The *Journal of Cotton Science 14:191–198 (2010)*
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# ARTHROPOD MANAGEMENT AND APPLIED ECOLOGY

## Mating Behavior of Wild *Helicoverpa zea* (Lepidoptera: Noctuidae) Males with Laboratory Females

Carlos A. Blanco*, Douglas V. Sumerford, Juan D. López, Jr., Gerardo Hernández and Craig A. Abel

**ABSTRACT**

**Studies conducted with the offspring of field-captured moths offer reliable indicators of natural behavior and response to tests. Utilizing techniques that can maximize mating frequency to obtain the greatest genetic representation of field-collected males is critical for developing representative data from laboratory studies. We studied techniques to maximize the potential genetic diversity of offspring from pheromone trap-captured *Helicoverpa zea* (Boddie) males (wild) enclosed with laboratory females for one to five days, compared with the enclosure of laboratory females and males. Females mated with wild males obtained their highest number of copulations, produced the largest proportion of fertile eggs, and lived longer when males were removed from their enclosure after two days. Higher female mortality was also observed after this time, further decreasing the potential genetic diversity of the offspring. Experiments conducted with two moth crowding ratios (30 and 60) for same-sex groupings indicated that copulations carried important negative longevity consequences for both sexes. Studies conducted with the offspring of 2-day moth enclosures may offer the greatest genetic diversity while reducing mortality of parental moths.**

The bollworm (corn earworm, tomato fruitworm) *Helicoverpa zea* [Boddie], is a pest of more than 30 cultivated plants and also has at least 76 wild hosts (Blanco et al. 2007). Partial control of this pest has relied on the adoption of genetically engineered (transgenic) corn (*Zea mays* L.) and cotton (*Gossypium hirsutum* L.) expressing insecticidal Cry proteins from the bacterium *Bacillus thuringiensis* Berliner (Bt). Because this pest develops in these two crops, and the increasing adoption of transgenic varieties in the North American agricultural landscape (James, 2006), the possibility of bollworm resistance developing to Bt is created (Tabashnik et al., 2003, 2008). The delay of *H. zea* resistance evolution to insecticidal Bt proteins is considered in 'the public good' by the U. S. Environmental Protection Agency (Matten and Reynolds, 2003), and this agency requires that companies commercializing Bt crops provide resistance management, and resistance monitoring plans.

Shifts in bollworm susceptibility to Bt proteins are usually measured by conducting laboratory bioassays (Ali et al., 2006; Andow et al,. 2008). Male moths collected from pheromone traps have been used to facilitate conducting these studies (Blanco et al., 2004). Although these individuals only represent half of the genetic diversity of this pest in the field, it is a simple way of obtaining moths that represent genotypes prevalent in field populations. Because the frequency of matings by bollworm under laboratory conditions has been documented to be relatively low (Jones et al., 1979; Carpenter et al., 1989), it is very important to find methods that can maximize mating frequency to obtain the greatest genetic representation of field-collected males. For example, mating frequency for the tobacco budworm (*Heliothis virescens* F.) significantly increases when mating feral males and laboratory females, as compared with copulations of feral males and females (Blanco et al., 2006, 2009).

Pheromone-captured bollworm males are of unknown age, plant host origin, disease susceptibility, etc. These factors and others, potentially affect their propensity to mate and contribute to the genetic diversity of offspring. The first problem is the high moth mortality (5 – 100%) during overnight delivery of the samples from the field to the laboratory (Blanco, unpublished). After live moths are received, mating success under laboratory conditions is influenced by several factors. Temperature and relative

C.A. Blanco, USDA-APHIS BRS, 4700 River road, Riverdale, MD 20737; D.V. Sumerford, USDA-ARS CICGRU, Iowa State University, Ames, IA 50011; J.D. López, Jr., USDA-ARS APMRU, 2771 F&B Road, College Station, TX 77845; G. Hernández, CINVESTAV, Sección de Teoría y Metodología de la Ciencia, Apartado Postal 14-740, México, D.F. 07000, Mexico; C.A. Abel, USDA-ARS CICGRU, Iowa State University,Ames, IA 50011
*Corresponding author: carlos.a.blanco@aphis.usda.gov

humidity affect the longevity and fecundity of moths and egg hatch (Ellington and El-Sokkari, 1986), and reproductive maturity and male to female ratios affect fecundity, oviposition, and adult longevity (Colvin et al., 1994; Jones et al., 1979). These important obstacles have the potential for lowering mating frequency and in turn decreasing the genetic diversity of the offspring resulting from field-collected males.

The goal of this study was to investigate the temporal dynamics of mating and oviposition patterns of *H. zea* reared in the laboratory. Data generated from this study could be used to develop an optimal strategy for mass-mating conditions and offspring production with the purpose of maximizing the genetic contribution of field-collected males.

## MATERIALS AND METHODS

Our study used laboratory reared male and female *H. zea* adults and field-collected moths captured in pheromone traps (hereafter referred to as wild). Wild males were collected near agricultural fields in Florida (Gasden County), Georgia (Decatur, Mitchell, Tift, and Appling counties) and Texas (Brazos County), and shipped in 0.47 L (pint-size) carton containers, (Neptune Paper Products, Inc., Newark, NJ, 8.5 cm Ø) using multiple overnight deliveries to USDA-ARS in Stoneville, Mississippi. Wild males per location were immediately mass enclosed with 24-36 h-old laboratory (King et al., 1985) females, at a ratio of 30♀ : 30♂. Concurrently, a laboratory moth trial (30♀ : 30♂, 24-36 h-old, referred to as laboratory) following the same method was set-up for comparison.

Mating arenas were 3.7 L (gallon-size) carton containers, (Neptune Paper Products, Inc., Newark, NJ, 17.0 cm Ø) covered on top with Batist cloth (Zweigart, Piscataway, NJ) and a sponge (4 x 6 x 1.5 cm) saturated with 10% sucrose solution was placed inside each container. The cloth was changed daily and the sponge was re-saturated, as needed, at this same time. Mating arenas were maintained in an incubator at 27 ±1°C, 75 ±10% RH and 14:10 h of light and darkness (concurrent with natural spring-summer light period of Mississippi) for 1 to 5 days (exposure time). Each arena was placed in a refrigerator (3°C) for a few minutes to diminish moth activity. Living and dead moths were counted, all males discarded and ≤25 females (depending on female mortality) were transferred individually to 470-mL containers with a plastic cup (37-mL [No. T-125 Solo plastic soufflés, Urbana, IL]) containing 15 mL of 10% sucrose solution and a tissue (EX-L, Kimwipes, Roswell, GA) stuffed into the cup to prevent the moth from drowning. The top of the container was covered with a piece of cloth. These oviposition containers were randomly rotated daily between shelves inside an incubator maintained under the previously described environmental conditions. Cloths from the oviposition containers containing eggs were changed daily for 6 days, and the number of eggs oviposited on cloth was assessed; female mortality was also monitored. Cloths with eggs were individually placed in plastic bags (No. 94600, Ziploc, Crawfordsville, IN) and maintained for up to 6 days in an incubator under the same previously described environmental conditions. Signs of fertilized eggs (dark-brown color eggs to hatched larvae) were recorded for each individual female for 6 consecutive days. Living and dead females were dissected at the end of the experiment to determine the number of spermatophores present.

To assess the possible role that crowding might have had on moth mortality, as well as the potential deleterious effect of mating on longevity, 30 and 60 same-sex 24-36 h-old laboratory moths were maintained under the previously described conditions for 5 days in 3.7 L containers. Mortality was assessed daily. This procedure was repeated four times in different dates.

Analyses were performed as randomized complete blocks. Each block was a temporal replicate of the experiment. Independent variables were origin of the male moths (laboratory vs. wild) and the length of time males and females were exposed to one another ("exposure time"). Restricted-maximum likelihood estimates of sources of variance were used to perform the two-way analysis of variance (ANOVA). Analyses were performed in Proc Mixed of SAS, version 8.2 (Littel et al., 1996). Fixed sources of variance included the origin of males, exposure time, and the interaction between these effects. Block effects were considered as a random source of variance. Least-square means and their standard errors are represented in text and graphs. Differences between wild vs. laboratory males were tested for each level of exposure time via the slice option of the LSmeans statement. Differences are represented within graphs (see figure captions). Dependent variables included the number of spermatophores per living female, the proportion of living females that were mated, the number of fertile eggs per living female, and the percentage of dead females.

Because mortality measurements were taken for each mating arena for five consecutive days (cumulative mortality), a repeated-measures design was used in the ANOVA (Proc Mixed, SAS, 2001). A heterogeneous, first-order autoregressive structure best modeled the covariance among subjects. Effects in the model included the four treatments described above, time (day 1 – day 5), and their interaction. Significant differences for the treatment effect were partitioned into three orthogonal contrasts: (1) comparison of cumulative percentage mortality in mating arenas with vs. without the opposite sex, (2) comparison of cumulative percentage mortality for 30 vs. 60 moths in single sex arenas, and (3) comparison of cumulative percentage mortality in mating arenas containing wild vs. laboratory males. Time effects were also completely partitioned into polynomial functional responses (i.e., linear, quadratic, cubic and remaining effects).

## RESULTS

**Mating dynamics:** The proportion of *H. zea* females that mated (evidenced by the presence of one or more spermatophores in their bursa copulatrix) varied with the origin of the males ($F = 214.54$; $df = 1, 27$; $P < 0.0001$). Laboratory females mated at a greater proportion with laboratory males (93.7±3.5%) than with wild males (36.5±3.5%). The amount of time males and females were exposed to one another ($F = 1.52$; $df = 4, 27$; $P = 0.2250$) and the interaction between origin × exposure time ($F = 0.95$; $df = 4, 27$; $P = 0.4523$) did not significantly affect the proportion of females that had mated (Figure 1).

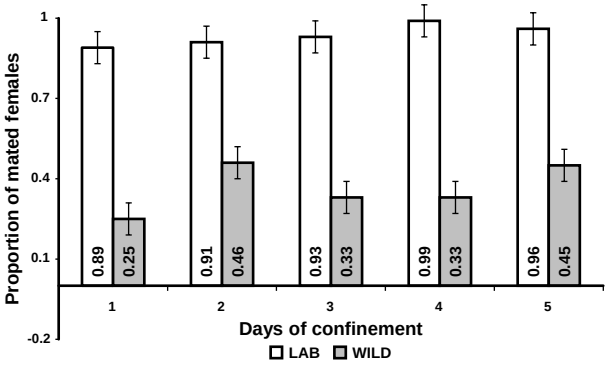


**Figure 1. Proportion of *Helicoverpa zea* females containing one or more spermatophores in their bursa copulatrix (mated) after 1-5 days of confinement with laboratory or wild (pheromone trap-captured) males. Bars represent mean ± SEM.**

The number of spermatophores per female significantly varied with the origin of the males ($F = 267.40$; $df = 1, 30$; $P < 0.0001$), the amount of time males and females were exposed to one another ($F = 9.21$; $df = 4, 30$; $P < 0.0001$), and the interaction between origin and exposure time ($F = 6.08$; $df = 4, 30$; $P < 0.0001$). The average number of spermatophores when females had access to laboratory males increased with exposure time to 3 days and leveled at ~2.0 thereafter (Figure 2). Conversely, females in the presence of wild males averaged less than one spermatophore for all exposure periods and there were no trends with the exposure periods.

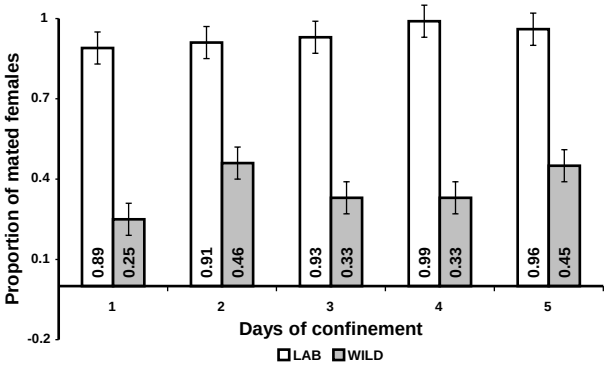


**Figure 2. Average number of spermatophores in the bursa copulatrix of *Helicoverpa zea* females confined for 1 to 5 days with laboratory or wild (pheromone trap-captured) males. Bars represent mean ± SEM.**

The number of fertile eggs per living *H. zea* female was significantly affected by the amount of time females were exposed to males ($F = 6.84$; $df = 4, 27$; $P = 0.0006$) and the source of males ($F = 48.53$; $df = 1, 27$; $P < 0.0001$) (Figure 3). Differences among exposure times on egg production were not dependent on the origin of males ($F = 0.96$; $df = 4, 27$; $P = 0.4465$). Females from mating arenas containing wild males averaged significantly fewer fertile eggs (27.3±5.2) than when the arenas contained laboratory males (70.7±5.2). Egg production was greatest during the first three days of exposure between males and females (exposure days 1-3 vs. days 4-5; $F = 24.65$; $df = 1, 27$; $P < 0.0001$). There were no other differences among exposure times for egg production (remaining exposure time effects; $F = 0.90$; $df = 3, 27$; $P = 0.4523$).

Mortality of *H. zea* females varied with the amount of time males and females were exposed to one another ($F = 6.03$; $df = 4, 27$; $P = 0.0013$) (Figure 4). Although the mortality of females averaged across exposure times was not significantly affected by the source of male

moths (F = 1.39; df = 1, 27; F = 0.2494), differences in female mortality in wild vs. laboratory males varied with exposure time (F = 3.44; df = 4, 27; P = 0.0215).

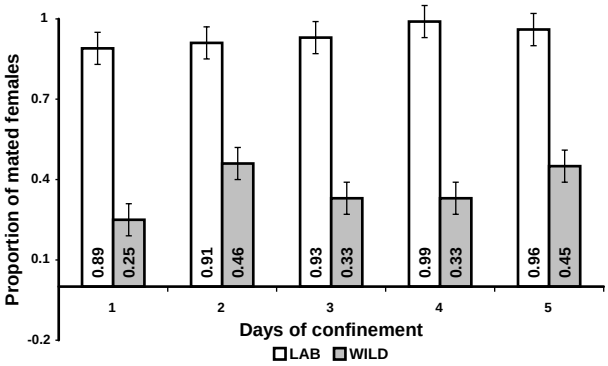


**Figure 3. Average number of fertile eggs oviposited over a 6-day period by *Helicoverpa zea* females confined for 1-5 days with laboratory or wild (pheromone trap-captured) males. Bars represent mean ± SEM.**

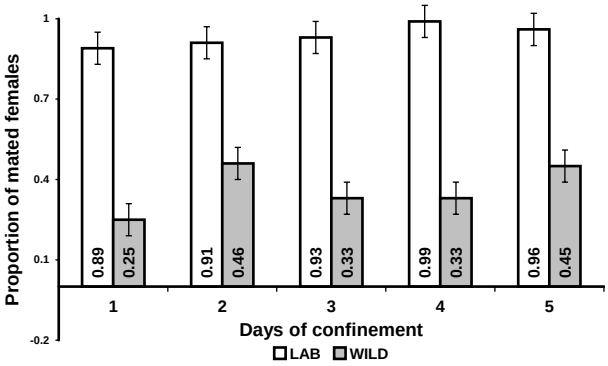


**Figure 4. *Helicoverpa zea* female mortality when confined for 1-5 days with laboratory or wild (pheromone trap-captured) males. Bars represent mean ± SEM.**

**Effects of crowding and mating on adult mortality:** The cumulative mortality of *H. zea* females was affected by wild or laboratory males, time, and their interaction (Figure 4, Table 1). There was no difference for cumulative mortality averaged over time when females were enclosed with laboratory males (22.0±4.1) vs. wild males (14.7±4.1). In the absence of males, there was no difference in mortality averaged over time in mating arenas with 30 females (9.8±4.1) vs. 60 females (9.5±4.1). The cumulative increase in mortality across time was best described by a linear response (Table 1). The dependence of treatment differences and time was due to different temporal responses of mating arenas with vs. without males, and different temporal responses in mating arenas with wild vs. laboratory males (Table 1; Figure 4).

The cumulative mortality of *H. zea* males was also affected by male origin, time, and their interaction (Table 2). Males not having access to females suffered less mortality than males exposed to females (Figure 5; Table 2). There were also significant differences between wild (44.8±4.6) vs. laboratory males (27.2±4.6) in cumulative mortality averaged over time when they had access to females. In the absence of females, there was no significant difference in mortality averaged over time in mating arenas with 30 males (14.7±4.4) vs. 60 males (4.6±4.4). The cumulative increase in mortality across time was best described by a linear response (Table 2).

**Table 1. Repeated-measures ANOVA for cumulative percentage mortality of *H. zea* females.**

| Source [z] | ndf | ddf | F | P |
|---|---|---|---|---|
| Treatment | 3 | 13.1 | 4.93 | 0.0167 |
| With vs. Without Females | 1 | 13.1 | 10.52 | 0.0064 |
| Without Males: 30 vs. 60 Males | 1 | 13.1 | 2.63 | 0.1289 |
| With Females: Wild vs. Laboratory Males | 1 | 13.1 | 1.64 | 0.2231 |
| Time | 4 | 20.1 | 25.76 | <0.0001 |
| Linear | 1 | 19.4 | 80.78 | <0.0001 |
| Quadratic | 1 | 23.7 | 4.81 | 0.0384 |
| Cubic | 1 | 24.8 | 0.11 | 0.7404 |
| Remaining | 1 | 34.9 | 1.64 | 0.2094 |
| Treatment x Time | 12 | 25.1 | 5.58 | 0.0001 |
| (With vs. Without Males) x Time | 4 | 20.1 | 7.43 | 0.0008 |
| (Without Males: 30 vs. 60 Females) x Time | 4 | 20.1 | 2.68 | 0.0611 |
| (With Males: Wild vs. Lab Males) x Time | 4 | 20.1 | 6.99 | 0.0011 |

**[z] Effects of mating-arena treatment, time, and their interaction were partitioned into *a priori* orthogonal contrasts to further explore causes of significance.**

**Table 2. Repeated-measures ANOVA for cumulative percentage mortality of *H. zea* males.**

| Source | ndf | ddf | F | P |
|---|---|---|---|---|
| Treatment | 3 | 13.4 | 19.76 | <0.0001 |
| With vs. Without Females | 1 | 13.4 | 51.95 | <0.0001 |
| Without Males: 30 vs. 60 Males | 1 | 13.4 | 0.09 | 0.7656 |
| With Females: Wild vs. Laboratory Males | 1 | 13.4 | 7.22 | 0.0183 |
| Time | 4 | 18.9 | 46.05 | <0.0001 |
| Linear | 1 | 28.4 | 128.42 | <0.0001 |
| Quadratic | 1 | 17.7 | 0.95 | 0.3437 |
| Cubic | 1 | 29.4 | 0.08 | 0.7821 |
| Remaining | 1 | 15.7 | 0.74 | 0.4024 |
| Treatment x Time | 12 | 23.2 | 11.89 | <0.0001 |
| (With vs. Without Males) x Time | 4 | 18.9 | 33.42 | <0.0001 |
| (Without Males: 30 vs. 60 Females) x Time | 4 | 18.9 | 0.15 | 0.9591 |
| (With Males: Wild vs. Lab Males) x Time | 4 | 18.9 | 2.87 | 0.0515 |

**Effects of mating-arena treatment, time, and their interaction were partitioned into *a priori* orthogonal contrasts to further explore causes of significance.**

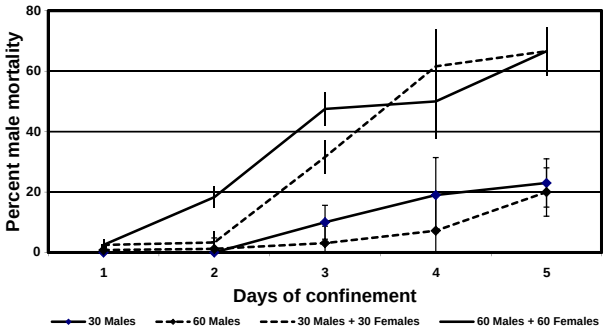


**Figure 5. *Helicoverpa zea* male mortality when confined for 1-5 days with laboratory females. Lines represent mean ± SEM.**

## DISCUSSION

*H. zea* is an insect that does not copulate with high intensity under laboratory (Callahan, 1962; Jones et al., 1979; Ellington and El-Sokkari, 1986; Colvin et al., 1994) or field conditions (Hendricks et al., 1970; Stadelbacher and Pfrimmer, 1973; Latheef et al., 1991). Understanding the main factors that reduce their mating frequency under mass mating conditions can allow a better use of an important resource such as pheromone trap-captured males. This information could aid in obtaining the highest genetic diversity of their offspring and the segregation of rare alleles in the $\geq F_1$ generation. A commonly used method for estimating moth copulations is dissecting female's bursa copulatrix and counting spermatophores (Jones et al., 1979; Carpenter et al., 1989; Blanco et al., 2006) after they have been exposed to males for a fixed number of days. This technique assesses the number of potential males that copulated, but since bollworm moths can copulate more than once, the increment of these male structures inside the bursa copulatrix gives an indication of the mating frequency. Thus, it does not mean that each copulation occurred with a different mate.

In this study, wild *H. zea* male copulation peak occurred on the second enclosure day (46% of females containing ≥ 1 spermatophores) while laboratory males achieved 89% copulation on the first day (Figure 1). This high proportion of copulations found in the laboratory moth matings demonstrates that females utilized in this study were highly sexually receptive. Laboratory *H. zea* moths copulated with a constant increment until the third mating day, a trend not repeated for wild-laboratory moth copulations (Figure 1). The average number of wild-male spermatophores per female reached 0.55 on the second day, while a greater average of spermatophores per female (2.04 – 2.09) was obtained from the third and fourth day in the laboratory matings (Figure 2); this is a day or two earlier than the peak copulation of 2.4 spermatophores / ♀ on the fifth day from a study conducted by Jones et al. (1979). As comparison, the number of spermatophores found in *H. zea* females captured in the field had more than two (Stadelbacher and Pfrimmer, 1973; Latheef et al., 1991). This high frequency of copulations reported here, similar to previously published reports of feral females, may indicate that genetics (paternity) might be switching every other day in the bollworm population. If

sperm precedence, the act of the subsequent sperm canceling the previous insemination, depends on the relative competitiveness of every ejaculation (Carpenter, 1992 but also see Blanco et al., 2008), this indicates that offspring paternity may be changing frequently.

Transferring only sperm, but not spermatophores during mating, has been documented in mated *H. virescens* (Henneberry and Clayton, 1985). The number of copulated females and the proportion of females that laid fertile eggs are important factors to consider when assessing the males' genetic diversity under mass mating conditions The trend observed for mated females and average number of spermatophores per female (Figures 1 and 2), together with the proportion of fertile females (Figure 6), gives a more complete picture of potential offspring production and diversity. In this study, the transfer of sperm without spermatophores occurred in 9.4% of the cases when these laboratory females mated with wild males and 1.5% when mated with laboratory males. The high proportion of fertile females with spermatophores (Figure 2) indicates that counting these structures, as suggested by Henneberry and Clayton (1985), is a reliable method of assessing mating frequency.

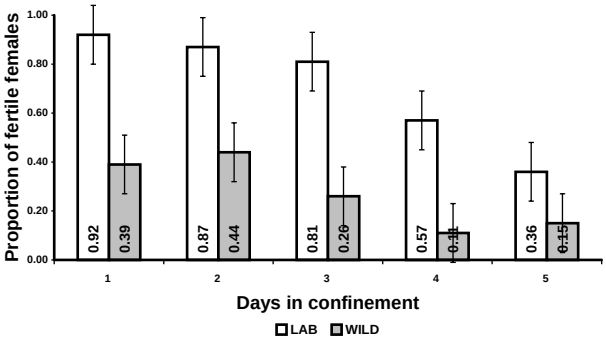


**Figure 6. Proportion of fertile *Helicoverpa zea* females confined for 1-5 days with laboratory or wild (pheromone trap-captured) males. Bars represent mean ± SEM.**

Highest fertile egg oviposition, when incorporated into the overall mating parameters is another important piece of information to assess maximum parental genetic diversity. Fertile egg production (Figure 3), the proportion of copulated females (Figure 1), and the proportion of females laying fertile eggs (Figure 6) closely correspond with each other, indicating that during the second day of enclosure, the offspring might have the greatest parental diversity. It is important to consider that paternal diversity is implied since typically one male only copulates a limited number of females (usually one) per night (Quaintence and Brues, 1905). Therefore, the increment change in the frequency of copulated females was attributed to matings from different males and not to the isolated cases of a male having multiple copulations daily. Also, females that are inseminated earlier in their life, as in the case of this study, utilize a bigger part of their stored energy for egg production (Proshold et al., 1982) and this in turn might be reflected in increased abundance of fertile eggs.

Another important aspect of the biology of this insect is the effect that copulation has on decreasing longevity (Jones et al., 1979). Sperm competition and secondary chemicals introduced by males during copulation have a negative effect on female longevity. Also in males, spermatophore, sperm and secondary chemistry production reduces their life span (Brookes, 2001). Figures 4 and 5 represent the trend of high mortality in both sexes as copulations increase. This information suggests that to obtain maximum genetic diversity and greatest female egg production, the offspring of wild males and laboratory females mass mated for two days should be used. These findings are relevant since offspring with greater genetic diversity may carry rare alleles, important for the detection of *Bacillus thuringiensis* resistance. Also, this information applies for the optimal reproduction of bollworm reared in laboratories. Removing males from the mating containers after two days of enclosure, although a labor intensive process, can enhance the longevity of fertile females, which in turn may produce a more genetically-diverse offspring, delaying inbreeding and the common genetic 'bottle neck' frequently encountered with bollworms reared in laboratories.

## ACKNOWLEDGEMENT

The authors wish to thank Drs. Randy J. Coleman and J. T. Vogt and three anonymous reviewers for their comments made to the manuscript.

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