change color even when larvae were exposed for as long as 120 hr. Also, when finely ground carbon was added to the medium, no particles were found in the gut after 120 hr. Small clumps of red material were occasionally seen in the midgut of 1st-stage larvae, but there is no evidence that this material was ingested orally, especially since our microscopic examinations showed that the foregut contained a narrow, collapsed but open lumen for its entire length. The minimum size of the lumen in cross section, 4× 30 μ (Cooke 1968[5]), appears to be a barrier to the passage of particulate matter the size of bovine red blood cells or larger. Moreover, we are not aware of any evidence that the foregut of the 1st instar is functional.

The foregut need not be functional for the midgut to be functional. Perhaps the midgut gradually accumulates certain metabolic wastes and serves as an excretory organ by voiding these wastes at the time of molting. Malpighian tubules have not been seen in young 1st-stage larvae, though they are present in the late 1st instars.

The rate at which radioactive phosphorus was accumulated by the tissues of 2nd-stage larvae indicated that phosphate was absorbed percutaneously and ingested orally at about equal rates. The rapid appearance of large quantities of phosphorus in the anterior portion of the midgut of 3rd instars, the much slower accumulation in the hemolymph, and the pattern of buildup in the other tissues all point to easy oral ingestion and little, if any, absorption of phosphorus through the integument. In test 1, the *p*H indicator phenol red was added to the medium. Thus, the appearance of a pink color in the midgut at 2 and 6 hr and in the hindgut of some larvae at 6 hr was regarded as further evidence of oral ingestion.

The high values of radioactivity occurring in all 3 instars strongly suggest absorption of the phosphate as an inorganic salt and also affinity of the phosphorus ion for the tissues. The hypothesis of decreased integumental absorption by 3rd-stage larvae compared with that of 1st and 2nd instars is consistent with the thick, strong body wall possessed by the more advanced larvae. An explanation for the large quantities of phosphorus accumulated by the midgut can be derived from the musculature of this organ and the possible differential accumulation of phosphorus for use in the metabolism of the muscles; if the tests had been conducted beyond 24 hr, other muscle tissues might have shown this accumulation.

The evidence indicates that the 1st-stage larvae of *H. lineatum* obtain important nutrients by percutaneous absorption and that little or no food is ingested orally by this form. Apparently the 3rd-stage larvae depend on oral ingestion almost completely, but our observations suggest that food is taken in by the 2nd instars by both oral ingestion and percutaneous absorption.

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Bioassay, Preliminary Purification, and Effect of Age, Crowding, and Mating on the Release of Sex Pheromone by Female *Tenebrio molitor*[1,2]

GEORGE M. HAPP[3] AND JAMES WHEELER[4]

ABSTRACT

The sex pheromone produced by female yellow mealworm beetles, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), was conveniently and quantitatively assayed by exposing males to air containing female scent. Although extracts of both sexes released a male response, males responded only to live females and not to live males. The female pheromone was purified 700-fold by column chromatography. By means of assays with live females it was demonstrated that pheromone emission reached a peak about 4 days after eclosion from the pupal skin. Crowding enhanced pheromone release in virgin females; after mating, the effect of crowding disappeared.

In a classic study of insect olfaction, Valentine (1931) demonstrated the importance of chemical stimuli in the mating of yellow mealworm beetles, *Tenebrio molitor* L. Valentine prepared an extract of female beetles, dipped a glass rod into the extract, and presented the coated rod to male *Tenebrio*. The males then were attracted by the scented rod. Filter paper or balls of cotton which had been left in containers with female beetles also served to excite the males. Recently Tschinkel et al. (1967) reexamined the role of this pheromone and employed a modification of the glass-rod presentation to provide a quantitative bio-

[1] Coleoptera: Tenebrionidae.

[2] Accepted for publication Oct. 23, 1968.

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assay for the titre of the pheromone in extracts of females. It is the intent of our paper: (1) to describe a new bioassay for the sex pheromone produced by female *Tenebrio,* (2) to report the results from some preliminary attempts to chemically purify the active material, and (3) to show that aging, crowding, and mating affect the release of pheromone by female beetles.

MATERIALS AND METHODS

Rearing.—*T. molitor* were maintained in the laboratory on a diet of chicken feed and brewer's yeast. Pupae were collected from these cultures, the sexes segregated, and the adults kept in pure cultures of only their own sex. Most adults were kept in crowded cultures with 400–500 beetles in a box about 8×10 in. For the studies on pheromone release isolated females were kept individually in 2-oz glass jars, and groups of 10 ♀ (crowded) were placed in similar jars.

Mass Collection of Female Scent.—After the newly eclosed females had darkened, they were transferred to large desiccators partially filled with oatmeal (Fig. 1). A slow stream of air was drawn through the desiccators, and volatile material in the effleunt was condensed in a trap cooled with dry ice:methanol. The trap, which contained mostly water, was rinsed daily with diethyl ether, the water continuously extracted with ether, and the ether layer dried. The resulting crude preparation of female scent was then used for chemical studies.

In addition, some beetles were homogenized in acetone to obtain the female scent. The acetone was separated from the solid residue, stripped off on a rotary evaporator, and methylene chloride was added.

Bioassay Apparatus.—Twenty small lucite chambers (15 cm long, see Fig. 2) were mounted on a lucite turntable (Fig. 3). Air from a compressed-air tank flowed into a box at the center of the turntable and then out through rubber tubes to the 20 test chambers. As the air streamed from the central box to each bioassay chamber, it passed through a removable eyedropper.

FIG. 1.—The system employed for the collection of female scent. Several thousand virgin females are confined in each desiccator. Air is passed through the desiccators and the volatile material is condensed in cold traps.

A group of 10 virgin ♂ was placed in each lucite chamber. All were more than 3 weeks old and were individually numbered with "Micro-Markers" (W. H. Brady, Milwaukee). While the males were in the bioassay chamber they were fed daily with pablum and a small piece of potato. All tests were made between the 4th and 10th hr of the daily 12-hr dark period.

Bioassay Procedure.—At least 1 hr before testing was started the air flow was turned on and adjusted so that the air in each test chamber was completely renewed in 1 min. During this hour the males tended to collect around the food which was placed near the outflow end of the chamber.

Chemical samples were introduced into an eyedropper with the aid of an Eppendorf micropipetter (10 μliter in methylene chloride); the solvent was allowed to evaporate for 30–45 sec, and the scented eyedropper was inserted into the air stream in place of the removable unscented blank. Live beetles could be placed in eyedroppers and similarly used as scent sources. The males were then watched for 5 min with the aid of dim red light. Each male was scored as responding positively when it rushed to the air inflow or when it attempted to mount another male and extruded its genital segments. Once an individual male had responded, its behavior during the remainder of a test period was ignored. At the close of each test the original eyedropper was put back in place and the turntable was rotated to bring the next chamber into position for observation. Results from 6 to 10 replicates for each sample were pooled and the totals expressed as a percentage of males responding. For comparisons of different chemical samples, each sample was tested at 2 concentrations. The relative potency of the 2 samples was then calculated by a "4-point parallel assay" technique of logit analysis (Berkson 1953). For any single sample, one may estimate the amount of material in micrograms required for a 50% response from the males; this quantity is termed the RD_{50}.

RESULTS

In the bioassay, the proportion of males which responded to a given scent varied with the concentration of the active chemical substance. Fig. 4 shows a plot of the logit of the response against the relative concentration of female scent collected by aeration (Berkson 1953). The bioassay results were extremely reproducible; pooled results from replicate tests with any given sample varied less than 5% from one assay to the next.

By the "4-point parallel assay" technique, it was readily demonstrated that not only female extracts, but also extracts of *males* released the male response. However, female extracts were 5–10 times more potent than male extracts. In contrast, live females but *not* live males excited the male beetles in the test chamber. In a typical experiment which utilized 3-

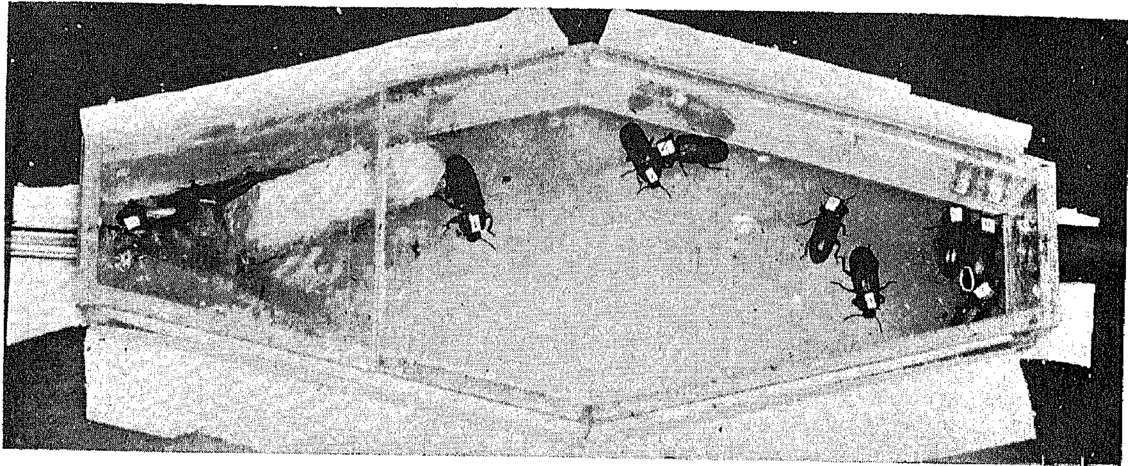

FIG. 2.—A bioassay chamber containing 10 ♂ *Tenebrio*. The air input is at the right.

week-old males or 3-week-old females as the scent sources, 67% of the males responded to female scent while only 5% responded to male scent. The latter response is at the level of random movement for males in the test chambers.

Preliminary Purification of the Pheromone.—By column chromatography on silicic acid, crude female scent may be purified 700-fold. Table 1 shows the results from 1 such column. Further analysis by TLC of fractions such as no. 5 in the table indicated that the material was a mixture of several substances. The nuclear magnetic resonance spectrum of this fraction was suggestive of a long chain ester in accord with the infrared absorption (5.75 μ). Mass spectra were inconclusive. Attempts at purification by TLC, alumina column chromatography, or gas chromatography led to loss of activity. Further purification of this material is currently in progress.

During the chemical studies, 1 pure compound that released male reproductive behavior was isolated from the condensate in the cold trap. This substance was identified as dibutyl phthalate by mass spectroscopy and TLC. The mass spectrum showed a parent ion at m/e 278.150 ($C_{16}H_5O_4$) and principal fragment ions at m/e 233.100 ($C_{12}H_{15}O_4$), 205.083 ($C_{12}H_{13}O_3$), and 149.023 ($C_8H_5O_3$). The fragmentation pattern and the mobility of the substance on TLC agreed precisely with results obtained from authentic dibutyl

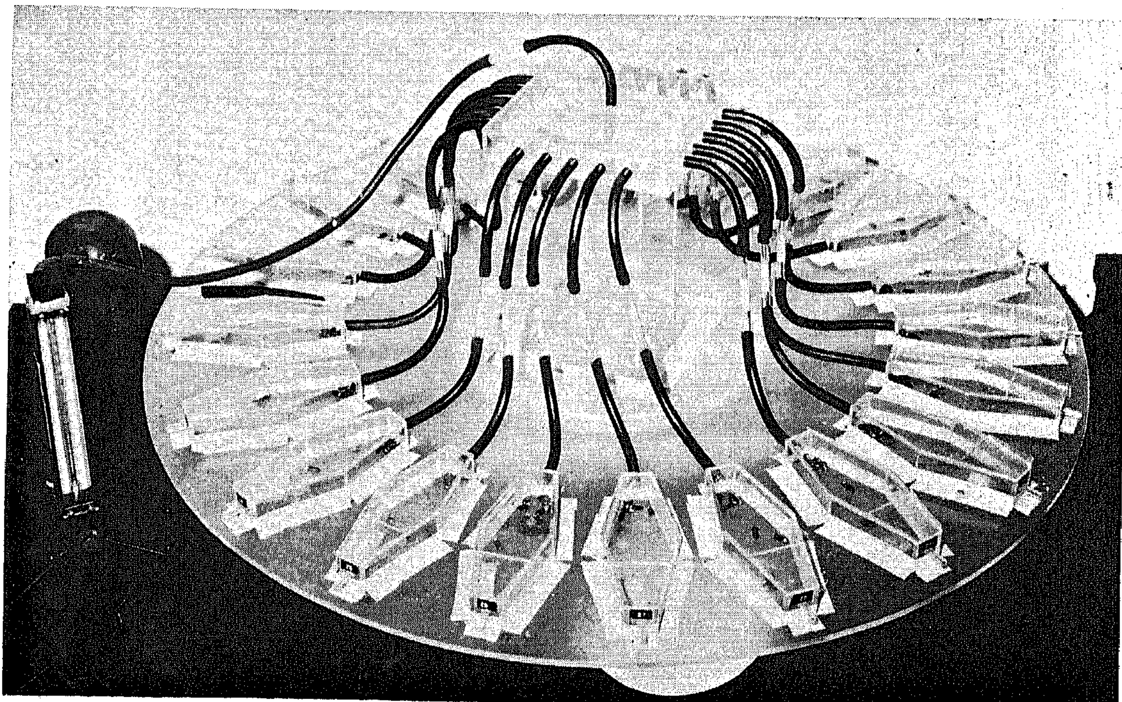

FIG. 3.—The entire bioassay apparatus. (See text for explanation.)

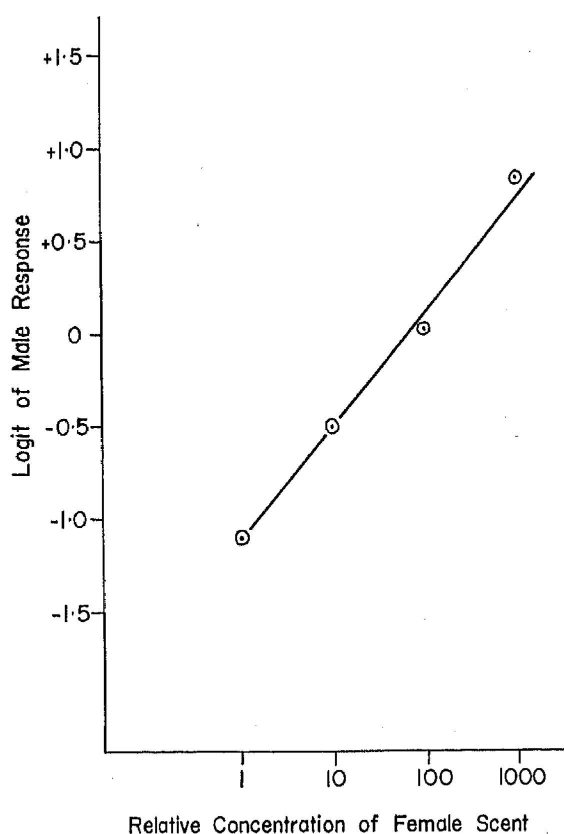

FIG. 4.—The dose-response curve for varying concentrations of female scent. The logits are based on pooled results of at least 10 replicates of each concentration. (See text for further explanation.)

phthalate. In the bioassay, the RD_{50} for dibutyl phthalate was found to be 8 μg, whereas that for the partially purified female scent, such as fraction 5 in the table, was 0.003 μg. The dibutyl phthalate was apparently contributed to the female scent preparation from the tygon® tubing in the collection system. It disappeared when the tygon was exchanged for rubber tubing.

Effects of Age and Crowding on Emission of Pheromone in Virgin Females.—Newly eclosed females released little sex pheromone. The attractiveness of these females to males increased steadily up to the 4th or 5th day and was reasonably constant thereafter in virgin females. The effect of crowding was dramatic; throughout most of the period of testing crowded females were much more attractive to male *Tenebrio* (Fig. 5) than were isolated females of the same age. The difference in attractiveness stemming from the 2 conditions is highly significant (by χ^2) from the 3rd day on. Although it is not of statistical significance, both crowded and isolated virgin females showed a dip in pheromone emission at about the 8th day, and this dip was seen repeatedly in every age analysis we made.

Effects of Age and Crowding on Emission of Pheromone in Mated Females.—Although the age effects, which presumably reflected sexual maturation, were certainly apparent in mated females (Fig. 6), there was relatively little difference between the attractiveness of crowded and isolated females, at least after the 4th day when mating occurred. In both groups, the decrease in pheromone emission at about the 8th day was quite pronounced.

DISCUSSION

Sex Specificity of the Pheromone.—Tschinkel et al. (1967) noted that not only extracts of females, but also extracts of males, released the male copulatory response in their bioassay. We had parallel results with our bioassay. However several esters, among them dibutyl phthalate, methyl benzoate, *n*-butyl butyrate, and methyl salicylate, also release the male response if they are present at high concentrations ($RD_{50} \geqslant 10$ μg). Recently, Regnier and Wilson (1968), in their investigations of the alarm response of the smaller yellow ant, *Acanthomyops claviger* Roger, found that biological activity of a variety of molecules is not so much related to functional groups and molecular shape as simply to molecular weight. In contrast, our data on the female sex pheromone in *Tenebrio* suggest that although a variety of chemicals can act to release the response, the natural material (albeit impure as in fraction 5 of Table 1) is more potent by at least 3 orders of magnitude. We tend to believe that the response of males to extracts of their own sex reflects their sensitivity to volatile chemical substances, different from the pheromone produced by the female, which are present in male extracts. Furthermore, in the bioassays with live beetles, males respond only to females but not to other males. Unquestionably, tests with live beetles are biologically more meaningful than experiments which employ whole extracts. Thus it appears most likely that *only* the female produces and releases this pheromone which attracts and excites the males of *T. molitor*.

Patterns of Pheromone Emission. — Pheromone emission in virgin female *Tenebrio* is affected by the stage of sexual maturation and by the presence of other females. To some extent the presence of other females is, in itself, acting to accelerate sexual maturation. Oöcyte growth is more rapid in crowded virgins than in isolated virgins (Mordue 1965). However, the level of pheromone emission per female in crowded females far exceeds that ever found in isolated virgins. Either pheromone emission is a function of the rate, rather than merely the stage, of sexual maturation, or crowding itself has some other influence on pheromone release independent of maturation. The mechanism by which crowding acts is now under investigation.

Although mating accelerates oöcyte growth in both isolated and crowded female *Tenebrio* (Mordue 1965), the effect of mating on pheromone production differs between these 2 experimental groups. Comparison of the isolated-virgin and the isolated-mated shows that mating accelerates pheromone production in single females, whereas in crowded females the virgins release more pheromone than do the mated females.

Table 1.—Column chromatography of scent of female *T. molitor* on silicic acid.

Fraction	Eluent[a]	Concentration (mg/ml)		Response		Relative activity[b]	RD50
		×	10×	×	10×		
1	0	0.37	3.7	0.276	0.505	0.2	10
2	2	.13	1.3	.263	.414	.2	10
3	2	.06	0.6	.333	.350	.1	20
4	4	.06	.6	.464	.637	4	0.5
5	4	.001	.01	.556	.768	700	.003
6	6	.007	.07	.667	.758	200	.01
7	6	.001	.01	.320	.677	200	.01
8	6	.02	.2	.280	.296	0.2	10
9	6	.01	.1	.303	.354	.8	2
10	10	.02	.2	.370	.724	10	0.2
11	20, 50, 100	.14	1.4	.566	.698	3	.6
Crude		.13	1.3	.337	.646	1	2

[a] % diethyl ether in pentane.
[b] Corrected for concentration differences.

In fact, mating appears to override the effect of crowding in both groups of mated females which scarcely differ from one another after the 3rd day of age. The results suggest that mating brings the pheromone emission patterns of the 2 groups into synchrony. In both the isolated and the crowded mated females pheromone production continues to rise through the 7th day, and then markedly falls for several days thereafter. This drop in release of the sex attractant correlates nicely with the onset of egg laying in *Tenebrio,* which occurs at 8 days of age (Dick 1937).

These patterns of pheromone emission must certainly be of adaptive significance. Aggregations of virgin females are much more atttractive to males than are isolated females, which fact would suggest

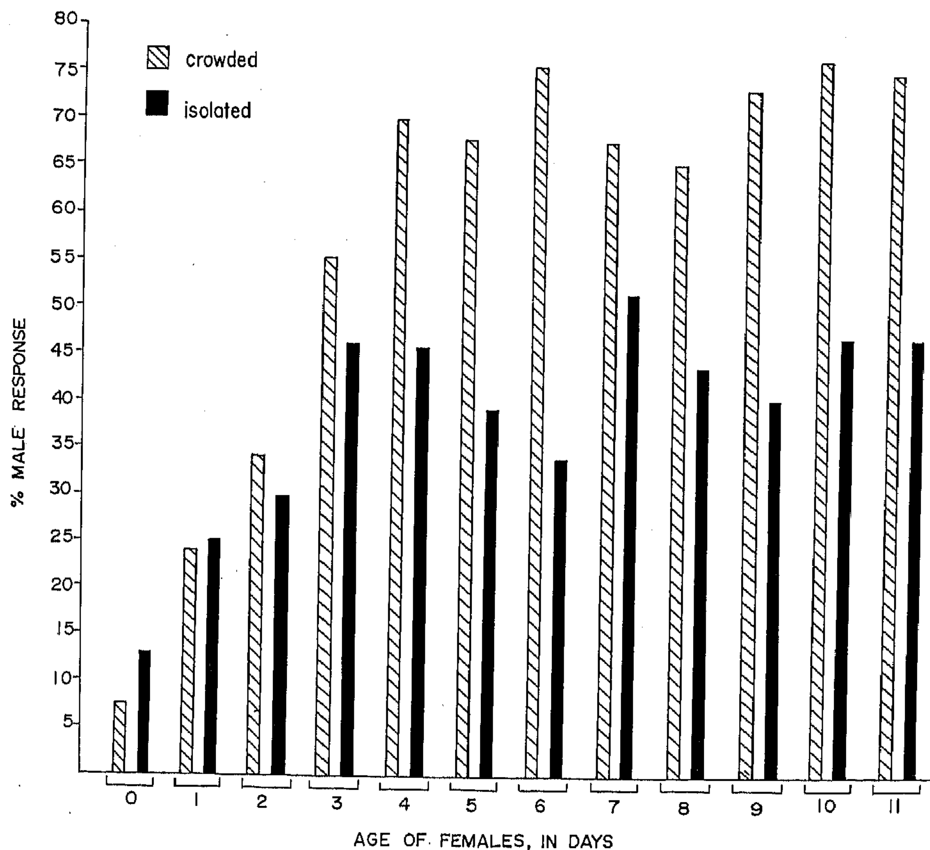

FIG. 5.—The response of males when exposed to virgin females of increasing age.

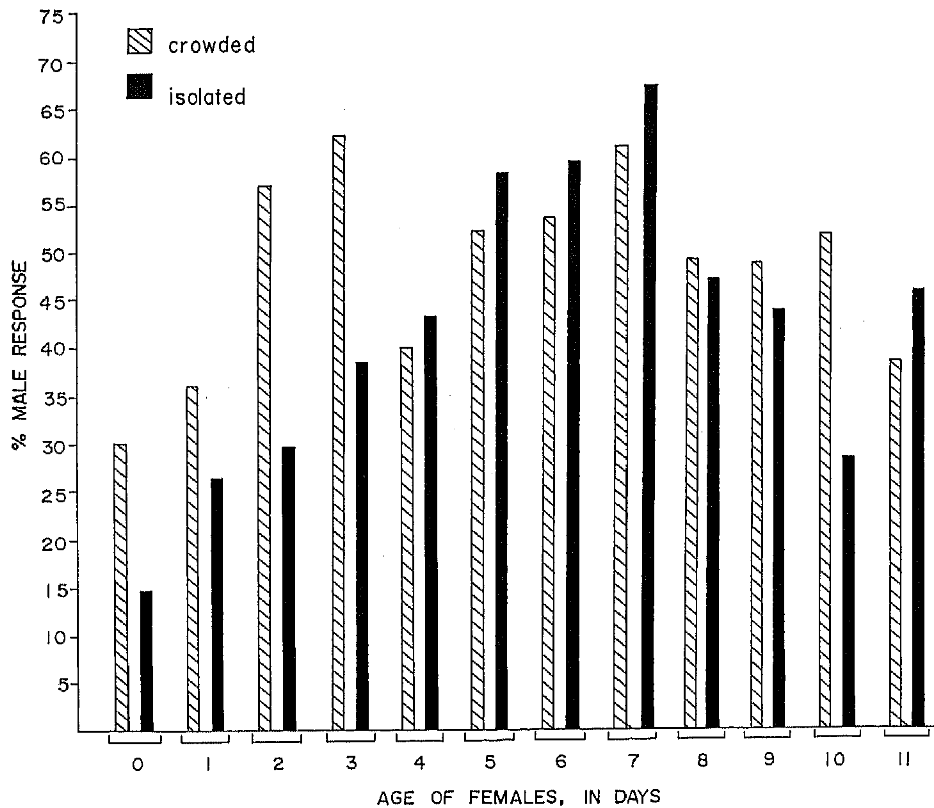

FIG. 6.—The response of males when exposed to mated females of increasing age.

that the males will tend to be drawn to the denser concentrations of females. Females nearing sexual maturation are more attractive than very young females, and the former remain highly attractive for several days after mating until the 1st cycle of oöcyte growth is completed. At that point, if the female is already mated, her rate of pheromone emission declines. However, if she has not mated she still remains highly attractive.

ACKNOWLEDGMENTS

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