

**Paternity Allocation in a Mutant *Heliothis virescens*<sup>1</sup> Colony**

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**Abstract.** Tobacco budworm, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), females can copulate multiple times creating the possibility for sperm competition. We used a colony lacking wild pigmentation on the wings (albino-type) for an experiment in which females double mated. Females copulated 2 days apart with two, 2-day-old males, one albino-type and one wild-type, or in the opposite sequence. A third of the females produced offspring from the first mate, and this group was significantly biased toward producing albino-type compared to wild-type progeny. A fourth of the females produced offspring from the second male exclusively and within this group was a significant bias toward wild-type compared to albino-type progeny. Almost half of the females produced offspring sired in equal proportions by both males simultaneously or in alternated paternities throughout all the reproductive life. These results suggest that regardless of the order in which moths mated, wild-type sperm had potential superior competitiveness. Therefore, sperm precedence is not the main driving force behind the paternity allocation mechanism in this strain of tobacco budworm.

**Resumen.** Las hembras del gusano tabacalero, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), tienen la capacidad de copular varias veces creando la posibilidad de que haya competencia entre el esperma dentro de ellas. Utilizamos una colonia de este insecto la cual no expresa la pigmentación normal en las alas de las palomillas normales (tipo albino) en un experimento en el cual las hembras tuvieron dos cópulas con dos días de separación, con dos machos de dos días de edad, uno de ellos del tipo albino y el otro del tipo normal así como otros tratamientos en los que la secuencia de machos fue contraria. Una tercera parte de las hembras produjeron progenie del primer macho y dentro de este grupo, hubo una tendencia significativa en producir más progenie del tipo albino que del tipo normal. Una cuarta parte de las hembras produjeron exclusivamente progenie del segundo macho, y dentro de este grupo hubo una tendencia significativa al producir más progenie del tipo normal que del tipo albino. Cerca de la mitad de las hembras produjeron progenie en proporciones similares de los machos normales y albinos simultáneamente, así como en patrones en los que alternaban la paternidad de la

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progenie a lo largo de sus vidas reproductivas. Estos resultados sugieren que sin importar la secuencia en que las palomillas fueron apareadas, el esperma de los machos normales mostró un potencial competitivo mayor y que la precedencia de esperma no es el mecanismo más importante en el momento de distribuir la paternidad en esta colonia de *H. virescens*.

## Introduction

Polyandry is the condition in which a female copulates with more than one male. This phenomenon is common across a variety of taxa (Simmons 2005) and has been documented in a variety of Lepidoptera species (Retnakaran 1974, Brower 1975, Etman and Hooper 1979, Watanabe 1988, Wool et al. 1988, Mason and Pashley 1991, Svård and McNeil 1994, Suzuki et al. 1996, Bissoondath and Wiklund 1997, LaMunyon 2000, Brakefield et al. 2001, Platt and Allen 2001). Moths of tobacco budworm, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), are promiscuous under natural and laboratory conditions. Dissections of field-collected females show they copulate as many as seven times (Raulston et al. 1975). Dissections of females from laboratory colonies show they copulate as many as 12 times during their relatively short adult life span ( $\leq 13$  days) (11 [Flint and Kressin 1968] to 12 times [Blanco and Groot, unpublished]).

When the sperm from two or more males is present in the female's reproductive tract, the progeny may be the result of eggs fertilized with (after Platt and Allen 2001): a) sperm of one male fertilizing all the eggs, b) sperm of the last male overcoming the sperm of the previous male(s), or c) a 'mixing pattern' of sperm from all males with which the female mated. The first two scenarios have been called 'sperm precedence' (Svård and McNeil 1994, Simmons 2001). The third scenario leads to multiple paternity (LaMunyon 2001, Simmons 2005).

Paternity allocation under polyandry is an active area of research in plants and animals. In Lepidoptera, this process has implications in different areas such as conservation of species (Brakefield et al. 2001), control programs using the sterile-male technique (Flint and Kressin 1968, Brower 1975, Pair et al. 1977), and susceptibility to insecticide (Wool et al. 1988). The last two topics are of special relevance for control of an important agricultural pest such as tobacco budworm because insecticide-resistant moths can mate multiple times, and depending on the paternity allocation, a proportion of the resulting progeny can also be resistant to insecticide, if a female copulates with a resistant and a susceptible male(s). Learning more information about paternity allocation in this important pest was the primary goal of this study.

Several studies investigated paternity allocation in multiply-mated females, including staging known matings with colonies with mutations (Retnakaran 1974, Etman and Hooper 1979, Wool et al. 1988, Brakefield et al. 2001), matings with males induced to be sterile (Flint and Kressin 1968, Pair et al. 1977), or by looking at genetic markers (Boggs and Watt 1981, LaMunyon 2001). Previous research on multiple-mated tobacco budworm females describes how paternity corresponds primarily to one male with a brief mixing of paternities when a subsequent male copulates with a female (Flint and Kressin 1968, Pair et al. 1977, LaMunyon 2001). Here we used a phenotypic marker in conjunction with staged matings to revisit the frequency at which sperm precedence occurs. We also investigated whether paternity mixing can last for more than a brief period of time in tobacco budworm, and we produced some measures of fitness cost associated with this kind of

mutation. This additional information can be used to calculate effective genetic diversity of tobacco budworm populations which might be useful for the refining plans for mitigation of resistance to insecticide and/or maintenance of strains reared in a laboratory.

### Materials and Methods

Experiments were conducted with a long-established colony of tobacco budworm maintained at USDA-ARS, Stoneville, MS. In 2005, one male from this colony gave rise to a pure-bred strain that lacked green coloration on the wings due to a homozygous recessive mutation resulting in white coloration on the wings, similar to the mutation described by Mitchell and Leach (1994). Hereafter, moths from this colony will be referred to as 'albino-type' (homozygous recessive, lacking green color on wings) in contrast to the 'wild-type' original USDA-ARS, Stoneville colony, which are homozygous dominant for producing green coloration (Fig. 1). All insects were reared on a wheat germ-based diet (Blanco et al. 2008) and controlled environmental conditions of  $27 \pm 2^\circ\text{C}$ ,  $75 \pm 10\%$  relative humidity, and photoperiod of 14:10 L:D hours.

**Experiment 1: Production of Offspring of Different Phenotypes.** To assess paternity allocation, newly emerged albino-type female moths ( $\leq 24$  hours) were caged in 3.8-liter cartons (Neptune<sup>®</sup>, Newark, NJ) capped with cloth (Batist



Fig. 1. Phenotypic differences between albino-type moths ('A', left group) and wild-type *Heliothis virescens* moths ('W', right group).

Zweigar<sup>®</sup>, Piscataway, NJ) and fed 10% sucrose solution for 2 days. Newly emerged albino-type and wild-type males ( $\leq 24$  hours) were divided into two feeding groups: a) half of the emerging males was fed 10% sucrose solution for 2 days in a 3.8-liter carton capped with cloth, and b) the other half was fed 0.1% rhodamine B (Sigma-Aldrich<sup>®</sup>, St. Louis, MO) dissolved in 10% sucrose solution for 2 days in a 3.8-liter carton capped with cloth. This procedure produced 'marked' males (Blanco et al. 2006). After the 2-day feeding period, moths were paired in 473-ml-cartons (Neptune<sup>®</sup>, Newark, NJ) capped with cloth and free access to sucrose solution. Four treatments were used, in which the first and second male were alternated according to the following scheme, while the females always were from the albino-type colony:

1. Albino-type male, unmarked – Wild-type male, marked
2. Albino-type male, marked – Wild-type male, unmarked
3. Wild-type male, unmarked – Albino-type male, unmarked
4. Wild-type male, marked – Albino-type male, marked

The first male mentioned in each treatment was introduced to a 2-day old female (2 days after emergence from the pupal case). After 24 hours, the first male was removed and the female was left alone for a day. On the third day, the second 2-day-old male was introduced in the container for a day. From the fourth day until her death, the female was left alone to oviposit on the cloth. Eggs were collected daily from the fourth day on, corresponding to what we refer to as 'first egg day' (Table 1).

Table 1. Age at which Albino-type Females Were Mated and Average Progeny (F<sub>1</sub> Moths) Produced (Experiment 1). Oviposition Pattern (Fertile Eggs) Differences Between Two Types of *Heliothis virescens* Strains.

	Female age (days)							
	3	4	5	6	7	8	9	10
	Experiment 1							
Sequence of male introduction	1 <sup>st</sup> male	2 <sup>nd</sup> male						
Eggs collected ('egg days')			1 <sup>st</sup>	2 <sup>nd</sup>	3 <sup>rd</sup>	4 <sup>th</sup>	5 <sup>th</sup>	$\geq 6^{\text{th}}$
Albino-type moth production			4.8	2.6	3.4	2.9	3.2	1.8
Wild-type moth production			7.1	8.2	10.0	8.2	7.1	3.2
	Experiment 2							
Fertile eggs of Albino♀ <sup>1</sup> mated with Albino♂	0	184	140	120	84	67	56	15
Fertile eggs of Albino♀ <sup>1</sup> mated with Wild♂	0	211	190	168	124	89	66	18
Fertile eggs of Wild♀ <sup>1</sup> mated with Albino♂	0	148	162	115	99	68	70	22
Fertile eggs of Wild♀ <sup>1</sup> mated with Wild♂	0	167	124	115	95	77	62	39

<sup>1</sup>Females were mated with only one male.

Removed males were frozen and discarded. Dead females were dissected, and their spermatophores examined under fluorescent light to detect the presence

of rhodamine B (Blanco et al. 2006) that originated from the marked males. This method determined whether females had mated with a marked and an unmarked male.

Sixty albino-type females were allowed to produce families for each treatment. Cloths containing eggs were placed in plastic bags (Sandwich 94600, Ziploc®, Crawfordsville, IN) from each family from the 'first egg day' until the female died or stopped laying eggs. Thirty randomly chosen neonates from each day were transferred individually onto 12-15 ml wheat germ-based insect artificial diet in plastic cups (37-ml [T-125 Solo® plastic soufflés, Urbana, IL]). Eggs in plastic bags and larvae on diet were maintained at the previously mentioned conditions in the laboratory. The genders were determined of moths that emerged from each family (referred to as progeny). The moths were recorded as albino-type or wild-type (Fig. 1). Progeny of females that did not contain two distinctive spermatophores (rhodamine-marked or not marked) in their bursa copulatrix were eliminated. Paternity allocation was assigned as 'first male' or 'second male' when the phenotype of all the progeny was the same as the first or second male, respectively. The 'mixing' pattern was assigned when the phenotype of both males was present on at least 2 egg days.

To determine whether albino females produced more offspring when mated with an albino or a wild-type male, we conducted an ANOVA using PROC MIXED (SAS Institute 2003). The fixed effects were treatment, day, and treatment\*day, while treatment\*replication was considered a random effect.

**Experiment 2: Fitness Measurements Used for Comparisons.** To measure egg production and fertility from a 2-day-old albino-type female that copulated for 1 day with either a 2-day old albino-type or 2-day old wild-type male, 21 single-pairs were put into 473-ml cartons capped with cloth and fed 10% sucrose solution. Also, wild-type females were singly-paired with either albino-type ( $n = 21$ ) or wild-type males ( $n = 21$ ) as a comparison, but these data were not included in the statistical analysis. Pairs were kept under the previously described conditions. Eggs oviposited on the cloth were recorded daily from the first 'egg day' (Table 1). Eggs were placed in plastic bags with the previously described environmental conditions, and hatching was recorded 72 hours later. To measure differences in body size between albino and wild-type moths, the front wing length and the dry-weight of 25, 2-day old females and 25, 2-day old males were recorded. A paired *t*-test was used for statistical analysis (SAS Institute 2003).

## Results

**Experiment 1: Production of Offspring of Different Phenotypes.** Thirty percent of the albino-type females mated with both males when the marked male was introduced first (i.e., Treatments 2 and 4 combined: 28 of 60 marked albino-type males and 11 of 60 marked wild-type males). Thirty-five percent of the albino-type females copulated with both males when the unmarked male was introduced first (i.e., Treatments 1 and 3 combined: 23 of 60 unmarked albino-type males and 20 of 60 unmarked wild-males). Twenty-nine percent of the females produced families with progeny exclusively from the first male, 23% exclusively from the second male, and 48% from mixed paternities when they copulated with both males ( $F = 10.29$ ,  $df = 2$ ,  $P = 0.079$ ). The interaction between albino-type or wild-type phenotype and mating order was significant ( $F = 7.07$ ,  $df = 7$ ,  $P = 0.032$ ). When the albino-type male copulated first, 14% of the females produced progeny exclusively

from the albino-type male, 31% from the wild-type male, and 55% were from mixed paternities (LSR = 2.5,  $df = 7$ ,  $P = 0.12$ ). When the wild-type male copulated first, 55% of the females produced wild-type progeny exclusively, 10% from the albino-type, and 35% of the progeny was from mixed paternities (LSR = 2.5,  $df = 7$ ,  $P = 0.08$ ). There was no overall treatment ( $P = 0.10$ ) or treatment\*day effect ( $P = 0.67$ ) on the number of offspring produced, but there was a significant day effect ( $P < 0.0001$ ). When comparing treatment\*day on Day 2, significantly more ( $P = 0.04$ ) offspring was produced when albino females mated with normal males ( $190.4 \pm 33.6$  SD) as compared with albino males ( $140.2 \pm 38.5$  SD). On Day 3, there was a similar marginally significant effect ( $P = 0.053$ ) when females mated with normal males ( $168.2 \pm 43.3$  SD) than when they mated with albino males ( $120.3 \pm 11.8$  SD).

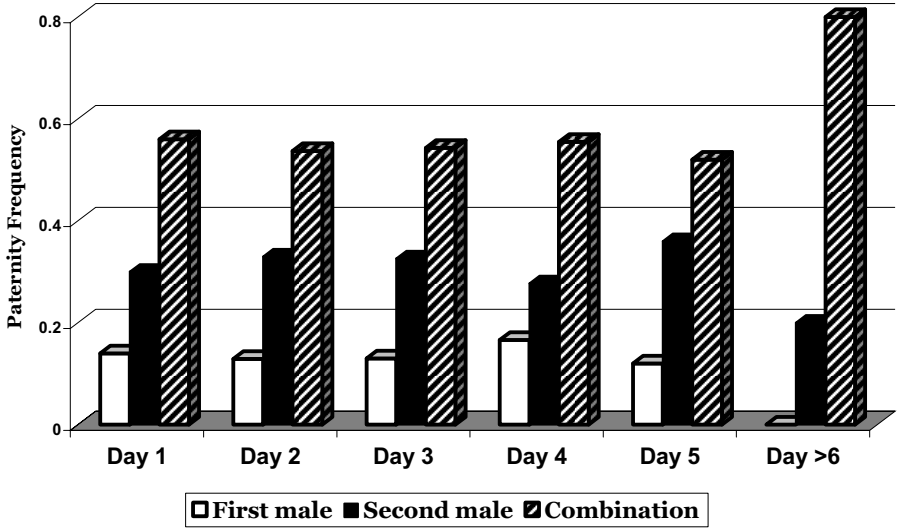
**Experiment 2: Fitness Measurements Used for Comparisons.** During their reproductive life, albino-type females that were mated with only albino-type males laid an average of 666 fertile eggs, compared to 866 fertile eggs when these albino-type females were mated only with wild-type males ( $t = 0.16$ ,  $df = 20$ ,  $P = 0.69$ ) (Table 1). In comparison, wild-type females that copulated with only wild-type males laid an average of 679 eggs while wild-type females that copulated only with albino-type males laid an average of 684 eggs. Albino-type males were significantly heavier (0.086 g) than wild-type males (0.062 g) ( $t = -7.63$ ,  $df = 24$ ,  $P < 0.0001$ ). The front wings of the albino-type males were also significantly larger (1.50 cm) than those of wild-type males (1.36 cm) ( $t = 4.97$ ,  $df = 24$ ,  $P < 0.0001$ ). Wild and albino-type females were not significantly different in weight (0.073 g albino-type ♀ vs. 0.068 wild-type ♀,  $t = 0.80$ ,  $df = 19$ ,  $P = 0.43$ ), or wing size (1.25 cm albino-type ♀ vs. 1.20 cm wild-type ♀,  $t = 1.29$ ,  $df = 19$ ,  $P = 0.21$ ).

## Discussion

Paternity allocation in twice-mated tobacco budworm females from this study allowed generation of additional information on the frequency and duration of sperm use. The first mating males were the exclusive fathers of all the progeny of 29% of the females, while the second mating males were the exclusive fathers of all the progeny by 23% of the females. The offspring of the remaining 48% of females were a mixture of both paternities that lasted for most ( $\leq 85\%$ ) of the reproductive life of the female. This indicates that the first or the second male had a  $\geq 71\%$  probability of fathering some of the offspring, and paternity mixing was as common as sperm precedence (Fig. 2).

These results are in contrast to previous studies in this system that showed the second (last) male generally obtained precedence over the first (Flint and Kressin 1968, Pair et al. 1977, LaMunyon 2001). This study shows the importance of long-term observations to assess paternity allocation. Using our data to focus only on the female's egg-laying days observed on two previous studies (Pair et al. 1977 and LaMunyon 2001), a paternity pattern closer to sperm precedence was observed. For example, five of the 39 females we categorized as having a mixture of sperm from both males, would be categorized as having a sperm precedence pattern when restricting these observations to any given two-consecutive oviposition days. But encompassing the entire reproductive output of these same females, it is more appropriate to classify their progeny as having paternity mixing because they exhibited paternity switches when using sperm of one male for one or more days and then from the other male for one or more days, or a mixture of sperm from both

**A. Albino Male followed by Normal Male**



**B. Normal Male followed by Albino Male**

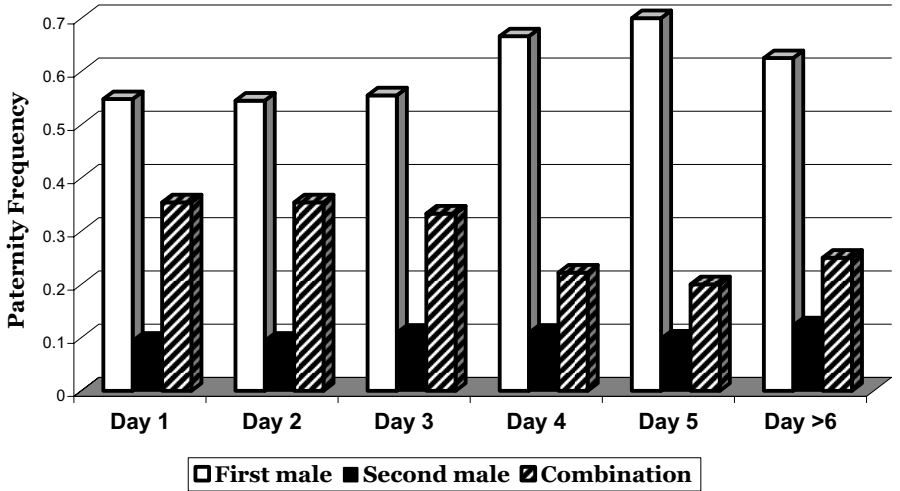


Fig. 2. Frequency of offspring sired by two *Heliothis virescens* males with different phenotypes. Days refer to the albino-type female reproductive life. A. The first male introduced was albino-type and the second was normal-type. B. The first male introduced was normal-type and the second was albino-type.



males for a number of days. When we examined data from only females that produced the offspring of both males for  $\geq 3$  consecutive days (71♀), first male paternity occurred in 35.9%, second male paternity in 21.5%, and mixing in 42.6%. Restricting our data analysis further to include only females that produced offspring from both males for  $\geq 4$  consecutive days (53♀), paternity allocation was: 40.6% of the offspring was sired by the first male, 19.7% by the second male, and 39.7% were from mixed paternity. These data manipulations allow us to confirm that last-male sperm precedence is not the rule and that paternity mixing in this strain of tobacco budworm was a consistent phenomenon during most of the reproductive life of the female.

The mixing of paternity during most of the female reproductive life (1-7 egg-laying days and 74% of the total egg output) in this mutant strain expands the knowledge of the reproductive biology of tobacco budworm. Progeny with a simultaneous mixture of paternities also has been observed in another Lepidoptera species (Brakefield et al. 2001) and in  $\approx 55\%$  of the insect species studied (Simmons and Siva-Jothy 1998). This mechanism might be influenced by at least one of the following factors: 1) age of the moths (LaMunyon and Huffman 2001), 2) interval between matings (Suzuki et al. 1996), 3) size of the female (LaMunyon 2001), 4) size of the male (Bissoondath and Wiklund 1997), 5) number of sperm transferred by a particular male (LaMunyon and Huffman 2001), and/or perhaps the type of mutation carried by one of the parents. Although this experiment controlled for the first three factors (double mating at different intervals were also attempted without obtaining sufficiently double-mated gravid females to include in this report), only male size varied (albino-type males had 9.0% bigger front wings and 27.9% heavier bodies than did wild-type males). A thorough evaluation of the potential fitness cost caused by the mutation for a lack of green color on wings awaits determination, but some fitness measures (reproductive output) indicate this mutation caused a  $\approx 25\%$  reduction in the viability of larvae that develop to adults. Expanding the measurement of paternity allocation in tobacco budworm presented in this report can assist, for example, in calculating how the offspring genotypes might result when an insecticide-resistant female copulates with a resistant and an insecticide-susceptible male. According to our data, there is only a  $\approx 25\text{-}30\%$  possibility that the progeny of this resistant hypothetical female would become completely susceptible to insecticide, so the 'dilution' of resistant genes the following generation would only occur in about one third of the resistant females.

Another observation from this study was that wild-type males fathered all the progeny (i.e., gained precedence) significantly more than albino-type males. When a wild-type male was the first to mate, it obtained precedence in  $\approx 55\%$  of the females, while when it was the second to mate it obtained precedence in  $\approx 30\%$  of the females (Fig. 2). In contrast, when the albino-type male was the first mate, it gained precedence in only  $\approx 13\%$  of the females, and when it was the second mate, it fathered the progeny in only  $\approx 10\%$  of the females. This was not because of fewer eggs fertilized by albino-type males, because the number of eggs produced by an albino-type female mated with an albino-type male was not significantly different from the number of fertile eggs by an albino-type female mated with a wild-type male (Table 1). Inbreeding avoidance (Zeh and Zeh 1997) due to genetic relatedness between albino-type females and males from a reduced gene pool of that strain, and the possibility of cryptic female choice can be part of the explanation. These are potentially important mechanisms in polyandry, which might



help females to enhance the survival of their offspring (Fisher et al. 2006, Simmons et al. 2006, Zeh and Zeh 2006).

In some cases, no paternity mechanism may be acting. The great incidence of mixed paternity in this study could have been created by two ejaculations entering the spermatheca of a tobacco budworm female (Raulston et al. 1975, LaMunyon 2001), allowing for the possibility of sperm mixing, which likely diminishes the possibility that females or males have control over fertilization of eggs. Facultative adjustment of the second ejaculation volume can influence the concentration ratio of first and second sperm inside the spermatheca (Ebenhard 1996, Raulston et al. 1975). Considering this possibility, while adjusting for the mutation fitness cost, we found significantly more wild-type progeny emerging from mixed paternity, which might indicate some sperm competition, inbreeding avoidance, and/or post-copulatory cryptic female choice.

Our results are based on determination of phenotypes of offspring (Fig. 1), using a mutant colony whose fitness has not been thoroughly characterized. The paternity allocation described should be interpreted within these boundaries. Inbreeding in the albino-type colony might have led to a competitive disadvantage of sperm, but it might reflect well other kinds of mutations (i.e., resistance to insecticide). Also, different mating intervals, mating frequency, age of moths, and use of a non-mutant colony, as used in previous reports, await further investigation in this mutant strain of tobacco budworm.

### **Disclaimer**

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