Metastatic carcinoma was often seen in the lungs, portahepatic lymph nodes, and omentum and peritoneum; however, metastases were also found in such organs as brain, ovary, kidney, thymus, spleen, and parathymic and parapancreatic lymph nodes.

Our conclusion is that methapyrilene hydrochloride is strongly carcinogenic for the liver of Fischer 344 rats of both sexes, inducing almost a 100 percent incidence of liver neoplasms after 43 to 64 weeks of treatment, many with metastases. Sodium nitrite in this experiment did not appear to affect the incidence or type of methapyrilene-induced tumors. The total dose of methapyrilene hydrochloride received by the male rats was between 9 and 13.5 g (22 to 34 g per kilogram of body weight), and by the females, 7 to 9 g (30 to 40 g per kilogram of body weight). The dose of methapyrilene hydrochloride recommended for humans in several over-the-counter sleep aids was 50 mg. Several million people may have taken this compound, some for several years. After we presented preliminary reports of our findings, the manufacturers withdrew methapyrilene from the market. Some compounds of similar structure are in use.

Methapyrilene is a potent liver carcinogen in rats, yet its chemical structure does not resemble that of any known class of carcinogen. It is possible, therefore, that it represents a new type of carcinogen. It is also notable that in the *Salmonella* mutagenesis test devised by Ames (*4*), methapyrilene has not been mutagenic when activated by rat liver microsomal fractions. Neither has methapyrilene transformed hamster embryo cells in culture when activated by liver microsomes in the test described by Pienta *et al.* (*5*).

W. Lijinsky, M. D. Reuber
B.-N. Blackwell

Chemical Carcinogenesis Program, Frederick Cancer Research Center, Frederick, Maryland 21701

References and Notes

1. W. Lijinsky and H. W. Taylor, *Food Cosmet. Toxicol.* **15**, 269 (1977).
2. The methapyrilene hydrochloride (Sigma) was more than 99 percent pure by chromatography. The compound was mixed with powdered Wayne Sterilizable Lab Meal in a blender and uniform admixture was assured by chemical analysis of aliquots. The sodium nitrite (Fisher Chemical) was similarly mixed with powdered food.
3. D. G. Goodman, J. M. Ward, R. A. Squire, K. C. Chu, M. S. Linhart, *Toxicol. Appl. Pharmacol.* **48**, 237 (1979).
4. A. W. Andrews, J. A. Fornwald, W. Lijinsky, *ibid.* **52**, 237 (1980).
5. R. J. Pienta, J. A. Poiley, W. B. Lebherz, *Int. J. Cancer* **19**, 642 (1977).
6. This work was supported by contract NO1-CO-75380 with the National Cancer Institute, NIH, Bethesda, Md. 20205.

29 April 1980

Induction of Follicle Separation in the Mosquito by Physiological Amounts of Ecdysterone

Abstract. *Physiological quantities of the molting hormone, ecdysterone, injected into female* Aedes aegypti *prematurely induced separation of incipient follicles in the ovarioles, an event that normally occurs only in blood-fed females. It was possible to stimulate this morphological event with physiological amounts of hormone by mimicking, with two injections, the timing of normal increases in endogenous hormone of blood-fed females.*

The development of insects is regulated by the interplay of juvenile hormone (JH), ecdysteroids, and hormones from the cerebral neurosecretory system. These same hormones are also produced by the adult insect. Both in larval stages and in the adult, JH is synthesized and secreted by the corpora allata. In the larval stage ecdysone is produced by the prothoracic glands, which degenerate in most adult insects. However, the presence of ecdysteroids in the ovaries of adult or pharate adult insects is well documented (*1*-*6*), and its heterogeneity between male and female insects has been demonstrated (*4*, *5*, *7*). Nevertheless, the function of ecdysteroids in female insects is still not clear because only a very high concentration of exogenous ecdysterone will produce a response in vivo (*8*, *9*). We now report on the stimulation of a normal morphological event, the formation and separation of new follicles in the mosquito ovary, by physiological amounts of ecdysterone.

Each ovary of *Aedes aegypti* has about 75 ovarioles, each of which consists of a single primary follicle and germarium. These primary follicles grow in unison. A few days after emergence of the adult, and prior to the first blood meal, a secondary follicle becomes visible in each germarium (Fig. 1A). About 20 hours after the blood meal, as yolk deposition is about half completed in the primary follicles, each secondary follicle separates from its germarium (Fig. 1, B and C). By the time the primary follicle becomes a mature egg and is laid, each

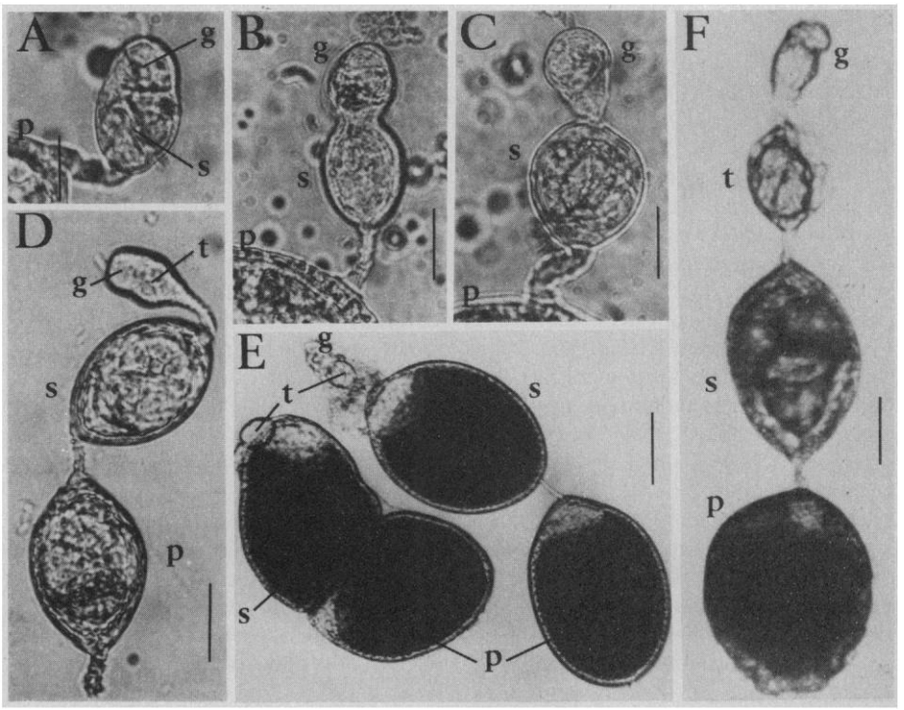

Fig. 1. Separation of the secondary follicle from the germarium in the ovary of *Aedes aegypti* (*19*). (A) Ovariole of unfed female. (B) Ovariole 17 hours after blood meal. (C) Separation complete 21 hours after blood meal. (D–F) Formation of additional follicles induced by ecdysterone: (D) Ovariole of unfed female after two injections of ecdysterone (as in Table 1, experiment A). (E) Same treatment as (D) but, in response to a blood meal taken 2 days after the first injection, yolk was deposited in both the primary and new secondary follicles. Small tertiary follicles had formed 24 hours after the meal. (F) Ovariole of female repeatedly injected with ecdysterone (*11*). A secondary and tertiary follicle have separated and another follicle is forming in the germarium. Vertical bars: 25 μm in (A) to (C), 50 μm in (D) and (F), and 100 μm in (E). Abbreviations: *g*, germarium; *p*, primary follicle; *s*, secondary follicle; and *t*, tertiary follicle.

0036-8075/80/0815-0819$00.50/0

secondary follicle is able to initiate yolk deposition as soon as the female takes a second blood meal. In this way the mosquitoes produce multiple batches of eggs.

Earlier investigators had found that injection of 1.0 to 10.0 μg of ecdysterone stimulated deposition of some yolk in the primary follicles of unfed *A. aegypti* (*8, 9*). While corroborating these accounts, we observed that 25 ng of ecdysterone injected into unfed females caused secondary follicles to separate from their germariums in many ovarioles (Table 1, experiment J). Although the dose we used was less than that required to stimulate yolk deposition (*8, 9*) or DOPA decarboxylase activity in vivo (*1, 9*), it was still far above the maximum amount of ecdysteroids reported in whole-body extracts of blood-fed females (*1, 2*). However, reducing the amount of ecdysterone administered in a single injection to a physiological level [250 pg (*2*)] greatly reduced the number of individuals in which new follicles separated from the germariums (Table 1, experiment H). The coincidence of the separation of new follicles and the rise in ecdysteroids after a blood meal gave credence to the idea that ecdysteroids normally stimulate the separation of the secondary follicle.

In *A. aegypti*, there is reportedly a slight and transient increase in ecdysteroids about 4 hours after a blood meal, followed by a major increase 12 to 14 hours later (*2*). We therefore mimicked the two increases in the ecdysteroid titer described for blood-fed females; we first injected 100 pg and 12 to 14 hours later, 275 pg of ecdysterone (*2, 10*). The secondary follicles in these females separated from the germariums in most cases (Table 1, experiment A; Fig. 1D). It was clear that two injections were more effective than an equal amount given as a single dose (Table 1, experiment B); the effect was optimal when the period between injections was 12 to 14 hours (Table 1, experiments A, C, and D). It was possible to stimulate follicle separation with injections of even less hormone (Table 1, experiments E, F, and G), evidence that an intrinsic amount of the ecdysteroids reported for whole-body extracts of *A. aegypti* (*2*) may be unavailable to the ovary.

These physiological quantities of ecdysterone did not provoke yolk deposition in either primary or secondary follicles. However, when secondary follicles formed as a result of these injections, both the primary and secondary follicles deposited yolk after the female fed on blood, an indication that the ecdysterone-induced secondary follicles were normal (Fig. 1E). Furthermore, when unfed females were given multiple injections of ecdysterone (*11*), more than one new follicle often formed and separated within the same ovariole (Fig. 1F). These experiments suggest that formation of new follicles within the germarium, as well as their separation from it, may be stimulated by the secretion of ecdysteroids in the normal blood-fed female mosquito (*12*).

Table 1. Separation of secondary follicles in unfed mosquitoes injected with ecdysterone.

Experiment	Amount injected* (pg)	No.	Percent with separation
A	100 and 275 (12 to 14 hours)	28	82
B	375	12	17
C	100 and 275 (3 hours)	21	38
D	100 and 275 (24 hours)	27	63
E	100 and 250 (16 hours)	19	83
F	10 and 25 (16 hours)	60	77
G	0.1 and 0.25 (16 hours)	11	9
H	250	11	27
I	2,500	29	59
J	25,000	17	100
K	Saline only	33	0

*Ecdysterone (Rohto Pharmaceutical Co., Japan) in 0.5 μl of saline. Numbers in parentheses indicate the interval between two injections.

Experiments with females from which the JH-producing corpora allata have been removed (allatectomized) (*13*) show that the separation of follicles is not dependent on JH (*14*). For example, 7 of 12 females allatectomized at emergence and then injected with ecdysterone (*15*) produced secondary follicles. We also found that females allatectomized shortly after emergence and later fed blood do not deposit yolk in their primary follicles, but the secondary follicles separate normally from the germariums, presumably in response to increases in endogenous ecdysteroids as in normal blood-fed females. In another experiment, 3-day-old females were decapitated (leaving the corpora allata in the thorax) and then injected with ecdysterone (*15*); secondary follicles separated in 25 of 28 females. Clearly, the effect of ecdysterone on separation of the follicles is independent of factors from the head and the corpora allata.

Whether ecdysteroids could have a similar role in the follicular development of other insects has yet to be demonstrated. It has been shown in *Bombyx mori* that an increase in the amount of ecdysteroid (*5, 6*) is critical to the initiation of oogenesis in the pupal female, but its specific role has not been described (*16*). Similarly, although the correlation was not made with the amount of ecdysteroid in intact animals, the presence of ecdysterone was necessary for the in vitro organization of follicles in the pupal ovaries of the mealworm, *Tenebrio molitor* (*17*). In vitro, the addition of ecdysterone accelerated the movement of follicles out of the ovary in the paedogenetically reproducing gall midge, *Heteropeza pygmaea* (*18*).

Because ecdysteroids stimulate the separation of follicles in the mosquito, we suggest that ecdysteroids in other insects and arthropods may have a similar function. Furthermore, the requirement for the specific timing of repeated exposures to the hormone may indicate a fundamental mechanism by which ecdysterone induces cellular responses in invertebrates.

ELIZABETH F. BECKEMEYER*
ARDEN O. LEA
Department of Entomology, University of Georgia, Athens 30602

References and Notes

1. D. A. Schlaeger and M. S. Fuchs, *Dev. Biol.* **38**, 209 (1974).
2. H. H. Hagedorn, J. D. O'Connor, M. S. Fuchs, B. Sage, D. A. Schlaeger, M. K. Bohm, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3255 (1975).
3. M. Lagueux, M. Hirn, M. de Reggi, J. A. Hoffmann, *C. R. Acad. Sci. Ser. D* **282**, 1187 (1976); C. Bordereau, M. Hirn, J.-P. Delbecque, M. de Reggi, *ibid.*, p. 885; A. Garen, L. Kauvar, J. -A. Lepesant, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5099 (1977); C. Bordereau, J. -P. Delbecque, M. Hirn, B. Lanzrein, H. Imboden, M. Luscher, J. D. O'Connor, in *Proceedings, VIIIth International Congress of the International Union for the Study of Social Insects*, J. de Wilde, Ed. (Agricultural Publishing Center, Wageningen, Netherlands, 1977), p. 290; A. Strambi, C. Strambi, M. de Reggi, *ibid.*, p. 19.
4. T. H. Hsiao and C. Hsiao, *J. Insect Physiol.* **23**, 89 (1977); M. Lagueux, M. Hirn, J. A. Hoffmann, *ibid.*, p. 109; M. de Reggi, *Recherche* **8**, 479 (1977); W. E. Bollenbacher, H. Zvenko, A. K. Kumeran, L. I. Gilbert, *Gen. Comp. Endocrinol.* **34**, 169 (1978).
5. B. Calvez, M. Hirn, M. de Reggi, *FEBS Lett.* **71**, 57 (1976); E. Ohnishi and F. Chatani, *Dev. Growth Differ.* **19**, 67 (1977).
6. J.-M. Legay, B. Calvez, M. Hirn, M. de Reggi, *Nature (London)* **262**, 489 (1976).
7. R. B. Hodgetts, B. Sage, J. D. O'Connor, *Dev. Biol.* **60**, 310 (1977).
8. A. Spielman, R. W. Gwadz, W. A. Anderson, *J. Insect Physiol.* **17**, 1807 (1971).
9. D. A. Schlaeger, M. S. Fuchs, S.-H. Kang, *J. Cell Biol.* **61**, 454 (1974).
10. The effectiveness of two unequal injections of ecdysterone in stimulating pupariation in *Sarcophaga argyrostoma* was greater when the first dose was smaller, according to J. Zdarek and G. Fraenkel [*Proc. Natl. Acad. Sci. U.S.A.* **67**, 331 (1970)].
11. Females were injected daily, first with 500 pg of ecdysterone and 17 hours later with 1000 pg, for 3 to 4 days.
12. We also find that ecdysterone induces premature follicle separation in the mosquitoes *Culex nigripalpus* and *Culex pipiens*. Massive amounts of injected ecdysterone result in degeneration of primary follicles in these insects, as reported for the black blowfly, *Phormia regina*, by C. Pappas and G. Fraenkel [*J. Insect Physiol.* **24**, 75 (1978)]. Follicle degeneration does not occur in *A. aegypti* injected with physiological amounts of ecdysterone.
13. Technique as in A. O. Lea [*ibid.* **9**, 793 (1963)].
14. Both primary and secondary follicles of allatectomized females remain small in the absence of JH, which is required for their normal pre-

vitellogenic growth, as reported by R. W. Gwadz and A. Spielman [*ibid.* **19**, 1441 (1973)].
15. Females, either decapitated or allatectomized, were injected first with 500 pg ecdysterone and 15 hours later with 1000 pg.
16. F. Chatani and E. Ohnishi, *Dev. Growth Differ.* **18**, 481 (1976).
17. A.-M. Laverdure, *Gen. Comp. Endocrinol.* **17**, 467 (1971).
18. D. F. Went, *J. Insect Physiol.* **24**, 53 (1978).
19. *Aedes aegypti* larvae, Segemaganga strain, were fed increasing daily portions of yeast, lactalbumin, and laboratory rat chow. "Unfed" adults were allowed 5 percent sucrose, maintained at 27°C, and were given the injections in the abdomen 2 to 5 days after emergence. While follicle separation started approximately 24 hours after the first injection, it was generally slower than after blood feeding; therefore we routinely examined the germariums 2 to 3 days after the last injection. The sheath of the ovariole was removed to confirm follicle separation. Females were scored positively after three or more follicles were identified as separated from the germariums. Generally, more than half of the ovarioles of positively scored females contained newly separated secondary follicles.
20. We thank D. M. Fendley for his technical assistance and expertise. Supported by NIH grant AI-09410 (to A.O.L.).
* Present address: Department of Zoology, Duke University, Durham, N.C. 27706.

18 January 1980; revised 13 March 1980

Puberty Delay by a Urinary Cue from Female House Mice in Feral Populations

Abstract. *Urine produced by wild female house mice, living in high- and low-density populations and confined to areas within a highway cloverleaf, was tested for its ability to delay puberty in juvenile female mice. Only urine collected from females in the dense population at its maximum density delayed puberty in test females. Urine collected when the population was less dense, or from a population that remained sparse, failed to delay puberty. These results suggest that a urinary factor present at high densities may delay puberty and thus help to slow further population growth.*

In confined populations of house mice (*Mus musculus*), population growth decreases as density increases through decreased reproductive rate, increased neonatal mortality, or both (*1*). A delay in the onset of puberty is a common cause of reduced reproductive rate in these populations. Delayed female puberty in albino mice (*M. musculus*) can result from the presence of grouped females or their urine (*2*). Similarly, soiled bedding from the cages of grouped wild female house mice retards puberty in females derived from wild *M. musculus* stocks (*3*). Chemical cues contained in urine may therefore play an important role in regulating natural rodent populations through controlling the rate of sexual maturation. This report provides, to our knowledge, the first evidence that wild female house mice living under natural conditions produce a urinary component that delays the onset of puberty in juvenile female mice coincident with density increases.

We observed populations of house mice living on "highway islands" (cloverleaf sections of Interstate 40, a major highway near Raleigh, North Carolina) over a 2-year period. Live trapping revealed restricted emigration and immigration (*4*), with relatively isolated rodent populations of varying densities and species compositions on highway islands at different successional stages (*5*). Because of this diversity, we removed all small mammals from two highway islands by snap trapping (*6*). Eight sexual pairs of second-generation laboratory-born house mice were introduced to each island in the spring of 1978 (*7*). Detailed floristic and faunistic descriptions of the cloverleafs, as well as data on house mouse emigration rates and population dynamics will be published elsewhere (*8*). Growth of the mouse populations was monitored for a year by trapping for 6 to 8 days within a 2-week period at least every 2 months. A grid of 51 live traps (Sherman) (6.5 by 16.5 by 5 cm) placed 10 m apart and lined with three pieces of filter paper (Whatman No. 1) (4 by 8 cm) to collect voided urine was used. On capture, we individually marked mice by toe clipping, recorded animals' weights and reproductive conditions, and removed the filter paper from the traps and stored it at −40°C. Schnabel (*9*) population estimates from individual mark-recapture data reveal changes in the population numbers over time (Fig. 1). Both populations peaked in December, but the number of individuals on each highway island varied considerably. The population estimate for population 1 in December was 16.0; that for population 2 was 73.7.

So that the puberty delaying potency of the urine of female mice from these populations could be assayed, we first determined whether urine from laboratory-housed wild female mice affects the onset of puberty in juvenile female albino mice (*10*). We housed four laboratory-born wild adult female mice (*11*) in 18 by 28 by 11 cm polypropylene cages with freely available food and water under a 14:10 light-dark cycle for 4 weeks. Individual females were removed and placed overnight in a Sherman trap lined with filter paper to absorb voided urine. A 2-cm^2 piece of filter paper impregnated with urine (*12*) was placed in the cage of a 25-day-old female albino mouse for 6 days (*13*). A second group of 25-day-old females receiving 2 cm^2 of clean filter paper for 6 days served as controls. After vaginal perforation, we lavaged the females' vaginas daily to determine the age of first estrus, which is designated by a completely cornified smear (*14*). Juvenile females exposed to the urine of grouped female wild mice attained puberty significantly later than control females (Table 1).

Once we established that urine from laboratory-housed wild females delays puberty in albino mice in a manner comparable to albino mouse female urine, we

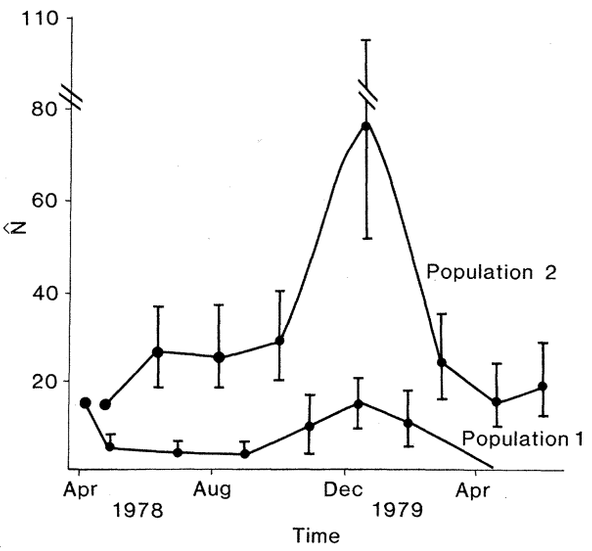

Fig. 1. Schnabel population estimates and their 95 percent confidence limits for two populations of house mice confined to highway islands. Mice were introduced to the highway islands in the spring of 1978, and the growth of the populations was monitored for 1 year (*7*).

0036-8075/80/0815-0821$00.50/0