Mating Success and Spermatophore Composition in Western Corn Rootworm (Coleoptera: Chrysomelidae)

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ABSTRACT Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) resistance management for transgenic (or *Bt*) corn hinges on understanding the mating behavior and biology of this adaptable insect pest. During mating, the male transfers sperm and additional, previously uncharacterized material, to the female in the form of a spermatophore. We investigated the composition of rootworm spermatophores. Proteins were found to be a major component, and the stable isotope ^{15}N was used to assess the fate of spermatophore nitrogen in mated female beetles and their eggs. We also performed longevity studies on mated and virgin females under three different diet treatments and investigated the relationships between morphometric characteristics and spermatophore volume of mating pairs of beetles. The stable isotope analysis determined that nitrogen provided to the female in the spermatophore was incorporated into the eggs. We found that virgin female beetles on a corn diet lived significantly longer than mated female beetles on the same diet. There were significant positive relationships between male size parameters (head capsule width, pronotum width, and elytral length) and spermatophore volume, and ampulla and spermatophylax volume.

KEY WORDS male investment, stable isotopes, female longevity, *Bt* corn, head capsule width

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte, first was documented as a pest of corn (*Zea mays* L.) in 1909 in Colorado (Gillette 1912). Since then, *D. v. virgifera* has spread across much of the Midwest (Ball 1957, Weekman 1961, Gould 1971) and Europe (Gray et al. 2009) and has developed resistance to a variety of control techniques, including crop rotation (Gray et al. 1998, Levine et al. 2002) and several classes of insecticides (Hamilton 1965, Levine and Oloumi-Sadeghi 1991, Meinke et al. 1998).

In 2003, rootworm-resistant transgenic corn hybrids expressing insecticidal proteins, derived from the bacterium *Bacillus thuringiensis* (*Bt* corn, Berliner), were released for rootworm control (EPA 2005). Use of *Bt* corn can reduce pesticide applications (Carpenter and Gianessi 2001, Shelton et al. 2002, Brookes and Barfoot 2005, Onstad et al. 2011), but insect resistance management (IRM) tactics are essential for maintaining the longevity of these technologies, specifically because none of the toxins expressed in current hybrids are considered high-dose (EPA 2005, Lefko et al. 2008). Recently, field-evolved resistance to the Cry3Bb1 toxin (one of the most widely-deployed toxins for management of the corn rootworm complex) was documented in several locations in Iowa, underscoring the fact that resistance management remains a critical issue for *Bt* corn (Gassmann et al. 2011). Resistance management of *Bt* corn is presently accomplished using the refuge strategy (EPA 2005), in which a portion of the *Bt* cornfield is planted with corn that is not resistant to rootworms (i.e., a refuge) (EPA 2005, 2010). The refuge is expected to yield a high population of susceptible beetles that will mate at random with potentially resistant beetles emerging from the *Bt* corn portion of the crop (Georghiou and Taylor 1977, Gould 1994, Gould 1998, Tabashnik et al. 2004). However, there are several critical unknowns in this mating system (reviewed by Spencer et al. 2009).

Although many resistance management models carry an assumption of random mating (Georghiou and Taylor 1977, Gould 1986, Gould 1994, Onstad and Gould 1998, Storer 2003), there are indications that mating in the western corn rootworm is not random. Although male western corn rootworms are capable of mating multiple times, they are less likely to mate as they age (Branson et al. 1977, Kang and Krupke 2009a). Females rarely mate a second time or beyond 24 h after emergence (Ball 1957, Cates 1968, Branson et al. 1977). There is some indication of size preferences as well: males that are larger often mate first (Quiring and Timmins 1990), and males tend to court large females more persistently (Kang and Krupke 2009b).

In western corn rootworms, copulation lasts from 1 to over 4 h (Cates 1968, Lew and Ball 1980, Kang and Krupke 2009a). During that time, the male deposits a spermatophore in the bursa copulatrix of the female

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(Lew and Ball 1980), which can represent up to 9% of the male's body mass (Quiring and Timmins 1990). The spermatophore has two portions: a gold-colored, partially sclerotized capsule (the ampulla), and a light-colored, gelatinous portion (the spermatophylax) (Lew and Ball 1980, Wedell and Arak 1989). The spermatozoa are deposited mainly in the ampulla and migrate to the spermatheca, which is attached to the bursa copulatrix, within ≈3 d after mating (Lew and Ball 1980). The spermatophore degrades from 3 to 7 d after mating (Branson et al. 1977, Lew and Ball 1980).

Spermatophores are commonly associated with either male investment, mate guarding, or both (Thornhill 1976, Vahed 1998). In bush crickets, the spermatophore constitutes ≈7–30% of male body mass and is offered to the female as a nuptial gift to facilitate and prolong sperm transfer (i.e., mate guarding) (Wedell and Arak 1989, Heller et al. 1998). Spermatophore composition and size may vary within and between species (Bissoondath and Wiklund 1995, Blanco et al. 2009, Crespo et al. 2010). For example, lepidopteran spermatophores vary in size between species, ranging from 1 to 11% of the male body mass (Boggs and Gilbert 1979, Boggs 1981, Bissoondath and Wiklund 1995). Those of bush crickets and butterflies have been found to contain large amounts of protein, and some carbohydrates, that are transferred to the female and provide additional nutrients for her offspring (i.e., male investment) (Boggs and Gilbert 1979, Bissoondath and Wiklund 1995, Heller et al. 1998). In a study of a congener of western corn rootworm, the spotted cucumber beetle, *Diabrotica undecimpunctata howardi* Barber, spermatophores of males that fed on cucurbits were found to contain protective compounds known as cucurbitacins (Tallamy et al. 2000). In some cases, the size of males and females in mating pairs covaries and serves as an indicator for spermatophore size in males or female fecundity in females (Wedell 1993a). However, there are no empirical data describing the constituents of the western corn rootworm spermatophore or their fate in females.

Spermatophores may serve a combination of several functions during and after mating (Thornhill 1976, Vahed 1998). If the spermatophore is mainly a form of male parental investment, it should: 1) limit the ability of males to mate again, and 2) significantly increase the fitness of his offspring (Trivers 1972, Sakaluk 1986, Parker and Simmons 1989). In contrast, spermatophores that function mainly for paternity assurance (i.e., mating plugs, aid the male in courting the female, etc.) should represent a relatively low cost to the male and thus be low in compounds like proteins (Thornhill 1976, Wedell 1993b). Males that minimize the cost of mate guarding efforts should, in theory, be able to inseminate more females and be favored selectively over males that have a longer refractory period because they produce spermatophylaxes with greater amounts of protein (Wedell 1993b, Vahed 1998). We sought to test the hypothesis that western corn rootworm spermatophores are high in nutrients (i.e., proteins) and that these compounds are used by both the female and her offspring.

If the spermatophore in western corn rootworms is a form of parental investment, variation between males in fitness and spermatophore size could influence the fitness of the female and her offspring, providing an opportunity for sexual selection (Thornhill 1976). Several male and female morphometric characters have been correlated with fitness, but have not been examined within the context of mating success. For example, elytral length, pronotum width, and head capsule width have been positively correlated with longevity in young adult males and females, as well as lifetime fecundity in mated females (Li et al. 2009). In the northern corn rootworm, *D. barberi* Smith & Lawrence, French and Hammack (2010) determined that larger females live longer and may be capable of producing more eggs. We sought to determine whether there is a relationship between spermatophore size and male and female morphometric characteristics.

The spermatophore represents an unexplored avenue for untangling mate choice and mating success in this widespread pest. Identifying factors, such as male or female preferences, will facilitate prediction of mating rates in *Bt*-refuge environments and will help assess the validity of the random mating assumption used in resistance management. This study was designed to further investigate the role of the spermatophore in the western corn rootworm mating system by quantifying several factors in both the laboratory and field, including: 1) characterization of the composition of western corn rootworm spermatophores; 2) the metabolic fate of western corn rootworm spermatophores; 3) the effects of mating (i.e., spermatophore transfer) on female longevity to test the hypothesis that females benefit directly from the components in the spermatophore; and 4) the relationships between several fitness parameters (head capsule width, pronotum width, and elytral length) and spermatophore size to explore potential characters under intersexual selection (i.e., are larger males consistently mating with larger females, producing larger spermatophores, or both?).

Methods

Insects. *Obtaining Spermatophores.* Mating pairs of beetles were collected from the field at two separate locations in Tippecanoe Co., IN, Throckmorton Purdue Agricultural Center (TPAC) and Agronomy Center for Research and Extension (ACRE), between 16 July and 2 August 2007 and 30 July and 20 August 2008. In an effort to avoid interrupting the mating process, mating beetles were placed carefully in 31- by 31- by 31-cm screen cages with fresh corn tassels for transport and maintenance in the lab. Pairs of beetles believed to have been interrupted were excluded. Beetles were permitted to finish mating and then held for 36 ± 12 h. They were then killed by freezing at −80°C, and were stored for subsequent dissection and chemical analysis.

Beetle Rearing. Beetles were reared partially in the laboratory for both the longevity study conducted in 2009, and the protein fate and fitness parameter studies conducted in 2010. In early to mid-June in 2009 and

2010, second- and third-instar western corn rootworm larvae were collected using methods similar to those described by Mabry et al. (2004) from blocks of unprotected non-*Bt* (i.e., containing no rootworm-resistant, *Bt*-toxins) corn hybrids at TPAC in Lafayette, IN, and Pinney Purdue Agricultural Center (PPAC) in Wanatah, IN. These hybrids included the following: Mycogen 2T777 and Mycogen 2T780 (Mycogen Seeds, Indianapolis, IN); DKC61–73 (Dekalb, St. Louis, MO); and Beck 5444RR (Beck's Hybrids, Atlanta, IN). The larvae were collected with 20-cm corn stalks, roots, and the accompanying soil (loosely packed) and placed in 1025-ml plastic cups (Fabri-kal Corp., Kalamazoo, MI) (Mabry et al. 2004). The cups were watered daily using a hand sprayer so that soil was kept slightly damp. Shortly before beetle emergence the stalks were cut to 1 cm in height. The cups were then covered with mesh lids and checked daily for emerging beetles.

Virgin beetles were also collected from the field using emergence cages at TPAC and PPAC in 2009 and TPAC in 2010. Cages were placed over individual, refuge (i.e., non-*Bt*) corn plants between 29 June and 9 July 2010. The emergence cages were modified versions of those described by Musick and Fairchild (1970). They measured 0.8 by 0.5 m and were constructed of fine wire mesh covering wood frames. The upper portion of the cage was fitted with a single funnel trap, secured, and maintained upright using a wooden stake. Cage bases were sealed by embedding them into the ground and then securing them with two tent pegs. Strips of foam were wrapped around the stalk to seal the opening around the plant. Cage contents were collected on Monday, Wednesday, and Friday in 2009. Collections were made daily in 2010.

Any emerging beetles were collected and sorted by sex into 473-ml or 947-ml glass canning jars with mesh lids (2009), or inverted 266-ml or 591-ml plastic cups (Gordon Food Services, Grand Rapids, MI) with $1{,}200 \pm 100$ mm^2 of ventilation holes covered with fine cloth mesh (2010). The containers were supplemented with a water source (moist cotton dental wick) and fresh, sliced, young corn ears, silks, and tassels replaced every Monday, Wednesday, and Friday in 2009, and every day in 2010. These colony containers were labeled with the date of emergence, source location, and sex and then placed in growth chambers (model I-36LLVL, Percival Scientific, Inc., Perry, IA) at a temperature of 21.0 ± 1.5°C, $80\% \pm 15\%$ RH in 2009, or 22.5 ± 2°C, $80\% \pm 5\%$ RH in 2010, and a photoperiod of 14:10 (L:D) h (Jackson and Elliot 1988).

Mating Assays. We obtained mated females for the longevity study in 2009, and the protein fate and fitness parameter studies in 2010, using the following procedure. Adults were mated by placing one virgin female and male beetle in an inverted 266-ml plastic cup as described above. Each pair was provided with young corn ears, leaves, silks, or both. The pairs were monitored at least every 30 min to check for mating activity. Pairs found in copula were noted and the time recorded. Beetles were monitored for ≈4 h for successful mating activity, or until mating ceased (Cates 1968, Kang and Krupke 2009a). A successful mating was defined as one that lasted at least 2.0 h (Sherwood and Levine 1993). When mating was complete, the time was again recorded.

Spermatophore Composition. *Sample Preparation.* Spermatophores were removed from mated females by dissection in a 0.75% saline solution (Sherwood and Levine 1993). Each spermatophore was rinsed five times in 5% chlorine bleach solution and then five times in 0.75% NaCl solution to remove any extraneous particles or contaminants, then stored in labeled vials at −80°C. Spermatophores were not removed from the bursa copulatrix as this tissue constituted a very minor portion of the sample and held the spermatophylax and the ampullae intact. Before analysis, spermatophores were weighed to the nearest 0.0001 g (model AE100, Mettler-Toledo, Columbus, OH) and then homogenized with 250 μl–500 μl of deionized water using a sonifier (Branson Sonifier 250, Branson Ultrasonics Corporation, Danbury, CT) at a setting of one (lowest intensity).

Protein and Carbohydrates. The protein content was estimated for 32 individual spermatophores using a Coomassie Plus Bradford Assay Kit (Product Number 23236, Thermo Scientific: Pierce Biotechnology, Rockford, IL). The assay was performed according to instructions provided by the manufacturer, and spermatophores were diluted with deionized water in proportion to mass (using the approximation 500-μl deionized water for every 0.01 mg of mass) to obtain measurable values. The Bradford assay was performed immediately after weighing, homogenization, and dilution to avoid significant protein degradation. The carbohydrate content of 46 spermatophores (processed in pairs to obtain quantifiable amounts of carbohydrates) was analyzed using a colorimetric anthrone assay (Koehler 1952, Mokrasch 1954). Paired spermatophores were collected on the same date and often from the same location.

Fate of Spermatophore Proteins. The beetles were collected from emergence cups (see Beetle Rearing) in the lab daily. The males were randomly assigned to one of two different feeding treatments: 1) L-Alanine enriched (control), or 2) ^{15}N L-Alanine enriched (treatment). Alanine was selected because it is the fourth most abundant amino acid in corn (George et al. 2004). The base diet for male beetles consisted of fresh field corn ears, silks, and tassels pureed with deionized water and enriched with either L-alanine (control; Cat. No. AC102830250, Fisher, Pittsburgh, PA) or ^{15}N L-alanine (treatment; Cat. No. NLM-454–0.5, Cambridge Isotope Laboratories, Andover, MA). Enrichments were added at a level of ≈0.5% fresh mass of the diet mixture (Petroski et al. 1994, James et al. 2006). This diet was either prepared and frozen until use (early in the study) or prepared fresh daily and delivered at a rate of 0.5 ± 0.05 g per container per day. Male diet was replaced daily in each colony container, which held no >12 male beetles. Female beetles were placed on a diet of fresh field corn ears, silks, and tassels and fed daily.

Only male beetles of 7 to 10 d old and <2-d-old females (Lew and Ball 1979) were placed in mating assays. No food was provided during the assay to maintain consistency in male diets. Once a successful mating (>2-h duration) was completed and the pair had separated, the males were removed and frozen. Females were returned to a diet of fresh field corn, changed every Monday, Wednesday, and Friday, for ≈9–14 d when egg development was estimated to be complete (Hill 1975, Lew and Ball 1980). Females were then frozen at −80°C. The mature eggs from each individual female were collected by first checking the surfaces in the enclosure, as females may oviposit on a variety of moist surfaces (Kirk et al. 1968), and by dissection (if she was frozen just before oviposition), and placed in a labeled 1.5-ml microcentrifuge tube. All eggs were collected using forceps, a micropipette, or both, and all dissections were performed as described previously. Before analysis, all the eggs from an individual female were dried for 72 h at 50 ± 5°C in a single-wall transit drying oven (model SW-17TA, Blue M Electrical company, Blue Island, IL), ground by hand using one end of a metal probe, and packed into 5- by 9-mm pressed tin capsules (Product No. 041077, Costech Analytical Technologies, Valencia, CA) at 0.500 ± 0.0400 mg per sample. Samples were submitted to Purdue Stable Isotope Facility (550 Stadium Mall Dr, West Lafayette, IN 47907) for ^{15}N content analysis and reported as $\delta\,^{15}N$-values, according to standard procedure (Peterson and Fry 1987).

Effects on Female Longevity. The study contained six treatments and was replicated 33 times. The treatments included: 1) mated female on a corn and water diet, 2) mated female on a water only diet, 3) mated female without access to food or water (no provisions), 4) virgin female on a corn and water diet, 5) virgin female on a water only diet, and 6) virgin female with no provisions. Food and water were changed or checked (respectively) three times each week, on Monday, Wednesday, and Friday. While corn was available from the field, each beetle in the corn and water treatments received 7.0 ± 0.1 g of fresh corn ears, silks, and leaves at each feeding. When corn was no longer available from the field, frozen corn (collected earlier in the season) was used in all treatments. As frozen and thawed corn was found to be more susceptible to mold and bacteria, it was first sanitized using a 5% solution of bleach, and then rinsed thoroughly before feeding it to the beetles. The rearing cups and water supply were also exchanged, washed in bleach water and rinsed thoroughly before reuse at each feeding when frozen corn was used.

Male beetles from 7 to 27 d old and females from 4 to 27 d old were placed in mating assays, as described previously. Mated female beetles were then placed individually in labeled 266-ml plastic cups and randomly assigned to one of three feeding treatments within 24 h of completing mating. Virgin female beetles were selected randomly from the remaining colony beetles with similar emergence dates (±3 d) as the mated females, and placed in the same feeding treatments to complete each replication. Cups were checked approximately once every 24 h to record the date of death.

Beetle Dissections. Dissections were performed as described by Sherwood and Levine (1993) in 0.75% saline solution using a compound scope equipped with a camera (Leica Microsystems, Inc., Bannockburn, IL). Using the Leica Application Suite program, morphometric characters and the reproductive tract (females only; mainly the bursa copulatrix, ovaries, or both) were photographed and measured to within 0.01 mm. The head capsule was measured at its widest point, from eye to eye. Measurements were taken when the bursa copulatrix contained a spermatophore. The volumes of the spermatophylax and ampulla were each determined separately, and then combined. Measurements were taken such that each width and length combination best represented an ovoid sphere, and calculated using the following equation, after Campbell (2009):

$$Volume = \frac{4}{3}\pi\left(\frac{length}{2}\right)\left(\frac{width}{2}\right)^2$$

Each photograph was labeled with the study and number of the beetle, as well as the date and location of collection.

Relationships Between Fitness Parameters. Male beetles of 7–10 d old and female beetles ≤ 2 d old (Lew and Ball 1979) that mated successfully in mating assays (see Mating Assays) were frozen as a pair, and stored in labeled vials at −80°C until dissection. Dissections were performed as described previously, except that morphometric characteristics (head capsule width, pronotum width, and elytral length) were photographed and measured in 70% ethanol before dissection. Elytral length measurements were taken from the right elytron from the dorsal view on each beetle. In total, 30 pairs of randomly selected beetles were dissected.

Field-Collected Pairs. Mating pairs were collected at PPAC and TPAC in individual 266-ml containers and provided with fresh food and water shortly after collection. At TPAC, 5–6 mated pairs were collected each day between 15 July and 21 July 2010 by searching rows of refuge corn for ≈1 h between 0730 and 1000 hours each day. The western corn rootworm population at TPAC in 2010 was extremely low compared with previous years (A.F.M., unpublished data). At PPAC, where beetles were more abundant, 15–20 pairs were collected over ≈1 h between 0830 and 1030 hours on 28 July and 29 July 2010. Mating pairs were allowed to finish mating and feed for 24 h after collection before they were frozen, placed in labeled vials, and stored at −80°C until dissection. For each location, 15 pairs were selected randomly for dissection. Beetles were dissected and photographed as described previously.

Data Analysis

Spermatophore Fate. The data for the spermatophore fate assay were not normal, even after trans-

Table 1. Protein and carbohydrate content of spermatophores of western corn rootworm collected in 2007 and 2008

	Proteins	Carbohydrates
Total fresh spermatophore mass (μg)	1158 ± 35.6 (n = 78)	
Total per spermatophore (μg)	84.56 ± 5.99	7.77 ± 0.32
Percent of spermatophore fresh mass	7.40 ± 0.27%	0.66 ± 0.02%
n	32	23[1]

These data were derived using spermatophores dissected from mated females collected while in copula from two different field locations.

1. 46 spermatophores were analyzed in 23 pairs to obtain quantifiable amounts of carbohydrates.

formation, so the treatments were compared using a Mann–Whitney test. The percent nitrogen by mass and the duration of mating were analyzed using linear regression.

Female Longevity. The mean number of days females survived on each feeding treatment (corn and water, water only, or no provisions) were analyzed individually using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests. The data for the "water only" and "no provisions" treatments had to be transformed using a Johnson transformation (before analysis because they failed to meet the normality assumptions necessary for ANOVA). A linear regression analysis was also performed to test the hypothesis that longevity was related to the duration of mating.

Relationships Between Fitness Parameters. The relationship between male or female morphometric characters and spermatophore size was analyzed using linear regression. Relationships between the size parameters of male and females in a given pair were also analyzed using linear regression. The data from pairs mated in the field and lab were analyzed separately. For the data from field-collected beetles, the variables of female head capsule width and female pronotum width were transformed with Box–Cox transformations, and female elytra width was transformed using a Johnson transformation, because they failed to meet the necessary normality assumptions. The volume of the ampulla and the spermatophylax were also analyzed with a linear regression analysis. All analyses were performed using Minitab Version 16.1.1 Statistical Software (Minitab Inc., State College, PA).

Results

Spermatophore composition was found to be 7.40 ± 0.27% proteins and 0.66 ± 0.02% carbohydrates (Table 1). The first mass of eggs from females that mated with males on the ^{15}N L-alanine diet had significantly greater $\delta\,^{15}$N-values compared with eggs from females that mated with control males (W = 378.0; $P < 0.001$) (Fig. 1). We found no relationship between the duration of mating and the percentage of nitrogen in the spermatophore (r = 0.000; F = 0.13; df = 1, 58; P = 0.720).

Virgin females on a corn and water diet lived significantly longer than mated females on the same diet (F = 24.51; df = 1, 66; $P < 0.001$), though no significant difference was found for virgin and mated females on a water only diet (Fig. 2). We found no relationship between the longevity of a female and the duration of mating, specifically for females on a corn and water diet (F = 0.00; df = 1, 31; P = 0.972).

The mean estimated volume of a spermatophore was 0.65 ± 0.043 mm^3 for field-collected pairs, and 0.80 ± 0.039 mm^3 for laboratory assays. No relationship was found between female head capsule width and spermatophore size in field (r = 0.000; F = 0.00; df = 1, 28; P = 0.981; n = 30) or lab studies (r = 0.173; F = 1.90; df = 1, 28; P = 0.179; n = 30) (Table 2). We did

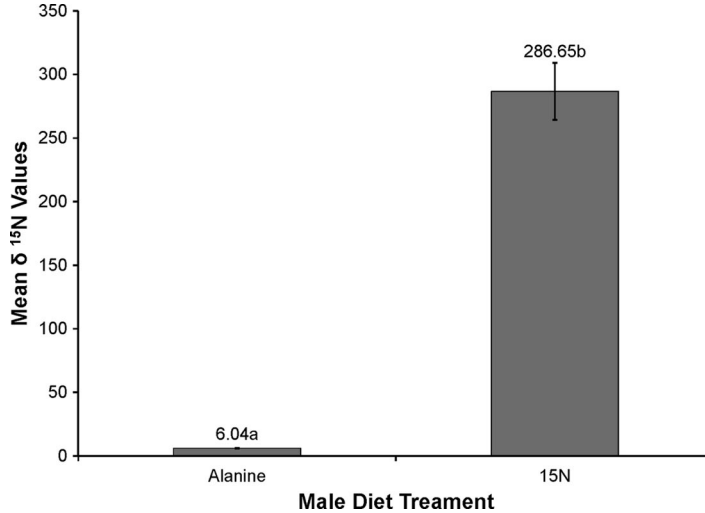

Fig. 1. The mean $\delta\,^{15}$N values (±SEM), in parts per thousand (permill), for the first mass of eggs from western corn rootworm females that mated with males on either an alanine-enriched diet or a ^{15}N-enriched diet. Differences are significant at a level of P = 0.05. For the alanine treatment, n = 27, for the ^{15}N treatment, n = 35.

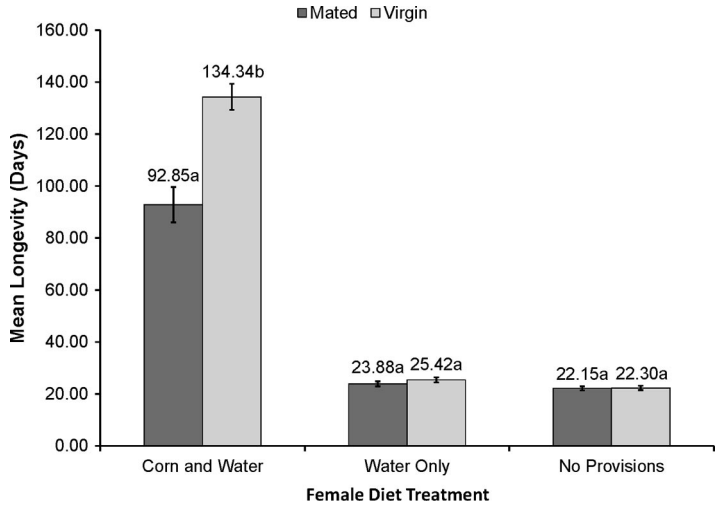

Fig. 2. Mean longevity (±SEM) of mated and virgin western corn rootworm females on three different diet treatments. Longevity was measured as days from eclosion. Values that do not share the same letter within a female diet treatment are significantly different. Differences are significant at a level of $P = 0.05$. For almost all mating status × diet combinations $n = 33$. For virgin females on a corn and water diet, $n = 35$.

not find any relationships between the head capsule widths, pronotum widths, or elytral lengths of mating males and females in either the lab or the field-collected pairs. However, there was a significant positive linear relationship between spermatophore size and male head capsule width in field-collected pairs ($r = 0.475$; $F = 9.45$; df = 1, 28; $P = 0.005$; $n = 30$), as well as a significant positive linear relationship between spermatophore size and male pronotum width ($r = 0.339$; $F = 4.77$; df = 1, 28; $P = 0.038$; $n = 30$). For beetles mated in the laboratory, there was a significant positive relationship between spermatophore size and male head capsule width ($r = 0.420$; $F = 7.22$; df = 1, 28; $P = 0.012$; $n = 30$); pronotum width ($r = 0.421$; $F = 7.25$; df = 1, 28; $P = 0.012$; $n = 30$); and elytral length ($r = 0.400$; $F = 6.54$; df = 1, 28; $P = 0.016$; $n = 30$). There was also a significant positive linear relationship between the size of the ampulla and the spermatophylax in field-collected beetles ($r = 0.407$; $F = 6.79$; df = 1, 28; $P = 0.015$; $n = 30$) and laboratory assays ($r = 0.379$; $F = 5.87$; df = 1, 28; $P = 0.022$; $n = 30$).

Discussion

Our results demonstrate that protein constitutes a majority of the nutrients in western corn rootworm spermatophores, given that spermatophores are typically over 80% water (Boggs 1981, Heller et al. 1998). The protein content of western corn rootworm spermatophores (7.4%) is within the range of some of the other known spermatophore compositions: 3.19% for *Pieris rapa* (Lepidoptera: Pieridae) (Bissoondath and Wiklund 1995), 4.23% for *Decticus verrucivorus* (Orthoptera: Tettigoniidea) (Heller et al. 1998), and 9% for *Poecilimon veluchianus* (Orthoptera: Tettigonioidea:) (Heller et al. 1998). Furthermore, there is evidence to suggest that males may be limited in their ability to mate again, particularly as they age (Kang

Table 2. Relationships between male and female fitness parameters and spermatophore size

Source	Parameters	Result
Field	Female head capsule width × male head capsule width	NS
Lab	Female head capsule width × male head capsule width	NS
Field	Female head capsule width × spermatophore size	NS
Lab	Female head capsule width × spermatophore size	NS
Field	Female pronotum width × spermatophore size	NS
Lab	Female pronotum width × spermatophore size	NS
Field	Female elytral length × spermatophore size	NS
Lab	Female elytral length × spermatophore size	NS
Field	Male head capsule width × spermatophore size	**
Lab	Male head capsule width × spermatophore size	*
Field	Male pronotum width × spermatophore size	*
Lab	Male pronotum width × spermatophore size	*
Field	Male elytral length × spermatophore size	NS
Lab	Male elytral length × spermatophore size	*

Only key comparisons were included. NS, not significant; *, $P < 0.05$; **, $P < 0.01$.

and Krupke 2009a), which may be due in part to the high costs of spermatophore production. These observations agree with the paternal investment theory, which stipulates that male contributions should be costly to produce (i.e., high in protein) and limit or reduce the number of subsequent copulations (Trivers 1972).

The results of ^{15}N mating assay and analysis provide further support for the hypothesis that male western corn rootworms contribute nutrients to their offspring. Our results demonstrate that nitrogen from male western corn rootworms is incorporated into the eggs of females. The limitations (i.e., we did not measure the ^{15}N in other portions of the female body or excrement) of our study make it impossible to determine what proportion of the proteinaceous material from the spermatophore was used for egg production, as we did not include analysis of the amount of ^{15}N contained in other female body tissues. Interestingly, our longevity study indicated no direct benefits of mating to females. These results concur with similar studies performed on mated northern corn rootworm; large females lived longer regardless of male size (French and Hammack 2010). Mating initiates vitellogenesis and ovarian development (Hill 1975), which diverts resources from the fat body in the female (Klowden 2002). If proteins supplied in the spermatophore were used by the female beetle for somatic maintenance, we would expect mated female beetles to live longer than virgin female beetles. The reverse was actually the case on a corn diet, and there was a significant decrease in the longevity of mated females compared with virgin females. Even under harsh conditions (i.e., no provisions), mated females did not survive any longer than virgin females, indicating that it is unlikely the spermatophore is used by the female for somatic maintenance. According to Hill (1975), virgin females lay considerably fewer eggs compared with mated females, and a similar pattern of longevity has been demonstrated for mated and virgin females of related *Diabrotica* spp. (Campbell 2009), *Eublemma amabilis* (Moore) (Varshney et al. 1971) and diamondback moth, *Plutella xylostella* L. (Wang et al. 2005), all of which transfer spermatophores during mating (Thornhill 1976, Yang and Chow 1978, Campbell 2009). We hypothesize that a variety of substances in the western corn rootworm spermatophore regulate and stimulate female vitellogenesis, resulting in differential reproduction and longevity, as found in several other insects (Wolfner 1997, Jin and Gong 2001, Shutt et al. 2010).

This study also provides evidence that male size may serve as an indicator of male investment because several male size parameters or indicators of male fitness (head capsule width, pronotum width, and elytral length), had a significant positive linear relationship with spermatophore volume. If size is a reliable indicator of potential male investment, one might predict that females would be more receptive to larger males. It has been demonstrated previously that male western corn rootworm size influences male mating success; large males mate more quickly than small males (Quiring and Timmins 1990). Before copulation, males will mount females, stroking them with their antennae and attempting to mate with them, until they are accepted (Lew and Ball 1979, Kang and Krupke 2009b). These behaviors likely serve a variety of purposes in mate selection, including an opportunity for both individuals to assess mate size and other indicators of mate quality.

This study has potential implications for resistance management. In a previous study, Murphy et al. (2011) found that males emerging from *Bt* corn had smaller head capsule widths compared with males emerging from refuge corn. We postulated that differences in male fitness might result in differences in male investment (i.e., spermatophore size), and the results of this study lend support to that hypothesis. Under the most recently approved modification to the refuge strategy, the seed mix refuge, refuge plants are mixed randomly throughout the field so that males and females emerging from refuge and *Bt* corn are closely associated in space and largely synchronized in time (Murphy et al. 2010). In view of the results presented here, larger males emerging from the refuge may produce larger spermatophores, which might provide discriminating females with a reproductive fitness advantage. If females are capable of discriminating between males on the basis of head capsule width, pronotum width, or elytral length, then larger males emerging from the refuge would have an advantage when competing with males emerging from *Bt* corn in this system. These are important considerations that could extend the predicted durability of *Bt* corn if included in resistance management models.

The widespread adoption of *Bt* corn has highlighted several opportunities for research in previously unexplored avenues of rootworm biology. Although maximizing insect control will always be an overarching goal, we are beginning to understand that stable and durable management programs require a more comprehensive knowledge of the biology of the target organism. For example, the underpinnings of IRM strategies for *Bt* corn targeting rootworms are mathematical models that are based largely on untested assumptions (i.e., random mating) and not empirical data. Many specifics of western corn rootworm mating remain unclear, yet they are critical to estimating the rates of gene flow between susceptible and putatively resistant beetles in the transgenic corn systems that dominate the North American landscape. Our work sheds light on one aspect of mating in this species by demonstrating that males may provide more than just gametes during sexual reproduction, and that the size of this investment varies with male size. However, many important questions remain unresolved. We know males provide material for egg production, but the precise nature of these materials (and whether they vary with larval host: *Bt* versus refuge corn), is unknown. Because spermatophores vary with male size, there is opportunity for female choice and nonrandom mating, which violates one key assumption of many IRM models. Given the variation previously documented in beetles emerging from *Bt* versus ref-

uge corn, it would be useful to directly compare the spermatophores produced by the males from each type of larval host. This would allow for an assessment of whether there are differences in mating success, fecundity, or both that would influence resistance management.

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References Cited

Ball, H. 1957. On the biology and egg laying habits of the western corn rootworm. J. Econ. Entomol. 50: 126–128.

Bissoondath, C. J., and C. Wiklund. 1995. Protein content of spermatophores in relation to monandry / polyandry in butterflies. Behav. Ecol. Sociobiol. 37: 365–371.

Blanco, C. A., M. G. Rojas, A. T. Groot, J. Morales-Ramos, and C. A. Abel. 2009. Size and chemical composition of *Heliothis virescens* (Lepidoptera: Noctuidae) spermatophores. Ann. Entomol. Soc. Am. 102: 629–637.

Boggs, C. L. 1981. Selection pressures affecting male nutrient investment a mating in Heliconiine butterflies. Evolution 35: 931–940.

Boggs, C. L., and L. E. Gilbert. 1979. Male contribution to egg production in butterflies: evidence for transfer of nutrients at mating. Science 206: 83–84.

Branson, T. F., P. L. Guss, and J. J. Jackson. 1977. Mating frequency of the western corn rootworm. Ann. Entomol. Soc. Am. 70: 506–508.

Brookes, G., and P. Barfoot. 2005. GM crops: The global economic and environmental impact: the first nine years 1996–2004. AgBioForum 8: 187–196.

Campbell, L. A. 2009. Hybridization in *Diabrotica barberi* Smith and Lawrence and *Diabrotica longicornis* (Say) (Coleoptera: Chrysomelidae): biology, behavior, field introgression, and a reevaluation of taxonomic status. Ph.D. dissertation, University of Nebraska, Lincoln, NE.

Carpenter, J. E., and L. P. Gianessi. 2001. Agricultural biotechnology: updated benefit estimates. (http://www.ncfap.org/documents/updatedbenefits.pdf).

Cates, M. D. 1968. Behavioral and physiological aspects of mating and oviposition by the adult western corn rootworm, *Diabrotica virgifera* Le Conte. Ph.D. dissertation, University of Nebraska Lincoln, NE.

Crespo, A.L.B., T. A. Spencer, S. Y. Tan, and B. D. Siegfried. 2010. Fitness costs of Cry1Ab resistance in a field derived strain of *Ostrinia nubilalis* (Lepidoptera: Crambidae). J. Econ. Entomol. 103: 1386–1393.

EPA. 2005. *Bacillus thuringiensis* Cry3Bb1 protein and genetic material necessary for its production (vector ZMIR13L in event MON863 corn (006484) fact sheet. (http://www.epa.gov/oppbppd1/biopesticides/ingredients/factsheets/factsheet_006484.htm#reports).

EPA. 2010. Biopesticides Registration Action Document: Optimum AcreMax B.t. Corn Seed Blends. (http://www.regulations.gov/search/Regs/home.html#docketDetail?R=EPA-HQ-OPP-2010–0405).

French, B. W., and L. Hammack. 2010. Reproductive traits of northern corn rootworm (Coleoptera: Chrysomelidae) in relation to female and male body size. Ann. Entomol. Soc. Am. 103: 688–694.

Gassmann, A. J., J. L. Petzold-Maxwell, R. S. Keweshan, and M. W. Dunbar. 2011. Field-evolved resistance to Bt maize by western corn rootworm. PLoS ONE 6: e22629.

George, C., W. P. Ridley, J. C. Obert, M. A. Nemeth, M. L. Breeze, and J. D. Astwood. 2004. Composition of grain and forage from corn rootworm protected corn event MON 863 is equivalent to that of conventional corn (*Zea mays* L.). J. Agric. Food Chem. 52: 4149–4158.

Georghiou, G., and C. E. Taylor. 1977. Operational influences in the evolution of insecticide resistance. J. Econ. Entomol. 70: 653–658.

Gillette, C. P. 1912. *Diabrotica virgifera* Lec. as a corn rootworm. J. Econ. Entomol. 5: 364–366.

Gould, F. 1986. Simulation models for predicting durability of insect-resistant germ plasm: a deterministic diploid, two-locus model. Environ. Entomol. 15: 1–10.

Gould, F. 1994. Potential and problems with high-dose strategies for pesticidal engineered crops. Biocontrol Sci. Technol. 4: 451–461.

Gould, F. 1998. Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. Annu. Rev. Entomol. 43: 701–726.

Gould, G. E. 1971. The corn rootworm problem in Indiana. Proc. Indiana Acad. Sci. 80: 267–272.

Gray, M. E., E. Levine, and H. Oloumi-Sadeghi. 1998. Adaptation to crop rotation: western and northern corn rootworms respond uniquely to a cultural practice, pp. 19–31. *In* S. G. Pandalai (ed.), Recent research developments in entomology, vol. 2. Research Signpost, Trivandrum, India.

Gray, M. E., T. W. Sappington, N. J. Miller, J. Moeser, and M. O. Bohn. 2009. Adaptation and invasiveness of western corn rootworm: intensifying research on a worsening pest. Annu. Rev. Entomol. 54: 303–321.

Hamilton, E. W. 1965. Aldrin resistance in corn rootworm beetles. J. Econ. Entomol. 58: 296–297.

Heller, K.-G., P. Faltin, P. Fleischmann, and O. v. Helversen. 1998. The chemical composition of the spermatophore in some species of phaneropterid bushcrickets (Orthoptera: Tettigonioidea). J. Insect Pathol. 44: 1001–1008.

Hill, R. E. 1975. Mating, oviposition patterns, fecundity and longevity of the western corn rootworm. J. Econ. Entomol. 68: 311–315.

Jackson, J. J., and N. C. Elliot. 1988. Temperature-dependent development of immature stages of the western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae). Environ. Entomol. 17: 166–171.

James, W. D., A. T. Showler, J. K. Westbrook, and J. S. Armstrong. 2006. Stable isotope tracer marking of individual boll weevils. J. Radioanal. Nucl. Chem. 269: 267–270.

Jin, Z.-Y., and H. Gong. 2001. Male accessory gland derived factors can stimulate oogenesis and enhance oviposition in *Helicoverpa armigera* (Lepidoptera: Noctuidae). Arch. Insect Biochem. Physiol. 46: 175–185.

Kang, J., and C. H. Krupke. 2009a. Likelihood of multiple mating in male western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae). J. Econ. Entomol. 102: 2096–2100.

Kang, J., and C. H. Krupke. 2009b. Influence of weight of male and female western corn rootworm (Coleoptera: Chrysomelidae) on mating behaviors. Ann. Entomol. Soc. Am. 102: 326–332.

Kirk, V. M., C. O. Calkins, and F. J. Post. 1968. Oviposition preferences of western corn rootworms for various soil surface conditions. J. Econ. Entomol. 61: 1322–1324.

Klowden, M. J. 2002. Physiological systems in insects. Academic, San Diego, CA.

Koehler, L. H. 1952. Differentiation of carbohydrates by anthrone reaction rate and color intensity. Anal. Chem. 24: 1576–1579.

Lefko, S. A., T. M. Nowatzki, S. D. Thompson, R. R. Binning, M. A. Pascual, M. L. Peters, E. J. Simbro, and B. H. Stanley. 2008. Characterizing laboratory colonies of western corn rootworm (Coleoptera: Chrysomelidae) selected for survival on maize containing event DAS-59122–7 J. Appl. Entomol. 132: 189–204.

Levine, E., and H. Oloumi-Sadeghi. 1991. Management of diabroticite rootworms in corn. Annu. Rev. Entomol. 36: 229–255.

Levine, E., J. L. Spencer, S. A. Issard, D. W. Onstad, and M. E. Gray. 2002. Adaptation of the western corn rootworm to crop rotation: evolution of a new strain in response to a management practice. Am. Entomol. 48: 94–107.

Lew, A. C., and H. Ball. 1979. The mating behavior of the western corn rootworm *Diabrotica virgifera* (Coleoptera: Chrysomelidae). Ann. Entomol. Soc. Am. 72: 391–393.

Lew, A. C., and H. J. Ball. 1980. Effect of copulation time on spermatozoan transfer of *Diabrotica virgifera* (Coleoptera: Chrysomelidae). Ann. Entomol. Soc. Am. 73: 360–361.

Li, H., S. Toepfer, and U. Kuhlmann. 2009. Relationship between phenotypic traits and selected fitness components of *Diabrotica virgifera virgifera.* Entomol. Exp. Appl. 131:254–263.

Mabry, T. R., J. L. Spencer, E. Levine, and S. A. Isard. 2004. Western corn rootworm (Coleoptera: Chrysomelidae) behavior is affected by alternating diets of corn and soybean. Environ. Entomol. 33: 860–871.

Meinke, L. J., B. D. Siegfried, R. J. Wright, and L. D. Chandler. 1998. Adult susceptibility of Nebraska western corn rootworm (Coleoptera: Chrysomelidae) populations to selected insecticides. J. Econ. Entomol. 91: 594–600.

Mokrasch, L. C. 1954. Analysis of hexose phosphates and sugar mixtures with the anthrone reagent. J. Biol. Chem. 208: 55–59.

Murphy, A. F., M. D. Ginzel, and C. H. Krupke. 2010. Evaluating western corn rootworm (Coleoptera: Chrysomelidae) emergence and root damage in a seed mix refuge. J. Econ. Entomol. 103: 147–157.

Murphy, A. F., N. J. Seiter, and C. H. Krupke. 2011. The impact of Bt maize as a natal host on adult head capsule width in field populations of western corn rootworm. Entomol. Exp. Appl. 139: 8–16.

Musick, G. J., and M. L. Fairchild. 1970. Field emergence cages for corn rootworm adults. J. Econ. Entomol. 63: 1710–1711.

Onstad, D. W., and F. Gould. 1998. Modeling the dynamics of adaptation to transgenic maize by European corn borer (Lepidoptera: Pyralidae). J. Econ. Entomol. 91: 585–593.

Onstad, D. W., P. D. Mitchell, T. M. Hurley, J. G. Lundgren, P. Porter, C. H. Krupke, J. L. Spencer, C. D. DiFonzo, T. S. Baute, R. L. Hellmich, et al. 2011. Seeds of change: corn seed mixtures for resistance management. J. Econ. Entomol. 104: 343–352.

Parker, G. A., and L. W. Simmons. 1989. Nuptial feeding in insects: theoretical models of male and female interests. Ethology 82: 3–26.

Peterson, B. J., and B. Fry. 1987. Stable isotopes in ecosystem studies. Annu. Rev. Ecol. Syst. 18: 293–320.

Petroski, R. J., R. J. Bartelt, and D. Weisleder. 1994. Biosynthesis of (2E,4E,6E)-5-ethyl-3-methyl-2,4,6-nonatriene: the aggregation pheromone of *Carpophilus freemani* (Coleoptera: Nitidulidae). Insect Biochem. Mol. Biol. 24: 69–78.

Quiring, D. T., and P. R. Timmins. 1990. Influence of reproductive ecology on feasibility of mass trapping *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae). J. Appl. Ecol. 27: 965–982.

Sakaluk, S. K. 1986. Is courtship feeding by male insects parental investment? Ethology 73: 161–166.

Shelton, A. M., J.-Z. Zhao, and R. T. Roush. 2002. Economic, ecological, food safety, and social consequences of the deployment of *Bt* transgenic plants. Annu. Rev. Entomol. 147: 845–881.

Sherwood, D. R., and E. Levine. 1993. Copulation and its duration affects female weight, oviposition, hatching patterns, and ovarian development in the western corn rootworm (Coleoptera: Chrysomelidae). J. Econ. Entomol. 86: 1664–1671.

Shutt, B., L. Stables, J. Aboagye-Antwi, J. Moran, and F. Tripet. 2010. Male accessory gland proteins induce female monogamy in anopheline mosquitoes. Med. Vet. Entomol. 24: 91–94.

Spencer, J. L., B. E. Hibbard, J. Moeser, and D. W. Onstad. 2009. Behaviour and ecology of the western corn rootworm (*Diabrotica virgifera virgifera* LeConte). Agric. For. Entomol. 11: 9–27.

Storer, N. P. 2003. A spatially explicit model simulating western corn rootworm (Coleoptera: Chrysomelidae) adaptation to insect-resistant maize. J. Econ. Entomol. 96: 1530–1547.

Tabashnik, B. E., F. Gould, and Y. Carrière. 2004. Delaying evolution of insect resistance to transgenic crops by decreasing dominance and heritability. J. Evol. Biol. 17: 904–912.

Tallamy, D. W., P. M. Gorski, and J. K. Burzon. 2000. Fate of male-derived cucurbitacins in spotted cucumber beetle females. J. Chem. Ecol. 26: 413–427.

Thornhill, R. 1976. Sexual selection and paternal investment in insects. Am. Nat. 110: 153–163.

Trivers, R. L. 1972. Parental investment and sexual selection, pp. 136–179. *In* B. Campbell (ed.), Sexual selection and the descent of man. Aldine Publishing Company, Chicago, IL.

Vahed, K. 1998. The function of nuptial feeding in insects: a review of empirical studies. Biol. Rev. 73: 43–78.

Varshney, R. K., U. P. Griyaghey, and R. M. Sundaram. 1971. Effects of food and mating on the longevity, fecundity and fertility in *Eublemma amabilis* (Noctuidae: Lepidoptera). Indian J. Agric. Sci. 41: 771–778.

Wang, X.-P., Y.-L. Fang, and Z.-N. Zhang. 2005. Effect of male and female multiple mating on the fecundity, fertility, and longevity of diamondback moth, *Plutella xylostella* (L.). J. Entomol. Nematol. 129: 39–42.

Wedell, N. 1993a. Spermatophore size in bushcrickets: comparative evidence for nuptial gifts as a sperm protection device. Evolution 47: 1203–1212.

Wedell, N. 1993b. Effort or paternal investment? Incorporation rate and cost of male donations in the wartbiter. Behav. Ecol. Sociobiol. 32: 239–246.

Wedell, N., and A. Arak. 1989. The wartbiter spermatophore and its effect on female reproductive output (Orthoptera: Tettigoniidae, Decticus verrucivorus). Behav. Ecol. Sociobiol. 24: 117–125.

Weekman, G. T. 1961. Problems in rootworm control. Proc. N.C. Br. Entomol. Soc. Am. 16: 32–34.

Wolfner, M. F. 1997. Tokens of love: functions and regulation of *Drosophila* male accessory gland products. Insect Biochem. Mol. Biol. 27: 179–192.

Yang, L., and Y. S. Chow. 1978. Spermatophore formation and the morphology of the reproductive system of the diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae). Bull. Inst. Zool. Acad. Sin. 17: 109–115.

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