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Effect of Copulation and Copulation Time on Female Reproductive Development in Diabrotica virgifera virgifera (Coleoptera: Chrysomelidae)

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EFFECT OF COPULATION AND COPULATION TIME ON FEMALE REPRODUCTIVE DEVELOPMENT IN *Diabrotica virgifera virgifera* (COLEOPTERA: CHRYSOMELIDAE)

Honors Research Project

by

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ABSTRACT

It has been established that a prolonged copulatory period of 3-4 hours is necessary for maximal insemination to occur in the western corn rootworn (WCR). This complete mating period has been suggested to speed ovarian development. The purpose of this study was to determine the relationship between copulation and ovarian development in the WCR. Using 11 and 12 day post-emergence virgin WCR beetles, four female groups of varying copulatory duration were established: (1) 15 min. in copula (2) 1 hr. in copula (3) 2 hrs. in copula (4) a control group mated until natural completion. After copulation, these beetles along with a control group of unmated females were placed in isolated cage vials. Frequency of egg-laying and post-copulation change in female weight was recorded up until the time of dissection. The beetles were then sacrificed between 21-24 days post-copulation and the effects on ovarian development were evaluated with the following criteria: (1) reproductive status (an ovarian rating system) (2) morphometric analysis of ovarian area (using an Olympus C-R Research Image Analyzer with an IBM/PC Microcomputer). The presence of spermatozoa in the spermatheca was also noted at the time of dissection. The results demonstrate significantly larger mean ovarian area and greater mean reproductive status rating ($P<0.05$) in the control group of mated until completion females compared with the control group of unmated females. Egg-laying frequency and mean post-copulation change in weight reflected fully developed ovaries present in the mated until completion beetles and not in the unmated beetles. The results with the groups of varying copulatory duration suggest that spermatozoa may play a role in the development of fully maturated ovaries. In addition, it appears that directly after copulation some factor other than spermatozoa is responsible for significant increases in reproductive development. This other factor appears relatively transient and does not maintain a significantly developed reproductive state past approximately 14 days post-copulation.

Key-words: _Diabrotica virgifera virgifera_, western corn rootworm, Coleoptera, Chrysomelidae, copulation duration, ovarian development, morphometric analysis.

INTRODUCTION:

The western corn rootworm (WCR), Diabrotica virgifera virgifera, and the northern corn rootworn (NCR), Diabrotica barberi, are considered the most serious corn pest species in the United States and Canada with estimated losses at $1 billion annually (Metcalf 1986). The adult beetle stage of both the WCR and the NCR damage corn by feeding on corn silks which can inhibit pollination and thus kernel development. The larvae of both species feed almost exclusively on corn roots and can cause extensive root damage (Levine and Oloumi-Sadeghi 1991).

WCR females have been reported as having a mean fecundity of >1,000 eggs, while NCR females have a mean fecundity of only approximately 274 eggs (Naranjo 1980). The tremendous egg-laying potential of the WCR has been proposed by Levine and Oloumi (1991) as a possible reason to account for the displacement of the NCR by the WCR in many parts of the United States where their ranges overlap. Based on general reproductive characteristics of insects and on what is specifically known about WCR reproduction, the sequence of events in WCR reproduction is thought to occur as follows:

(1) Copulation--an elaborate process lasting 3-4 hrs. (Lew and Ball 1979) during which the male deposits a complete spermatophore, a pear-shaped gelatinous sac (Lew and Ball 1980), inside the female's bursa copulatrix. The spermatophore forms inside the male during copulation and is then transferred into the female. Lew and Ball observed that a spermatophore is transferred to the female WCR between .5 hrs. and 4 hrs. into copulation. The spermatophore encloses the spermatozoa from the male. Lew and Ball's results (1980) also demonstrate that spermatozoa are deposited into the spermatophore inside the female between 1.5 and 4 hrs. into copulation. It has been observed that most WCR females normally mate only once (Cates 1968, Hill 1975, Branson 1977). In addition, it has been shown that female WCR beetles can store sperm for long durations in the spermatheca (>50 days), and that only one mating provides the spermatozoa necessary for the duration of a female's life (Branson and Johnson 1973).

(2) Transfer of Spermatozoa--spermatozoa migrate by an unknown mechanism (Wigglesworth 1974) during the later parts of copulation and post-copulation from the bursa copulatrix to the spermatheca of the female. Lew and Ball (1980) observed female's spermatozoa in the spermatheca as early as 2 hrs. into copulation and as late as 3 days post-mating still in the spermatophore inside the female's bursa copulatrix. Spermatophores inside mated females begin to degenerate five days post-mating and are completely gone by 7 days (Lew and Ball 1980). The spermatheca stores and nourishes the spermatozoa until used for fertilization (Wigglesworth 1974).

(3) Fertilization--has not been studied in the WCR; however, it

is assumed to occur in the WCR as in most insects, i.e., long after copulation and most likely just prior to egg laying (Wigglesworth 1974).

The ovaries of newly emergent female beetles contain only small undeveloped oocytes. Most females naturally mate within the first few days following emergence. In order for fertilization to occur, it is thought that undeveloped oocytes must mature into fully developed eggs. This process is referred to as ovarian development. Hill (1975) suggested that mating apparently speeds ovarian development, and that unmated beetle's ovaries appeared not to develop as rapidly.

This study was conducted to document and determine the relationship between copulation and ovarian development in the WCR.

METHODS:

A non-diapausing strain of Diabrotica virgifera virgifera (Branson 1976) obtained from the USDA Northern Grain Insects Laboratory, Brookings, South Dakota, was used in this study. All of the beetles used emerged on 30 December 1989 and were immediately segregated based on sex in Brookings, South Dakota. These beetles were then sent to the Illinois Natural History Survey, Champaign, Illinois, where the sexes where maintained in separate cages in isolated environmental chambers at 27 degrees celsius and 14L:10D photoperiod,

At days 11-12 post emergence (2/10/1990-2/11), these virgin WCR beetles were mated and four female groups of varying copulatory duration were established: (1) 15 min. in copula (N=10) (2) 1 hr. in copula (N=9) (3) 2 hrs. in copula (N=9) (4) mated until natural copulatory completion (group mean duration= 3 hrs. 14 min. N=9). Copulation times were established by considering the beginning of copulation to be when the male's aedeagus was fully inserted into the female's vagina (Lew and Ball 1980, see also illustrations in Lew and Ball 1979).

After copulation, the female beetles along with one group of unmated females (N=10) were weighed and then caged in isolated vented plastic vials (Naranjo and Sawyer 1987) These WCR beetles were maintained under a temperature of 25 degrees celsius with a 12:12 (L:D) photoperiod. All beetles had access to the diet described by Branson and Jackson (1988) with the addition of the following ingredients to prevent mold: .09% sorbic acid, .18% methyl paraben, .14% mold inhibitor, .23% formaldehyde (37.5% solution). The diet was stored in 3-ml shell vials inserted 1 cm from the top of the rearing vial cages (Naranjo and Sawyer 1987).

Egg-Laying Patterns

The cage vials were inserted through the holes in the lids of plastic boxes. Approximately one-third of each vial was below the surface of the lid. The vials rested on a foam pad on the bottom of the plastic boxes. Each foam pad had a black cloth covering it which was kept moist for the duration of the experiment (See Naranjo and Sawyer 1987) Egg-laying patterns were examined over time by observing the presence or absence of eggs oviposited on the black cloths.

Post-Copulation Changes in Weight

All beetles were weighed immediately after copulation using a digital analytical balance. Each beetle was subsequently weighed an additional five times before dissection. The differences between the five separate post-copulation times and the weight directly after copulation was then determined to evaluate changes in female weight over post-copulation time in relation to duration of copulation.

Reproductive Status and Ovarian Area

All beetles were sacrificed between 21-24 days post-copulation. A dissection was carried out by first crushing a

beetle's head with forceps, and then placing the beetle in a 0.75% NaCl solution under a dissection microscope. The beetle was then oriented with its ventral surface facing upward. A microforceps was used to hold the beetle in place, while a tungsten needle was inserted between the thorax and abdomen. This needle was then manipulated back and forth carefully to cut the connections between the thorax and abdomen so that the abdomen could be pulled free from the rest of the body. A tungsten needle was then run from the anterior end of the abdomen along the boundary between the dorsal and ventral surface to open up the abdomen and expose the ovaries. The reproductive tract was then removed using microforceps and cleaned of fat body with a fine 0000 M. Grumbacher 178 paint brush.

The presence of spermatozoa in the spermatheca was noted at the time of dissection by placing the spermatheca with a drop of 0.75 % NaCl on a slide and crushing it with a cover slip as described by Lew and Ball (1980). The presence or absence of spermatozoa was then determined by placing the slide under a compound microscope and checking for spermatozoa.

The ovaries were first categorized using a 1-5 rating system that Hill (1975) utilized. Stage 1 ovaries have none or very few immature oocytes present. Stage 2 ovaries have more, as well as larger oocytes which occupy less than one-half of the ovary. In stage 3, the ovary has even larger oocytes present which fill more than half of the ovary. Stage 4 ovaries have completely mature eggs present, and it is the stage in which oviposition is considered to occur. In stage 5, the ovary is fully spent and no oocytes are present.

In addition to rating the ovaries according to an arbitrary scale, morphometric analysis techniques were performed to evaluate ovarian development. At the time of dissection, each ovary was photographed using Ektachrome tungsten 160 film by a 35mm camera attached to the dissection microscope. A stage micrometer was also photographed with each dissected ovary. Each ovary was cut free at the lateral oviduct and positioned under the microscope so that the surface opposite the calyx was photographed. The slide film was developed and then analyzed for total ovarian surface area photographed using the following morphometric analysis equipment: an Olympus Corporation C-R Research Image Analyzer with CUE-4 Program along with an IBM/PC Microcomputer and Image Monitor. The photographed stage micrometer was used to calibrate the program. It was found, however, that recalibrating the system for each slide was unnecessary. Calibrating any stage micrometer for a given microscope power photographed, produced the same results as calibrating for the individual slide.

Data Analysis

Selected mean post-copulation weight changes were analyzed using a t-test. Data from ovarian area and reproductive status (ovarian ratings) were analyzed using analysis of variance (ANOVA) techniques to establish the probability of varying copulatory duration groups deviating significantly. In

addition, the means of ovarian area and rating from the unmated females and mated until completion females were compared to other copulatory duration groups using t-tests.

RESULTS:

Presence of Spermatozoa

Analysis of spermatheca contents at the time of dissection revealed the following:

(1) All females that were allowed to mate until natural copulatory completion contained spermatozoa in the spermatheca (N=9).
(2) 66% of the 2 hrs. in copula females contained spermatozoa (N=6), while the remaining 33% did not (N=3).
(3) All of the remaining copulatory duration groups and the unmated control females lacked spermatozoa in the spermatheca.

Egg-Laying Patterns

The presence or absence of eggs deposited on the black cloths was noted daily. Eggs were first observed being oviposited by some females on 19 January (8 days post-copulation). The number of females ovipositing per copulatory group was recorded and daily percentages of females ovipositing calculated (refer to Table 1).

The 2 hrs. in copula females were divided into two groups in table #1 based on the presence of spermatozoa at the time of dissection: 2 hrs. in copula with sperm (2 hrs. W/S, N=6) and 2 hrs. in copula without sperm (2 hrs. W/O, N=3). If the females that were allowed to complete mating are taken to represent normal egg laying, it appears from table #1 that the 2 hrs. W/S females did not differ from this control group in egg-laying pattern. In both groups there was an initial large number of females ovipositing on 19 January through 23 January (9-13 days post-copulation), followed until the time of dissection by a consistent but lower percentage of females laying eggs daily.

The 2 hrs. W/O females and the 1 hr. in copula group (N=10) had some females ovipositing on 19 January through 23 January (9-13 days post-copulation), but at a lower percentage than the 2 hrs. W/S females and mated to completion females. On January 24 (13 or 14 days post-copulation), however, the percentage of females in the 2 hrs. W/O and the 1 hr. in copula females ovipositing decreased dramatically to a level in table #1 similar to that of the unmated control females and 15 min. in copula females. Very few females that were unmated or mated 15 min. in copula oviposited.

Post-Copulation Changes in Weight

Changes in female weight following copulation demonstrate (Figure #1) that both the mated until completion females and the 2 hrs. W/S females had mean increases in weight that were significantly greater than the mean change in weight of the unmated control females ($P<0.05$) at all times measured post-copulation. In addition, comparing the mean change in weight in the mated until completion females and the 2 hrs. W/S females showed no significant differences for all weights recorded ($P>0.05$). The food vials in all cages were changed on 27 January

(17 or 16 days post-copulation). This probably accounts for the increase in weight observed for the 2 hrs. W/S females and the mated to completion females on 28 January (18 days post-copulation for those females).

From figure #1, it is also apparent that the 1 hr. in copula females demonstrate a significant increase in mean weight at day 5.5 post-copulation from the mean weight of the control females ($P<0.05$) which were arbitrarily assigned a copulation date of 10 January and thus a post-copulation date of 6 days. The 1 hr. in copula females were assigned a value of 5.5 days post-copulation because 6 of the females in the group were mated late in the afternoon on 10 January (i.e. six days post-copulation), while the remaining 3 females of the group were mated early morning on 11 January (i.e. five days post-copulation). These results suggest that the 1 hr. in copula females received some sort of stimulus from the male during the 1 hour copulation duration. This would account for the significant increase in weight over the unmated females. In addition, a comparison between the mean weight of the 1 hr. in copula beetles at 5.5 days post-copulation and the mated to completion beetles at day 6 revealed no significant differences ($P>0.05$). Therefore, it appears that a copulation duration of 1 hour elicits the same weight increase that a fully mated female undergoes. It would be expected that the 2 hr. W/O females would have undergo the same increase in weight associated with copulation as the 1 hr in copulation females. Although the mean weight increase following copulation at day 6 post-copulation is lower than the 1 hr. in copula females, a t-test between the two means of these group revealed no significant differences ($P>0.05$). The 2 hrs. W/O group had a very high standard deviation at day 6 post-copulation which was probably associated with the groups small sample size. At day 10 post-copulation, however, the mean increase in weight of the 1 hr. in copula females decreased, as did the large standard deviation of the 2 hrs. W/O females, to a level similar to that of the unmated females and 15 min. in copula females. The 15 min. in copula females showed no significant changes in post-copulation weight throughout the experiment as compared to the unmated females.

OVARIAN AREA AND REPRODUCTIVE STATUS

The results on ovarian area and reproductive status rating are summarized in figures 2 and 3, respectively. T-tests performed for both mean ovarian area and reproductive status rating showed no significant differences ($P>0.05$) between the mated until completion control group and the 2 hrs. W/S group, while t-tests comparing the remaining four groups to the mated until completion group did showed in all cases that the mean ovarian rating and the mean ovarian area was significantly greater ($P<0.05$) in the females allowed to copulate until completion. In addition, t-tests of mean ovarian area and rating comparing the unmated females to the mated to completion females and 2 hrs. W/S females showed in both cases significant differences ($P<0.05$). No significant differences occurred between the other 3 remaining groups compared to the unmated

females with respect to mean ovarian area and rating.

These results suggest that two groups existed based on ovarian area and ovarian rating:

Group I: (labeled a in figures 2 & 3) composed of the control females mated until natural completion and the female mated 2 hours which contained spermatozoa at the time of dissection.

Group II: (labeled b in figures 2 & 3) composed of the remaining females that had a copulation duration of 2 hours and lacked spermatozoa, plus all other copulatory duration groups and the control females which were unmated.

T-tests comparing the means for both ovarian area and reproductive status between the two groups in group I revealed no significant differences ($P>0.05$). In addition, analysis of variance (ANOVA) tests were conducted for mean ovarian areas and reproductive status ratings for female copulatory duration in group II. These also revealed no significant differences ($P>0.05$). Therefore, the existence of two separate groups was suggested.

GROUP	DATE 2/19	2/21	2/22	2/23	2/24	2/25	2/26	2/27	2/28	3/1	3/2	3/3
Mated until Completion	88	100	00	56	22	22	22	33	22	33	00	22
2 Hrs. in copula w/sperm	57	86	50	50	17	50	33	50	50	00	33	33
2 Hrs. in copula w/o sperm	33	33	33	66	00	00	00	00	00	00	00	00
1 Hr. in copula	44	44	33	56	11	00	00	11	00	11	00	00
15 Min. in copula	00	10	00	20	10	00	00	00	00	10	00	00
unmated	00	10	10	00	00	00	00	00	00	00	00	00

Table #1. The relationship between copulation time and percentage of females laying eggs in WCR mated 10 or 11 January, 1990. The mated for 2 hrs. group has been divided into two separate groups to demonstrate the difference in percentage of females laying eggs between those individuals that contained spermatozoa at the time of dissection and those that did not. Mated until completion N=9, 2 hrs. in copula w/sperm N=6, 2 hrs. in copula w/o sperm N=3, 1 hr. in copula N=9, 15 min. in copula N=10, unmated N=10.

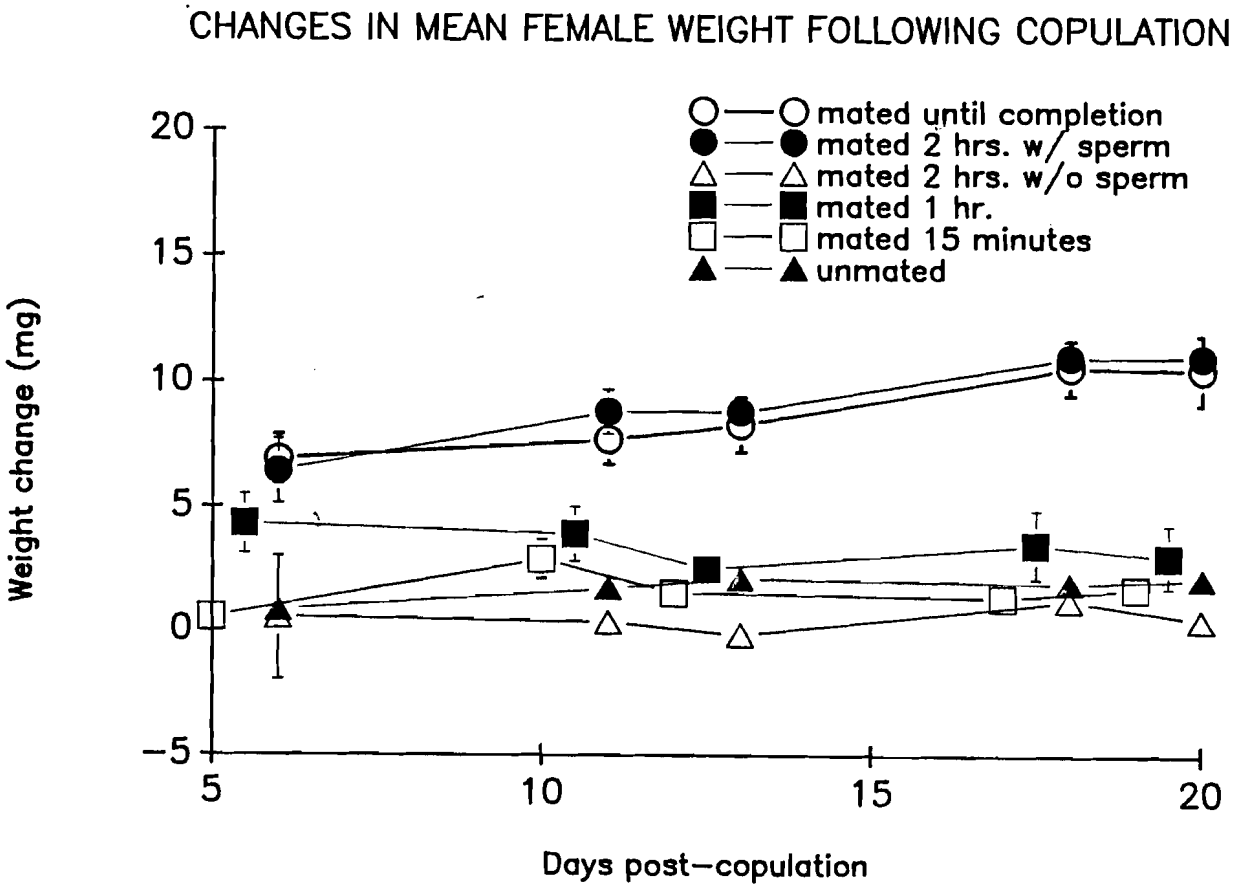

Figure #1. The relationship between copulation time and mean change in post-copulation weight of WCR beetles weighed over a period of 20 days after copulation. The mated for 2 hrs. group has been divided into two separate groups to demonstrate the difference in mean post-copulation change in weight between those individuals in that group that had spermatozoa present in the spermatheca at the time of dissection and those that did not. Mated until completion N=9, mated 2 hrs. w/sperm N=6, mated 2 hrs. w/o sperm N=3, mated 1 hr. N=9, mated 15 minutes N=10, unmated N=10.

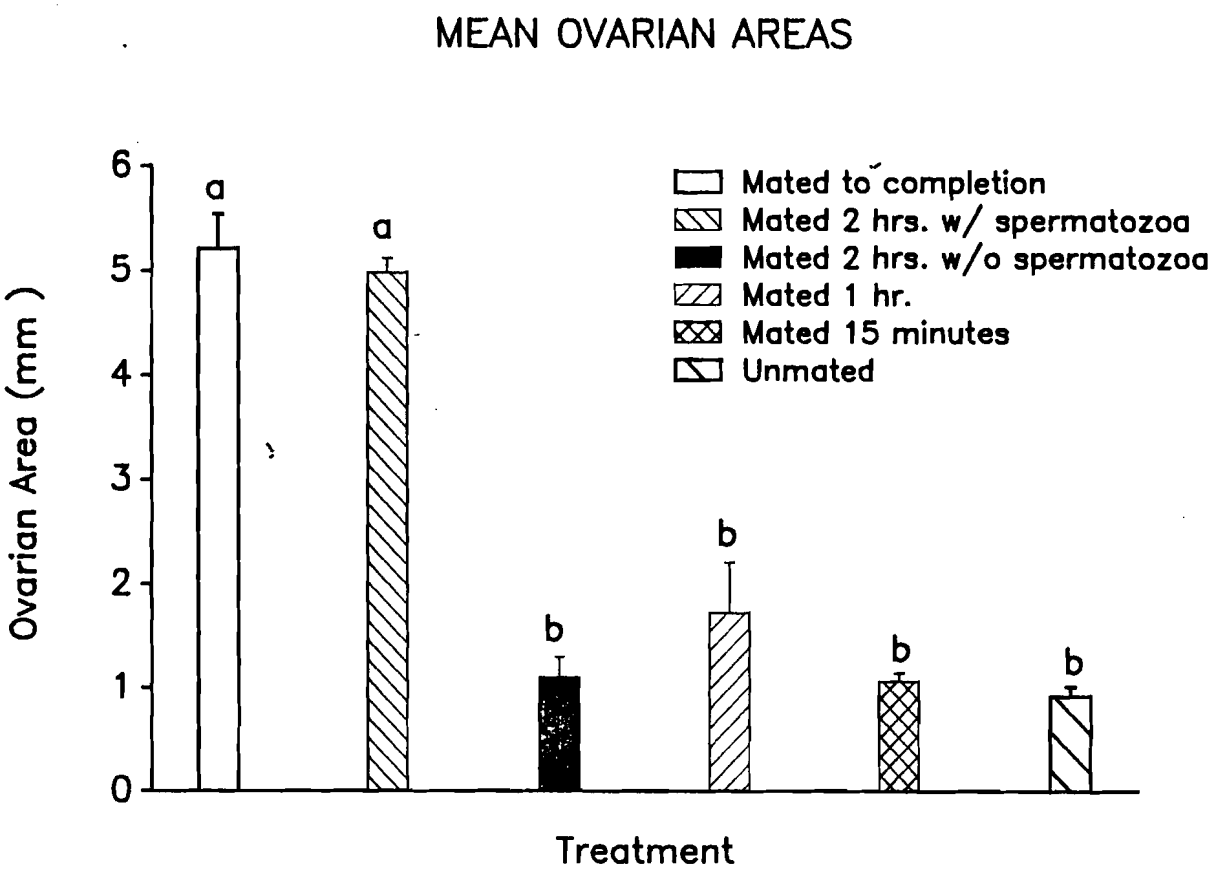

Figure #2. The relationship between copulation time and mean ovarian area in dissected 32-36 day post-emergence (21-24 day post-copulation) western corn rootworms, Diabrotica virgifera virgifera. The mated for 2 hrs. group has been divided into two separate groups to demonstrate the difference in ovarian area between those that had spermatozoa in the spermatheca and those that did not (a= $P<0.05$ to umated group and $P>0.05$ to mated to completion group; b= $P<0.05$ to mated to completion group and $P>0.05$ to unmated group). Mated until completion N=9, mated 2 hrs. w/sperm N=6, mated 2 hrs. w/o sperm N=3, mated 1 hr. N=9, mated 15 minutes N=10, unmated N=10.

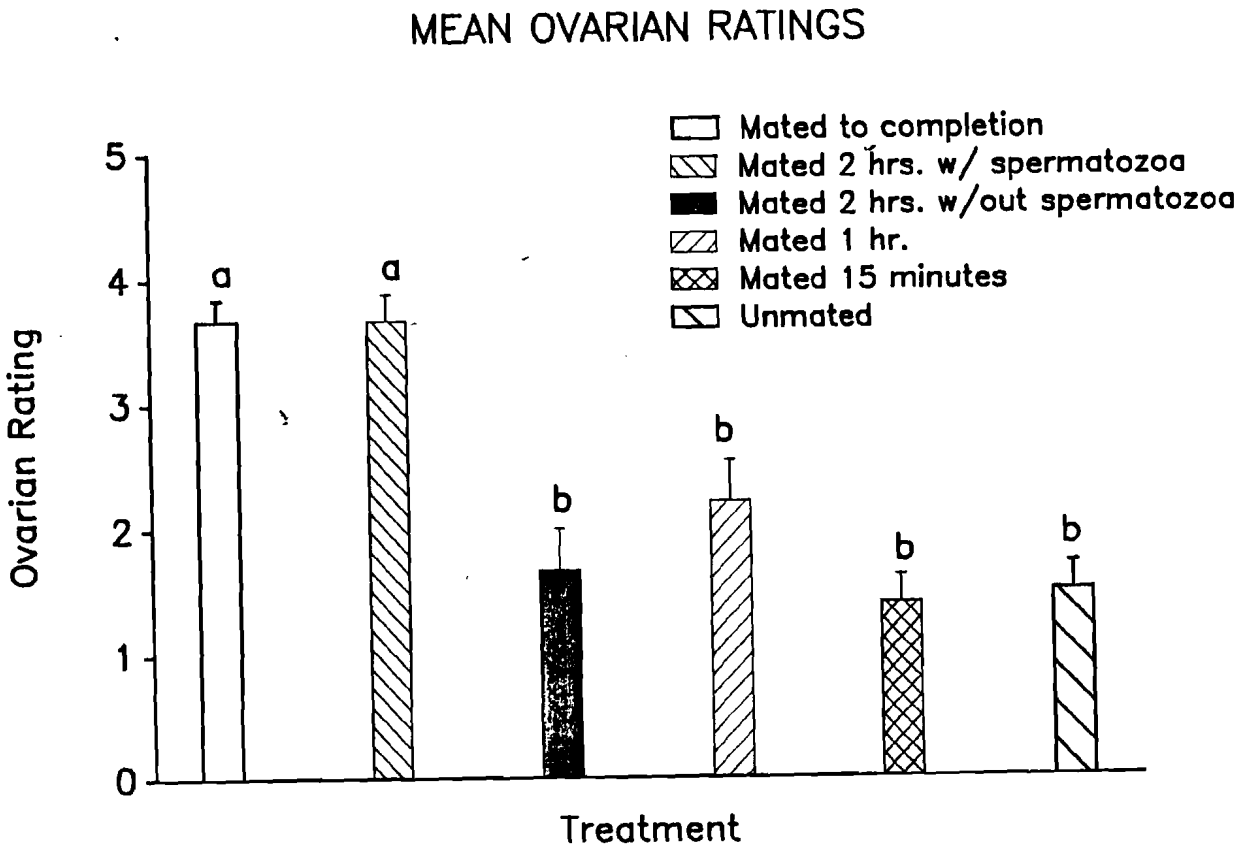

Figure #3. The relationship between copulation time and mean ovarian rating (reproductive status) in dissected 32-36 day post-emergence (21-24 day post-copulation) western corn rootworms, Diabrotica virgifera virgifera. The mated for 2 hrs. group has been divided into two separate groups to demonstrate the differences in ovarian rating between those individuals in that group that had spermatozoa present in the spermatheca and those that did not (a= $P<0.05$ to unmated group and $P>0.05$ to mated to completion group; b= $P<0.05$ to mated to completion group and $P>0.05$ to unmated group). Mated until completion N=9, mated 2 hrs. w/sperm N=6, mated 2 hrs. w/o sperm N=3. mated 1 hr. N=9, mated 15 minutes N=10, unmated N=10.

DISCUSSION

Two new criteria were developed in this study to evaluate female reproductive development in the WCR: (1) post-copulation changes in female weight and (2) ovarian area using morphometric analysis equipment. These criteria seemed to correlate very well with traditional methods of analysis of reproductive development, which are respectively, percentage of females laying eggs and reproductive status. The use of post-copulation changes in weight for evaluating reproductive development needs to be looked into further. In this study the number of eggs laid by females was not recorded, and this is an important factor that would need to correlate with post-copulation changes in weight for this new criteria to be useful in accurately analysing female reproductive developmetn. Ovarian area using morphometric analysis equipment, appears to be a very accurate way to quantify ovarian development in the laboratory. There seems to be no agreement on a single reproductive status rating system, and ovarian area may be an excellent alternative.

The results from this study strongly suggest that a complete copulation period speeds ovarian development. The unmated females used were dissected 36 days after emergence, and arguably no ovarian development appeared to occur in this time period. Hill (1975) reported that out of 3 WCR females that were left unmated in the laboratory 2 oviposited in their lifetime (one oviposited 22 days post-emergence and the other 41 days). Hill's sample size was very small, however, and it might be appropriate in a future study to use a larger sample size of unmated WCR females and record the degree of ovarian development that occurs in the absence of mating for the duration of an unmated female's lifetime.

Higher mean ovarian ratings and greater ovarian areas reflect the presence of fully developed oocytes in the ovaries. Therefore, the results on ovarian area and reproductive status from the females mated for 2 hours suggest that spermatozoa (or something directly associated with the presence of spermatozoa) are likely involved in the signal which causes undeveloped oocytes to develop to fully maturated eggs at around days 20-24 post-copulation. Spermatozoa have been suggested to speed egg development in other insects (Wigglesworth 1974), and it might be interesting to compare the evolutionary relationship between insects which demonstrate this characteristic.

The egg-laying pattern data and post-copulation weight results reveal that if the presence of spermatozoa is also a signal for reproductive development directly after copulation, it is not the only one. Lew and Ball (1980) reported that WCR females at the earliest begin to receive spermatozoa from the male after 1.5 hours into copulation. They also observed that during the first hour of copulation the female receives a large quantity of a milky-colored gelatinous substance deposited into the anterior lobe of the bursa copulatrix. Therefore, the significant differences in egg-laying patterns and mean changes

in post-copulation weight between the females mated for 1 hour and the control unmated females suggests that spermatozoa are most likely not the trigger and that either the physical tactile stimulus of copulating 1 hour or some factor present in the substance Lew and Ball described probably stimulates reproductive development. The dramatic decrease in number of females in the 1 hr. group ovipositing at approximately 13 days post-copulation and the decrease in post-copulation weight at day 10.5 suggests, however, that the stimulus which caused initial reproductive development was transient. In addition, it appears, based on ovarian area and reproductive status ratings, that at the time of dissection these 1 hr. in copula female's ovaries had reverted back to the undeveloped state with no significant differences from the unmated females.

From the egg-laying patterns and changes in post-copulation weight, it appears that the females without sperm at the time of dissection which were mated for 2 hrs. may not have received spermatozoa during copulation. The results from this group on egg-laying and post-copulation changes in weight seem to correlate well with the 1 hr. in copula group which should have never received sperm. The number of females in the 2 hrs. W/O group was so small, however, that the experiment should probably be repeated with a larger number of females present in this group. There is no evidence that a copulation duration of 15 min. has any effect on reproductive development in the WCR.

Branson and Guss (1984), discuss a method to sterilize male WCR, and this might produce an interesting future study with copulatory duration groups that would support the role of spermatozoa in speeding egg development. It would be necessary before conducting this study, however, to evaluate what is effected in the male by the sterilization process.

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