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Variation in Sexual Communication of the Tobacco Budworm, *Heliothis virescens*

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Abstract. Females of the tobacco budworm, *Heliothis virescens* (F.), exhibit distinct geographical and temporal variation in sex pheromone composition, but the causes and significance of this variation are largely unexplored. Here we assessed whether 1) female pheromone variation was related to the host plants of origin, and 2) pheromone lures with varying amounts of Z9-14:Ald or 16:Ald were differentially attractive to males. Variation in female pheromone did not seem to be related to the host plants from which the eggs or larvae were collected, which may be because field-collected larvae were reared for three to five larval stages on artificial diet. By varying the concentration of Z9-14:Ald within the range in the female pheromone gland, we found males were more attracted as the amount increased from 1 to 10% relative to Z11-16:Ald, but significantly less with the highest concentration of 25%. In contrast, with 16:Ald, similar numbers of tobacco budworm males were caught in all traps where 16:Ald ranged from 0 to 200%. These results show that variation in Z9-14:Ald but not 16:Ald is evolutionarily significant and likely subject to stabilizing selection in the field.

Resumen. Las hembras de *Heliotis virescens* presentan marcadas variaciones geográficas y temporales en la composición de sus feromonas sexuales, sin embargo las causas y significado de dicha variación en general no han sido estudiados. Este artículo evalúa 1) si la variación de feromonas de las hembras se relaciona con la planta huésped de origen y 2) si las trampas de feromonas con cantidades variables de Z9-14:Ald o 16:Ald tienen un atractivo diferencial para los machos. La variación de la feromona de las hembras no pareció estar relacionada con la planta hospedera de la que los huevecillos o larvas fueron colectados, lo que podría deberse a que las larvas colectadas en el campo fueron criadas con dieta artificial durante 3 a 5 estados larvales. Al variar las concentraciones de A9-14:Ald dentro del rango que se encuentra en la glándula de feromonas de la hembra encontramos que se atraía más a los machos a medida que la cantidad aumentaba del 1 al 10 % con respecto a Z11-16:Ald pero significativamente menos con la concentración más alta del 25%. En contraposición, en el experimento con 16:Ald se capturó un número similar de machos de *H.virescens* en todas las trampas en las que 16:Ald varió de 0 a 200%. Estos resultados muestran que la variación que se

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observó en Z9-14:Ald es importante en términos evolutivos y es probable sujeto de selección estabilizadora en el campo, mientras que este no es el caso de 16:Ald.

In the tobacco budworm, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), there is considerable geographical and temporal variation in the female-produced sex pheromone (Groot et al. 2009a). However, the variation was less when field-collected insects were reared in a laboratory, indicating some of the variation was caused by environmental factors (Groot, A.T. unpubl. res.). Because tobacco budworm is a generalist herbivore feeding on more than 37 plant species from 14 families (Sheck and Gould 1993), variation in the sex pheromone in the field may be due to the host plants on which the females fed as larvae. In most moths, sex pheromone components are synthesized *de novo* rather than derived from dietary components. However, because production of sex pheromone involves similar enzymes used in general metabolism, particularly those of fatty acid metabolism (Jurenka 2004, Rafaeli 2005), it is possible that host plant adaptation might affect components of this pathway that are shared between pheromone production and general fatty acid metabolism. In this communication we address two questions: a. Could the variation in sex pheromone composition in tobacco budworm females be related to the host plants on which tobacco budworm was collected in the field? b. How important is the variation in female sex pheromone for male response?

Materials and Methods

Tobacco budworm eggs and larvae were collected from three field sites (Clayton, NC (35°39'58" N, 78°30'36" W, hereafter NC), Stoneville, MS (33°25'04" N, 90°54'37" W, MS), and College Station, TX (30°38'22" N, 96°21'39" W, TX) from three plants (tobacco, *Nicotiana tabacum* Linné, Solanaceae) in North Carolina and Mississippi, garbanzo (*Cicer arietinum* L., Fabaceae, Sierra variety) in Mississippi and Texas, and velvetleaf (*Abutilon theophrasti* Medik, Malvaceae) in Mississippi, during 2 years (2007 and 2008). All larvae were reared to adult on artificial wheat-germ diet at North Carolina State University. Seven to 72 pheromone glands were extracted from 2-5 day old virgin females injected with 7.5 pmol PBAN during the photophase to stimulate pheromone production (see Groot et al. 2005 for a detailed description; see Fig. 1 for the number of glands extracted per group). All pheromone glands were extracted for 20-30 minutes in 50 µl hexane containing 20 ng 1-pentadecyl acetate as an internal standard. Samples were reduced to 1-2 µl under a gentle stream of N_2 and injected into a splitless inlet of a HP6890 gas chromatograph coupled with a high resolution polar capillary column and a flame-ionization detector. Before and after each gas chromatograph sequence, we injected authentic standards of all the pheromone components to assess column performance as well as check the retention times of each of the components. We corrected all integration results by the differential response of the FID to the standards. Significant differences between the pheromone blends of the seven groups of females were determined in SAS, with analysis of variance (PROC GLM) and Tukey adjustments for multiple comparisons for each of the compounds (SAS 2002-2003).

Male response to varying sex pheromone blends was assessed in field trapping experiments in Clayton, NC, using *Heliothis* mesh traps arranged in a completely randomized block design (see Groot et al. 2007 for more details). Two synthetic lure experiments were done, where the relative amount in the lure was

varied of the: 1) critical secondary sex pheromone component Z9-tetradecenal (Z9-14:Ald) and 2) compound hexadecanal (16:Ald). The latter compound is present in tobacco budworm pheromone glands in relatively large amounts (e.g., Tumlinson et al. 1975, 1982; Klun et al. 1980; Heath et al. 1991; Groot et al. 2009a), but its behavioral significance has not been established. Lures consisted of 300 µg of Z11-16:Ald set to 100%. In the first experiment, the relative amount of Z9-14:Ald added was 1, 2, 5, 10, or 25% of the major component (this is within the range of variation in the female pheromone gland), which equaled 3, 6, 15, 30, or 75 µg per lure. This experiment was done in a cotton field and in a tobacco field. In the second (16:Ald) experiment, all lures consisted of 300 µg Z11-16:Ald (100%) and 15 µg Z9-14:Ald (5%). The relative amount of 16:Ald added was 0, 5, 25, 100, or 200%. Again, this is within the range of variation in the female pheromone gland, including females that exhibit an unusual profile with 16:Ald as the major component (A. T. Groot and C. Schal unpublished results). Because the total amount of pheromone was greater in the lures that contained 200% (i.e., 600 µg) 16:Ald, we included another treatment where lures were loaded with a total of 390 µg (similar to lures loaded with 100% Z11-16:Ald, 5% Z9-14:Ald, and 25% 16:Ald); this amounted to 128 µg Z11-16:Ald, 6.4 µg Z9-14:Ald, and 256 µg 16:Ald. This experiment was done in a tobacco field. Differences in trap catches were analyzed using an ANOVA in SAS after square-root transforming the data to stabilize the variance. The means were separated using a Tukey's studentized range (HSD) (SAS 2002-2003).

Results and Discussion

Variation in the Pheromone Composition of Tobacco Budworm Females. We found significant variation for all sex pheromone compounds except 14:Ald (Fig. 1). Considering the critical secondary sex pheromone component Z9-14:Ald, the variation ranged from 2.3 to 40% (108 females contained between 2 and 10%, 83 females contained between 10 and 20%, and 11 females contained >20%). The minor compound 16:Ald varied along a much greater range, from 5 to 241%. Variation in the alcohol Z11-16:OH may be because it serves as precursor to its aldehyde and acetate derivatives (Tillman et al. 1999, Jurenka 2004 Rafaeli 2005), because we previously found that greater amounts of aldehyde and acetate products are usually associated with lesser amounts of the corresponding alcohol (Groot et al. 2005, 2009b; Sheck et al. 2006).

The variation in the sex pheromone in female pheromone glands does not seem to be related to the host plants from which the eggs or larvae were collected (Fig. 1a). For example, females collected from garbanzo in Mississippi contained significantly more Z9-14:Ald than females collected from garbanzo in Texas. Also, females originally collected from tobacco in North Carolina contained significantly more 16:Ald than females from the same species of plant in Mississippi. A correlation between the variation and location or year was also not apparent. It is possible that host plant effects have been obscured because field-collected larvae were reared for three to five larval instars on artificial diet. Therefore, it will be interesting to assess variation in the pheromone when larvae are reared on different plant materials. It is also possible that larvae on different crops reflect genetic polymorphism in female oviposition preferences, independent of larval host adaptation or differences in pheromone production. Recently, Blanco et al. (2008) found significant variation in the ability of two genetically-independent tobacco

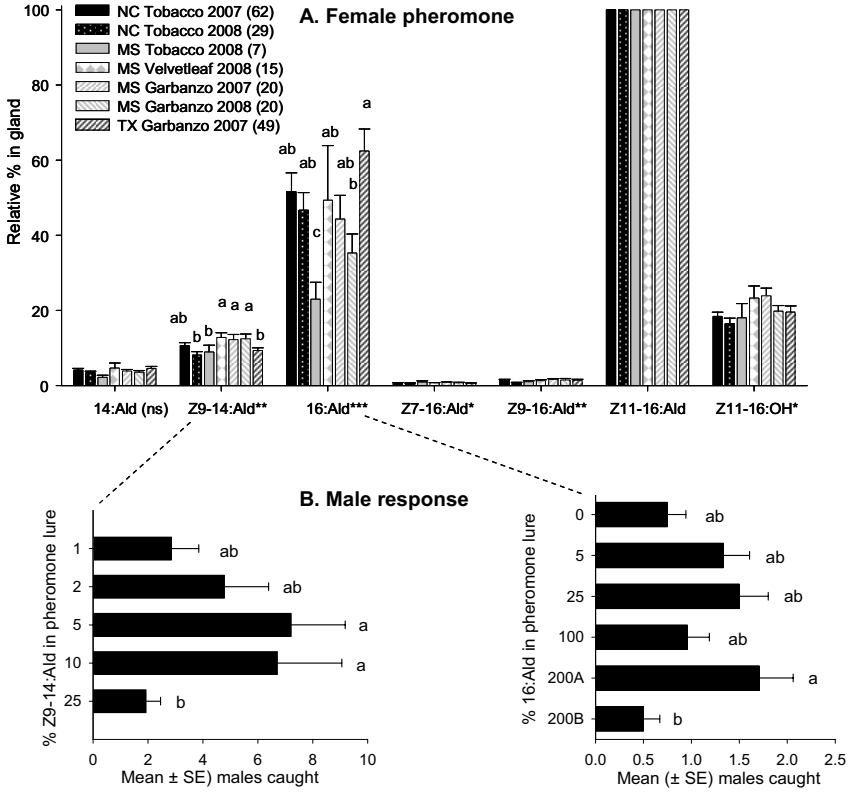

Fig. 1A. Variation in the female sex pheromone blend in the seven groups of females collected as larvae in four States and on three host plants. The relative amounts of all compounds are calculated by setting the major component Z11-16:Ald to 100%. The numbers in parenthesis in the legend are the number of pheromone glands extracted in each group. On the x-axis significant differences in relative amounts for each compound, based on ANOVA, are indicated. NS: non-significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. 1B. Percentage of males caught in pheromone traps containing varying pheromone lures, as indicated on the y-axis. In experiment 1, varying Z9-14:Ald, we caught a total of 329 *Heliothis virescens* males, in experiment 2, varying 16:Ald, we caught a total of 162 males.

Fig. 1A. Variación en la mezcla de feromonas sexuales en siete grupos de hembras colectadas como larvas en 4 estados larvales y en 3 plantas hospederas. La cantidad relativa de todos los compuestos se calcula fijando el componente principal Z11-16:Ald a 100%. Los números entre paréntesis en la leyenda (pie de grabado) representan el número de glándulas de feromona extraídas en cada grupo. En el eje de la x se indican diferencias significativas en cantidades relativas para cada compuesto, basadas en ANOVA. NS: no significativas, *$p < 0.05$, **$p < 0.001$. 1B. Porcentaje de machos capturados en trampas de feromonas que contenían diversos cebos de feromonas como se indica en el eje de la y. En el experimento 1, con concentraciones diversas de Z9-14:Ald capturamos un total de 329 machos de *Heliothis virescens*, en el experimento 2 con diversas concentraciones de 16:Ald, capturamos un total de 162 machos de *Heliothis virescens*.

budworm laboratory strains to develop and grow on cotton, *Gossypium hirsutum* L., or garbanzo, suggesting genetic adaptation and differentiation in plant use. We are currently investigating whether females from the two strains also differ in sex pheromone composition.

Male Response. Because in the Z9-14:Ald experiment, location (cotton or tobacco field) did not have a significant effect on the trap catches, we show the pooled data of both fields. There was a significant difference in the number of tobacco budworm males caught in traps in both the Z9-14:Ald (P = 0.0007) and 16:Ald experiments (P = 0.014). Specifically, in the Z9-14:Ald experiment, trap catch of tobacco budworm males increased as the amount of Z9-14:Ald in the lure increased from 1 to 10%, but decreased significantly by 71% (P = 0.007) in lures with the greatest concentration of 25% Z9-14:Ald (relative to 100% Z11-16:Ald, Fig. 1B). Thus there is an optimum range of concentrations of Z9-14:Ald for a female to release, from the standpoint of attracting males. This is much narrower than the observed range of concentrations in female pheromone glands, implying stabilizing selection should be acting on this component. In the 16:Ald experiment, similar numbers of tobacco budworm males were caught in all traps where 16:Ald ranged from 0 to 200%. Only when lures were loaded with less of the major component (treatment 200b), were significantly fewer males attracted than when 200% 16:Ald was added to the minimal blend. This result indicates the large variation in the amount of 16:Ald in the female pheromone gland is not subject to the same kind of stabilizing selection as Z9-14:Ald.

In conclusion, the variation in the production of tobacco budworm sex pheromone as well as in the tobacco budworm male response is greater than was previously assumed but does not seem to be related to the host plants on which eggs or larvae were collected, at least when larvae were subsequently reared on artificial diet. Future research will assess the amount of variation in the sex pheromone when larvae are reared on different plants. Comparing the amount of variation in the female pheromone to the amount of variation in the male response elucidates the evolutionary significance of this variation by highlighting which components are under the strongest selection.

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