Journal of Insect Physiology 55 (2009) 1058–1064

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Cadherin gene expression and effects of Bt resistance on sperm transfer in pink bollworm

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ARTICLE INFO

Article history:
Received 15 April 2009
Received in revised form 21 July 2009
Accepted 30 July 2009

Keywords:
Bacillus thuringiensis
Bt resistance
Cadherin
Fitness costs
Pectinophora gossypiella
Sperm competition
Sperm transfer

ABSTRACT

Cadherin proteins bind *Bacillus thuringiensis* (Bt) toxins in lepidopteran midguts but their inherent function remains unclear. In pink bollworm, *Pectinophora gossypiella*, three recessive mutations in a cadherin gene (*BtR*) are tightly linked with resistance to Bt toxin Cry1Ac. Here we examined patterns of transcription of this gene and the association between cadherin genotype and sperm transfer in pink bollworm. Cadherin RNA was most abundant in larvae, but was also found in adults and embryos. In fourth instar larvae, cadherin RNA was most abundant in the gut, yet its presence in the testes indicates a potential role in sperm production. Previously, we found reduced first-male paternity in pink bollworm males homozygous for cadherin mutations conferring resistance to Bt, when a resistant and susceptible male competed for access to a female. However, the number of offspring sired by resistant and susceptible males was similar without competition. Male Lepidoptera produce both fertile eupyrene sperm and anucleate, non-fertile apyrene sperm, suggesting that apyrene sperm may contribute to male reproductive success when sperm competition occurs. Accordingly, we hypothesized that cadherin-based resistance to Bt entails fitness costs that reduce apyrene sperm transfer. To test this hypothesis, we compared apyrene and eupyrene sperm transfer in males from four strains of pink bollworm. Transfer of apyrene and eupyrene sperm was lower in homozygous resistant than in susceptible males. Furthermore, homozygous resistant males weighed less than susceptible males, which could have diminished sperm transfer by resistant males directly, or via a positive association between male weight, spermatophore weight and sperm transfer. While data suggest that cadherin mutations induced a recessive fitness cost affecting apyrene sperm transfer, these mutations also generated recessive costs that affected other traits and could have lowered first-male paternity.

1. Introduction

Insect resistance to toxins from *Bacillus thuringiensis* (Bt) often involves costs that lower the fitness of resistant individuals relative to susceptible individuals in the absence of Bt toxins (Gassmann et al., 2009). Such fitness costs select against resistance in environments where Bt toxins are absent, counterbalancing selection that favors an increase in resistance in fields of transgenic Bt crops (Carrière and Tabashnik, 2001; Carrière et al., 2005; Gould et al., 2006; Tabashnik et al., 2005, 2008; Crowder and Carrière, in press). Expression of costs is commonly affected by environmental conditions (Gassmann et al., 2009). Thus, a better understanding of the physiological basis of costs and of factors that affect their expression could be useful for enhancing resistance management (Gassmann et al., 2009).

In the pink bollworm, *Pectinophora gossypiella*, and two other major lepidopteran pests of cotton (*Heliothis virescens* and *Helicoverpa armigera*), resistance to Bt toxin Cry1Ac is linked with mutations in a gene encoding a cadherin protein that binds Cry1Ac in the larval midgut (Gahan et al., 2001; Morin et al., 2003; Xu et al., 2005). Cadherin-based resistance to Cry1Ac is associated with various fitness costs (Gassmann et al., 2009) and much is known about cadherins in other organisms (Angst et al., 2001; Purohit et al., 2004; Cyr et al., 2008). However, the inherent function of Cry1Ac-binding cadherin in insects is not known, and information on expression of the pink bollworm cadherin has not been reported. Here we examined patterns of transcription of the cadherin gene in larval tissues and throughout development and the association between cadherin genotype and sperm transfer in pink bollworm.

Pink bollworm exhibits first-male paternity, whereby the first male to mate typically sires >80% of offspring (Bartlett and Lewis,

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doi:10.1016/j.jinsphys.2009.07.013

1985; Higginson et al., 2005). Cadherin-based resistance to Cry1Ac in pink bollworm involves costs affecting male reproductive success with but not without competition for access to females (Higginson et al., 2005). Without competition between males, fecundity of susceptible females did not differ between those that mated with homozygous resistant males and those that mated with homozygous susceptible males. However, when a resistant and susceptible male competed for matings with a susceptible female, resistant males that mated first sired significantly fewer offspring than susceptible males that mated first. As females mated several times during the competition experiment, the reduced first-male paternity of resistant males indicated that cadherin-based resistance to Bt involves fitness costs that reduce sperm precedence (Higginson et al., 2005).

Males of most lepidopteran species produce two types of sperm (Swallow and Wilkinson, 2002; Friedländer et al., 2005; Till-Bottraud et al., 2005). Apyrene sperm is thinner and shorter and does not contain chromatin, while eupyrene sperm is longer and carries a normal haploid complement of chromosomes. Eupyrene sperm is produced during the penultimate or final larval instar, while apyrene sperm is produced at or after pupation, and the switch from eupyrene to apyrene sperm production is controlled by a specific hemolymph factor active near pupation (Swallow and Wilkinson, 2002; Friedländer et al., 2005). Sperm heteromorphism may contribute to male competitive success when females mate more than once with different males (Swallow and Wilkinson, 2002; Friedländer et al., 2005; Till-Bottraud et al., 2005). Under such sperm competition, apyrene sperm could improve male reproductive success by increasing the ability of related eupyrene sperm to fertilize eggs (e.g., by displacing or killing unrelated eupyrene sperm), or preventing unrelated eupyrene sperm from reaching eggs (e.g., by blocking access of rival sperm).

As resistance to Bt toxin Cry1Ac did not affect reproductive success of *P. gossypiella* males in the absence of competition (Higginson et al., 2005), we hypothesized that cadherin-based resistance to Bt has minor effects on eupyrene sperm production. However, because resistance to Bt reduced first-male paternity, we hypothesized that cadherin mutations reduce the production of apyrene sperm, and hence the ability of resistant males to prevent access to eggs by eupyrene sperm of unrelated males. To test these hypotheses, we compared the number of apyrene and eupyrene sperm transferred to virgin females by males of different cadherin genotypes.

2. Materials and methods

2.1. Pink bollworm strains

Insect strains were maintained on wheat germ diet at a population size >1000 per generation (Carrière et al., 2004). Selection of strains with Cry1Ac was done by feeding larvae on diet containing 10 μg of toxin per ml of diet, a concentration that kills all susceptible and heterozygous individuals (Carrière et al., 2007). The source of Cry1Ac was MVP-II (Dow Agrosciences, San Diego, CA), a liquid formulation containing protoxin encapsulated in *Pseudomonas fluorescens* (Tabashnik et al., 2002).

Females used to quantify apyrene and eupyrene sperm transfer and males and females used in RT-PCR experiments originated from APHIS-S, a susceptible strain reared in the laboratory for >20 years without exposure to toxins. Males used to quantify apyrene and eupyrene sperm transfer originated from four strains: two strains containing only Bt-resistant individuals (MOV97-R4 and SAF97-R4) and two related hybrid strains (MOV97-H4 and SAF97-H5), each containing a mixture of Bt-resistant, susceptible and heterozygous individuals.

Hybrid strains MOV97-H4 and SAF97-H5 originated from MOV97 from the Mohave Valley in western Arizona and SAF97 from Safford in eastern Arizona. The MOV97 and SAF97 strains were started from field-collected insects in 1997 (Tabashnik et al., 2000). Laboratory selection of MOV97 and SAF97 with Cry1Ac rapidly produced the resistant strains MOV97-R and SAF97-R that are equally able to survive on Bt cotton (Carrière et al., 2006). MOV97-R had the cadherin alleles *r1* and *r3*, while SAF97-R had *r1* and *r2* (Morin et al., 2003).

The first hybrid strains (MOV97-H1 and SAF97-H1) were created by pooling offspring from MOV97-R and MOV97 or SAF97-R and SAF97, respectively (Carrière et al., 2006). Because the frequency of resistance alleles declined in hybrid strains reared on non-Bt diet (Carrière et al., 2004), hybrid strains were periodically selected by feeding larvae with a diet containing 10 μg of Cry1Ac toxin per ml of diet. The surviving Bt-resistant insects were crossed with insects from hybrid strains to generate new hybrid strains with a higher frequency of *r* alleles. MOV97-H4 was derived from MOV97-H1 by three iterations of this process. SAF97-H4 was created by crossing insects from the F8 generation of the resistant strain SAF97-H1R and the susceptible strain SAF97-H1S, which originated from SAF97-H1 (Gassmann et al., 2008). MOV97-R4 and SAF97-R4 were obtained by selecting larvae of MOV97-H4 and SAF97-H4 with a diet containing 10 μg of Cry1Ac toxin per ml of diet. The *r1* allele was absent in SAF97-H5 and SAF97-R4, likely because too much time had elapsed before SAF97-H4 was selected with Cry1Ac.

F7–F10 males from MOV97-H4, F4–F6 males from SAF97-H5, F4–F7 males from MOV97-R4 and F4–F6 males from SAF97-R4 were used in experiments to quantify apyrene and eupyrene sperm transfer. Forty-five generations had elapsed between the creation of MOV97-H1 and the F7 generation of MOV97-H4, while 15 generations had elapsed between the creation of SAF97-H4 and the F4 generation of SAF97-H5. Every additional generation in hybrid strains contributes in reducing linkage between cadherin alleles and alleles at other loci. Thus, the use of insects from MOV97-H4 and SAF97-H5 increased the chances of detecting fitness costs arising from pleiotropic effects rather than linkage with deleterious alleles (Carrière et al., 2006). We refer collectively to the MOV97-H4 and MOV97-R4 as the Mohave strains, and to SAF97-H5 and SAF97-R4 as the Safford strains.

2.2. RT-PCR and real-time qRT-PCR for assessing cadherin expression

To determine the developmental expression of the pink bollworm cadherin gene, total RNA was extracted from 35 to 100 mg of tissue from embryos, first to fourth instar larvae, pupae, and adults using TRIzol (Invitrogen, Carlsbad, CA). The number of individuals used in these RNA extractions ranged from several hundred (embryos and first instars) to four or five (fourth instars and later development stages). To assess cadherin expression in different tissues, total RNA was obtained from fourth instar larval tissues using TRIzol reagent. Intact gut tracts were obtained from 10 males and 10 females. Fifteen females were used to obtain head tissue, foreguts (including salivary glands), midguts, hindguts (including Malpighian tubules), and fat body tissue. Testes were dissected from 45 males.

cDNA was produced using random hexamer primers and First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD). For the amplification of a 557-bp fragment from the cadherin gene (*BtR*), RT-PCR was performed using the gene *BtR*-specific primers 3306F (5′-GTTCGACCACGGTATTCCT-3′) and 3862R (5′-GACAGTCGT-CAGTGCAGAGT-3′). To eliminate the possibility of amplification of PCR products from contaminating genomic DNA, PCR primers used to amplify the *BtR* amplicon in both RT-PCR and qRT-PCR (see below) were designed to span at least one predicted intron/exon

junction. A 343 bp fragment of the pink bollworm gene encoding ribosomal protein subunit 7 (*RPS7*) was amplified as an internal control using the gene-specific primers RPS7F (5′-ACAACAAGAAGTCAATCATCATCTA-3′) and RPS7R (5′-TTGTGTTCAATGGTGGTCTGCTG-3′). PCR was performed on 1 μL cDNA templates with 0.5 μM primer solutions and 0.625 units of ExTaq premix DNA polymerase (Takara Bio USA, Madison, WI). The DNA was initially denatured for 3 min at 94 °C, and the PCR amplification included 25–30 cycles of 30 s denaturing at 94 °C, 30 s annealing at 54.8 °C for *BtR* and 57.8 °C for *RPS7*, and 1 min extension at 72 °C in an Eppendorf Mastercycler Gradient thermal controller (Hamburg, Germany). RT-PCR products were separated on 1.25% agarose gels and stained with ethidium bromide.

For quantitative real-time PCR (qRT-PCR), QuantiTect custom primers and probes were produced by Qiagen (Valencia, CA). For the amplification of the 99 bp *BtR* amplicon, the 20× primer solution supplied by Qiagen contained the sense primer (5′-GAACCAGACATTCGCCAT-3′) and antisense primer (5′-CGGTCCGTTGCTATTACCTT-3′). The 20× Quantiprobe solution contained the FAM-labelled probe (5′-CCAAAACATCAAGCTC-3′). For the amplification of the 150 bp *RPS7* reference amplicon, the 20× primer solution contained the sense primer (5′-CCGTGAGTTGGAGAAGAA-3′) and the antisense primer (5′-AGGATAGCGTCGTACACTGA-3′). The 20× Quantiprobe solution contained the FAM-labelled probe (5′-AACAAACAGAAGAGGC-3′). One microliter of cDNA template (undiluted, 1:10 diluted, 1:100 diluted, 1:1000 diluted, 1:10,000 diluted) was mixed with primer/probe solutions (total reaction volume of 25 μL) in a 96-well plate according to the manufacturer's instructions. qRT-PCR was conducted in an ABI-PRISM 7000 with an initial activation step at 95 °C for 15 min, followed by 45 cycles of three-step cycling (30 s extension at 76 °C, 15 s denaturation at 94 °C, and 30 s annealing at 56 °C). Fluorescence data were collected during the final annealing step of each cycle.

The relative gene expression values of *BtR* in different developmental stages and tissues were determined by the modification of methods of Hauton et al. (2005). Threshold values (Ct) were obtained from each sample when the emitted fluorescence value exceeded the arbitrary threshold established at the beginning of the logarithmic phase of PCR amplification. qRT-PCR efficiencies (*E*) for each gene were calculated from the slope (*S*) of lines generated by plotting the Ct value against the $\log_{10}$ of the dilution factor for each gene, using the equation:

$$E = 10^{(-1/S)}$$

Gene expression in different tissues was normalized against values from fat body tissue according to the equation:

$$\text{tissue expression ratio} = \frac{(E_{BtR})^{\Delta \text{Ct } BtR}}{(E_{RPS7})^{\Delta \text{Ct } RPS7}}$$

where E_{BtR} and E_{RPS7} are the corresponding real-time PCR efficiencies for *BtR* and *RPS7*, respectively, and ΔCt *BtR* and ΔCt *RPS7* are the differences in Ct values between fat body and another tissue (e.g., $\text{Ct}_{\text{fat body}} - \text{Ct}_{\text{head}}$) for *BtR* and *RPS7*, respectively. Gene expression in different development stages was normalized against values in embryos according to the same equation, where E_{BtR} and E_{RPS7} are the efficiencies, but ΔCt is the Ct difference between the embryo and another developmental stage (e.g., $\text{Ct}_{\text{embryo}} - \text{Ct}_{\text{pupa}}$).

Expression ratios calculated with undiluted, 1:10 diluted, and 1:100 diluted cDNA were used to evaluate changes in cadherin expression across development stages and tissues. As ratios obtained for a given stage or tissue are not statistically independent (samples for a particular development stage or tissue were pooled to perform a single RNA extraction), the mean ratios were compared qualitatively rather than statistically.

2.3. Transfer of apyrene and eupyrene sperm

Moths were weighed within 24 h of eclosion and virgin moths less than 3 days old were used in experiments. A single male from one of the four strains was paired with an APHIS-S female in 470 mL paper cup cages with transparent lids. Moths were observed every 30 min beginning 6 h after the onset of the scotophase (Higginson et al., 2005). After the termination of copulation (mean duration = 1.4 h, range 0.5–2.5 h), males were preserved individually in ethanol at −20 °C for genotyping, while females were transferred to 30 mL cups on ice. To avoid sperm migration from the spermatophore to the spermatheca (Cook and Wedell, 1996), females were decapitated and dissected less than 30 min after the termination of copulation. The bursa copulatrix was extracted and the spermatophore that it contained removed. Females with a ruptured spermatophore or multiple spermatophores were discarded. Spermatophores were weighed (accuracy 0.0001 mg).

Spermatophores were placed in a drop of phosphate-buffered saline solution on a gridded microscope slide. Spermatophores were pierced and squeezed with forceps to expel sperm. Freshly transferred spermatophores contain loose apyrene sperm and bundles of eupyrene sperm (Lachance et al., 1975; Cook and Wedell, 1996). After gently mixing, the number of eupyrene sperm bundles was counted at 50× magnification with a dissecting microscope. After counting sperm bundles, the slide was washed into a 15 mL tube with 1 mL of distilled water. Sperm samples were diluted 1:10 with distilled water, gently mixed, and three 10 μL aliquots were pipetted onto two microscope slides each containing three non-overlapping circles (1.8 cm diameter) traced with a permanent marker. Aliquots were spread within the boundary of circles with a clean pipette tip, allowed to dry for 2 h under a dust cover, and slides were stored at 4 °C until apyrene sperm was counted using dark field microscopy at 100× magnification.

2.4. Male genotyping

DNA was extracted using DNAzol (Molecular Research Center, Inc., Cincinnati, OH), and males were genotyped at the cadherin locus (*BtR*, AY198374) using PCR (Morin et al., 2004). Males from MOV97-H4 and MOV97-R4 were tested for *r1* and *r3*, while males from SAF97-H5 and SAF97-R4 were tested for *r1* and *r2*. We tested males from SAF97-H5 and SAF97-R4 for *r1* to confirm previous results indicating that *r1* had been lost from the Safford strains.

2.5. Statistics

The number of matings involving and APHIS-S female and males of each resistance genotype are presented in Table 1 for each combination of strain origin (Mohave or Safford) and strain type (resistant or hybrid strain). Because males of some homozygous resistant genotypes were rare, we considered the homozygous resistant genotypes *r1r1*, *r2r2*, *r1r3*, *r3r3* as *rr*, and the heterozygous genotypes *r1s*, *r2s*, and *r3s* as *rs* in statistical analyses. The number of apyrene sperm for each male was the average of the six counts. All statistical analyses were performed with JMP version 7.0 (JMP, 2007). The explanatory and response variables were normally distributed and did not require transformation before analysis. As detailed below, we used multiple regression models to assess factors affecting transfer to females of apyrene sperm and eupyrene sperm bundles, as well as weight of spermatophores and males.

In the multiple regression models assessing variation in transfer of apyrene sperm or eupyrene sperm bundles, initial factors considered were strain origin, strain type, and cadherin

Table 1
Number of matings between susceptible APHIS-S females and males of each cadherin genotype for combinations of strain origin (Mohave or Safford) and strain type (hybrid or resistant strain). The frequency of *r1*, *r3*, and *s* was respectively 0.09, 0.19, and 0.72 in MOV97-H4, while the frequency of *r2* and *s* was respectively 0.25 and 0.75 in SAF97-H5.

Strain origin	Strain type	Genotype	*N*
Mohave	Hybrid	*r1r1*	0
	Hybrid	*r1r3*	1
	Hybrid	*r3r3*	1
	Hybrid	*r1s*	10
	Hybrid	*r3s*	20
	Hybrid	*ss*	27
	Resistant	*r1r1*	3
	Resistant	*r1r3*	4
	Resistant	*r3r3*	20
Safford	Hybrid	*r2r2*	3
	Hybrid	*r2s*	19
	Hybrid	*ss*	28
	Resistant	*r2r2*	10

genotype (*ss*, *rs*, or *rr*) of males; and weights of males, females and spermatophores. In a first step, a model containing all possible interactions (up to fourth order) among strain origin, male cadherin genotype, and male, female and spermatophore weight was fit. Because resistant strains only contain *rr* genotypes (Table 1), interaction terms involving strain type and male genotype could not be evaluated, and strain type was only included as a main effect in the model with interaction terms. In a second step, an extra-sum-of-squares *F*-test was used to compare the model with the interaction terms to a model with main effects only. The resulting *F*-test assessed whether any interaction terms are needed to model variation in the number of apyrene sperm or eupyrene sperm bundles transferred to females (Ramsey and Schafer, 2002). Interaction terms were not significant and thus were not needed to model variation in transfer of apyrene sperm ($F_{32,106} = 1.01$, $p = 0.47$) or eupyrene sperm bundles ($F_{32,106} = 1.34$, $p = 0.14$). In a third step, an extra-sum-of-squares *F*-test was used to assess the effects of colony type and female weight jointly, as these factors were not significantly associated with both response variables ($p > 0.05$) in models including main effects. Colony type and female weight were not significantly associated with transfer of apyrene sperm ($F_{2,138} = 0.49$, $p = 0.61$) or eupyrene sperm bundles ($F_{2,139} = 2.37$, $p = 0.097$) and these factors were removed from main effects in the multiple regression models.

In the multiple regression model assessing variation in spermatophore weight, factors initially considered were strain origin, strain type, male genotype, and male and female weight. As above, interaction terms were not significant and thus were not needed to model variation in spermatophore weight ($F_{18,121} = 1.54$, $p = 0.087$). Colony type and female weight were not significantly associated with the transfer of eupyrene sperm bundles ($F_{2,139} = 2.37$, $p = 0.097$) and these factors were removed from main effects in the multiple regression model.

In the multiple regression model assessing variation in male weight, factors initially considered were strain origin, strain type, and male genotype. The interaction between strain origin and male genotype was not significant ($F_{2,139} = 0.69$, $p = 0.50$) and thus not considered in the final multiple regression model.

To assess the dominance of fitness costs affecting transfer of apyrene sperm and eupyrene sperm bundles, we used linear contrasts between least squares means to contrast *rr* vs. *rs* males and *rs* vs. *ss* males. Because spermatophore weight and male weight affected sperm transfer (see Section 3), we also used linear contrasts between least squares means to compare male and spermatophore weight between male genotypes.

3. Results

3.1. Cadherin gene transcription

Gene-specific PCR primers and RT-PCR were used to determine the relative abundance of cadherin (*BtR*) RNA across developmental stages and in different tissues. As expected, the primer set 3306F and 3862R amplified a 557 bp RT-PCR product from cDNA (Figs. 1, 2A, and 3A). This primer set amplified a much larger product (approximately 3 kb) from genomic DNA (gDNA), indicating that the primers span a region containing at least one intron and the 557 bp RT-PCR product is not a result of gDNA contamination (Fig. 1). The *BtR* primers used in qRT-PCR were also designed to span predicted intron/exon junctions. Although these primers were not tested with gDNA, the lack of bands (agarose gel electrophoresis analysis) or peaks (melting curve analysis) beyond those expected for a single 557 bp fragment suggests that only cDNA was amplified with the *BtR* primers (results not shown).

RPS7 transcript levels were relatively uniform in cDNA samples from all developmental stages (Fig. 2A). The abundance of *BtR* transcripts was highest in first to third instar larvae, intermediate in fourth instars and adults; and lowest in embryos and pupae (Fig. 2). In fourth instar larvae, *BtR* transcript abundance was lower in males than females, but the opposite occurred for adults (Fig. 2). *BtR* transcript abundance in fourth instar larvae was highest in the foregut and midgut, intermediate in the hindgut and testes, and lowest in the head and fat body (Fig. 3).

3.2. Effects of male genotype and other traits on apyrene and eupyrene sperm transfer

Apyrene sperm transfer varied among male genotypes ($F_{2,140} = 6.17$, $p = 0.0027$). Apyrene sperm transfer was lower for homozygous resistant males (*rr*) than for susceptible (*ss*) males (Table 2: one-tailed linear contrast, $F_{1,140} = 10.4$, $p = 0.0008$). Apyrene sperm transfer did not differ between *ss* and heterozygous (*rs*) males (two-tailed linear contrast, $p = 0.91$). Thus, a recessive fitness costs affected apyrene sperm transfer in both strains. Apyrene sperm transfer was greater in males from Mohave (Least squares means = 100.1, SE = 2.8) than Safford (Least squares means = 71.4, SE = 3.6) ($F_{1,140} = 37.1$, $p < 0.0001$). Apyrene sperm transfer was positively associated with male weight (slope = 3869.7 sperm per g, $t_{1,140} = 3.00$, SE = 1291.5, $p = 0.0032$)

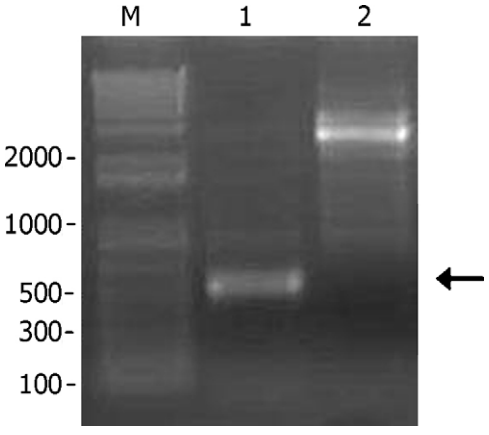

Fig. 1. PCR amplification of pink bollworm cadherin fragment from whole-body fourth instar larval cDNA (Lane 1) and genomic DNA (Lane 2) using 3306F and 3862R PCR primers. Lane M contains 1 kb Plus DNA Ladder (Invitrogen) and corresponding sizes in bp are shown.

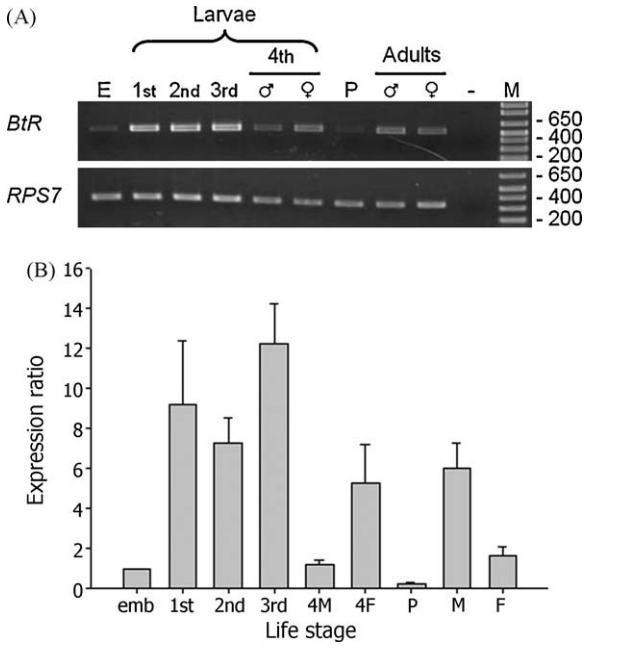

Fig. 2. RT-PCR (A) and qRT-PCR (B) amplification of cadherin (*BtR*) and *RPS7* fragments from different developmental stages of pink bollworm, including embryos (emb), first to third instar larvae, fourth instar larvae males (4M) and females (4F), pupae (P), and adult males (M) and females (F). qRT-PCR values from each developmental stage were normalized against embryos. Bars in (B) are standard errors.

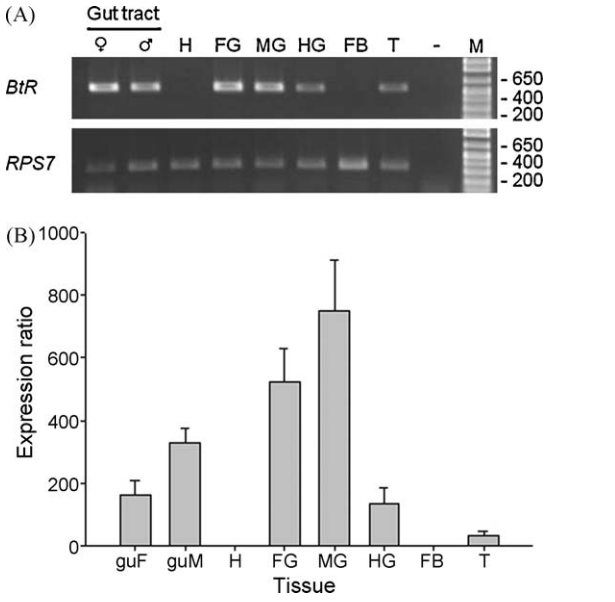

Fig. 3. RT-PCR (A) and qRT-PCR (B) amplification of cadherin (*BtR*) and *RPS7* fragments from different tissues of pink bollworm fourth instar larvae, including fat body (FB), intact male and female gut tract (guM and guF), head (H), foregut (FG), midgut (MG), hindgut (HG), and testis (T). qRT-PCR values from each tissue were normalized against fat body. Expression ratio for fat body and head was respectively 1 and 0.21. Bars in (B) are standard errors.

and spermatophore weight (slope = 77.1 sperm per mg, $t_{1,140} = 4.37$, SE = 17.7, $p < 0.0001$).

Linear contrasts indicated that transfer of eupyrene sperm bundles was lower for *rr* than *ss* (Table 2: two-tailed linear contrast, $F_{1,140} = 5.34$, $p = 0.022$). However, the contrast between *rs* and *ss* males did not reveal a fitness cost ($p = 0.51$). Thus, a recessive fitness cost affected eupyrene sperm transfer in both strains. The transfer of eupyrene sperm bundles was not significantly affected by strain origin ($p = 0.44$), male weight ($p = 0.12$), or male genotype ($p = 0.068$). Spermatophore weight was positively associated with eupyrene sperm transfer (slope = 40.5 bundle per g, SE = 15.3, $t_{1,140} = 2.63$, $p = 0.0094$).

Spermatophore weight was lower in males from Mohave (Least squares mean = 0.44, SE = 0.013) than Safford (0.51, SE = 0.016) ($F_{1,141} = 11.0$, $p = 0.0011$). A positive association occurred between male weight and spermatophore weight (slope = 31.0 mg spermatophore per g male weight, SE = 5.6, $t_{1,141} = 5.56$, $p < 0.0001$). While spermatophore weight was positively associated with transfer of apyrene and eupyrene sperm (see above), no significant difference in spermatophore weight occurred between *ss* and *rr* (Table 2: $p = 0.078$). Thus, the recessive costs affecting apyrene and eupyrene sperm transfer did not occur because the *rr* males produced lighter spermatophores than the *ss* males. Linear contrasts revealed similar spermatophore weight in *rs* and *ss* males ($p = 0.68$).

Weight of males from Mohave (Least squares means = 8.9, SE = 0.24) was less than weight of males from Safford (Least squares means = 10.1, SE = 0.30) ($F_{1,141} = 15.1$, $p = 0.0002$), as males from hybrid strains (Least squares means = 7.7, SE = 0.28) were lighter than males from resistant strains (Least squares means = 11.3, SE = 0.61) ($F_{1,141} = 18.5$, $p < 0.0001$). Costs affecting apyrene sperm transfer could have resulted from the production of smaller *rr* than *ss* males (see above). Indeed, linear contrasts revealed that *rr* males weighed less than *ss* males (Table 2: $F_{1,141} = 9.74$, $p = 0.0022$). Weight did not differ between *rs* and *ss* males ($p = 0.060$). Thus, recessive fitness costs affected male weight in both strains.

4. Discussion

Previous results showed that when susceptible and cadherin-based resistant pink bollworm males competed for matings and females mated repeatedly, the homozygous Bt-resistant males that mated first with a virgin APHIS-S female sired significantly fewer offspring than susceptible males that mated first (Higginson et al., 2005). Here, we found that transfer of apyrene sperm to virgin APHIS-S females was lower in homozygous resistant males than in susceptible males (Table 2), supporting our hypothesis that cadherin mutations conferring resistance to Cry1Ac reduce the transfer of apyrene sperm. However, a recessive fitness cost affecting transfer of eupyrene sperm was also detected (Table 2). Lepidopteran sperm bundles typically contain 256 eupyrene sperm (Cook and Wedell, 1996; Koudelova and Cook, 2001). Because homozygous resistant males still transferred many sperm bundles to females (Table 2), the number of eupyrene sperm they

Table 2
Least squares means for the number of apyrene sperm and eupyrene sperm bundles transferred to APHIS-S susceptible females. Least squares means for the weight of the male genotypes and weight of spermatophore transferred to females are also shown. *N* refers to the number of matings involving *P. gossypiella* males of each cadhering genotype. Standard errors are provided in parentheses.

Genotype	*N*	Apyrene sperm	Eupyrene sperm	Male weight (mg)	Spermatophore weight (mg)
rr	42	74.3 (4.2)	58.1 (3.6)	8.0 (0.4)	0.50 (0.02)
rs	49	91.2 (3.8)	66.2 (3.3)	9.9 (0.5)	0.47 (0.02)
ss	55	91.8 (3.5)	69.1 (3.0)	10.6 (0.5)	0.46 (0.02)

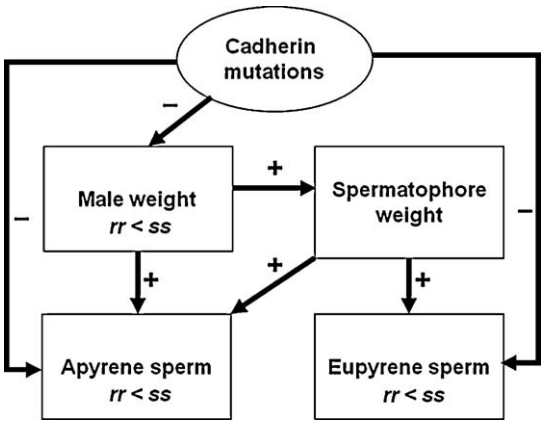

Fig. 4. Factors affecting apyrene and eupyrene sperm transfer from pink bollworm males to susceptible females. Arrows indicate a significant association between factors. Plus or minus signs beside arrows indicate positive or negative associations. Results of significant contrasts between genotypes ($rr < ss$) are shown below factors.

transferred likely exceeded the number of available eggs. Thus, the recessive cost affecting eupyrene sperm transfer detected here is consistent with previous results that female fecundity was similar when females mated with a single resistant or susceptible male (Higginson et al., 2005).

A summary of results reported here shows that sperm transfer was affected by male weight, spermatophore weight, and recessive fitness costs associated with resistance to Cry1Ac (Fig. 4). Spermatophore weight was positively associated with the number of eupyrene bundles and apyrene sperm transferred to females in both strains, a situation apparently uncommon in Lepidoptera (Friedländer et al., 2005). However, no recessive costs affected spermatophore weight (Fig. 4), indicating that the fitness costs affecting first-male paternity (Higginson et al., 2005) did not result from effects of the cadherin mutations on spermatophore production. Male weight was positively associated with spermatophore weight, a common trend in the Lepidoptera (Bissoondath and Wiklund, 1996; Marcotte et al., 2003). Male weight was also positively associated with the transfer of apyrene sperm, but not with transfer of eupyrene sperm. Importantly, cadherin mutations induced recessive costs on male weight (Fig. 4). Such costs could have reduced apyrene sperm transfer in homozygous resistant males because male weight was positively associated with transfer of apyrene sperm. Furthermore, recessive costs affecting male weight could also have reduced apyrene and eupyrene sperm transfer in homozygous resistant males via the link between male weight, spermatophore weight, and sperm transfer (Fig. 4). Thus, while our results support the hypothesis that the reduction in first-male paternity in Bt-resistant males is mediated by indirect effects of cadherin mutations on apyrene sperm transfer, cadherin mutations also induced recessive costs on other traits (male weight and eupyrene sperm production) that could have affected first-male paternity.

RT-PCR revealed that cadherin transcripts are not abundant in embryos, increased to high levels in the first three larval instars, dropped dramatically in fourth instar males but less in females, reached low levels in pupae, and increased again in adult males but less in females (Fig. 2). Production of eupyrene and apyrene sperm typically occurs before and after pupation, respectively (Swallow and Wilkinson, 2002; Friedländer et al., 2005). If this were the case in pink bollworm, the relatively high cadherin transcription in third instars and adult males would be consistent with significant effects of cadherin mutations on eupyrene and apyrene sperm transfer, respectively. Similar to other studies in Lepidoptera (Midboe et al., 2003; Aimanova et al., 2006), RT-PCR confirmed that cadherin transcription was most prevalent in the larval gut tract of pink bollworm, although the significant levels of *BtR* transcript observed in testes suggest that cadherin could also play a role in sperm production (Fig. 3).

Cadherin mutations conferring resistance to Bt have been identified in three key lepidopteran pests of cotton, but the inherent function of these cadherin proteins remains unclear (Gassmann et al., 2009). Although *Drosophila melanogaster* does not appear to have an orthologue to the lepidopteran cadherin Bt receptors, the Cad99C cadherin is important for regulating the length of apical microvilli in *Drosophila* follicle cells (D'Alterio et al., 2005). Cadherin proteins that bind Bt toxins in the insect midgut are most similar to the classical cadherins (type 1) distributed in many mammalian tissues, which confer calcium-dependent cell–cell adhesion (Gessner and Tauber, 2000; Aimanova et al., 2006). Midboe et al. (2003) hypothesized that insect midgut cadherin proteins guide cell proliferation and differentiation, contributing to the maintenance of the structural integrity of the larval digestive tract during development. However, several types of classical cadherins are expressed in mammalian testes, where they contribute to the structural integrity of the epididymis (the coiled tube connecting testicles to the vas deferens) and support normal sperm maturation (Cyr et al., 2008). Furthermore, classical cadherins are also found on plasma membranes of spermatozoa and oocytes and may play an important role in gamete interactions (Ziv et al., 2002; Purohit et al., 2004). Therefore, the established role of classical cadherins in sperm maturation and gamete interactions in mammals, along with transcription of a midgut cadherin in the testes of pink bollworm (Fig. 3), are in agreement with the finding that cadherin mutations conferring resistance to Bt reduced both apyrene and eupyrene sperm transfer in pink bollworm (Fig. 4). Nevertheless, the cadherin mutations had negative effects on male weight, indicating that such mutations could have detrimental effects on digestive physiology, and thus affect the expression of many reproductive traits (Fig. 4).

As we did not quantify apyrene and eupyrene sperm in the duplex (the sperm storing region of the ejaculatory duct), it is unknown whether apyrene and eupyrene sperm were transferred to the female spermatheca in the same proportion that they were stored in the duplex. Data on apyrene and eupyrene sperm in the duplex of Bt-resistant and susceptible pink bollworm males will be needed before concluding that the cadherin mutations reduced the production of apyrene and eupyrene sperm in homozygous resistant males. Although the transfer of eupyrene sperm was not affected by strain origin, males from Mohave were generally smaller, had smaller spermatophores, and transferred relatively more apyrene sperm than males from Safford. Such differences were likely genetically based, as the strains had been reared under identical laboratory conditions for several generations before these experiments. Because the Mohave and Safford strains have been reared in the laboratory since 1997 (Tabashnik et al., 2000), we do not know if these differences existed in the field or arose in the laboratory.

Tight physiological regulation of apyrene and eupyrene sperm differentiation and the reduction in fertilization opportunity associated with production of chromatin-free apyrene sperm suggest an adaptive role of sperm heteromorphism (Swallow and Wilkinson, 2002; Friedländer et al., 2005; Till-Bottraud et al., 2005). While it has been proposed that apyrene sperm contribute to male competitive success in the presence of sperm competition, the several hypotheses that explain sperm heteromorphism have been difficult to evaluate. The present finding that cadherin mutations affect transfer of apyrene and eupyrene sperm in pink bollworm suggests that cadherin genes could provide molecular markers associated with variation in sperm heteromorphism in insects. Such markers could be useful for designing studies that

will more precisely elucidate the adaptive value and function of apyrene sperm.

Acknowledgements

This research was partly supported by USDA Agriculture Biotechnology Risk Assessment Research Grant 2003-04371 and USDA National Research Initiative Grant 2006-35302-17365. Mention of a commercial or proprietary product does not constitute a recommendation by the USDA. We thank Dawn Higginson and two anonymous reviewers for comments that improved the manuscript.

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