer. The observed proportion was considerably less than the expected proportion using the calculation procedures of Colless (1958) and Krafsur (1977).

The incidence of rural malaria in Punjab Province, Pakistan, is classically seasonal, with peak transmission occurring during the monsoon season, August through mid-October, and a secondary increase occasionally occurring during late spring, April to mid-May (Russell et al. 1963). The bimodality of malaria transmission presumably reflects the well-documented bimodal seasonal abundance patterns of the primary vector species, *Anopheles culicifacies* Giles (e.g. Afridi and Puri 1940, Pal 1945, Ansari and Nasir 1955, Rafi 1955). However, vector survivorship as well as abundance is important in malaria transmission. Horizontal studies on longevity have been conducted in nature (Afridi et al. 1940) and large outdoor insectaries (Russell and Rao 1942); however, vertical age-grading studies on wild populations to estimate daily survivorship have not been conducted in Pakistan.

Vertical methods of estimating survivorship in nature are dependent upon the morphological changes which occur in the reproductive system of the female during oögenesis and oviposition (Detinova 1962). One of the simpler methods of determining oviparity relies on the degree of coiling of the ovarian tracheoles; however, only the unfed or freshly fed portion of the population whose ovaries are at Mer's (1936) Stages I or early II may be scored because yolk deposition during oögenesis occludes microscopical examination and progressively uncoils the skeins of the tracheoles.

In conjunction with a series of routine collections to describe temporal population dynamics of *An. culicifacies* at several Punjabi villages near Lahore (Reisen 1978), subsamples of females taken during afternoon indoor resting collections were dissected according to the procedures outlined in Wld. Hlth. Org. (1975) to determine insemination, ovarian condition and oviparity using the tracheolation method. Most of the females dissected were half-gravid or gravid, precluding observation of the tracheolar skeins; and those few females which were unfed were mostly nulliparous. The small numbers of unfed, parous females collected prompted subsequent laboratory experimentation on the time in life of blood feeding and its relation to the initiation of ovarian development, the time of oviposition during the diel, the degree of refeeding on the night of oviposition and the relationship between refeeding and female survival. The relationships of these findings to *An. culicifacies* bionomics and survivorship in

nature are discussed and the possibility of outdoor resting considered.

EXPERIMENTAL METHODS AND RESULTS

INITIATION OF OVARIAN DEVELOPMENT. To determine the time when the 1st blood meal was taken under "midsummer" insectary conditions (LL:DD=16.8 with 1.5 hr simulated crepuscular periods, temperature = 28°C to 30°C, relative humidity = 70 to 80%), about 250 mixed male and female pupae were allowed to emerge into a 1-gal (3.8 liter) carton cage containing a tethered laboratory mouse as a blood meal source, and a 3% sucrose solution available in vials with sponge wicks. The night of emergence (night 0), 127 females and 110 males emerged, but only 1 female took a partial blood meal, based on the external appearance of the abdomen (Fig. 1). Possibly the newly-emerged females may have engorged on the sugar solution which may be less readily available in nature. Therefore, the procedure was repeated, except that the sugar bottle was removed. Here, 116 females and 112 males emerged on night 0, and similarly only 4 females took a partial blood meal. Thus the presence of a sugar source did not seem to make an appreciable difference in the degree of blood feeding at emergence, since these results were not significantly different using a 2 × 2 contingency χ^2 test ($P > 0.05$).

The non blood-fed (unfed) females in the sugar group were placed back in the cage with the males and offered a blood meal again on night 1 (Fig. 1). During night 1, 53 (44.2%) of the 120 surviving females took a full blood meal, 49 (40.8%) took a partial blood meal, and 18 (15.0%) appeared to be unfed based on the degree of abdominal distention. Ten of the fully fed females were dissected at 1500 hrs, and were found to have their eggs

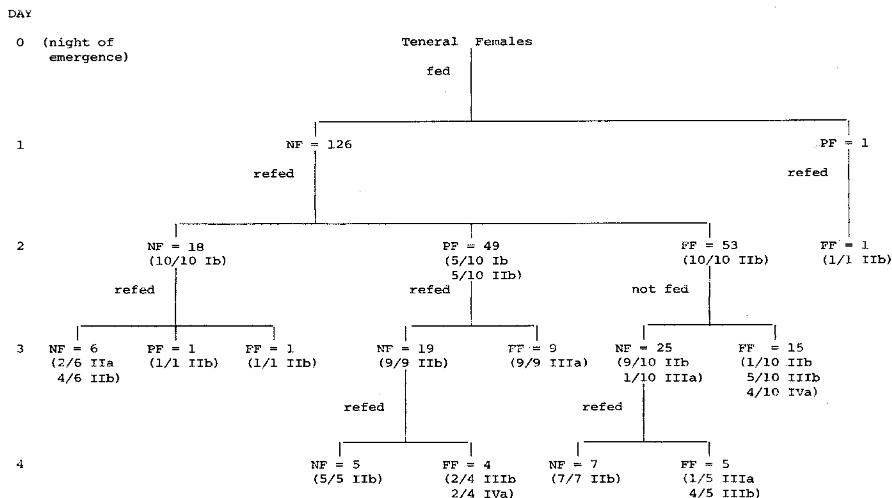

Fig. 1. The physiological effects of blood feeding on ovarian maturation. NF = not fed, PF = partially fed, FF = fully fed; Ib to IVa refer to the ovarian classification of Mer (1936) which is based on the quantity of yolk deposition in the developing follicles.

matured to ovarian stage IIb, the anopheline resting stage using Mer's (1936) modification of Christophers' (1911) classification scheme as summarized in Clements (1963). The remaining fully-fed females were held in the insectary in a separate cage until the following afternoon and were not offered an additional blood meal. At this time, 25 females appeared unfed with no remaining blood and 15 appeared half-gravid. Of 10 females dissected in each group, 9 of the females appearing unfed were at Stage IIb and 1 was at IIIa, while 5 of the half-gravid females were at Stage IIIb and 4 were at IVa. Thus, about 40% of the females taking a full blood meal on night 1 (estimated 21.2 of 53) successfully matured their eggs past the resting stage IIb without refeeding. The remaining unfed females were offered another blood meal; 5 fed fully and 7 did not feed (Fig. 1). The 5 full-fed females developed their ovaries to Stage III (IIIa = 1; IIIb = 4), while the 7 which did not re-feed remained at Stage IIb.

Of the 49 females which took a partial meal during night 1 and were offered an additional blood meal on night 2, 9 re-fed and appeared fully-fed (Fig 1). All of the fully-fed females were at ovarian State IIIa, while the unfed females remained at Stage IIb. Ten of the remaining unfed females were again offered a blood meal. Those feeding developed their ovaries to Stage III, while those appearing unfed remained at Stage IIb. Females not feeding on night 1 or dissected on day 2, were offered another opportunity to feed (Fig. 1). On day 3, 6 appeared unfed, 1 partially fed and 1 fully fed; none had developed their ovaries past Stage IIb. Clearly, for most nulliparous females (82.0% in the present cohort, 98.8/120.0), a 2nd blood meal was required to stimulate the development of the ovaries past Stage IIb, thus verifying the existence of a pregravid phase similar to that described for *An. gambiae* by Gillies and Wilkes (1965). Ovarian maturation beyond Stage IIb was independent of mating as only 6 of the 72 females whose spermathecae were examined were found to be inseminated (only 2 of 11 with ovaries matured beyond Stage IIb).

PROPORTION OF FEMALES UNFED. To quantitate the proportion of unfed females present on any given day starting at emergence, about 350 pupae were allowed to emerge into a 1 gal cage containing a tethered mouse and a 3% sugar solution in vials with sponge wicks. Each day, starting the morning after emergence, all females were removed, lightly etherized, and their abdominal appearance examined under a dissecting microscope and scored as unfed, partially fed, half gravid or gravid. Sugar solution was continually present and a tethered mouse was added to the cage each night. An oviposition cup was added to the cage the night after the 1st gravid females were observed and was continuously present thereafter.

The proportion of unfed females present in the test cohort decreased rapidly from 98.2% on the morning after the night of emergence (day 0) to 20.3% by day 2 to 6.1% by day 6 (Fig. 2). These data provided a significant ($P < 0.01$) fit for a negatively curvilinear regression equation of the form $\ln y = 4.613 - 1.192 (\ln x)$, where y = the percent of the population appearing unfed, and x = the time in days after emergence. On days 1 and 2, 42.0 and 20.3% of the females appeared partially fed, respectively, decreasing to less than 5% for the rest of the study (Fig. 2). As indicated in the previous section, many nulliparous *An. culicifacies* females first take a partial blood meal to develop their ovaries to resting Stage IIb and then imbibe a replete blood meal to initiate oögenesis.

TIME OF OVIPOSITION. About 150 pairs of the Sattoki strain of *An. culicifacies* were held together for 2 nights after emergence to allow mating and then were offered a mouse as a blood source for 2 nights. One hundred fed or gravid females were then placed in a 1-gal cage where they were offered a 6-cm diameter plastic cup filled with well-water and lined with filter paper as an oviposition sub-

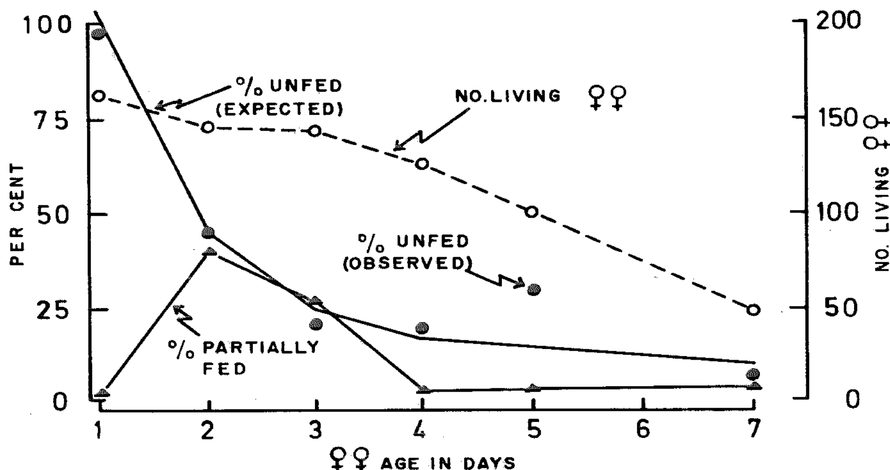

Fig. 2. The number of females alive and the proportion of them which appeared unfed (y) and partially fed on each day plotted as a function of female age in days after emergence (x). The predicted proportion unfed was calculated for each day from the regression equation, $\ln y = 4.613 - 1.192 (\ln x)$.

strate for the next 2 successive nights. Each hour from 1900 to 0800 the cup was replaced and labelled as to time. The following morning, the eggs in each cup were counted and considered an index of female oviposition activity.

In the insectary, the rhythm of *An. culicifacies* oviposition activity was essentially bimodal, with increases after dusk and during dawn, and decreases occurring during early morning (0200 hrs) and with the onset of photophase (Fig. 3). No eggs were deposited during photophase, even though the oviposition cup was continuously present. A total of 1865 and 3817 eggs were laid on nights 3 and 4, respectively. Thus, based on the mean fecundity of isolated females of the Sattoki strain ($n = 25$, $\times \bar{x}$ S.E. $= 120 \pm 7$ eggs/female) about 16 and 32 females oviposited on each night, respectively.

EFFECT OF REFEEDING ON SURVIVAL. Female *An. culicifacies* were held with males for 2 nights for mating and then offered a tethered laboratory mouse for 1 night as a blood meal source. The following morning 4 groups of 100 freshly-fed females were isolated into 1-gal carton cages and held for 2 nights. Sugar solution (3% sucrose) on sponge wicks was available continuously. On the evening of the 3rd night, all dead females were removed and counted, the sugar bottle removed, and all 4 groups offered oviposition cups. A tethered mouse was offered to groups A and C, and a cotton pad soaked in water was placed on the gauze tops of the cages of groups A and B. The following morning, the oviposition cups were removed, the number of eggs counted, the number of females dying recorded, and all females scored by abdominal condition. Questionable females were dissected and scored by ovarian condition. In addition, 25 freshly-fed females in group A and 25 unfed females in group B were held in the insectary without food or water until 1700 and the numbers dying recorded.

Seventy-one of 72 and 53 of 55 females in groups A and C which survived the night refed (Table 1). Of these, 20 and 21

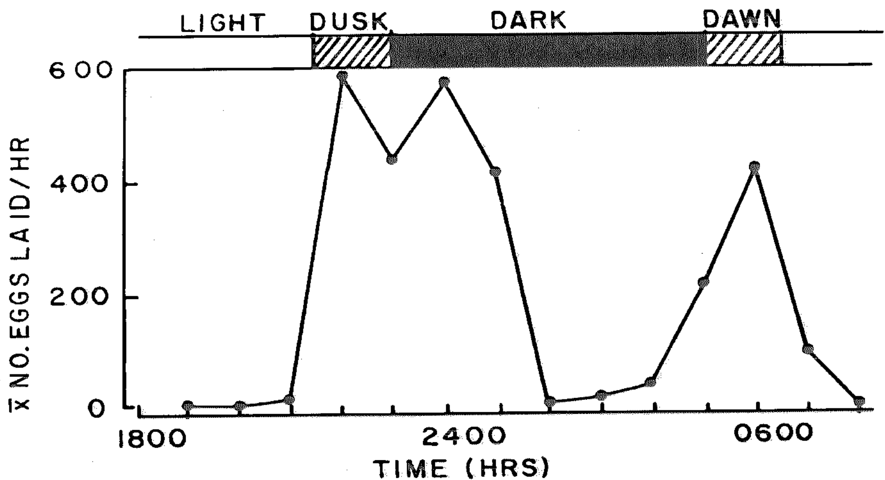

Fig. 3. The mean number of eggs oviposited during each 1 hour period plotted at the beginning of each period.

refed without ovipositing and appeared half-gravid (HG). The mortality in blood-fed (A and C) and non blood-fed (B and D) groups was statistically comparable ($P > 0.05$), although significantly more females died in groups C and D which did not have a water pad ($P < 0.005$). Of the 25 females held without water until 1700 hrs, 8 and 6 from groups A and B died. These results suggested that *An. culicifacies* females readily re-feed the same night that they oviposit; however, immediate re-feeding did not significantly increase survival in the insectary until the following afternoon ($P > 0.05$), the time of our routine indoor resting collections. Presumably similar numbers could also survive the additional 3 hrs until sunset when the unfed females would have had a chance to re-feed.

RELATION TO BIONOMICS AND SURVIVORSHIP

In the insectary, most *An. culicifacies* adults emerged during scotophase and mated on their second or third night of life (Reisen et al. 1979). Blood feeding was independent of mating and about 50% of the unfed females fed on their second night of life. These results agreed well with field observations where virgin females were collected at bovid baits (16% of 574♀♀ dissected in 1976–1977) and fed females were collected pairing at male swarms (Reisen and Aslamkhan 1976; 71.8% of 44♀♀). In the present study, more than 80% of the nulliparous *An. culicifacies* females studied did not develop their follicles past Mer's (1936) Stage IIb after their initial blood meal, but rather required a replete second meal to initiate oögenesis.

Although the oviposition rhythm was bimodal with a primary early evening peak and a secondary morning peak, practically all ovipositing females re-fed before photophase in the insectary. The bimodality of the oviposition cycle was unexpected, since in nature most females feed after midnight (Ludlam 1971, Reisen et al. 1976a, Reisen and Aslamkhan 1978) with little increase in biting activity during dawn. Since few unfed parous females were collected in diurnal indoor resting collections during summer (1 of

Table 1. Effect of refeeding the same night on the survivorship of uniparous *An. culicifacies* females.

	Abdominal condition[1]	Water Pad: Blood source	Water Pad: No blood source	No Water Pad: Blood source	No Water Pad: No blood source
Group:		A	B	C	D
No. ♀♀ at start:		77	72	64	63
Dead:	NF	0	3	2	6
	FF	2	0	5	0
	G	3	3	2	4
	Total:	5	6	9	10
Alive:	NF	0	55	2	34
	FF	51	0	32	0
	HG	20	0	21	0
	G	1	11	0	19
	Total:	72	66	55	53
Proportion Alive(%)[2]:		97.5[a]	91.7[a]	85.9[b]	84.1[b]
No. eggs laid:		1859	3723	2425	3302

[1] NF=Not fed, Stage I to IIa, FF=fresh fed Stage IIIa, HG=half gravid, fresh blood but ovaries at Stage V, G=gravid Stage V.

[2] Proportions followed by the same letter were not significantly different by Chi Square analyses ($P>0.05$).

887 ♀♀ dissected = 0.1%), dawn ovipositing females in nature must also re-feed just prior to, or during the dawn ingress into the indoor resting sites (Reisen et al. 1976a); or, alternatively, the amplitude of the dawn oviposition peak may be somewhat reduced in nature. Under insectary conditions, unfed, parous females readily survived until late afternoon and thus would have been available for afternoon resting collections, provided they did not take a blood meal on the night of oviposition. These data implied that in nature, as well as in the insectary, practically all females re-feed the same night that they oviposit.

During summer the gonotrophic cycle required somewhat less than 2 days (Reisen et al. 1976b); that is, once the ovaries attained the resting Stage IIb, females matured their eggs and oviposited on the 2nd night after a replete blood meal. Thus, assuming that ½ of the females fed on the first and second night after emergence and refed again on the following night, the proportion of the population appearing unfed could be estimated by Colless's (1958) formula, $u = 1 - p^n$, where u = the proportion unfed, p = daily survivorship, and n = the time from emergence to first blood meal, here 1.5 days $\{(0.5 \times 1) + (0.5 \times 2) = 1.5$ days$\}$. In the present study, p was estimated from the ratio of the number of Stage V, gravid, to blood-fed, Stages III and IV, females since the length of the gonotrophic cycle was 2 days and all females are presumed to rest indoors (Reisen 1978); thus $p = 394/458 = 0.86$. Our estimate of p was slightly higher than that of Russell and Rao (1942) using release-recapture methods (when recalculated from their original data using least squares, $p = 0.707$), but was much higher than p estimated using the tracheolation method to calculate the proportion parous to be used in the formula of Davidson (1954); where $p = m^{i/g}$ and g = the length of the gonotrophic cycle and m = the proportion of oviparous females; in the present study, g = 2 days, m = 1 parous ♀♀ of 35 unfed ♀♀ dissected = 0.028, and $p = 0.169$. Thus, applying Colless's formula with $p = 0.86$ and $n = 1.5$, $u = 0.202$, considerably higher than the observed value of $u = 35/887 = 0.039$. Alternatively, using the calculation methods of Krafsur (1977), u was recalculated as u =

$\frac{1}{2}(p^1 + p^2)/x$, with $x = \frac{1}{2}(p^1 + p^2) + (p^3 + p^4) + \ldots + (p^{t-1} + p^t)$, $p = 0.86$, and t = female life expectancy, here considered to be about 12 days (Reisen and Mahmood unpubl.); $u = 0.173$, again considerably higher than the observed value of 0.039. Similar discrepancies between expected and observed proportions of unfed *An. gambiae* females have been attributed to exophillic resting by the pre-gravid females (Krafsur 1977). Exophilly has been described for *An. culicifacies* females in Gujarat, India, using a pit-shelter technique (Shalaby 1971); however, in Pakistan males or females have rarely been encountered in routine outdoor resting collections using battery-powered sweepers (indoor: 14,524 ♀♀ – 4,897 ♂♂ in 547 man hours of collection; outdoor: 1 ♀♀ – 2 ♂♂ in 434 man hours; Reisen 1979). Alternative explanations for the discrepancies between the observed and expected proportions of pregravid females include a shorter duration of the pre-gravid phase, the use of a spuriously high estimate of daily survivorship, p, and/or changes in female survivorship with age.

The present results did indicate the need for more intensive investigations using vertical and horizontal life table methods to estimate survivorship in both the field and the laboratory. We did conclude from the present study that the ovarian tracheolation method was not suitable for age-grading *An. culicifacies* populations during the summer, since the estimates would be essentially restricted to the pregravid portion of the population, thus yielding gross underestimates of daily survivorship.

ACKNOWLEDGMENTS

We thank the members of the ecology department for their technical assistance. Dr. R. K. Sakai made helpful comments and suggestions and provided adults of the Sattoki strain for experimentation. This research was funded by Grant No. AI-10049 from the U.S. National Institute of Allergy and Infectious Disease, N.I.H. The administrative assistance of the U.S. Agency for International Development in Pakistan is acknowledged.

Literature Cited

Afridi, M. K. and I. M. Puri. 1940. Studies of the behaviour of *Anopheles culicifacies*. Part I. Review of the literature. J. Mal. Inst. India 3:1–22.

Afridi, M. K., A. Majid and I. A. Shah. 1940. Studies on the behavior of *Anopheles culicifacies*. Part II. J. Mal. Inst. India 3:23–51.

Ansari, M. A. R. and A. S. Nasir. 1955. A preliminary note on anophelism of Lahore suburbs. Pak. J. Hlth. 4:212–223.

Christophers, S. R. 1911. The development of the egg follicle in anophelines. Paludism 2:73–89.

Clements, A. N. 1963. *The physiology of mosquitoes*. Pergamon Press, New York. 393 p.

Colless, D. H. 1958. Relationship between certain biological constants and trophic condition in mosquito populations. Nature (London) 182:951–952.

Davidson, G. 1954. Estimation of the survival-rate of anopheline mosquitoes in nature. Nature (London) 174:792–793.

Detinova, T. S. 1962. *Age-grouping methods in Diptera of medical importance*. Wld. Hlth. Org., Geneva, 210 pp.

Gillies, M. T. and T. J. Wilkes. 1965. A study of the age-composition of populations of *Anopheles gambiae* Giles and *A. funestus* Giles in north-eastern Tanzania. Bull. Entomol. Res. 56:237–262.

Krafsur, E. S. 1977. The bionomics and relative abundance of *Anopheles* species with respect to the transmission of *Plasmodium* of man in Western Ethiopia. J. Med. Entomol. 14:180–194.

Ludlam, K. W. 1971. Feeding behavior of six common anophelines of West Pakistan. Doctoral Diss., Univ. Md., College Park. 67 pp.

Mer, G. G. 1936. Experimental study on the development of the ovary in *Anopheles elutus* Edw. (Diptera, Culicidae). Bull. Entomol. Res. 27:351–359.

Pal, R. 1945. On the bionomics of *Anopheles culicifacies* Giles, Part III. The behaviour of the adults. J. Mal. Inst. India. 6:217–238.

Rafi, S. M. 1955. Resting places inside homes of *A. culicifacies* and *A. stephensi*, malaria vectors of the Punjab. Pak. J. Hlth. 5:146–154.

Reisen, W. K. 1978. A quantitative mosquito survey of 7 villages in Punjab Province, Pakistan with notes on bionomics, sampling methodology and the effects of insecticides. S.E. Asia J. Trop. Med. Publ. Hlth. 9: (In press).

Reisen, W. K. and M. Aslamkhan. 1976. Observations on the swarming and mating behavior of *Anopheles culicifacies* Giles in nature. Bull. Wld. Hlth. Org. 54:155–158.

Reisen, W. K. and M. Aslamkhan. 1978. Biting rhythms of some Pakistan mosquitoes. Bull. Entomol. Res. 68:313–330.

Reisen, W. K., Y. Aslam and T. F. Siddiqui. 1977. Observations on the swarming and mating of some Pakistan mosquitoes in nature. Ann. Entomol. Soc. Amer. 70:988–995.

Reisen, W. K., M. Aslamkhan and Z. A. Naqvi. 1976b. Observations on the resting habits and diel changes in the ovarian condition of some Punjab mosquitoes (Diptera: culicidae). Biologia 22:79–88.

Reisen, W. K., M. Aslamkhan, M. Suleman and Z. A. Naqvi. 1976a. Observations on the diel activity patterns of some Punjab mosquitoes (Diptera: Culicidae). Biologia 22:68–77.

Reisen, W. K., F. Mahmood and T. Parveen. 1979. Laboratory observations on the time of mating of *Anopheles culicifacies* Giles. Mosquito News, 39:328–333.

Russell, P. F. and T. R. Rao. 1942. Observations on longevity of *Anopheles culicifacies* imagines. Amer. J. Trop. Med. 22:517–533.

Russell, P. F., L. S. West, R. D. Manwell and G. Macdonald. 1963. *Practical Malariology*. Oxford University Press, London. 750 pgs.

Shalaby, A. M. 1971. Sampling of outdoor resting populations of *Anopheles culicifacies* and *Anopheles fluviatilis* in Gujarat State, India. Mosquito News 31:69–73.

Wld. Hlth. Org. 1975. *Manual of practical entomology in malaria. Part II. Methods and techniques.* Geneva. 191 pgs.

DIFLUBENZURON INHIBITS CHITIN SYNTHESIS IN *CULEX PIPIENS* L. LARVAE

NICOLAS P. HAJJAR[1]

Pesticide Chemistry and Toxicology Laboratory, Department of Entomological Sciences, University of California, Berkeley, California 94720

ABSTRACT. Diflubenzuron (DFB) inhibits growth and development of *Culex pipiens* L. larvae at 4 ppb. With 42 hr exposure, a period allowing two molts, it causes a dose-dependent reduction in body weight and chitin content, such that at 9 ppb these are only 66 and 27% of control respectively. There is also a dose-dependent increase in the instar duration and mortality. Following [^{14}C]glucose feeding, DFB has no effect on the transport of [^{14}C] glucose and its [^{14}C] metabolites into the integument of fourth instar larvae, but causes a dose-dependent decrease in their incorporation into the newly-formed chitin.

INTRODUCTION

Diflubenzuron (DFB), 1-(2, 6-difluorobenzoyl)-3-(4-chlorophenyl) urea, is a potent insecticide that acts on a wide variety of insects species (Verloop and Ferrell, 1977). It is, however, particularly effective against a number of medically important mosquito species at very low doses under field (0.025–0.05 lb/acre) and laboratory (0.4 ppb) conditions (Arias and Mulla 1975, Mulla and Darwazeh 1976). Recently we reported on the *in vitro* inhibition of chitin synthesis by DFB in *Oncopeltus fasciatus* abdomen systems and demonstrated that inhibition proceeds very rapidly without hormonal involvement. DFB blocks the terminal polymerization step in chitin formation (Hajjar and Casida 1978, 1979). The present paper investigates the *in vivo* inhibition of chitin synthesis by DFB in *Culex pipiens* L. larvae.

MATERIALS AND METHODS

Studies were conducted on larvae from an autogenous strain reared in this lab-

[1] Present address: Toxicology Laboratory, Department of Entomology, Box 5215, North Carolina State University, Raleigh, North Carolina 27650.