

# Electrophoretic Confirmation of Sperm Mixing in Mated Colorado Potato Beetles (Coleoptera: Chrysomelidae)

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**ABSTRACT** Sperm precedence in the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), was investigated using phosphoglucomutase (Pgm) allozymes as genetic markers. Females were mated to 2 males carrying different Pgm alleles, and the proportion of offspring sired by each male was determined by cellulose acetate gel electrophoresis. Our results indicate that Pgm alleles in the Colorado potato beetle are inherited in the Mendelian fashion, and there is no change in sperm utilization over time. We also were able to confirm that sperm precedence in this insect is incomplete, with  $\approx 72\%$  of the larvae fathered by males at the 2nd mating. Possible implications of incomplete sperm precedence for insecticide resistance management are discussed.

**KEY WORDS** *Leptinotarsa decemlineata*, sperm precedence, allozymes, cellulose acetate gel electrophoresis, phosphoglucomutase

COLORADO POTATO BEETLE, *Leptinotarsa decemlineata* (Say), is the major defoliating insect pest of potato in North America (Weber and Ferro 1994). This species has a remarkable ability to develop resistance to a wide variety of insecticides, with some materials failing after only 1 yr of application (Forgash 1985). Currently, the general approach to resistance management in Colorado potato beetle populations is to minimize beetle exposure to insecticides through the use of integrated pest management (IPM) practices such as crop rotation (Roush et al. 1990), refugia (Whalon and Ferro 1998), and proper timing of insecticide applications (Weber and Ferro 1994). Because insecticide resistance is almost never completely dominant (Roush and Daly 1990), individuals that are heterozygous for the resistant allele are likely to be eliminated by application of high insecticide doses. Therefore, successful Colorado potato beetle control depends on a sufficiently large proportion of resistant individuals mating to susceptible individuals. As a result, an understanding of beetle mating behavior is important for the management of this pest.

Colorado potato beetle is a polygamous species, with both males and females copulating multiple times with different partners (Szentesi 1985). Furthermore, at least 3 matings are required to fill the female's spermatheca completely, and multiple copulations increase female fertility (Boiteau 1988). If a resistant female mates to a resistant male within the main crop, and then mates again with a susceptible male arriving from a refugium, information on the proportion of that female's offspring sired by the 2nd mate is important for resistance management (Haubruege et al. 1997). Previous studies of polygamous Coleoptera have shown sperm precedence, with the last male to mate fathering most offspring (Huettel et al. 1976, Eady and

Tubman 1996). However, this precedence is almost never complete, and the sperm from earlier matings usually fertilizes at least some of the offspring (Lindquist and House 1967, Vick et al. 1972, Huettel et al. 1976, Eady and Tubman 1996). Using black- and white-colored morphs, Boiteau (1988) showed that when a Colorado potato beetle female mates to 2 different males, the sperm of the 2nd male fertilizes 32–53% of the eggs. This is valuable information; however, Boiteau's (1988) choice of color morphs as a genetic marker required using highly inbred laboratory strains of nonallelic recessive mutants. Unfortunately, inbreeding is known to affect sperm precedence in some insects (Hughes 1997).

Phosphoglucomutase (Pgm) allozymes have been used commonly as markers in the studies of insect population genetics (Dinardo-Miranda and Contel 1996, Owusu et al. 1996), and sperm precedence in polygamous insects (Huettel et al. 1976, McCauley and O'Donnell 1984). These allozymes are determined by codominant alleles and usually are inherited in a simple Mendelian fashion (Huettel et al. 1976). Preliminary screening of the Colorado potato beetle population revealed 2 Pgm allozymes which differed in relative speed of migration during cellulose acetate gel electrophoresis. In the current study, we attempted to confirm Boiteau's (1988) findings using wild-type Colorado potato beetles as test subjects, and Pgm allozymes as a genetic marker.

## Materials and Methods

**Beetle Maintenance and Sampling Procedures.** General Colorado potato beetle adults were obtained from our laboratory colony. Large colony size and regular infusion of field-collected individuals allowed

Table 1. Inheritance of phosphoglucosmutase (Pgm) allozymes in the Colorado potato beetle

Pair	♂ ♂	♀ ♀	Progeny ratio		P <sup>a</sup>
			Observed	Expected	
1	aa	ab	23aa:25ab	24aa:24ab	0.8382
2	bb	ab	23ab:25bb	24ab:24bb	0.8382
3	bb	ab	19ab:29bb	24ab:24bb	0.3048
4	ab	bb	20ab:28bb	24ab:24bb	0.4126
5	ab	ab	12aa:25ab:11bb	12aa:24ab:12bb	0.9686
6	ab	ab	19aa:21ab:8bb	12aa:24ab:12bb	0.2752
7	bb	aa	48ab	48ab	—
8	aa	bb	48ab	48ab	—

a, slow migrating electromorph; b, fast migrating electromorph.

<sup>a</sup> P values for chi-square goodness-of-fit tests. The null hypothesis tested is that the observed ratio of progenies fathered by each male is not different from the ratio expected in the case of Mendelian segregation of Pgm alleles.

us to keep genetic composition of the colony close to the genetic composition of local field populations. Throughout the experiment, all beetles were maintained at a photoperiod 16:8 (L:D) h. Pgm alleles carried by the individual adult beetles were determined by excising one middle leg and analyzing it by cellulose acetate gel electrophoresis (see below). After the operation, the beetles were isolated in separate ventilated plastic Nalgene boxes (12 by 7 by 5.5 cm) containing 1 cm of soil on the bottom. The containers were kept in the environmental chamber at 19°C for 24 h and then transferred to a 27°C environmental chamber. We used the lower temperature for 24 h to decrease the rate of beetle metabolism in an attempt to prevent possible excessive bleeding. At the end of the experiment, the electrophoresis data collected for individuals from leg samples were verified by analyzing larger tissue samples (beetle heads and pronotae).

**Relative Fitness of Operated Beetles.** To determine if leg amputation deleteriously affected reproductive fitness, we compared fertility, fecundity, and survivorship of operated and intact beetles. Oviposition of 25 operated and 25 intact mixed-sex beetle pairs was monitored for 2 wk starting the day beetles emerged from the soil. Eggs were collected daily and incubated at 27°C until hatching. The numbers of beetles laying eggs, eggs laid by each beetle, and larvae that hatched from the laid eggs were recorded. Experimental data were analyzed using Student *t*-test (PROC *t*-test, SAS Institute 1989). Survivorship was tested by placing 30 operated and 30 intact mixed-sex beetle pairs on caged potato plants in the greenhouse, where they were maintained at 23 ± 3°C. The number of beetles surviving after 1 mo was recorded. This experiment was repeated twice; in total, 120 beetles per treatment were tested. The data were pooled, and the proportion of surviving beetles was compared between the treatments using the chi-square test (PROC FREQ, SAS Institute 1989).

**Allozyme Inheritance.** To determine if Pgm allozymes were inherited in a Mendelian fashion, 8 beetles (9 d old) of each sex carrying known Pgm alleles were arranged into mixed-sex pairs to provide a number of different crosses involving both homo- and heterozygous individuals (Table 1). Each pair was placed for

24 h in a large petri dish (14 by 2.5 cm) lined with moistened paper towels, provisioned with excised potato foliage, and kept under 25 ± 2°C. Preliminary observations (A.V.A.) revealed multiple matings performed by the beetles within a 24-h period under similar conditions.

After mating, the males were stored at -80°C. Females were returned to Nalgene containers kept at 27°C for oviposition. Egg masses were collected daily over a 2-wk period and incubated at 27°C until hatching. Larvae (1 d old) were placed on excised potato leaflets inserted in small vials containing tap water. The leaflets were then placed inside ventilated Nalgene containers lined with paper towels. Larvae were reared at 27°C until they reached late 2nd-early 3rd instar, when they were frozen at -80°C. Forty-eight progeny of each female were sampled at random from at least 4 different egg masses and analyzed using allozyme acetate gel electrophoresis. Chi-square goodness-of-fit tests were used to compare observed and expected (based on Mendelian inheritance) progeny ratios (PROC FREQ, SAS Institute 1989).

**Sperm Precedence.** Beetles (9 d old) were arranged according to their genetic markers into 15 mating triplets, each consisting of 2 males with different Pgm alleles and 1 female. In each triplet, a male beetle was placed together with a female beetle for 24 h of mating, then replaced for another 24 h by the 2nd male. The mating beetles were kept in large petri dishes lined with moistened paper towels and provisioned with excised potato foliage. A number of different crosses involving both homo- and heterozygous males and females was performed (Table 2).

After mating to both males, female beetles were returned to their Nalgene containers at 27°C for oviposition. Mated males were stored at -80°. Egg masses laid by the females were collected daily for 1 mo. Collected eggs were incubated at 27°C until hatching. Larvae (1 d old) were placed on cut potato leaflets inserted in small vials with tap water inside ventilated Nalgene containers lined with paper towels.

Larvae were reared at 27°C until they reached late 2nd-early 3rd instar, and then frozen at -80°C. We sampled, at random, 36 larvae from at least 3 different egg masses laid by each female during the 1st week

Table 2. Sperm precedence in the Colorado potato beetle

Triplet	1st ♂	2nd ♂	♀	Observed progeny ratio	Expected progeny ratio ( <i>P</i> ) <sup>a</sup>			% fatherhood	
					No precedence	50:50% mix	100% precedence	1st ♂	2nd ♂
1	aa	bb	bb	1ab:71bb	72ab	36ab:36bb (0.0000)	72bb	1.38	98.52
2	aa	bb	ab	7aa:32ab:33bb	36aa:36bb	18aa:18ab:36bb (0.0117)	36ab:36bb	19.44	80.56
3	bb	aa	ab	28aa:44ab	36ab:36bb	18aa:36ab:18bb (0.0000)	36aa:36ab (0.1797)	22.22	77.78
4	ab	bb	bb	70bb	35ab:35bb	17.5ab:53.5bb	70bb	0	100
5	ab	bb	bb	4ab:68bb	36ab:36bb (0.0000)	18ab:54bb (0.0012)	72bb	11.11	88.89
6	ab	bb	bb	17ab:54bb	35.5ab:35.5bb	17.75ab:53.5bb (0.8456)	71bb	47.89	52.11
7	bb	ab	bb	28ab:44bb	72bb	18ab:54bb (0.0739)	36ab:36bb	22.22	77.78
8	bb	ab	bb	26ab:44bb	70bb	17.5ab:53.5bb (0.1308)	35ab:35bb	25.72	74.28
9	bb	ab	bb	7ab:64bb	71bb	17.75ab:53.5bb (0.0172)	35.5ab:35.5bb (0.0000)	80.28	19.72
10	bb	ab	bb	24ab:46bb	72bb	18ab:54bb (0.2461)	36ab:36bb	33.33	66.77
11	bb	ab	bb	28ab:44bb	72bb	18ab:54bb (0.0739)	36ab:36bb	22.22	77.78
12	bb	ab	bb	6ab:66bb	72bb	18ab:54bb (0.0073)	36ab:36bb	83.33	16.67
13	bb	ab	bb	32ab:40bb	72bb	18ab:54bb (0.0143)	36ab:36bb (0.5043)	11.11	88.89
14	bb	ab	bb	39ab:33bb	72bb	18ab:54bb (0.0003)	36ab:36bb (0.6168)	0	100
15	bb	ab	bb	23ab:49bb	72bb	18ab:54bb (0.3559)	36ab:36bb	36.11	63.89

a, slow-migrating electromorph; b, fast-migrating electromorph.

<sup>a</sup> *P* values for chi-square goodness-of-fit tests. The first null hypothesis tested was that the observed ratio of larval genotypes was not different from the ratio expected for the Mendelian segregation of Pgm alleles if both males in a triplet sired equal numbers of offspring. If the first null hypothesis was rejected, the second null hypothesis tested was that all the offspring were fathered by one of the males.

after mating. An additional 12 larvae per week per female were sampled for the next 3 wk of the experiment. All the sampled larvae were analyzed using allozyme acetate gel electrophoresis. In total, 70–72 larvae were analyzed for each female.

The number of offspring sired by one of the males in a triplet was calculated according to the Mendelian law of inheritance from the number of offspring carrying a Pgm allele unique to this male. The rest of larvae were assumed to be sired by the 2nd male in the triplet. Chi-square goodness-of-fit tests (PROC FREQ, SAS Institute 1989) were used to compare observed and expected (based on Mendelian inheritance) progeny ratios. The null hypotheses was that both males in a triplet sired equal numbers of offspring. If the observed ratio was significantly different from the ratio expected assuming equal sperm mixing, we next tested the hypothesis of complete sperm precedence or absence of sperm precedence (depending on which male was heterozygous). Similarly, we used chi-square goodness-of-fit tests to investigate if there was a change in sperm utilization over time. The null hypothesis was that the proportion of offspring sired by each male did not change between the 1st and the following 3 wk after mating.

**Allozyme Gel Electrophoresis.** All tissue samples obtained during the study were homogenized in

20–40  $\mu$ l (depending on the sample size) of distilled water and centrifuged for 6 min at 6,000 rpm. Eight  $\mu$ l of supernatant were transferred to applicator wells (Super Z-12 applicator kit, Helena Laboratories, Beaumont, TX) and then applied to cellulose acetate plates (Titan III, Helena Laboratories) soaked for 30 min in L-buffer (6.06 g of Trizma base, 0.37 g of Na<sub>2</sub>EDTA, 0.20 g of anhydrous MgCl<sub>2</sub>, and 2.32 g of maleic acid dissolved in 1 liter of distilled water). Acetate plates were placed into a horizontal electrophoresis chamber (Zip Zone, Helena Laboratories) and run for 1.5 h at 175  $\pm$  3 V using L-buffer as electrode buffer. The plates were then removed from the chamber and stained using a mixture of 1.0 ml Tris HCl (pH = 8), 2 ml of agar, and the following water solutions: 1.5 ml NAD (2 mg/ml), 5 drops MgCl<sub>2</sub> (20 mg/ml), 5 drops of glucose-1-phosphate (50 mg/ml), 5 drops of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (10 mg/ml), 5 drops of phenazine methosulphate (2 mg/ml), and 20  $\mu$ l of glucose-6-phosphate dehydrogenase (80 U/ml) (Hebert and Beaton 1993).

## Results

**Relative Fitness of Operated Beetles.** On average, intact females produced 185.68  $\pm$  13.64 eggs (mean  $\pm$  SE) and 110.60  $\pm$  8.70 larvae; operated females pro-

duced  $183.68 \pm 13.84$  eggs and  $126.68 \pm 8.50$  larvae. Leg excision had no significant effect on the number of eggs laid (Student *t*-test,  $T = 0.1029$ ,  $df = 48$ ,  $P = 0.9184$ ) or 1st instars produced (Student *t*-test,  $T = 1.2315$ ,  $df = 48$ ,  $P = 0.1926$ ). Twenty-four percent of intact and 22% of operated beetles died over a 1-month period, a difference that was not statistically significant ( $\chi^2 = 0.21$ ,  $df = 1$ ,  $P = 0.6450$ ).

**Allozyme Inheritance.** Experimental results are shown in Table 1. All females produced viable offspring, and the ratio of larvae carrying different Pgm alleles did not differ significantly from that expected based on the Mendelian law of inheritance.

**Sperm Precedence.** Experimental results are shown in Table 2. A certain degree of sperm precedence (or sperm mixing) was observed in all females. On average,  $\approx 72\%$  of the larvae were fathered by the 2nd males, which produced more progeny in all but 2 triplets. There was no statistically significant difference between the observed offspring ratios and the ratios expected in the case of complete sperm precedence in 26.67% of the broods, indicating that all the larvae in those broods could have been sired by 2nd males. At the same time, in 40% of the tested broods, the proportion of progeny produced by the 2nd male was not significantly different from 0.5. Except for 1 triplet, the degree of sperm precedence did not seem to change with time because there were no significant differences in larval genotype ratios between the 1st and the following 3 wk of oviposition (chi-square tests,  $P > 0.05$ ). In 1 triplet, ( $\chi^2 = 5.94$ ,  $df = 1$ ,  $P = 0.0148$ ), the 2nd male fathered 2.7 times more larvae during the 1st wk of oviposition than during the following 3 wk.

## Discussion

Pgm allozyme acetate gel electrophoresis proved to be an efficient technique for studying sperm precedence in the Colorado potato beetle. Excising one middle leg did not affect beetle survival or reproduction, and allowed us to determine Pgm genetic markers before mating. This information dramatically reduced the number of gels we had to analyze compared with those required had beetles taken from populations with different allozyme frequencies been mated as suggested by Huettel et al. (1976).

Results of the current study confirm the existence of incomplete sperm precedence in summer generation (long-day) Colorado potato beetles, with sperm from the 2nd mating somewhat prevailing over sperm from the 1st mating. The proportion of larvae fathered by the 2nd males in our study ( $\approx 72\%$ ) was higher than the proportion recorded by Boiteau (1988) (32–53%). Nevertheless, the 1st mating still resulted in the production of a significant number of offspring, with the proportion of larvae sired by the 1st males being  $\approx 2$  times higher than similar proportions reported for other insects (Vick et al. 1972, Huettel et al. 1976 [see Coffelt 1975 in references], Eady and Tubman 1996). There was considerable variation in sperm precedence among beetle triplets, with the 2nd males fathering from 16.67 to 100% of the offspring. Variability

in sperm precedence among the conspecific males is known to occur (Hughes 1997), and could be explained by genetic variation in molecular mechanisms of sperm displacement (Clark et al. 1995), different numbers of copulations secured by the 1st and 2nd males (Boiteau 1988, Clark et al. 1995), different numbers of sperm transferred by different males at a single copulation (Newport and Gromko 1984), and by unknown environmental influences and sampling errors (Hughes 1997). Each of these factors could account for the discrepancy between our results and those of Boiteau (1988). Another factor contributing to the observed discrepancy could have been the difference in beetle strains used in the 2 discussed studies, because Boiteau (1988) used as genetic markers black-colored mutants obtained from a laboratory strain originating from only 2 field-collected black females mated to wild-type males (Boiteau 1985), and inbreeding is known to have a detrimental effect on the 2nd male sperm precedence (Hughes 1997). Despite certain numerical differences, the results of our study and the study by Boiteau (1988) strongly support the existence of the same phenomenon—incomplete sperm precedence in the Colorado potato beetle.

Together with distributing eggs in space and time (Solbreck 1978, Voss and Ferro 1990) and mating to multiple partners (McCauley and Reilly 1984, Szentesi 1985), incomplete sperm precedence might be one of the components of an overall bet-hedging strategy used by the Colorado potato beetle to maximize the number of surviving offspring. Mixing sperm of different males in female spermathecae will contribute to genetic variability of the resulting progeny, which can have important implications for the evolution of insecticide resistance in this species.

Most of the currently recommended resistance management programs rely on the refugia–high-dose strategy, promoting mating between resistant and susceptible insects and then exterminating heterozygous progeny of these crosses. When the size of refugia is appropriate for maintaining a sufficiently large susceptible population, and their spatial location favors gene flow between resistant and susceptible populations, this strategy provides a significant delay in resistance development (Gould 1998, Whalon and Ferro 1998). However, Gould (1998) pointed out that success of this approach is hindered by the ability of the Colorado potato beetle to mate in the vicinity of the place of its eclosion from a pupa. He argued that such an ability will increase the probability of a resistant female mating with a resistant male and leaving at least some homozygous-resistant offspring before mating to a susceptible male arriving from a refugium. Incomplete sperm precedence will further elevate the frequency of resistant homozygotes within beetle populations, because it means that a resistant female mated with a resistant male will continue producing some resistant offspring even after a subsequent mating with a susceptible male. Therefore, a successful resistance prevention program should be directed toward complete curtailment of mating between resistant individuals, a goal very hard to achieve under field

conditions. Fortunately, insecticide resistance in the Colorado potato beetle often is linked to the reduced fitness, including prolonged time of development (Argentine et al. 1989, Trisyono and Whalon 1997). Furthermore, the rate of development for surviving beetles is slowed by insecticide exposure (Ferro et al. 1997). As a result, when resistant beetles emerge from the pupae, the main crop could already be colonized by the susceptible adults immigrating from refugia, and the probability of 2 resistant beetles mating with each other is diminished. We suggest that, depending on local conditions and developmental characteristics of resistant strains, this effect could be further enhanced by the early planting of refugia. Success of this approach will largely depend on a thorough understanding of dynamics of resistant and susceptible populations within the main crops and refugia. Additional investigations are required before arriving at any further conclusions.

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