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Cadherin mutation linked to resistance to Cry1Ac affects male paternity and sperm competition in *Helicoverpa armigera*

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Abstract

Several lepidopteran pests of cotton have cadherin-based resistance to the *Bacillus thuringiensis* (Bt) toxin Cry1Ac. Cadherins are transmembrane proteins that mediate cell-cell adhesion and tissue morphogenesis, suggesting that fitness costs associated with cadherin mutations may be present in many aspects of life history. To evaluate whether cadherin-based resistance is associated with fitness costs reducing male paternity in *Helicoverpa armigera*, we examined the effects of a major cadherin resistance allele on sperm competition within and between male ejaculates. When homozygous resistant and susceptible males competed for fertilization of a homozygous resistant or susceptible female, fertilization success was high in males with a different cadherin genotype than females and low in males with the same cadherin genotype as females. Single matings between heterozygous males and susceptible females produced offspring within typical Mendelian ratios. Heterozygous males mated to resistant females, however, resulted in a disproportionate number of heterozygous offspring. While these results show that cadherin-based resistance to Cry1Ac has significant impacts on paternity in *H. armigera*, there was no evidence that costs associated with resistance consistently reduced male paternity. Rather, effects of cadherin-based resistance on paternity depended on interactions between male and female genotypes and differed when males or sperm competed for fertilization of females, which complicates assessment of impacts of cadherin resistance alleles on resistance evolution.

Keywords

Bt resistance; cadherin; fitness costs; sperm competition; non-Mendelian inheritance

1. Introduction

Transgenic crops producing *Bacillus thuringiensis* (Bt) toxins are increasingly used in developing and developed countries (James 2013), providing improved pest suppression and reduced use of insecticides (Pray et al., 2002; Carrière et al., 2003; Cattaneo et al., 2006; Wu

et al., 2008; Hutchison et al., 2010; Lu et al., 2012; Shi et al., 2013), but continued use of Bt crops is threatened by the evolution of resistance in target pests (Tabashnik et al. 2013). To delay resistance evolution, the refuge strategy has been proposed and widely adopted. The logic of this strategy is that the rare resistant individuals from Bt crops mate with the relatively abundant susceptible individuals produced from nearby non-Bt host plants. Provided that resistance is recessively inherited, the progeny from such matings will die on the Bt crops, thereby reducing the heritability of resistance (Carrière et al., 2010; Tabashnik et al., 2013). Fitness costs occur when resistance alleles have negative indirect (i.e., pleiotropic) effects on fitness components in absence of Bt toxin (Gassmann et al., 2009). Accordingly, the capacity of refuges to delay resistance is not only affected by the production of susceptible insects for mating with resistant insects, but also by fitness costs that select against resistance in refuges (Carrière and Tabashnik, 2001; Alphey et al., 2008; Carrière et al., 2010; Onstad and Carrière, 2014).

Fitness costs of resistance to Bt are common and can affect several fitness components, including body mass, development time, survival, female fecundity and male virility (Gassmann et al., 2009). In most insect species, females mate multiply with several males and competition between ejaculates and cryptic female choice of sires is widespread (Eberhard 1996; Simmons, 2001). Nevertheless, few studies have analyzed the potential impacts of Bt resistance on sperm competition. In pink bollworm (*Pectinophora gossypiella*) where first-male sperm precedence occurs, males homozygous for resistance alleles that mated first with a virgin Bt-susceptible female sired significantly less offspring than Bt-susceptible males that mated first (Higginson et al., 2005). The reduced first-male paternity in homozygous resistant males was associated with lower transfer of sperm, although the resistance alleles also affected other traits that could have lowered mating success (Carrière et al., 2009).

In three major lepidopteran pests of cotton (cotton bollworm, *Helicoverpa armigera*; tobacco budworm, *Heliothis virescens*; and *P. gossypiella*), mutations in a cadherin gene that encodes a larval midgut protein binding the Bt toxin Cry1Ac is linked with resistance to this toxin (Gahan et al., 2001; Morin et al., 2003; Xu et al., 2005). Cadherins comprise an important class of transmembrane proteins that mediate cell-cell adhesion and many aspects of tissue morphogenesis (Halbleid and Nelson 2006). Several types of cadherin are expressed in reproductive tissues of mammals (Rowlands et al., 2000; Xiao et al., 2013) and are present on both spermatozoa and oocytes, where they are thought to play a role in gamete adhesion (Goodwin et al., 2000; Ziv et al., 2002; Purohit et al., 2004; Marín-Briggiler et al., 2008). Among insects, cadherin resistance alleles are expressed in guts and testes (Carrière et al., 2009). While presence of resistant cadherin proteins on sperm or oocytes of pink bollworm remains unknown, the role of cadherins in tissue-sperm and sperm-egg interaction in mammals raises the possibility that reduced paternity by resistant males is mediated by reduced sperm numbers or cryptic female choice.

In China, Bt cotton producing the toxin Cry1Ac has been planted since 1997 to control major lepidopteran pests including the cotton bollworm, *H. armigera*. Although Bt cotton has continued to provide substantial control of *H. armigera*, increases in the frequency of cadherin-based resistance to Cry1Ac have been reported in some field populations from

northern China, where Bt cotton has been intensively planted (Li et al., 2007; Liu et al., 2010; Zhang et al., 2011, 2012). The small but statistically significant increases in the frequency of resistance to Cry1Ac provide an early warning of resistance that could eventually result in practical problems (Zhang et al., 2011, Tabashnik et al. 2014).

In the present study, we evaluate effects of a major cadherin resistance allele of *H. armigera* on sperm competition within and between ejaculates. Bimodal distribution of sperm use, where one male sires all or nearly all offspring, is common throughout Lepidoptera (Simmons and Siva-Jothy 1998; Simmons, 2001), suggesting that conserved mechanisms of sperm handling underlie sperm use patterns. Specifically, second-male sperm precedence occurs in *H. armigera* and typically all offspring of a female mated with two males are sired by a single male (Teng and Zhang, 2009, Yan et al., 2013). If the cadherin resistance allele in *H. armigera* displays similar fitness costs to that of pink bollworm, resistant males are expected to fertilize all offspring of females less often than susceptible males when competing against the ejaculates of susceptible males. To separate potential fitness costs of sperm numbers from sperm genotype, we further examine patterns of sperm use of resistant and susceptible allele-bearing sperm within the ejaculates of heterozygous males when fertilizing virgin females. While the cadherin resistance allele was not consistently associated with costs reducing male paternity, it significantly affected sperm competition between males and within ejaculates of heterozygous males, indicating the presence of sperm $\times$ female or sperm $\times$ egg interactions in determining sperm use patterns.

2. Materials and Methods

2.1 Insects

The susceptible SCD strain was collected from Cote D'Ivoire in the 1970s and was kindly provided by Bayer Crop Science (Yang et al., 2009). Since 2001, this strain was maintained without exposure to any insecticides or Bt toxins and is homozygous (*ss*) for the wild type allele of *HaCad* (*H. armigera* cadherin gene). The GYBT strain is a laboratory-selected strain with about 500-fold resistance to Cry1Ac, and is homozygous for a truncated allele (*r₁*) of *HaCad* (Xu et al., 2005). The allele *r₁* was shown to be the most common of several resistance alleles in populations of *H. armigera* from northern China (Zhan et al., 2012). To control for genetic background, the *r₁* allele in the GYBT strain was introgressed into the genetic background of the susceptible SCD strain to create a near-isogenic resistant strain, SCD-r1 (Yang et al., 2009). The SCD-r1 strain, homozygous for the *r₁* allele of *HaCad* (*r₁r₁*), showed 438-fold resistance to Cry1Ac when compared with the SCD strain.

Larvae were reared on an artificial diet based on wheat germ and soybean powder at $27\pm1^{\circ}\text{C}$ with a 16:8 (L:D) photoperiod. Adults were held under the same temperature and light conditions at an RH of 60–70% and supplied with a 10% honey water solution.

2.2 Interejaculate sperm competition

Two-day-old virgin moths were used throughout the experiments for mating in transparent plastic cups (300 mL) supplied with 10% honey water solution. Four combinations of trios were set to evaluate impacts of *r₁* on male paternity (detailed in Table 1). For each trio, a female moth and the first male moth was put in a cup for the first night, and the first male

was removed from the cup after a successful mating (i.e., lasting for more than 30 min); the second male was put into the cup for the second night, and the second male was removed from the cup after a successful mating. Eggs were collected for a period of three days following the second mating. After egg collection, the female was dissected to check for the presence of spermatophores. If the female had two spermatophores, the trio was considered an effective trio for paternity analyses. Eggs produced by each effective trio were hatched and reared separately, with each hatched larvae reared individually on artificial diet to third instar. For each of the four combinations of trios, a subset of effective trios was arbitrarily selected for genotyping offspring. Twenty third instars per trio were arbitrarily selected for *HaCad* genotyping. Mortality of larvae before individuals were sampled for genotyping was negligible. The parents in each effective trio were also genotyped.

2.3 Intraejaculate sperm competition

Thirty males of SCD (*ss*) and 30 females of SCD-*r1* (*r₁r₁*), and 30 females of *ss* and 30 male *r₁r₁* were respectively pooled to produce F₁ offspring. Two-day-old virgin *r₁s* male moths from the F₁ offspring were paired individually with an *ss* or *r₁r₁* female moth (detailed in Table 2). For each combination of matings, 130 pairs were set and 40 pairs that had successfully mated on the first night were arbitrarily selected to quantify the sperm type (*s* or *r₁*) that fertilized eggs. As before, eggs were collected for a period of 3 days following mating and hatched larvae were reared separately for each pair, by feeding artificial diet to each larvae individually until they reached third instar. Mortality of larvae before individuals were sampled for genotyping was negligible. For each pair, 18–20 randomly selected third instars and their parents were preserved for *HaCad* genotyping.

2.4 PCR screen of the cadherin genotypes for individual larvae

Genomic DNA from individual larvae or adults was prepared using a genomic DNA extraction kit according to the manufacturer's instructions (Axygen Biosciences, Union, CA). Allele specific PCR (AS-PCR) was used to amplify and discriminate between the susceptible (*s*) and *r₁* mutant alleles of *HaCad* with the methods from Yang et al. (2006). Considering the *r₁* allele of *HaCad* has a deletion between Exon 8 and Exon 25, a common forward primer (5'-CTTCACACATGATGTTCTCG-3') for both alleles was designed in Exon 8. Two reverse primers were designed to flank the mutant sites: s-R (5'-CTATGTAGAACGCCTCGTGAG-3', located on Exon 9) for susceptible allele *s* and r1-R (5'-CTTCACACATGATGTTCTCG-3', located on Exon 25) for the mutant *r₁* allele. The 25 µl PCR reaction system contained 2.5 µl of 10× PCR buffer, 2 mM of MgCl₂, 1 µM of each for the three primers, 150 µM of dNTP, 1 U of rTaq polymerase and 100 ng genomic DNA. PCR was performed for 30 cycles of 30 s at 94 °C, 1 min at 55 °C and 2 min at 72 °C. PCR products were separated by 1.5% gel electrophoresis and visualized with ethidium bromide staining. The susceptible homozygote (*ss*) has a single band of 650 bp (Figure 1), the resistant homozygote (*r₁r₁*) has a single band of 410 bp, and the heterozygote (*r₁s*) has two bands (both 410 bp and 650 bp).

2.5. Statistical analyses

Interejaculate sperm competition—In the present experiment, the progeny of individual females was always sired by a single male genotype. To evaluate the presence of second-male sperm precedence and of costs affecting sperm competition, we compared fertilization success of males (resistant and susceptible) competing for a single virgin female (resistant or susceptible). Logistic regression for binary data was used to evaluate whether the percentage of females exclusively fertilized by $r_I r_I$ males was affected by female genotype, second male genotype, or the interaction between these factors. In this analysis, a significant second male genotype effect would indicate the presence of second-male sperm precedence if a higher percentage of females exclusively fertilized by $r_I r_I$ males occurred when $r_I r_I$ males mated second than when ss males mated second. We did not find any evidence for a significant effect of second male genotype, but did find a significant effect of female genotype (see Results). To further assess the significant effect of female genotype and the presence of costs, paternity data were pooled for the two combinations of matings ($ss\sigma^7$ first; $r_I r_I\sigma^7$ second or $r_I r_I\sigma^7$ first; $ss\sigma^7$ second), and the percentage of females exclusively fertilized by $r_I r_I$ males was calculated for each female genotype (either ss or $r_I r_I$). For each female genotype, a likelihood ratio X^2 test was used to evaluate whether the percentage of females exclusively fertilized by $r_I r_I$ males was significantly different from 50%.

Intraejaculate sperm competition—To evaluate more directly the capacity of r_I and s sperm to fertilize females, we quantified fertilization success of the sperm types when heterozygous males mated with a single virgin female (resistant or susceptible). Logistic regression for binomial counts was used to assess whether the percentage of r_I sperm fertilizing eggs was affected by the type of cross producing the $r_I s$ male, female genotype, and the interaction between these factors. In a second step, paternity data were pooled for each female genotype and the percentage of r_I sperm siring eggs was calculated. For each female genotype, a likelihood ratio X^2 test was used to evaluate whether the percentage of r_I sperm siring eggs was significantly different from 50%. All statistical analyses were performed in JMP (2010).

3. Results

3.1. Interejaculate sperm competition

The genotype of the second male to mate (either ss or $r_I r_I$) did not significantly affect the percentage of females exclusively fertilized by $r_I r_I$ males (Table 3, $X^2 = 1.23$, $P = 0.27$), showing a lack of second-male paternity advantage. However, female genotype significantly affected the percentage of females exclusively fertilized by $r_I r_I$ males ($X^2 = 23.07$, $P < 0.0001$). The fertilization advantage of $r_I r_I$ males with ss females, and the fertilization advantage of ss males with $r_I r_I$ females (Table 3), was similar irrespective of the order in which the male genotypes mated (female genotype $\times$ second male genotype interaction, $X^2 = 2.65$, $P = 0.10$). The percentage of ss females exclusively fertilized by $r_I r_I$ males (83.3%) was significantly higher than 50% ($X^2 = 17.46$, $P < 0.0001$), indicating a fitness advantage of $r_I r_I$ compared to ss males when males competed for ss females. In contrast, the percentage of $r_I r_I$ females exclusively fertilized by $r_I r_I$ males (36.7%) was significantly

lower than 50% ($X^2 = 4.32$, $P = 0.038$), indicating a fitness disadvantage of $r_I r_I$ compared to ss males when mating with $r_I r_I$ females.

3.2. Intraejaculate sperm competition

The type of cross producing the $r_I s$ male did not significantly affect success of the r_I sperm (Table 4, $X^2 = 0.012$, $P = 0.91$). However, female genotype significantly affected the percentage of r_I sperm fertilizing her eggs ($X^2 = 16.01$, $P < 0.0001$). The difference in fertilization success of r_I sperm between ss and $r_I r_I$ females was similar for the two types of crosses producing the $r_I s$ male (type of cross producing the $r_I s$ male $\times$ female genotype, $X^2 = 0.059$, $P = 0.81$). Overall, the percentage of r_I sperm fertilizing eggs of ss females (49.6%) was not significantly different from 50% (Figure 2, $X^2 = 0.091$, $P = 0.76$). However, the percentage of r_I sperm fertilizing eggs of $r_I r_I$ females (42.5%) was significantly lower than 50% (Figure 2, $X^2 = 35.48$, $P < 0.0001$). Thus, when heterozygous males mated with a virgin female, r_I and s sperm had equal fertilization success of ss females but r_I sperm had lower fertilization success of $r_I r_I$ females than s sperm.

4. Discussion

The results presented here show that cadherin-based resistance to Cry1Ac has significant impacts on paternity in *H. armigera*. In the experiment involving sperm competition between two males for a single female, fertilization success was high in males with a different cadherin genotype than females and low in males with the same cadherin genotype as females. Costs were clearly not present when males competed for ss females because $r_I r_I$ males had a significant paternity advantage over ss males, but costs were present when males competed for $r_I r_I$ females. Results from the experiment involving sperm competition within the ejaculate of a heterozygous male show that the progeny from ss females was as likely to carry a paternal r_I or s allele, but the progeny from $r_I r_I$ females was more likely to carry a paternal s than a r_I allele, again indicating asymmetrical costs only present when males mated with $r_I r_I$ females. Taking results from both experiments together, we conclude that costs of cadherin-based resistance to Cry1Ac did not generally reduce male paternity in *H. armigera*, as was previously found in *P. gossypiella* (Higginson et al., 2005). Furthermore, effects of cadherin-based resistance on paternity differed when males or sperm competed for fertilization of females, which complicates assessment of impacts of cadherin resistance alleles on resistance evolution.

Cadherins are implicated in gametic interactions, gamete production and many aspects of tissue morphogenesis (Goodwin et al, 2000; Ziv et al., 2002; Purohit et al., 2004; Halbleid and Nelson 2006; Marín-Briggiler et al., 2008). In this study, we found complex interactions between Bt resistance cadherin genotypes in females, males and sperm in determining paternity success. The paternity advantage of males with different cadherin genotypes than females in the interejaculate competition experiment could be due to higher use of cadherin hetero-allelic sperm by females, or higher viability of $r_I s$ offspring than ss or $r_I r_I$ offspring. However, these hypotheses were only partially supported by results from the experiment involving sperm competition within the ejaculates of heterozygous males. When $r_I s$ males mated with ss females, there was no evidence of differential sperm use or lower viability of ss than $r_I s$ offspring. However, when $r_I s$ males mated with $r_I r_I$ females, results were

consistent with the hypotheses of higher use of cadherin hetero-allelic sperm or that $r_I r_I$ offspring have lower viability than $r_I s$ offspring. However, it is unlikely that viability of $r_I r_I$ and $r_I s$ offspring differed because mortality of larvae was very low before they were selected for genotyping. Furthermore, egg hatching is similar in the SCD (54.4 %) and SCD- r_I (50.1 %) strains (Yang et al. 2009), and our analysis of the data reported in Yang et al. (2009) reveal no significant difference in egg survival between these strains ($df = 18$, $t = 1.20$, $P = 0.25$). Accordingly, other factors than sperm competition and offspring viability caused the consistent paternity advantage of males with a different cadherin genotype than females in the intejaculate sperm competition experiment.

The consistent use of cadherin hetero-allelic sperm by females found here supports the hypothesis that differential sperm retention or displacement was mediated by male $\times$ female interactions (Eberhard, 1996; Simmons, 2001). Genetically based male $\times$ female interactions are important determinants of paternity success in mice, fruit flies and beetles (Pitnick et al., 2009). Within mice, biased fertilization is the result of sperm-egg interactions (Wedekind et al., 1996), but for the most part the underlying mechanisms are unknown (Pitnick et al., 2009). In the context of *H. armigera*, further experiments are needed to evaluate whether female cadherin genotype affects sperm output of males, or sperm cadherin genotype affects sperm retention or use in females.

Using microsatellite markers, Yan et al. (2013) found evidence for second-male sperm precedence in *H. armigera* when two males competed for access to individual females. This type of sperm competition was consistent with the rapid loss of sperm from the spermatheca and storage of about one seventh of the ejaculate volume less than 24 h after copulation, which could have facilitated displacement of sperm from the first male to mate (Yan et al., 2013). Interestingly, in the present experiment conducted after introgressing the cadherin mutation r_I in the genetic background of the Bt-susceptible SCD strain, there was no evidence that the order of mating of the male genotypes affected fertilization success. This could indicate that female sperm choice or changes in ejaculate composition based on cadherin genotype of females overrides second-male precedence in *H. armigera*. An alternative hypothesis, however, is that the extensive period of laboratory rearing of the SCD strain resulted in evolutionary changes in sperm precedence. To reconcile results from the present and Yan et al. (2013) study, it will be important to evaluate whether second-male precedence still occurs within the SCD strain.

The non-Mendelian inheritance of the r_I allele in crosses between $r_I s$ males and $r_I r_I$ females is a surprising result. Differential success of r_I and s sperm could be explained by differences in the performance or function of sperm bearing different cadherin genotypes, or underrepresentation of r_I sperm in the ejaculates of heterozygous males. Meiotic drive systems frequently result in few or no sperm bearing the non-driving gene or allele being transferred to females (reviewed in Lyttle, 1991; Lyon, 2003; Taylor and Ingvarsson, 2003; Immler, 2008). However, results from this study provide no evidence of reduced r_I sperm numbers in heterozygous *H. armigera* males: when mated with ss females, $r_I s$ males sired offspring within typical Mendelian ratios. Furthermore, sperm phenotypes are primarily determined by testicular gene expression (Eddy, 2002), and even in the presence of postmeiotic gene expression, sharing of gene products among syncytial spermatids results in

sperm reflecting the diploid phenotype of the male (Braun et al., 1989; Erickson, 1990; Joseph and Kirkpatrick, 2004). However, there are two examples in mice of the haploid genotype of sperm influencing sperm performance or function. First, males hemizygous for the *t*-locus meiotic drive system produce wild-type sperm with reduced motility and poor egg penetration, resulting in the *t*-locus being transmitted to the majority of offspring (Lyon 2003; Véron et al., 2009). Second, sperm deficient in the sperm adhesion molecule (*Spam1*), due to allelic variation among spermatids, have reduced cumulus penetration and egg-sperm binding (Zheng et al., 2001a). In both the *t*-locus and *Spam1* examples, phenotypic differences among sperm are the result of gene products being retained within developing spermatids without diffusion through the syncytial cytoplasm (Zheng et al., 2001b; Véron et al., 2009). Thus, given the prominent role of cadherins in cell adhesion (Halbleid and Nelson 2006), it is possible that enhanced fertilization success by *s* relative to *r_I* sperm of *r_I r_I* females is a consequence of improved *s* sperm retention and positioning within the female tract or superior egg penetration. Further studies are required to test the functional non-equivalence of *r_I* and *s* sperm, including verification of cadherin expression and localization within developing spermatids.

In contrast to *P. gossypiella* from Arizona in which costs reducing male paternity were consistently associated with cadherin resistance alleles (Higginson et al., 2005; Carrière et al., 2009), results from this study indicate that cadherin resistance alleles have more complex effects on male paternity in *H. armigera*. Specifically, the effect of *r_I* on reproductive success of competing males and sperm was context dependent and influenced by the cadherin genotype of both males and females. In addition, the outcome of male and sperm competition on transmission of the *r_I* and *s* alleles from males to offspring was different. However, another layer of complexity occurs because it was recently found that many different cadherin resistance alleles can segregate within populations of *H. armigera* and *P. gossypiella* (Zhan et al., 2012; Fabrick et al., 2014). For example, eight different cadherin resistance alleles were identified in eight larvae from populations exposed to Bt cotton in India, and individual larvae carried several different transcript isoforms of particular cadherin alleles (a total of 19 cDNA isoforms in the 8 larvae). The implication of these findings is that extensive experimental work will be needed to evaluate how cadherin resistance alleles interact with paternity to affect the trajectory of resistance evolution. Nevertheless, the significant effects of cadherin resistance alleles on paternity of *P. gossypiella* and *H. armigera*, coupled with potentially high genetic variation at cadherin loci within some populations of these pests, indicate that the cadherin gene linked to resistance to Bt could be an important contributor to sperm-female or sperm-egg interactions.

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Figure 1.
Banding patterns of PCR products for *HaCad* genotyping.

Figure 2.
Distribution of percentage of eggs fertilized by r_1 sperm in SCD (*ss*) or SCD- r_1 (r_1r_1) females mated with a r_1s male.

Table 1

Four combinations of mating trios for assessing effects of cadherin-based Cry1Ac resistance on sperm competition between a resistant ($r_I r_I$) and susceptible (ss) male competing for a single resistant or susceptible virgin female

Combination	No. of trios set	No. of effective trios tested	Larvae tested per trio
$ss\text{♀} \times ss\text{♂} / r_I r_I\text{♂}$	130	20	20
$ss\text{♀} \times r_I r_I\text{♂} / ss\text{♂}$	82	16	20
$r_I r_I\text{♀} \times ss\text{♂} / r_I r_I\text{♂}$	180	30	20
$r_I r_I\text{♀} \times r_I r_I\text{♂} / ss\text{♂}$	172	30	20

Table 2

Four combinations of matings for assessing competition between r_I and s sperm when a heterozygous male ($r_I s$) mated with a single resistant ($r_I r_I$) or susceptible (ss) virgin female

Cross producing $r_I s$ males	Female genotype	No. of single pairs set	No. of tested single-pairs	Larvae tested per single pair
$r_I r_I \text{♀} \times ss \text{♂}$	ss	130	40	18–20
	$r_I r_I$	130	40	19–20
$ss \text{♀} \times r_I r_I \text{♂}$	ss	130	40	19–20
	$r_I r_I$	130	40	19–20

Table 3

Effects of female genotype and order of mating of the male genotypes on the percentage of females exclusively fertilized by r_1r_1 males

Female genotype	Mating order		No. of effective trios tested	Number of females fertilized by r_1r_1 male	% females fertilized by r_1r_1 males
	First male	Second male			
ss	ss	r_1r_1	20	15	75
ss	r_1r_1	ss	16	15	94
r_1r_1	ss	r_1r_1	30	12	40
r_1r_1	r_1r_1	ss	30	10	33

Table 4Effects of type of cross producing $r_I s$ males and female genotype on percentage of eggs fertilized by r_I sperm

Cross producing $r_I s$ males	Female genotype	No. of tested single-pairs	% eggs fertilized by r_I sperm ($\pm$ SE)	95 % confidence interval
$r_I r_I \text{Q} \times ss \text{O}^{\text{g}}$	ss	40	49.9 (2.0)	46.0–53.9
	$r_I r_I$	40	42.4 (1.8)	38.8–45.9
$ss \text{Q} \times r_I r_I \text{O}^{\text{g}}$	ss	40	49.2 (1.8)	45.7–52.8
	$r_I r_I$	40	42.7 (1.7)	39.3–46.1