Inhibition of Pupation due to Crowding in Some Tenebrionid Beetles[1]

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ABSTRACT Pupation of mature larvae of several species of tenebrionid beetles is inhibited by crowding. The magnitude of the inhibition varies according to species. The primary factor causing this inhibition is the mechanical stimulation resulting from inter-larval contacts. Cannibalism of prepupae, pupae and teneral adults is discussed as the probable chief factor responsible for the evolution of the inhibitory effect of crowding.

Of the many possible effects of population density on growth and development, those of greatest importance vary greatly depending on species and developmental stage (Allee, '34). Among insect larvae, the time required for completion of the larval stage may be shortened or lengthened by grouping, the effect sometimes being dependent on the degree of crowding. In a few cases, the principal stimulus and receptor mechanisms for these phenomena have been elucidated. For example, the acceleration of maturation in the desert locust, *Schistocerca gregaria,* is caused by a pheromone secreted by adult males of this species (Norris, '54; Loher, '60). By contrast, the acquisition of gregarious behavior in young *Schistocerca* and *Locusta* hoppers can be effected by purely mechanical stimulation (Ellis, '59), and mechanical stimulation is also a principal factor in the determination of winged forms in certain species of aphids (Johnson, '65; Lees, '67).

This report was prompted by the observation that fully grown larvae of the large Central American tenebrionid beetle *Zophobas rugipes* Kirsch pupated rapidly in isolation, but failed to pupate under the crowded conditions of mass rearing although larval-larval molts continued to occur. Qualitatively similar behavior is displayed by larvae of several other tenebrionid species. To our knowledge, this is the first demonstration of an important effect of mechanical stimulation in the timing of pupal development.

MATERIALS AND REARING

The adults of the species studied were from the following sources: *Tenebrio molitor* L.: laboratory stock culture; *Tenebrio obscurus* F.: supplied by Mr. John Doyen; *Eleodes armata* LeC.: Panoche Creek, San Benito Co., California; *Argoporis alutacea* Csy.: Guaymas, Mexico; *Amphidora littoralis* Esch.: Antioch, California; *Neatus tenebrioides* Beauv.: Glastonbury, Connecticut. Larvae of *Zophobas rugipes* Kirsch were collected from a man-made cave near San Jose, Costa Rica, where they were living in fruit-bat guano. The larvae and adults of all species were maintained and reared satisfactorily on wheat bran and water.

METHODS AND RESULTS

Z. rugipes larvae of the same weight range as adults (0.75-0.95 gm) were transferred from crowded cultures in which no pupations had taken place to large petri dishes (155 mm diameter) at five densities (1, 2, 4, 8 and 16 larvae per dish). A damp piece of filter paper and 30 gm of fresh wheat bran were

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added to each dish. The number of larvae in each group varied from 18 to 64. Pupae were recorded and removed as they appeared,[4] and within each group the remaining larvae were combined to keep their densities as close as possible to the original density. For example, if one larva remained in each of two dishes of the two-per-dish group, these were combined in a single dish. Under these conditions, 100% of the isolated larvae pupated within 20 days. At higher densities, the rate of pupation was progressively lower (fig. 1). In figure 2 the time (in days) required for 50% of a particular group to pupate (P_{50}) is plotted against density. The slope of the lines is in direct relation to the sensitivity to crowding.

More bran is consumed and more frass produced per unit time in dishes containing larger numbers of larvae; the results shown in figure 1 might be due to one or both of these factors. Three more larval density series were therefore constructed, in which the bran substrate was largely replaced by neutral material (sand, cellulose) or by larval frass. As shown in figure 2, the pupation rate on larval frass was slightly lower at every density than the rate on pure bran. The pupation rate on sand or cellulose is slightly higher than on pure bran at the two densities at which these were tested. Relative to the mechanical effects discussed below, these effects are small and were not further studied.

It is also possible to manipulate the relative population density by changing container size while keeping the number of larvae constant. Twenty-five of each of three sizes of petri dish (155 mm, 100 mm and 50 mm diameter) were set up with proportional amounts of bran and two larvae per dish; as a control, a similar series was furnished with one larva per dish and the two series were run simultaneously. As shown in table 1, isolated larvae pupated at a constant rate, regardless of dish size, while in the 2-larvae groups, the rates of pupation were retarded, particularly in the smallest containers. Thus it appears that, within these limits, the number of individuals per unit space is a controlling factor, whereas the amount of space available to an isolated individual is of little consequence.

It seemed possible that the density effect was due to a volatile pheromone

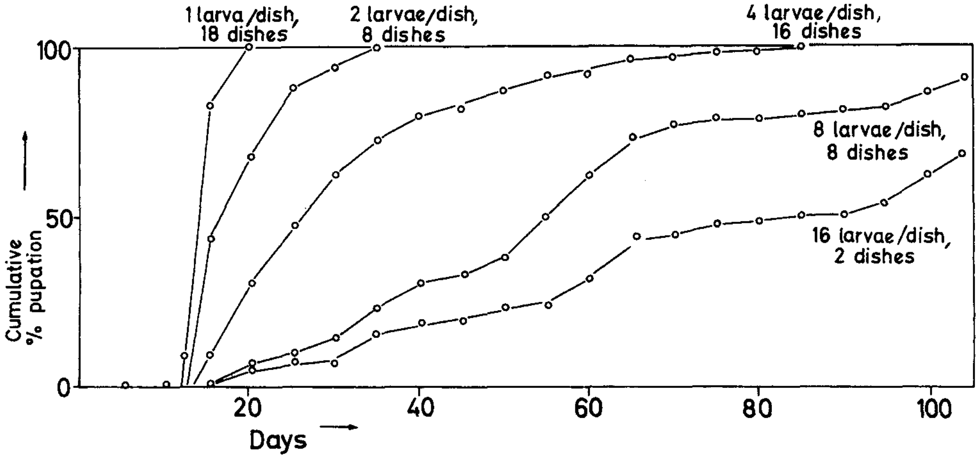

Fig. 1 Onset of pupation of *Zophobas rugipes* larvae in relation to their density in 150 mm petri dishes.

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[4] The sequence of events of pupation in *Z. rugipes* is as follows: shortly after isolation, the larva excavates a chamber (4.0 cm × 2.0 cm) in the bran or soil. Within this chamber, it curls into a crescent shape, and in two to five days becomes quiescent and turns into a "prepupa." The prepupa no longer responds to agitation by walking but by flicking the body in the same manner as a pupa. At room temperature (25°C.) larval-pupal molt takes place between 11 and 17 days after isolation and at this point pupation is completed. The pupal-adult molt follows 10 to 15 days later. The adult digs out of its chamber when its cuticle is fully hardened and its defensive glands are filled (about 7 days).

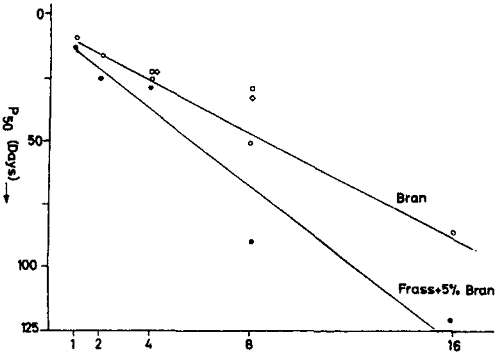

Fig. 2 Effects of density and nutritive materials on the pupation of mature Z. *rugipes* larvae (weight range: 0.75 - 0.95 gm). P_{50} values represent time in days at which 50% of the larvae in each group had pupated. Pupae were recorded and removed at five day intervals. Four diets were employed: fresh bran, *open circles;* 95% larval frass - 5% bran, closed circles; 95% sea sand - 5% bran, squares; 95% powdered cellulose - 5% bran, diamonds.

TABLE 1

Relation of P_{50} *to size of the petri dish when larvae are isolated or paired*

Dish diameter	P_{50} (days)	
	2 larvae/dish	1 larva/dish
mm		
155	16	13
100	17	14
50	34	14

secreted by the larvae themselves. To test this possibility, a number of large (155 mm diameter) petri dishes were modified by the addition of a transparent central lucite ring, 60 mm in diameter, cut to a height of 1 to 2 mm less than the inside height of the corresponding petri dish. When closed by the lid, an inner and an outer chamber were thus created, between which air, but not larvae, could circulate freely.

Bran was added to 50 such dishes and these were divided into two groups of 25. In the first group, a single larva was placed in the central chamber and 15 larvae in the peripheral chamber of each dish (1 + 15 series), while in the control group, one larva was placed in the central chamber and none in the peripheral chamber (1 + 0 series). In both groups, the isolated larvae in the central chambers pupated at very similar rates (1 + 15 series, P_{50} = 13 days; 1 + 0 series, P_{50} = 14 days). Thus, an airborne pheromone does not appear to be involved, nor do auditory or visual stimuli, since these would have been communicated just as readily. Especially visual stimuli are unlikely to play a role, for the larvae have no eyes.

Although the foregoing experiments suggest that the inhibition of pupation observed among crowded Z. *rugipes* larvae must involve primarily mechanical cues, proof of this hypothesis required that the effect be achieved by artificial means. Four centimeter sections of no. 6 metal bead chain (approximating the length and weight of mature Z. *rugipes* larva) were suspended in a radial row from the inside of the lids of large plastic petri dishes (155 mm diameter) in such a manner that when fitted to a dish, each chain rested for most of its length on the bottom of the petri dish. When the lids were revolved, the chains dragged over and disturbed the entire inside area of the dish. The petri dish bottoms were each attached to a plywood board by a single stove bolt which extended through the center of the lid and served as a bearing. A plastic ring was fastened to the top of each petri dish to serve as a pulley, and the lids were rotated by means of rubber drive belts cut from an inner tube. The belts were driven by one of three steel rods which were activated through a system of pulleys by an electric motor connected to a variable speed gearbox. An electric clock was modified to serve as an automatic switch-timer (plate 1); this was replaced in some experiments by a Dayton timing switch.

Since vibration is a form of mechanical agitation, it was expected that both the rotation of the lids and vibration from the motor might in themselves cause a retardation of pupation. To monitor this possibility, every experiment involved three groups of 15 larvae. The board itself held 30 dishes, 15 equipped with chains as described above, and 15 as controls with shortened chains so that larvae contained in these were not touched or disturbed by direct contact with the chains. A third group of 15 petri dishes rested on a non-vibrating surface nearby. These three groups will be referred to as "chains," "vibrating controls" and "non-vibrating controls," respectively.[5]

During a continuous regime of ten rotations per hour (2 rpm for 5 minutes of each hour or 240 touches/day, since the larva is touched once every revolution), no pupation or formation of prepupae was observed among mature *Z. rugipes* larvae stimulated by chains. Stimulation by vibration alone also retarded pupation, but to a much lesser degree. A typical result is shown in figure 3. The inhibition of pupation imposed upon larvae in the "chains" group had no effect on their ability to pupate subsequently; after the machine was halted, these larvae pupated normally after the usual delay.

Thus, vibration alone can reduce pupation rate in *Z. rugipes* larvae. Nevertheless, our interest centered on the effects of direct tactile stimulation, for two reasons. First, the lowering of pupation rate caused by vibration alone became complete inhibition when reinforced by contact with chains, thus indicating direct tactile stimulation as a factor of great importance. Second, observations during and after collection of the larvae suggested that within the fruit-bat guano which constitutes their natural substrate, larval contact was frequent, whereas vibration *per se* would be highly damped and consequently unlikely to be important.

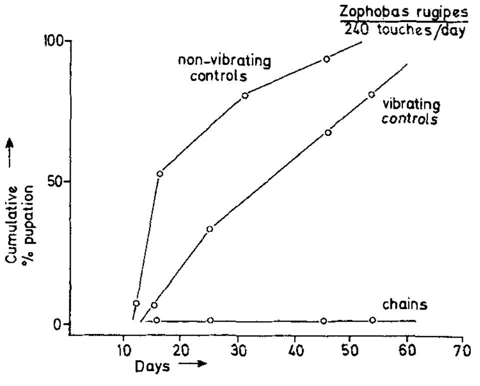

Fig. 3 Pupation of *Z. rugipes* larvae in the mechanical stimulation machine, compared with non-vibrating controls. One larva and 10 gm of bran were added to each dish; 15 larvae in each experimental group. All larvae were healthy-appearing, nine month old individuals, weighing between 0.75 and 0.95 gm. Lids of stimulated groups were rotated for five minutes of each hour at 2 rpm.

It was therefore of interest to measure the results of tactile (chain) stimulation in the absence of vibration. Although due to the construction of the machine, rotation of the chains necessarily entailed some simultaneous vibration, the total vibrational stimulus could be reduced by simply operating the motor for a smaller proportion of time during a given experimental period. Thus, when the motor was operated at one-half rpm for only five minutes of every four hours (15 touches/day; a 16-fold reduction over the previous experiment shown in fig. 3), there was no longer any apparent difference in pupation rate between the vibrated and non-vibrated control larvae. The retardation of pupation seen in the "chains" group in figure 4 may therefore be attributed entirely to direct tactile stimulation.

Experiments using lower rates of stimulation allowed a varying percentage of larvae to pupate and indicated that the total number of touches per unit time determines the fraction of larvae which pupate. Within limits, it appears to make little difference whether the stimuli are spread out over several periods or given in a single period per day. A straight line results when the logarithm of the number of touches per 24 hours is plotted against the fraction of the chain group which molted into pupae within 20 days (fig. 5).[6] Thus, it appears that each larva has an upper threshold of disturbance above which it will not enter the prepupal state, and it is the spread of

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[5] There was also a possibility that larval contact with bead chain might itself somehow cause a change in pupation rate. Therefore, in some experiments, pieces of bead chain similar in size and quantity to those attached to the lids of dishes in the "chains" group were added to the bran substrate of the "non-vibrating controls." However, no significant differences in pupation rate or mortality were observed as a result of passive contact with bead chain and this factor was not further investigated.

[6] No additional prepupae form after about seven days and the data suggest that all larvae able to pupate under the prevailing conditions do so within 20 days.

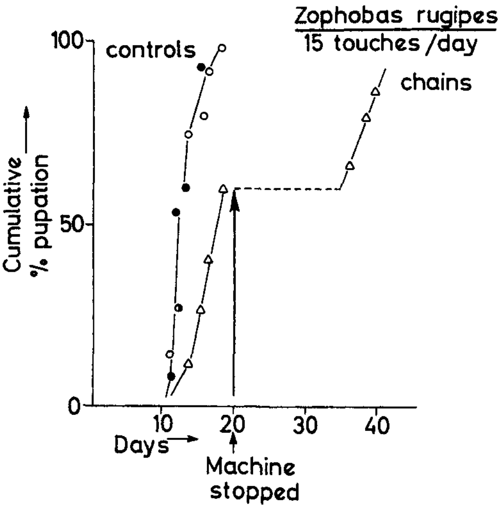

Fig. 4 Pupation of *Z. rugipes* larvae in the mechanical stimulation machine. All conditions as in figure 3 except that rotation occurred for five minutes every four hours (0.5 rpm). The machine was stopped after 20 days; the remaining larvae pupated normally after about 16 days delay. "Non-vibrating controls," open circles; "vibrating controls," closed circles.

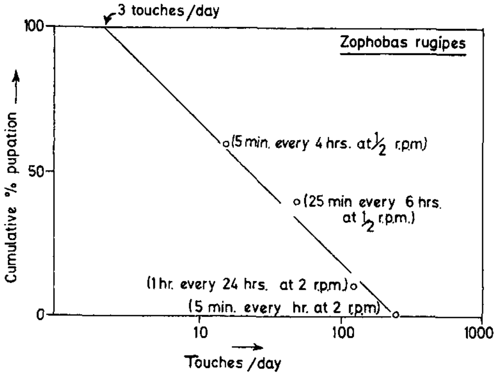

Fig. 5 Relation between the logarithm of the number of touches per day and the per cent of the larvae pupating within 20 days. Temporal distribution of stimulation is given in parentheses.

these thresholds which gives rise to the phenomenon which is the subject of this paper.

The effect of mechanical stimulation is upon formation of prepupae and, once these are formed, further stimulation does not prevent larval-pupal molt, even when the level of stimulation is sufficient to inhibit completely the formation of prepupae from active larvae. Of 20 prepupae subjected to mechanical stimulation (240 touches per day, 5 minutes every hour at 2 rpm) within 48 hours after their formation, 95% pupated normally and 5% died. Therefore, further metamorphosis of the prepupa is apparently no longer subject to inhibition by mechanical disturbances.

The inhibition of pupation by crowding implied that the population density should have an effect on the size of the mature larva and hence the adult. One hundred very small larvae of *Z. rugipes* (average weight 8.2 mg each: age approximately 2 weeks) were placed into individual glass vials with bran, but were otherwise treated the same as the mass cultures. When the larvae grew too large for the vials, they were transferred to 100 mm petri dishes. The weights of these larvae at pupation ranged from 0.3 to 0.6 gm (mean 0.423, S.E. = 0.012). Pupation began about three months after isolation and was complete after seven months (80 pupae recovered; average time to pupation: 140, s = 28 days). In contrast, larvae in crowded mass cultures attained a mean final weight of 0.811 gm (S.E. = 0.014) (almost twice the weight of isolated larvae), but none pupated up to 18 months. This experiment emphasizes that the effect of crowding is not upon growth and molting, but upon pupation. At ages of over 12 months, some larvae in the cultures became moribund, flattened and inactive and appeared to have resorbed much of their fat body. These larvae were usually partly cannibalized. After 18 to 20 months most of the larvae in crowded cultures had died without pupating. Even among old larvae which appeared healthy, there was increased mortality when they pupated after isolation (table 2).

The larvae of another tenebrionid beetle, *Tenebrio molitor*, form pupae even under grossly crowded conditions, and we assumed initially that this species lacked the pupation-inhibition response described above for *Z. rugipes*. However, when *T. molitor* larvae were placed in 100 mm petri dishes at several densities, an effect of crowding on pupation rate was immediately apparent (fig. 6). Iso-

TABLE 2

Effect of larval age on success of pupation: status of 60 individuals 30 days after isolation from young and old mass cultures

Stages	Age of mass culture	
	6 to 7 months	10 to 12 months
Prepupae	19	4
Pupae	44	26
Active larvae	4	11
Dead larvae	4	19

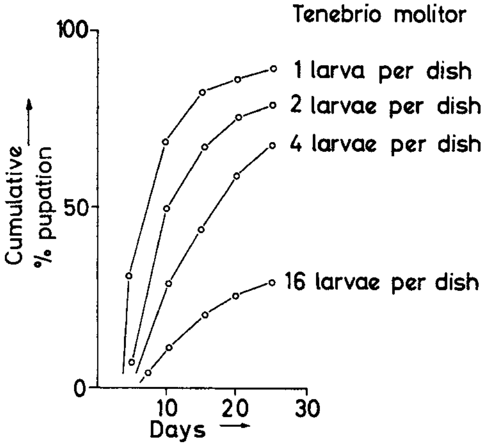

Fig. 6 Onset of pupation of *T. molitor* larvae in relation to their density in 100 mm petri dishes. Larval weight ranged from 0.14 to 0.19 gm.

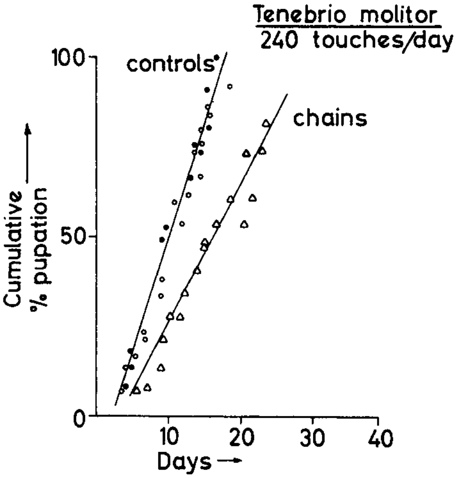

Fig. 7 Pupation of *T. molitor* larvae in the mechanical stimulation machine. Data from two experiments are plotted together. Each dish contained one mature larva (weight range: 0.14 - 0.19 gm) and 212 gm of bran. The lids were rotated for five minutes every hour at 2 rpm. "Non-vibrating controls," open circles; "Vibrating controls," closed circles.

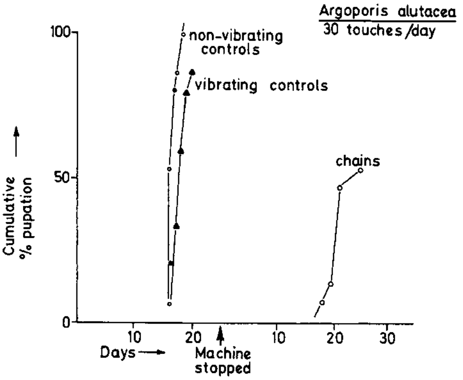

Fig. 8 Pupation of *A. alutacea* larvae in the mechanical stimulation machine. One larva and 2.5 gm of bran were added to each dish; 15 larvae in each group. (Weight range: 0.094 - 0.127 gm). Light (no. 3) bead chain was used for stimulation in the "chains" group. Lids were rotated at 1 rpm for five minutes every four hours. The machine was stopped after 25 days; the remaining larvae pupated normally.

lated larvae stimulated by chains were also delayed in pupation (fig. 7).

Comparing figures 3 and 7, it is clear that although the response to mechanical disturbance is similar in both species, larvae of *T. molitor* are much less sensitive to both vibration and direct tactile stimulation. Preliminary experiments in the mechanical stimulation machine with a third tenebrionid species, *Argoporis alutacea*, resulted in considerable mortality among the chain-stimulated larvae until the original no. 6 bead chain was replaced by a lighter size (no. 3). A relatively low intensity of stimulation (30 larva-chain contacts/24 hours) resulted in complete inhibition of pupation among *A. alutacea* larvae (fig. 8). Although due to the chain size substitution and the different intensity of stimulation it is impossible to compare this experiment directly with any of those described previously, we feel that it is safe to say that *A. alutacea* larvae are even more sensitive to tactile stimulation than are larvae of *Z. rugipes*.

Discovery of this spectrum of response to mechanical agitation prompted us to survey several other tenebrionid species available to us. Experiments in petri dishes showed inhibition of pupation as a function of crowding in *Neatus tenebrioides* Beauv., *Eleodes armata* LeC., and in *Amphidora littoralis* Esch. In actual situations of crowding, the activity of the larvae (which varies from species to species) as well as their sensitivity to disturbance determines the observed level of inhibition. However, in the absence of stimulation experiments on isolated larvae, it is not possible to conclude that these effects are due to mechanical stimulation. It should be recalled that the pupation of *Z. rugipes* larvae was slightly delayed by the presence of high concentrations of larval frass (fig. 2). Crowding is also known to delay pupation among larvae of the tenebrionid *Tribolium castaneum* Herbst (Karten, '65) and *T. confusum* Duval. In the latter case it has been shown that the effect is due almost entirely to larval conditioning of the substrate; if any response to mechanical stimuli is present in this species, it must be slight (Park, '38).

DISCUSSION

Noting that crowding affects pupation and not larval-larval molts, one may speculate on the physiological bases for these phenomena as follows: It has been shown in a number of insects that the relative level of juvenile hormone (JH) is intimately related to the process of metamorphosis; if the level of JH is artifically increased in a mature larva, the next molt will result in a further larval instar in place of the expected pupa (Wigglesworth, '64; Novak, '66; Sehnal and Meyer, '68). JH levels may be playing a similar role in the development of tenebrionid larvae (Radtke, '42), which tend to have variable numbers of larval instars in any case (Cotton, '27). Cases have been described in which neurosecretory activity and hormone levels are affected by crowding (Mordue, '65; White, '68), and grouping has recently been suggested as a factor repressing molting in cockroach nymphs (Wharton et al., '68) and the crab *Gecarcinus* (Bliss, '64). We now propose that in tenebrionid species whose larvae are affected by mechanical stimulation, a heightened internal secretion of JH is induced by the nervous impulses arising from external irritation. Isolation (cessation of stimulation) would then result in a reduction of the JH level, eventually allowing pupation

Johansson ('58) has noted that starvation of *Oncopeltus* females inhibits the secretion of hormone from the corpora allata. In the context of pupation this may explain the slight accelaration when a small amount of food is diluted with sand or cellulose (fig. 2).

Farnesol, which has JH activity, has been isolated from *Tenebrio molitor* frass (Schmialek, '61), and could be partly responsible for the effect of crowding in some tenebrionids. The concentrations of farnesol in the frass of other tenebrionids is not known.

Although various mechanisms are involved, inhibition of pupation by crowding appears to be a widespread phenomenon among the tenebrionids. What evolutionary advantage may be conferred by this ability to delay pupation in the presence of other active organisms or their excreta? Extensive studies by Park et al. ('65, '68) and others (Ho and Dawson, '66; Lloyd, '68) on the flour beetles *Tribolium confusum* and *T. castaneum* have shown that their population dynamics are dominated by cannibalism. Much less is known about the ecology of the species examined in this study, but we can say from our own observations that cannibalism is a frequent occurrence. In the case of *Z. rugipes*, for example, large active larvae rarely attack one another, but feed readily on the relatively immobile and defenseless prepupae, even preferring them to pupae (Willson, unpublished). Furthermore, newly eclosed adults are eaten by other adults, especially under dry conditions. Thus cannibalism may have provided the selective pressure for the delaying of pupation until the larva has found a secluded niche, free of tactile contact with other organisms.[7] A lack of frass in the area

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[7] Intraspecific predation during a vulnerable period of the life cycle can be solved in other ways. Petri dish crowding experiments failed to show any effect on *Tenebrio obscurus* larvae. Pupation in this species appears to be regulated by quite different factors, possibly photoperiod (Cotton, '30). On several occasions, nearly all of our mature *T. obscurus* larvae pupated within a few days of one another. Since few large larvae remained under these circumstances, very few of the pupae were lost by cannibalism. *Tribolium* populations likewise produce "waves" of pupae which "satiate" the remaining cannibalistic adults and larvae. This has been noted as an important survival mechanism in *Tribolium* and may have application elsewhere (Lloyd, '68; Park et al., '68).

might also be a helpful indication of seclusion. The ecology of each species would determine which of these cues would assume importance.

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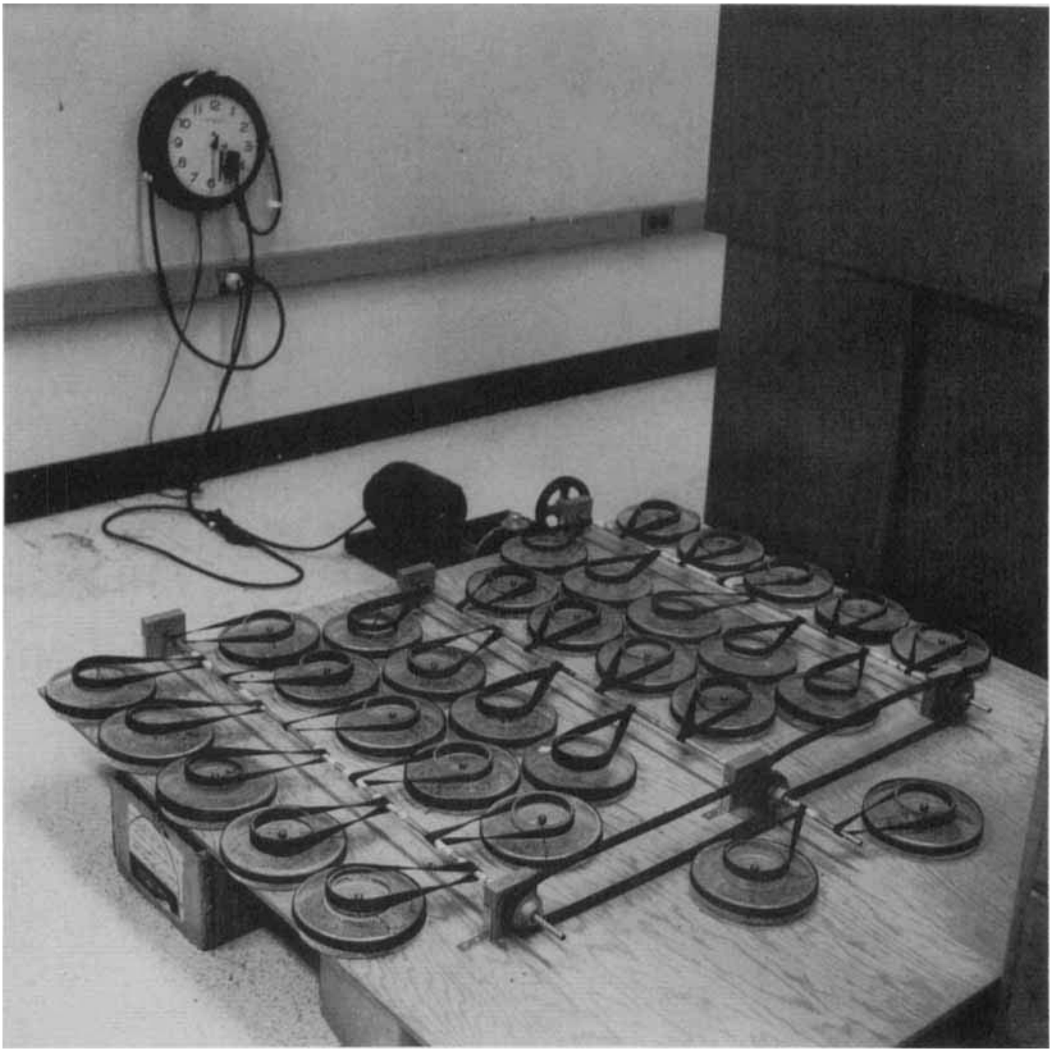

EXPLANATION OF FIGURE

The mechanical stimulation machine. The modified petri dish lids are rotated simultaneously by a variable-speed motor controlled by a timing clock.