Size and Chemical Composition of *Heliothis virescens* (Lepidoptera: Noctuidae) Spermatophores

CARLOS A. BLANCO,[1,2] M. GUADALUPE ROJAS,[3] ASTRID T. GROOT,[4] JUAN MORALES-RAMOS,[3] AND CRAIG A. ABEL[1]

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ABSTRACT The tobacco budworm, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), is a polyandrous species of economic importance on the American continent. This sexual behavior allows for the presence of multiple spermatophores inside a female and the possibility of different males fertilizing the female's offspring, which can make insecticide resistance management or sterile insect release programs particularly challenging. The presence of spermatophores in a female can greatly influence her behavior, physiology, and offspring production. The role that these reproductive structures have is directly influenced by their size and the amount and type of substances that they contain as they are passed into the female during copulation. In this study, we investigated the role that male feeding has on mating potential, including the basic chemical composition and coloration of three sequentially produced spermatophores by male moths that were fed nothing, water, sucrose solution, or nectar. Male moth feeding had a direct influence on spermatophore weight, which was used as an indicator of polyandrous behavior. Nectar-fed moths produced heavier spermatophores and copulated in greater proportion than moths exposed to the other treatments. The total sugar and protein content of spermatophores was not influenced by the type of male feeding. Red or pink spermatophores were more prevalent in the first-produced spermatophores, diminishing in proportion on the second, and increasing again on the third-produced spermatophore, but this coloration proportion was prevalent of males not fed or fed only water. There were no differences in the chemical composition of the different colored spermatophores. These results indicate that polygynous behavior on *H. virescens* can be influenced by the type of male feeding.

KEY WORDS tobacco budworm, polygyny, male feeding, spermatophore weight, spermatophore color

Lepidoptera sexual "life style" can be classified as monogamous and polygamous behavior. In the former category, males and females tend to mate a limited number of times, usually with one partner, whereas in the latter category, moths have several sexual encounters with different mates. Polyandry allows for the possibility of multiple paternities in a given female's offspring, whereas males can fertilize eggs of multiple females in polygyny. These phenomena bring diverse intergender and intragender interactions because sperm of multiple males inside a female compete for egg fertilization. These fertilization scenarios include sperm competition (Parker 1970), parental investment for the successful development of offspring (e.g., nuptial nutritional "gifts" given at mating (Thornhill and Alcock 1983), accessory gland secretions passed during copulation (Leopold 1976, Boggs and Gilbert 1979, Simmons and Parker 1989), sexual selection (e.g., females discriminating among suitable partners; Lyengar and Eisner 1999), or the preferential use of certain sperm that gives her offspring reproductive advantage (Orteiza et al. 2005), and even the "assessment" of the energy used in refusing the sexual advances of males (Rowe et al. 1994, Holland and Rice 1998, Moore et al. 2001).

The study of polygyny and polyandry in an economically important species such as the tobacco budworm, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), can have direct practical implications. For example, this knowledge can help better understand insecticide resistance management and sterile insect releases, because the multiple copulations a female can have in her lifetime (putatively with different resistant and susceptible males) could directly influence how insecticide resistance is passed to the next generation. Also, the possibility that progeny might or might not be sterile may depend on the number of

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[1] USDA–Agricultural Research Service, Southern Insect Management Research Unit, 141 Experiment Station Rd., Stoneville, MS 38776.

[2] Corresponding author, e-mail: carlos.blanco@ars.usda.gov.

[3] USDA Agricultural Research Service, Biological Control of Pests Research Unit, 141 Experiment Station Rd., Stoneville, MS 38776.

[4] Department Entomology, Max Plank Institute for Chemical Ecology, Hans-Knoell Strasse 8, 07745 Jena, Germany.

males (putatively sterile and nonsterile) to which a female mates. Assessing the degree of polyandry can give us an approximation of how many different males' sperm might be inside a female reproductive system. The tobacco budworm exhibits polyandrous behavior because females captured in the field have had up to seven spermatophores inside the bursa copulatrix (Raulston et al. 1975), putatively from different males, whereas under laboratory conditions the record has been 11 (Flint and Kressin 1968) to 12 (C.A.B. and A.T.G., data not shown) spermatophores. The capacity of a tobacco budworm male to copulate multiple times has not been reported to our knowledge. Besides fertilization, spermatophores are the main conduit for Lepidoptera males to pass nutritional (Thornhill 1976, Boggs 1981), physiological (Park et al. 1998), behavior-modifying (Raabe 1986, Sadek 2001), and even toxic (Chapman et al. 1995) substances that aid males, affect females during offspring production, or both.

This study had three basic objectives. First, we wanted to document the effect of male tobacco budworm moth feeding on spermatophore production and mating potential. It has been demonstrated that the acquisition of resources (mainly nectar) can influence egg and spermatophore production in Lepidoptera (Stjernholm and Karlsson 2006, Lewis and Wedell 2007). 2) Having obtained spermatophores from different feeding and mating manipulations, we then wanted to revisit the finding that asserts that the first spermatophore produced in *H. virescens* is always red (Henneberry and Clayton 1984), whereas successive spermatophores are not. And third, we were interested in documenting basic chemical composition of consecutive spermatophores of tobacco budworm.

Materials and Methods

Insects. *H. virescens* moths were obtained from two laboratory colonies: 1) the Agricultural Research Service (ARS) colony, established in 1971 from larval collections in wild plant hosts, has been maintained on soybean–wheat germ-based insect artificial diet (Blanco et al. 2009) at the USDA–ARS facility in Stoneville, MS; and 2) the YDK colony of the North Carolina State University (NCSU), Raleigh, NC, was established in 1988 from a field collection in Yadkin County, NC (Gould et al. 1995) and maintained on corn–soybean-based insect artificial diet modified from Burton (1970).

Mating Potential, Spermatophore Production, and Coloration in Once-Mated Females. To obtain daily-produced spermatophores inside different females and assess mating potential, four cohorts of 25 newly emerged (≤24-h-old) female moths from each of the two colonies were enclosed for 2 d in 500-ml containers (42505LY, Consolidated Plastic Co., Twinsburg, OH) with free access to distilled water in a 37-ml cups (T-125, Solo, Urbana, IL) with a paper tissue (Kleenex, Roswell, GA) stuffed in it. Newly emerged (≤24-h-old) males in four 25-moth cohorts per colony per 500-ml container were fed, for 2 d, the following feeding treatments dispensed in 37-ml cups with paper tissue: 1) nothing (control, empty 37-ml cup with paper tissue), 2) distilled water, 3) 10% sucrose solution, or 4) nectar solution that consisted of 0.267 g of praline, 0.066 g of threonine, 0.1 g of histidine, 0.134 g of tyrosine, 0.034 g of glycine, 0.034 g of alanine, 0.034 g of serine, 0.034 g of asparagine, and 0.034 g of lysine (P-5607, T-8441, H-6034, T-1145, G-6388, A-3534, S-5511, A-4159, and L-1262, respectively, Sigma, St. Louis, MO), 15 g of fructose (3500, BioServ, Frenchtown, NJ), and 15 g of glucose (39894, BulkFoods.com, Toledo, OH), dissolved in 69.2 g of water (Morales-Ramos et al. 1996). Moths were maintained in incubators at 28 ± 0.4°C, 75 ± 10% RH, and a photoperiod of 14:10 (L:D) h at the ARS facility during the feeding period. At the end of the 2-d moth feeding period, males of each treatment cohort were weighed individually in a microbalance (BP210 D, Sartorius Ag, Göttingen, Germany) and individually enclosed in separate 500-ml containers (inside an empty 37-ml cup with paper tissue) with an individually weighed female for 24 h, conforming a treatment pair. Pairs (18 per feeding treatment) were maintained in incubators under the previously described conditions. After the initial 24-h mating period, each (first) female was individually placed in a 1.5-ml centrifuge tube (RU-06333-50, Cole-Parmer Instrument Co., Vernon Hills, IL) and immediately frozen (−13.8 ± 2.8°C). A 2-d-fed female (second) was then immediately enclosed with each previous male for 24 h. The second female also was collected after 24 h of enclosure, placed in a centrifuge tube, and frozen. The same process was repeated with a consecutive (third) 2-d-fed female on the third enclosing day. Frozen females were dissected, and the spermatophores inside the bursa copulatrix were counted and weighed. Their coloration was designated as "red" for those ranging from hue 7.5, chroma 8–12, and value 4–7; "pink" for hue 7.5, chroma 8, and value 8 (Munsell book of color yr 1973, Baltimore, MD); or "clear" for not presenting color. When a female did not have a spermatophore, it was discarded from the analysis, and the subsequent female, if contained a spermatophore, was considered as the first or second female, depending on the order she was copulated. Spermatophores obtained from these females were individually placed in clean centrifuge tubes and stored frozen until their chemical analyzes were done. The whole process of introducing three different females to each male of the four feeding treatments was repeated five times at different dates.

Mating Potential, Spermatophore Production, and Coloration in Once-Mated versus Twice-Mated Females. In a separate experiment, we also investigated the spermatophore color frequency but now in single- versus twice-mated females. This experiment followed what was described previously, except that the first female was enclosed for 2 d, and on the third day, the first female was removed and a virgin (second) female was introduced to that particular male for a day. The first and second females were frozen as described above, the spermatophores were extracted and weighed, categorized as red, pink or clear; placed in clean centrifuge tubes; and stored frozen until their

chemical analyzes were done. Every feeding treatment consisted of 18 males with their respective females, a process repeated twice at different dates.

Frequency of Color in Spermatophores Inside the First and Second Enclosed Females Belonging to Two Tobacco Budworm Colonies. To find out whether the origin of an *H. virescens* colony or the insect artificial diet they were reared on was not an important factor in determining the frequency of red spermatophores, a separate experiment was conducted. Larvae of the ARS and YDK colonies were simultaneously reared on soybean–wheat germ-based or corn–soybean-based insect artificial diets under the previously described environmental conditions at the USDA–ARS facility and at 28 ± 0.4°C, 70 ± 10% RH, and a photoperiod of 14:10 (L:D) h at the Department of Entomology, NCSU. Emerging moths from both diets and colonies were fed 10% sucrose solution for 2 d and enclosed in pairs (500-ml containers at the USDA facility and 3,785-ml containers at the Department of Entomology, NCSU). A male was enclosed with two consecutive females, each female enclosed for only 24 h as described previously. All possible combinations involving the two different *H. virescens* colonies, moth sexes, and the two insect artificial diets were set up at each laboratory. Each female was frozen as described above in centrifuge tubes, dissected, and the spermatophores were counted and classified as red, pink, or clear. Six to 19 pairs were set up for each of the 16 possible treatment combinations at the ARS and NCSU facilities.

Polygyny Assessment. To determine the effect that male feeding might have on the size of the female's bursa copulatrix after mating, as an indication of the transfer of the 'nuptial nutritional gifts' or the accessory gland secretions weight passed during copulation, a separate experiment was conducted. A female fed water for two days as previously described was enclosed with a male fed nothing (control), water, sucrose or nectar for two days. Pairs (18 pairs per feeding treatment) were enclosed as described for the 'mating potential and spermatophore production and coloration' experiment described above. Moths were individually weighed before the feeding period commenced and before and after each 24-h pair-enclosing periods. After males have been enclosed with three different females for the three consecutive days or if he died before the three days on a previous enclosure, they were individually placed in centrifuge tubes and frozen. Frozen females were dissected, the bursa copulatrix was extracted and the fresh weight of this reproductive structure was recorded. Each bursa was then placed individually in a new centrifuge tube. Frozen males and extracted bursa copulatrix were placed individually in centrifuge tubes with the lid open inside an oven at 45°C for 72 h and their dry weight was recorded.

Spermatophore Chemical Analyzes. The chemical analyzes of spermatophores extracted from the first two experiments consisted on the determination of the spermatophore protein, free amino acids, salts, and carbohydrates content. For protein analyses, samples of spermatophores categorized by color were individually weighed (PB303-S Mettler Toledo, Fisher Scientific, Suwanee, GA) into 15.0- by 1.0-cm glass vials and diluted to a 1:10 concentration with an aqueous 0.9% sodium chloride (S-5886, Sigma) solution, and mixed using a tissue tearor (model 985-370, Biospec Products Inc., Vernon Hills, IL) for 5 min each. The protein content was then determined using DC protein assay (500–0116, Bio-Rad, Hercules, CA) as reported by Blanco et al. (2008b). Amino acid content by spermatophore color was done based on weight. Each sample was diluted to a 1:10 concentration with a 0.1 N HCl (9544-02, Mallinckrodt Baker, Inc., Phillipsburg, NJ) in water. Each sample was further diluted to a 1:100 final concentration with another solution containing 100 μl of a mixture of: 0.052% L-norleucine (N-6877, Sigma) in 0.1 N HCl, added to 49.9 ml of aqueous 0.002% sodium azide (S-8032, Sigma). Twenty-five microliters per sample was analyzed using a Dionex DX500 system equipped with an autosampler and an electrochemical detector. AminoPac PA10 column, eluent, and detector parameters were followed as specified by the manufacturer (055406, Dionex Co., Houston, TX). Identification of the sample peaks was done based on comparison with amino acid standard solution (AA-S-18, Sigma) also diluted with the norleucine, sodium azide mixture to a 1:50 concentration. Anion and carbohydrate content in spermatophores also was analyzed using the Dionex system as described above. Samples adjusted by weight were diluted 1:10 with Milli-Q water (Millipore, Billerica, MA), and 5 μl samples were individually analyzed using IonPac, AS11-HC (052961) for anion and CarboPac (057180) for carbohydrates. All specified parameters were followed as described by the manufacturer. Three repetitions per analysis were done. The data were organized, and percentage of content was calculated using Excel 2003 (Microsoft, Redmond, WA).

Experimental Design and Statistical Analyzes. The experiments were set up as split-plot design in which the main units were four treatments and the subunits were females. Analysis of variance (ANOVA) and general linear model (GLM) were used to analyze spermatophore weight data by using JMP version 7 software (SAS Institute 2007). Mean weights (least square means for GLM analysis) were compared in different ways: 1) among food treatments, 2) among females (first, second, and third), 3) among treatments by female, 4) among females by treatment, and 5) among spermatophore color by female. Means were compared using Tukey–Kramer honestly significant difference test. Spermatophore color data were analyzed by repeated measures univariate type by looking only at red-colored spermatophores and then looking only at clear spermatophores. Spermatophore production was analyzed as repeated measures with PROC MIXED and used a covariance structure for heterogeneous compound symmetry (SAS version 7 software), which allows for unequal variances between female treatments.

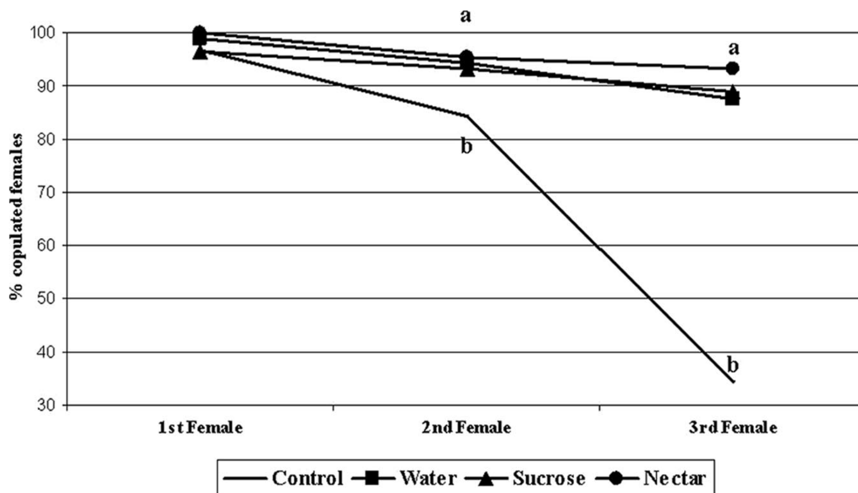

Fig. 1. Percentage of first, second, and third females copulated by *H. virescens* males fed different substances. Percentages of copulated second and third females in control males are significantly different ($P \leq 0.05$) from the rest of the treatments.

Results

Male and female moth weight was not significantly different among treatments before the initiation of feeding (♂ 0.1590 ± 0.0014 g [mean ± SEM], $F = 11.3$, df = 3, 210, $P = 0.34$/♀ 0.1567 ± 0.0010 g, $F = 1.76$, df = 3, 606, $P = 0.15$). After the 2-d feeding and before the first copulation, nectar-fed males were significantly heavier (22% heavier than sucrose to 45% heavier than control) than the rest of the treatments ($F = 74.1$; df = 3, 68; $P < 0.0001$), a trend that continued after the first (23% heavier than sucrose to 43% heavier than control, $F = 44.4$, df = 3, 60, $P < 0.0001$), second (28% heavier than sucrose to 54% heavier than control, $F = 47.4$, df = 3, 59, $P < 0.0001$), and third (20% heavier than sucrose to 42% heavier than water [none of the control males had a third copulation], $F = 20.4$, df = 2, 20, $P < 0.0001$) copulation. There were no significant weight differences between females allocated to produce first, second, or third spermatophores ($F = 2.8$; df = 2, 568; $P = 0.061$).

The percentage of copulated females was significantly reduced on the second and third female when all the feeding treatments were combined ($F = 25.0$; df = 2, 568; $P < 0.001$), because control males copulated significantly less than the rest of the treatments ($F = 44.5$; df = 2, 58; $P < 0.001$) (Fig. 1).

Weights of the first and third spermatophores were significantly heavier than second spermatophores ($F = 5.65$; df = 2, 537; $P = 0.003$). Weights of the second spermatophore were significantly lighter in control males ($F = 3.3$; df = 3, 190; $P = 0.02$). Weights of the third spermatophore also were significantly lower in control, water, and sucrose-fed males than in nectar-fed males ($F = 8.5$; df = 3, 155; $P < 0.0001$). Overall, nectar-fed males produced heavier first, second, and third spermatophores than control, water, or sucrose-fed males ($F = 10.92$; df = 3, 546; $P < 0.0001$) (Fig. 2).

Red/clear spermatophore frequency varied among treatments and females ($F = 4.3$; df = 6, 568; $P = 0.002$). In control ($F = 7.0$; df = 2, 16; $P < 0.0001$) and water-fed males ($F = 7.4$; df = 2, 16; $P < 0.0001$), the proportion of red spermatophores was lower on the second-produced than on the first- and third-produced spermatophores, whereas in the sucrose-fed ($F = 3.2$; df = 2, 16; $P = 0.005$) and nectar-fed males ($F = 3.5$; df = 2, 16; $P = 0.002$), the proportion of red spermatophores was significantly higher only in the first-produced spermatophore (Fig. 3). Red or clear spermatophores' weight was not significantly different between first- (red, 2.16 ± 0.16 mg ([mean ± SEM]; clear, 2.16 ± 0.17), second- (red, 1.79 ± 0.23; clear, 1.77 ± 0.08), and third-produced (red, 1.57 ± 0.16; clear, 1.76 ± 0.23) spermatophores ($F = 0.83$; df = 3, 546; $P = 0.47$).

The spermatophores' color frequency (red, pink, or clear) in a female copulated twice with the same male for two consecutive days and the color of the third spermatophore in a female copulated once on the third day was not significantly different among the four different treatments fed to males ($F = 0.69$; df = 6, 24; $P = 0.66$).

The frequency of red spermatophores produced by males from two different tobacco budworm colonies was significantly influenced by male colony (ARS or NCSU) and the insect artificial diet on which males and females were reared. ARS males reared on NCSU diet produced significantly more red spermatophores (99.5%) than NCSU males fed on NCSU diet (83.9%) ($F = 7.96$; df = 1, 203; $P = 0.005$). This interaction also was influenced by the diet that the first female was fed with (ARS diet, 83.9% red spermatophores; NCSU diet, 97.6% red spermatophores) ($F = 2.55$; df = 1, 203; $P = 0.01$).

Male weights of the polygyny assessment experiment were not significantly different before the feeding period ($F = 1.68$; df = 3, 68; $P = 0.17$). Male weight after the 2-d feeding period was significantly higher on those moths

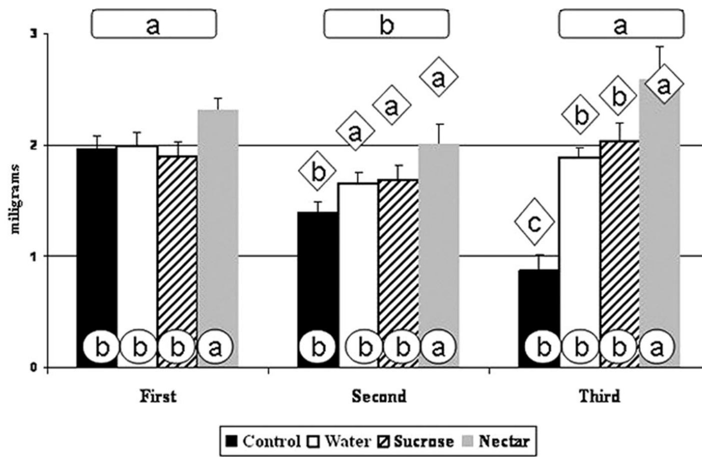

Fig. 2. Weight (+ SE) of three consecutively produced spermatophores of *H. virescens* males fed different substances. Bars with different letters in circles indicate significant ($P < 0.05$) differences among the three spermatophores weights combined. Different letters in diamonds indicate differences among male feeding treatments for second- and third-produced spermatophores. Different letters in rectangles indicate differences in weights between all treatments combined by first-, second-, and third-produced spermatophores.

fed nectar (0.08031 ± 0.00268 g [mean ± SEM]) than of those fed sucrose (0.05837 ± 0.00278 g). The sucrose-fed males were significantly heavier than water-fed males (0.04405 ± 0.00163 g) and control (0.04366 ± 0.00127 g) males ($F = 60.5$; df = 3, 66; $P < 0.0001$). Fresh weight of the bursa copulatrix of females mated with control males (0.00869 ± 0.00096 g), water-fed males (0.01026 ± 0.00060 g), sucrose-fed males (0.01102 ± 0.00063 g), or nectar-fed males (0.01058 ± 0.00044 g) were not significantly different ($F = 2.08$; df = 3, 46; $P = 0.11$). The same trend of no significant differences among feeding treatments was observed with dry weights ($F = 2.98$; df = 3, 46; $P = 0.33$).

Total protein content was not significantly different among control (147.0 ± 9.8 mg [mean ± SEM]), water-fed (185.2 ± 33.9), sucrose-fed (177.1 ± 24.4), or nectar-fed (160.6 ± 12.0) spermatophores. The analysis of 11 amino acids of spermatophores produced by the four different treatments revealed that only serine content was significantly higher on control than on sucrose-fed or nectar-fed males spermatophores ($F = 3.8$; df = 3, 32; $P = 0.01$) (Fig. 4).

Red and clear spermatophores were not significantly different in their amount of sulfate ($F = 0.05$; df = 1, 5; $P = 0.83$), phosphate ($F = 0.78$; df = 1, 5; $P = 0.41$), nitrate ($F = 2.18$; df = 1, 5; $P = 0.19$), fluoride

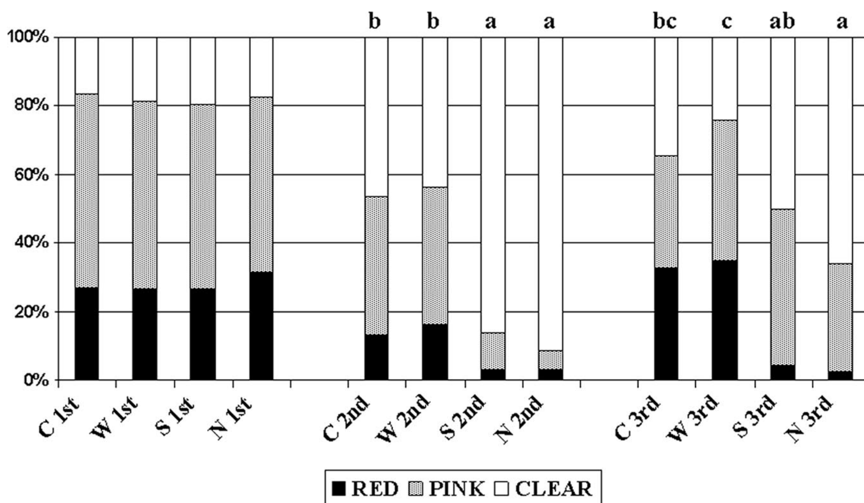

Fig. 3. Percentage of incidence of clear (white portion), pink (gray portion), and red (black portion) first-, second-, and third-produced spermatophores by *H. virescens* males fed nothing (C), water (W), sucrose (S), and nectar (N). Bars of second- (second) and third (third)-produced spermatophores with different letters on top indicate significantly differences ($P \leq 0.05$) of the proportion of color in spermatophores.

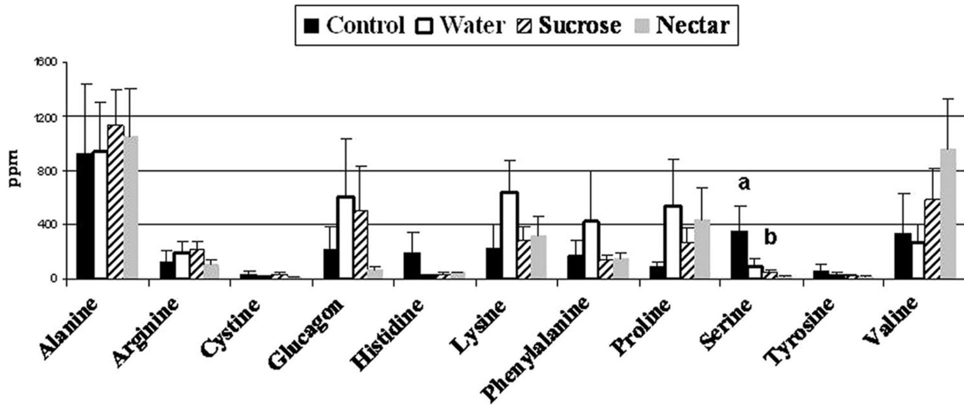

Fig. 4. Average amount of amino acids in spermatophores produced by *H. virescens* male moths fed four different substances. Bars on top on serine with different letters are significantly different at $P \leq 0.05$.

($F = 1.10$; df = 1, 5; $P = 0.34$), or chloride ($F = 0.86$; df = 1, 5; $P = 0.39$) (Fig. 5), or in the total amount of sugars (red, 627.3 ± 33.3 ppm; clear, 668.0 ± 169.8) ($F = 0.04$; df = 1, 5; $P = 0.84$). Of the eight individual sugars analyzed, only the content of arabinose was significantly different between red and clear spermatophores ($F = 21.3$; df = 1, 5; $P = 0.005$) (Fig. 6).

Discussion

Male food not only influenced the size of spermatophores in this study but also the capacity of the moths to produce daily spermatophores. Ninety-eight percent of virgin males, regardless of feeding treatment, produced first spermatophores. This proportion slightly dropped to ≈93% for the second spermatophore of those males fed water, sucrose, or nectar and significantly lower (≈85%) on control males. Males that had something to drink produced a significantly higher number of third spermatophore than control males (Fig. 1). It seems that liquid, more than nutritional or caloric components of sucrose or nectar made a difference in the number of copulated females.

Although it is expected that *H. virescens* moths feed on nectar, pollen, or both by observations made on the head of field-collected moths (G. Jones, personal communication) and by moths having been observed resting on ergot (*Claviceps* spp.) of flowering *Paspalum dilatatum* Poir. and *Lolium multiflorum* Lam. at night (J. Lopez, personal communication), no empirical data have been generated to date that confirm this possibility. This study strongly suggests that some type of feeding, specially nectar, makes a difference in the mating potential of males and the spermatophore size, which in turn can

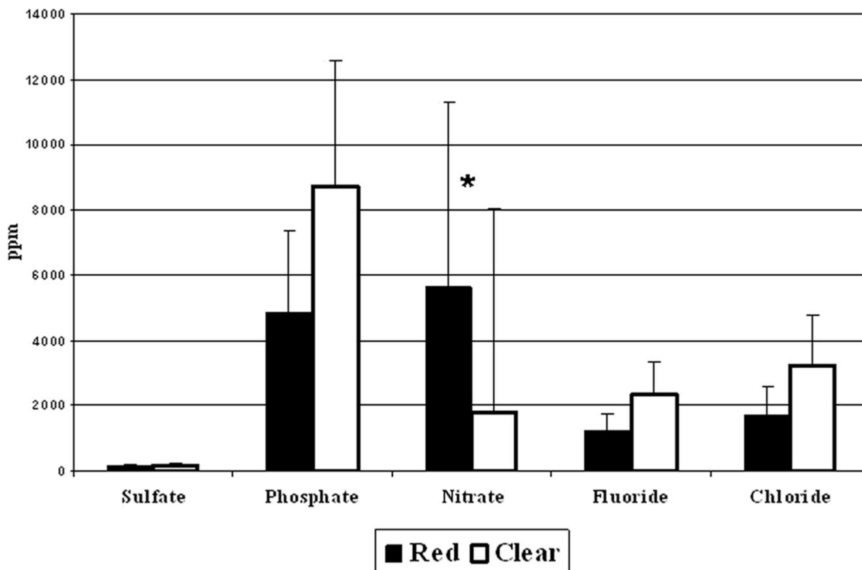

Fig. 5. Average concentration of different substances in red and clear *H. virescens* spermatophores. Asterisk (*) indicates concentration of nitrate is expressed as ppt.

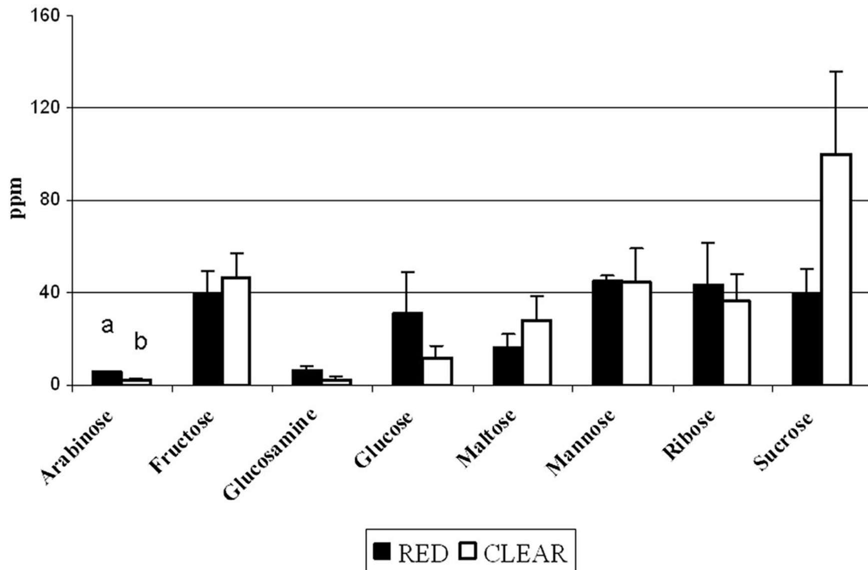

Fig. 6. Carbohydrate content in red and clear *H. virescens* spermatophores. Arabinose concentration was significantly ($P \leq 0.05$) different between clear and red spermatophores.

have a direct influence estimating the degree of polyandry described by Svärd and Wiklund (1989). The influence of sugary substance intake in *H. virescens* females influences their longevity, flight, and reproduction capacities (Willers et al. 1987), but the role of these substances on the male's "investment" in offspring has not been investigated in this species.

Counts of spermatophores inside field-collected *H. virescens* females indicate that these insects copulate an average of 1.6 (Hendricks et al. 1970) to 2.7 (Raulston et al. 1975) times, but the use of sperm and/or the mechanism of sperm competition among these two to three males is not thoroughly understood in this species (Pair et al. 1977, LaMunyon 2001, Blanco et al. 2008). Spermatophore counts in tobacco budworm females (≤2.7, Raulston et al. 1975) are directly influenced by the type of substance that the male had access to (Fig. 2). Assuming that each spermatophore belongs to a different male, it indicates that this species is polyandrous compared with the average of only 1.2 spermatophores per females evaluated in 18 different species reported in the seminal study of Ehrlich and Ehrlich (1978). But if *H. virescens* is compared with *Pieris rapae* L. (Bissoondath and Wiklund 1996a) or *Danaus plexippus* L. (Svärd and Wiklund 1989), it may look as if tobacco budworm behaves more as a monogamous species. Therefore, the number of spermatophores found per female does not necessarily offer the complete picture of polyandrous behavior. The assessment of physiological mechanisms needs to be incorporated into a more complete study of mating systems. For example, the size of spermatophores has been used as an indication of paternal investment in offspring. Very polyandrous *P. rapae* males (Bissoondath and Wiklund 1996a) produce consecutive spermatophores that are similar in mass and nutrition. It has been postulated that in most polyandrous species a selection force must be acting to produce subsequent spermatophores of similar size (Boggs 1981, Karlsson 1996, Molleman et al. 2004), but the direct influence of these subsequent spermatophores on the production of offspring is not completely apparent (Molleman et al. 2004). More Lepidoptera species tend to produce smaller second (and third) spermatophores (Svärd and Wiklund 1991, Royer and McNeil 1993, Karlsson 1996, Torres-Vila and Jennions 2005, Lauwers and Van Dyck 2006, Knight 2007) as was partially the case reported in this study of *H. virescens* (Fig. 2). The second spermatophore of *H. virescens* was ≈18% lighter than the first spermatophore, but the third spermatophore was 17% heavier than the second and only 1% lighter than the first, unique results as far as we can tell for tobacco budworm. However, these spermatophore weight differences were influenced by male feeding treatment. Average weights of first, second, and third spermatophores were significantly different only in control males (1.96, 1.40, and 0.87 mg, respectively), showing in this case a decreasing tendency that is common in less polyandrous or monandrous species. Males fed water, sucrose, or nectar had slightly reduced weights between first and second (13%) and an increase between first and third (4%) spermatophores, which denotes a clear polyandrous behavior (Bissoondath and Wiklund 1996a,b) (Fig. 2).

Protein content in spermatophores has been used as another cue for assessing polyandrous behavior. According to Bissoondath and Wiklund (1996a,b) in the polyandrous genus *Pieris*, the protein content of subsequent spermatophores has a low variation in certain species. Our data, although somewhat limited ($n = 23$), indicate that the second *H. virescens* spermatophore contains ≈27% more protein than the first and the third spermatophore has ≈50% more than the first spermatophore. Total protein content was not signif-

icantly affected by male feeding treatment, except in the case of the amino acid serine that was significantly higher on control than in nectar-fed male spermatophores (Fig. 4).

One of the factors creating these differences may be male's weight because heavier male moths tend to produce large spermatophores (Bissoondath and Wiklund 1996b). In this study, male weights were significantly affected by feeding treatment, but male moths had similar weights before feeding. Our data on spermatophore and moth weights indicate that *H. virescens* is a species with high level of polyandry compared with the estimates of Svärd and Wiklund (1989). The ratio that results of dividing a particular male weight by the weight of the bursa copulatrix in a female that he copulated was 7.0 ± 0.003% ([mean ± SEM]) in *H. virescens.* In highly polyandrous Lepidoptera families such as Pieridae, this ratio is ≈10 (range, 4.2–15.5%), whereas in a less polyandrous family (Satyridae), it is ≈2.5, ranging from 1.4 to 5.1. The same relationship for highly polyandrous butterflies such as *D. plexippus* and *Papilio machon* L. is only 5.3 and 3.5%, respectively (Svärd and Wiklund 1989).

In a few reports (Callahan and Cascio 1963, Proshold et al. 1975, Park et al. 1998), it has been observed that red substances are transferred into females during the copulation with virgin males. Henneberry and Clayton (1984) mentioned that virgin *H. virescens* males had this red substance before mating and did not recur after they have copulated. They proposed that a way to identify virgin males was to look for this red substance in the male's primary simplex that once transferred into females remains clearly visible for the rest of the female's life. Further exploring if the presence of this red substance is only transferred by virgin males, we observed the color of first-, second-, and third-produced spermatophores in 1) three independent females or in 2) females that had the first and second spermatophore of a single male and a third independent female containing the third-produced spermatophore and 3) on those produced by two independent colonies fed two different insect artificial larval diets. Our results indicate that there is a high proportion of red/pink spermatophores produced by virgin males, but it only averages 85%. The disappearance of this red substance on the nonvirgin male copulations also averaged 85% of the cases when males were fed sucrose solution (as with the Henneberry and Clayton 1984 study). When males were kept without a drinkable substance or only water was provided to them, the red/pink substance only "disappeared" in ≈45% of the cases. The reappearance of the red/pink substance occurred in higher proportion (≈30%) on the third spermatophore of control and water-fed males (Fig. 3). The proportion of red versus clear spermatophores when a male produced two spermatophores in a single female follows the same coloration pattern as for spermatophores that are produced in three independent females. The possibility that a particular *H. virescens* colony or larval food might be one of the factors causing the presence of red coloration on the first and its disappearance on subsequent spermatophores indicate that food provided to the first female (the one that had a higher proportion of red spermatophores), had a direct influence in our results.

Based on our results, we hypothesize that the protein of the red spermatophores could be classified as a phosphoprotein and that the binding site of the phosphate groups esterified to serine (Stryer 1988). Further research on this subject is being conducted.

The tobacco budworm exhibits a high level of polyandry under laboratory conditions that may not be apparent by looking only at mating frequency, but also by considering all the different physiological aspects of the reproductive biology of *H. virescens* and comparing them with those of other lepidopteran species. Highly polyandrous species, such as tobacco budworm, offer additional challenges to insecticide resistance management and to the implementation of sterile insect release programs.

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