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GEOGRAPHIC AND TEMPORAL VARIATION IN MOTH CHEMICAL COMMUNICATION

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In moth pheromone communication signals, both quantitative and qualitative intraspecific differences have been found across geographic regions. Such variation has generally been hypothesized to be due to selection, but evidence of genetic control of these differences is largely lacking. To explore the patterns of variation in pheromone signals, we quantified variation in the female sex pheromone blend and male responses of two closely related noctuid moth species in five different geographic regions for 2–3 consecutive years. We found significant variation in the ratios of sex pheromone blend components as well as in male response, not only between geographic regions but also within a region between consecutive years. The temporal variation was of a similar magnitude as the geographic variation. As far as we know, this is the first study reporting such temporal variation in moth chemical communication systems. The geographic variation seems to at least partly be controlled by genetic factors, and to be correlated with the quality of the local chemical environment. However, the pattern of temporal variation within populations suggests that optimization of the pheromonal signal also may be driven by within-generation physiological adjustments by the moths in response to their experience of the local chemical environment.

KEY WORDS: **Experience, female sex pheromone, *Heliothis subflexa*, *Heliothis virescens*, male response, phenotypic plasticity, selection.**

Prezygotic behavioral isolation is a major component of speciation (e.g., Mayr 1963; Dieckmann and Doebeli 1999; Ritchie et al. 1999; Kondrashov and Kondrashov 1999; Jiggins et al. 2001; Kirkpatrick and Ravigne 2002). However, the evolution of behavioral isolation is poorly understood (Coyne and Orr 2004). To gain insight into the evolution of prezygotic behavioral isolation it is essential to quantify intraspecific variation in the premating signals, on which selection may operate, and biotic and abiotic factors that contribute to this variation.

Premating signals are generally hypothesized to be under stabilizing selection (Gerhard 1991; Löfstedt 1993; Butlin 1995; Linn and Roelofs 1995; Butlin and Trickett 1997; Phelan 1997; Zhu et al. 1997; Shaw and Parson 2002; Bürger et al. 2006).

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For example, in the well-studied moth *Ostrinia nubilalis*, females with altered pheromone blend ratios are much less attractive to conspecific males than are normal females (Glover et al. 1991; Liu and Haynes 1994; Linn et al. 1997; Zhu et al. 1997). Specifically, in moth sexual communication systems, divergence in these long distance mate finding signals has been postulated to be possible through so-called asymmetric tracking (Löfstedt 1993; Phelan 1997; Roelofs et al. 2002): females with a genetic alteration in the emitted blend ratio could evolutionarily be tracked by males because of a putative wide response window of males, observed mainly in laboratory experiments (e.g., Linn and Roelofs 1995; Roelofs et al. 2002). However, this scenario contrasts with findings in the field, showing that the range of male response is restricted and there is a narrow window of ratios that elicits peak response (Löfstedt 1990; Linn and Roelofs 1995; Zhu et al. 1997). Hence, the frequency of females with an altered blend or males with an altered response (e.g., Linn et al. 2003, 2007) would not be expected to increase in the population, unless some other ecological factors—parasitoids or predators, for example (e.g., Raffa and Dahlsten 1995), the presence of other species with similar signals (e.g., Groot et al. 2006)—strongly selected for such a change, or stochastic events prevailed over selection (Wright 1931, 1932; Wade and Goodnight 1998).

In visual and acoustic communication systems, mate recognition signals have been shown to differ among populations and these differences appear related to selection by environmental factors that affect the efficiency of specific mate recognition signals (e.g., Marchetti 1993; Endler 1995; Ritchie et al. 2001; Slabbekoorn and Smith 2002; Magalhaes et al. 2008). Even though chemical communication signals also have been shown to be affected by abiotic environmental factors (e.g., Cardé et al. 1975; Webster and Cardé 1982; Haynes and Birch 1984; Linn et al. 1988, 1991; Dumont and McNeil 1992; Murlis et al. 2000; Raina 2003), and interspecific competition is recognized as playing a major role in the partitioning of pheromone communication channels in moths (reviewed by Cardé and Baker 1984), so far local environmental conditions have not been explored as a factor that may cause divergence of chemical communication systems (Johansson and Jones 2007).

Sources of variation in the habitat that likely affect chemical communication systems are the presence and abundance of species with similar chemical cues, because they may either affect the signal-to-noise ratio (e.g., Eizaguirra et al. 2002; Haynes et al. 2002; Gemeno et al. 2006; Eizaguirra et al. 2007; Solé et al. 2008) or generate communication interference (e.g., Cardé et al. 1977; Löfstedt et al. 1991; Butlin 1995; Saetre et al. 1997; McElfresh and Millar 1999; Gries et al. 2001; Jiggins et al. 2001; McElfresh and Millar 2001; Groot et al. 2006), both of which would result in selection for females with the most distinct, optimized pheromone blend (i.e., negative frequency-dependent selection). Such local natural selection forces may alternate directions or be unidirectional, similar to what has been found for beak sizes in the Galapagos finches (Grant and Grant 2002). Only when specific local environmental conditions persist may selection forces from the environment result in directional or divergent selection. We will refer to variation related to selection across a persistent environmental gradient as the "selection hypothesis."

An alternative, more speculative hypothesis to explain variation in chemical communication signals is that females and males exhibit phenotypic plasticity in sexual signaling, and experience—either by immature stages or by early adults—shapes the expressed phenotype. That larval memory of odors can persist into adults—even in holometabolous insects in which neural tissue is reorganized in the pupal stage—has been demonstrated in *Drosophila* (Tully et al. 1994), in the fly *Musca domestica* (Ray 1999), and recently also in *Manduca sexta* (Blackiston et al. 2008). Early adult (postimaginal) experience has been shown to be an important factor in female oviposition preference, referred to as the neo-Hopkins selection principle (Jaenicke 1983, 1988; van Emden et al. 1996; Barron 2001). Similarly, in male moths, preexposure to specific sex pheromone blends has recently been shown to affect their subsequent responses to sex pheromone (Anderson et al. 2003; Andersson et al. 2007). The question is whether preexposure to specific pheromone blends and other semiochemicals could also cause females to alter the blend that they produce. Many female moths can perceive their own pheromone as well as pheromone compounds of other species (e.g., Ljungberg et al. 1993; Schneider et al. 1998; Groot et al. 2005b; Hillier et al. 2006). Also, most female moths produce their sex pheromone de novo every night (e.g., Raina 1989; Rafaeli 2002; Jurenka 2003), and can modulate the time and temporal patterning of pheromone release (calling) in relation to environmental conditions (Schal and Cardé 1985; Lim et al. 2007) and presence of conspecifics (Conner et al. 1980; Lim and Greenfield 2007, 2008). Hence, it may be possible that females can vary their biosynthesized as well as emitted pheromone blend to some extent depending on the prevailing olfactory cues in their habitat. We will refer to this hypothesis as the "experience hypothesis."

The experience hypothesis may be distinguished from the selection hypothesis in the following way. If variation in the pheromone blend is due to selection, then the direction of change in the pheromone blend in year X compared to year X–1 is likely to correspond to the selection pressure in year X–1 (i.e., relative densities of species that share pheromone components or interfere in pheromone communication). If variation in the pheromone blend is due to female experience, then the blend in year X should directly reflect the relative species densities in year X. Of course, it is possible that stochastic events, selection pressure, and phenotypic plasticity resulting from experience may

Table 1. Pheromone composition of *Heliothis virescens* (Hv), *Heliothis subflexa* (Hs), and *Helicoverpa zea* (Hz).

Component	Hv	Hs	Hz
14:Ald	2	0.3	
Z9–14:Ald	**5.5+++**	0.3	
16:Ald	20	5	**7+**
Z7–16:Ald	1	0.5	**1+**
Z9–16:Ald	0.5	**18+++**	**2+++**
Z11–16:Ald	**60++++**	**45++++**	**90++++**
Z7–16:OAc		**1+**	
Z9–16:OAc		4	
Z11–16:OAc	–	**12+**	–
Z9–16:OH		20	
Z11–16:OH	11+/–	**12+++**	–

The numbers for Hv and Hs refer to a mean relative percentage of these compounds in the pheromone gland of females collected as larvae from the field in NC in 2004–2006. Percentages for Hz are means from the literature (Klun et al. 1980; Pope et al. 1984). Bold numbers in each column indicate the sex pheromone components that have been shown to be important in the attraction of conspecific males (shown for Hv by Roelofs et al. 1974; Tumlinson et al. 1975; Klun et al. 1979, 1980a; Tumlinson et al. 1982; Vetter and Baker 1983; Ramaswamy et al. 1985; Teal et al. 1986; Vickers and Baker 1997; shown for Hs by Teal et al. 1981; Klun et al. 1982, Heath et al. 1990; Vickers and Baker 1997; Groot et al. 2007; shown for Hz by Klun et al. 1980b; Pope et al. 1984). ++++ Major sex pheromone component; +++ Critical secondary sex pheromone component; + Additional sex pheromone component; – Repellent (shown for Z11–16:OAc in Hv by Vickers and Baker 1997, and in Hz by Fadamiro and Baker (1997) and Quero and Baker (1999); shown for Z11–16:OH in Hz by Vetter and Baker 1984); +/– Attractive at low amounts (<1%), repellent at higher amounts (Ramaswamy et al. 1985).

operate concurrently, making it difficult to distinguish among these processes.

We explored these hypotheses by sampling two closely related noctuid moth species, *Heliothis virescens* (Hv) and *H. subflexa* (Hs), in five different regions for 2–3 consecutive years. Hv is a New World generalist herbivore, feeding on over 37 plant species from 14 different families (Sheck and Gould 1993), whereas Hs is a New World specialist herbivore, feeding only on plant species in the genus *Physalis* (McElvare 1941). Both species produce multi-component sex pheromone blends, with Z11–16:Ald as the major sex pheromone component (see Table 1). The critical secondary sex pheromone component of Hv is Z9–14:Ald. This component is critical because without it conspecific males are not attracted (Roelofs et al. 1974; Tumlinson et al. 1975; Klun et al. 1979, 1980a,b; Pope et al. 1982; Tumlinson et al. 1982; Vetter and Baker 1983). Hs females produce Z9–14:Ald as well, but in much smaller amounts than Hv (Klun et al. 1982; Groot et al. 2005a). The critical secondary sex pheromone components of Hs are Z9–16:Ald and Z11–16:OH (Teal et al. 1981; Heath et al. 1990; Vickers 2002). The latter component has also been shown to inhibit the attraction of both Hv (Vetter and Baker 1983) and *Helicoverpa zea* (Hz), a heliothine moth with a similar sex pheromone blend (Quero and Baker 1999). Three compounds are produced only by Hs: Z7–16:OAc, Z9–16:OAc and Z11–16:OAc, to which we will refer as the acetates. At least one of the acetates, Z11–16:OAc, has a dual function as well: it increases attraction of conspecific Hs males but inhibits attraction of Hv males (Vickers and Baker 1997; Vickers 2002; Groot et al. 2006, 2007). The other compounds present in the pheromone glands of both species have not been systematically tested, so their potential roles are unclear either in the attraction of conspecific males and/or behavioral antagonism of heterospecific males. In this study, we will concentrate on the pheromone components that have been shown to be important in the attraction of Hv (Z9–14:Ald) and Hs (Z9–16:Ald) and/or in the repellence of Hv and Hz (the acetates and the alcohol Z11–16:OH).

Based on the different roles of the pheromone components in the sexual communication of Hv and Hs, we can explore the following hypotheses. If variation in the pheromone signals is due to *selection*, we would expect: 1) an increase in Z9–14:Ald in Hv females in year X when the relative abundance of Hs and/or Hz was relatively high in year X–1, because Hv females with an increased amount of Z9–14:Ald would have been at a selective advantage; 2) an increase in Z9–16:Ald in Hs females in year X when the relative abundance of Hz was relatively high in year X–1, because Hz females produce this component as well, only in much smaller amounts relative to the major component Z11–16:Ald (see Tables 1 and 3) an increase in Z11–16:OH and/or the acetates in Hs in year X when the relative abundance of Hv and/or Hz was relatively high in year X–1, as these components not only increase the chance of conspecific attraction (Teal et al. 1981; Heath et al. 1990; Vickers 2002; Groot et al. 2006, 2007), but also decrease the chance of heterospecific attraction. If, on the other hand, variation in pheromone blends is due to *experience*, we would expect all these changes in the same years. Alternatively, if variation is due to other factors or due to genetic drift, then no correlations between the relative amounts of these components and the relative abundance of the three species are expected. We quantified the pheromone gland contents of 17–54 females per region annually, to obtain an estimate of the geographic variation as well as temporal variation of the sex pheromone signals.

As for male response, recent studies have shown that preexposure of male moths to specific sex pheromone blends can change their response (Anderson et al. 2003; Andersson et al. 2007), as mentioned previously. This suggests that the male response can be coupled to current local conditions, and could explain how female production and male response can be behaviorally coupled (Gray and Cade 1999). If this is true we would expect: a) variation in male response within one region, and b) covariation of male response with female pheromone production. We determined the attraction of local males to live females for 2

Table 2. Sites, dates, and host plants from which eggs and larvae were collected.

	Heliothis virescens (Hv)			Heliothis subflexa (Hs)2	
Year site[1]	2004	2005	2006	2004	2005
Clayton, North Carolina (35°39′58″N, 78°30′36″W)	Eggs (tobacco) June	Eggs (tobacco) June		Larvae June	Larvae June
Stoneville, Mississippi (33°25′04″N, 90°54′37″W)		Larvae (chickpea) June	Larvae (chickpea) August		
College Stn., Texas (30°38′22″N, 96°21′39″W)	Larvae (chickpea) June	Larvae (chickpea) May	Larvae (chickpea) August	Larvae June	Larvae May
Tampico, **MXE** (22°13′02″N, 97°50′40″W)		Larvae (cotton) Oct	Larvae (cotton) August		Larvae Oct
Chamela, **MXW** (19°31′49″N, 105°03′47″W)					Larvae Oct

[1]MXE is Eastern Mexico and MXW is Western Mexico.

[2]All Hs larvae were collected from fruits of *Physalis* spp. (in NC mostly from *P. angulata*, and in MX from *P. philadelphica*).

consecutive years, in the field, as a measure of variation in male response to female signals, and correlated their response to the different pheromone components mentioned previously.

Material and Methods

VARIATION IN FEMALE PHEROMONE PRODUCTION

Insect collections and cultures

Eggs of Hv and larvae of Hv and Hs were collected in the field in five different regions in 3 and 2 consecutive years, respectively, as summarized in Table 2. The collection sites were Clayton, North Carolina; Stoneville, Mississippi; College Station, Texas; Tampico in the Province of Tamaulipas, Mexico; and Chamela in the Province Jalisco, Mexico. Tampico is on the East coast of Mexico and we refer to this site as MXE, whereas Chamela is on the West coast and is referred to as MXW. Larvae collected in the field were reared to adults on artificial diet at North Carolina State University (NCSU). Hs larvae were given *Physalis angulata* fruits in addition to the artificial diet; pupae were separated by sex and checked daily for emergence. Pheromone glands were extracted from two to five days old virgin females that emerged from the field-collected larvae and/or from their female offspring (i.e., females that were reared in the lab for one generation).

Pheromone gland extractions

The relationship between gland content and volatile emission appears to be linear (Heath et al. 1991), so the pheromone blend profile in the pheromone gland can be a good and much more convenient approximation of the pheromone blend emitted by the female. A total of 266 Hv pheromone glands were extracted and analyzed; 92 in 2004 (38 from NC and 54 from TX), 117 in 2005 (30 from NC, 19 from MS, 37 from TX, and 31 from MXE), and

Table 3. Total number of males caught in traps with live virgin females.

Year	Males[2]	Males captured in female-baited traps[1]		Ratio Hv/Hs males
		Hv females	Hs females	
2004[3]	Hv males	**411** (52)	2 (58)	
	Hs males	4 (52)	**103** (58)	**3.99**
	Hz males	67 (52)	24 (58)	
2005	Hv males	**104** (77)	0 (52)	
	Hs males	0 (77)	**216** (52)	**0.49**
	Hz males	7 (77)	0 (52)	
2006	Hv males	**1249** (214)	11 (218)	
	Hs males	0 (214)	**633** (218)	**2.05**
	Hz males	22 (214)	56 (218)	

[1]In parentheses is the total number of live-female traps used. Numbers in bold indicate conspecific attraction.

[2]Heliothis virescens (Hv), Heliothis subflexa (Hs), and Helicoverpa zea (Hz)

[3]Detailed data published in Groot et al. (2006).

57 in 2006 (18 from MS, 22 from TX, and 17 from MXE). A total of 151 Hs glands were analyzed, 39 in 2004 (21 from NC, 18 from TX) and 112 in 2005 (30 from NC, 31 from TX, 20 from MXE, and 31 from MXW). In 2004, all female pupae were transferred to a room with reversed light:dark cycle (lights on from 18.00–08.00). Pheromone glands from two- to five-day-old virgin females were extracted during the 6th hour of scotophase. In 2005 and 2006, all female pupae remained at an ambient light:dark cycle (lights on from 05.00–19.00). Two to five days old virgin females were injected with 7.5 pmol PBAN during the photophase to stimulate pheromone production (see Groot et al. 2005a). All pheromone glands were extracted for 20–30 min in 50 μl hexane containing 20 ng 1-pentadecyl acetate as an internal standard. Extracts were stored at −25°C. The hexane was reduced under a gentle stream of N_2 to 1–2 μl, taken up into 2 μl octane, and placed in a 50-μl glass insert within a crimp-capped vial. Using a 7683 automatic injector, the entire volume of extract was injected into a splitless inlet of a HP6890 gas chromatograph (GC) coupled with a high resolution polar capillary column (DB-WAXetr [extended temperature range]; 30 m × 0.25 mm × 0.5 μm) and a flame-ionization detector (see Groot et al. 2005a for further details). Before and after each GC sequence, which was generally every one–two days, we injected authentic standards of all the pheromone components to assess column performance as well as to check the retention times of each of the components. We corrected all integration results by the differential response of the FID to the various authentic standards. Samples were chromatographed as they became available, to alleviate decomposition in storage, especially of the aldehydes, but a subset of samples was reanalyzed (i.e., integrated and calculated) in one consecutive session to confirm that the analysis was consistent over time.

Quantitative and statistical analysis

Because there is high variance among female moths in total pheromone gland content, even within treatments, most researchers analyze differences between the amount of each component after converting amounts to percentages relative to either 1) the single most abundant compound (i.e., the "major" component) (e.g., Heath et al. 1991, Teal and Tumlinson 1997), or 2) the total amount of all of the pheromone components in the gland (e.g., Heath et al. 1991). We chose to remove the major component Z11–16:Ald from the analysis to examine each other component as a percentage of the total of all "minor" components, because a number of these compounds are present in relatively small amounts (e.g., 14:Ald and Z9–14:Ald comprise only up to about 1% of the pheromone blend in Hs; see Table 1). Therefore, the variation in these compounds becomes more obvious when the major component (comprising about 60% of the total blend in Hv and 45% of the total blend in Hs) is removed (see also Sheck et al. 2006). We refer to these percentages as the percent of all minor components. It is important to note that "minor" refers only to representation of a pheromone component in the blend, and not to its function in attracting males.

Previously we found only minor differences in pheromone composition between glands extracted from virgin females in the scotophase and glands extracted in the photophase from mated females injected with PBAN (Groot et al. 2005a). Also, in this study, the total amount of pheromone in the glands was similar between glands extracted in 2004 and those extracted in 2005 and 2006 (see Fig. S1). Therefore, in this study, differences in pheromone composition were compared between years, regions, and their interaction by species, even though females were treated differently between years (i.e., without PBAN injections in 2004 and with PBAN injections in 2005 and 2006). Values were log transformed as needed to stabilize the variance. To determine whether years and regions had an overall effect on the pheromone composition, a multivariate analysis of variance (MANOVA) was conducted within each species using the computer program SAS, version 9.1 (SAS Institute, 2002–2003). After finding a significant overall year effect ($P < 0.0001$) as well as a significant overall region effect ($P < 0.0001$) in each species, a separate MANOVA was conducted by year or by region, that is, treating the years or the regions as fixed effects, respectively, to separately assess the magnitude of each effect on the pheromone variation. The means were separated using least significant differences (LSD).

VARIATION IN MALE RESPONSE

Live female baited traps

To assess variation in male response, live females were used as lures in trapping experiments conducted in 2 consecutive years, 2005 and 2006, at the NCSU Central Crops Research Station in Clayton, North Carolina. Attraction of naturally existing Hv and Hs males to females was measured using Hartstack wire-mesh cone traps (Hartstack et al. 1979). Traps were distributed at least 15 m apart throughout a field. One live one- to three-day-old virgin female was placed in a small open cylinder, sealed with gauze on both sides, which was placed at the opening of each trap. Males caught in the traps during one–two nights were sorted by species under a microscope, and counted. When lures (containers with females) were left in the field for two nights, all containers were rotated among all trap locations after one night to minimize position effects and odorant contamination.

In 2005, containers were deployed with virgin females that were collected as eggs or larvae in the field (see above). From Aug 29 to Sep 29, a total of 73 Hv females (34 Hv-NC and 39 Hv-TX females) were tested in North Carolina. Of these females, the pheromone glands of 29 Hv-NC and 33 Hv-TX females were extracted and analyzed. In the same period and at the same field station, a total of 73 Hs females were tested (38 Hs-NC females and 35 Hs-TX females). Of these females, the pheromone glands

of 27 Hs-NC and 21 Hs-TX females were extracted and analyzed. In 2006, virgin females were used that had been reared in the lab at NCSU for four–six generations; thus, this was a different set of females than the set examined for variation in pheromone composition, as described previously. From July 30 to September 2, a total of 214 Hv females and 218 Hs females were tested in North Carolina. The glands of all tested females were extracted and analyzed by GC, as described previously. However, because differences persisted among females originating from different regions even when reared in the lab for four–six generations (A. Groot, unpubl. ms.), in this analysis we only included females originating from North Carolina ($n = 103$ Hv females and 61 Hs females).

Statistical analysis

The females used in 2005 were collected as eggs or larvae in the field, and reared to adults under the same conditions. We first determined the level of assortative attraction, that is, whether naturally existing males in North Carolina were differentially attracted to females that originated from North Carolina over females originating from other regions, irrespective of their pheromone composition. An analysis of variance (ANOVA) was conducted in SAS 9.1 (SAS, Cary, NC) using PROC GLM, where region was the class and the mean number of males caught per female per night was the variable. To test the correlation between the relative percent of each of the pheromone components and the mean number of Hv or Hs males caught per female per night, we used PROC CORR in SAS 9.1, after the numbers of males caught were square-root transformed to stabilize the variance. Because we found significant differences in pheromone composition between females from different regions, this correlation analysis was conducted using the females originating only from North Carolina. The pheromone gland composition of North Carolina females used in 2005 might have differed from that of females used as lures in 2006, which could affect the correlations between number of males caught and the relative amount of the different pheromone components. Therefore, we conducted an ANOVA to test for differences between the females tested in 2005 and those tested in 2006 in those components that showed a significant correlation with trap catch in either year.

Results

VARIATION IN Hv PHEROMONE GLAND COMPOSITION

Geographic variation

When year was treated as fixed variable, the overall pheromone composition was marginally different between North Carolina and Texas in 2004 ($P = 0.05$) in which Hv-NC females contained relatively more Z9–16:Ald and less of the alcohol Z11–16:OH than Texas females (Fig. 1A). However, significant differences were found in the overall pheromone compositions among the four and three regions examined in 2005 ($P < 0.0001$) and 2006 ($P = 0.0042$), respectively. In 2005, each of the minor pheromone components differed significantly between at least two of the four regions. Most importantly, the pheromone glands of Hv-NC females contained the highest percentage of the critical secondary pheromone component Z9–14:Ald, whereas Hv-MXE females contained the least. In 2006, again, Hv females from MXE contained significantly less Z9–14:Ald than Hv-MS females, but similar to Hv-TX females (Fig. 1A). Hence, in both 2005 and 2006, females from the Eastern part of the United States (NC and MS) produced more of the critical secondary pheromone component Z9–14:Ald than females from the west (TX and MXE).

Variation within regions

When region was treated as fixed variable, significant differences between years were found in females from all four regions ($P < 0.0001$ for all; Fig. 1B). The most consistent variation between years within regions was in Z9–14:Ald (Fig. 1B). The difference between the 2004 and 2005 samples was similar to the difference between the 2005 and 2006 samples, confirming that the differences between years was not related to a difference in gland extraction procedure, that is, whether females were injected with PBAN (in 2005 and 2006) or not (in 2004).

VARIATION IN Hs PHEROMONE COMPOSITION

Geographic variation

When year was treated as fixed variable, significant differences were found in 2004 between Hs females originating from North Carolina and Texas, and in 2005 among the four regions. Specifically, in 2004 North Carolina and Texas females significantly differed in their overall pheromone composition ($P = 0.0314$), with Hs-NC females containing significantly more of two of the three acetates (Z9–16:OAc and Z11–16:OAc) than Hs-TX females, and significantly less of both C-16 alcohols (Fig. 2A). In 2005, the pheromone composition significantly differed among the four regions analyzed ($P < 0.0001$), which was due to a significant difference in each of the minor pheromone components between at least two of the four regions. Most interestingly, the percentage of the critical secondary sex pheromone component of Hs, Z9–16:Ald, was significantly lower in Hs-NC and Hs-TX females than in the Mexican females. As in 2004, Hs-NC females contained significantly more of the two 16-carbon acetates than Hs females in the other regions.

Variation within regions

When region was treated as a fixed variable, significant differences were found between years in females from North Carolina ($P < 0.0001$), but not in females from Texas ($P = 0.27$). In 2005,

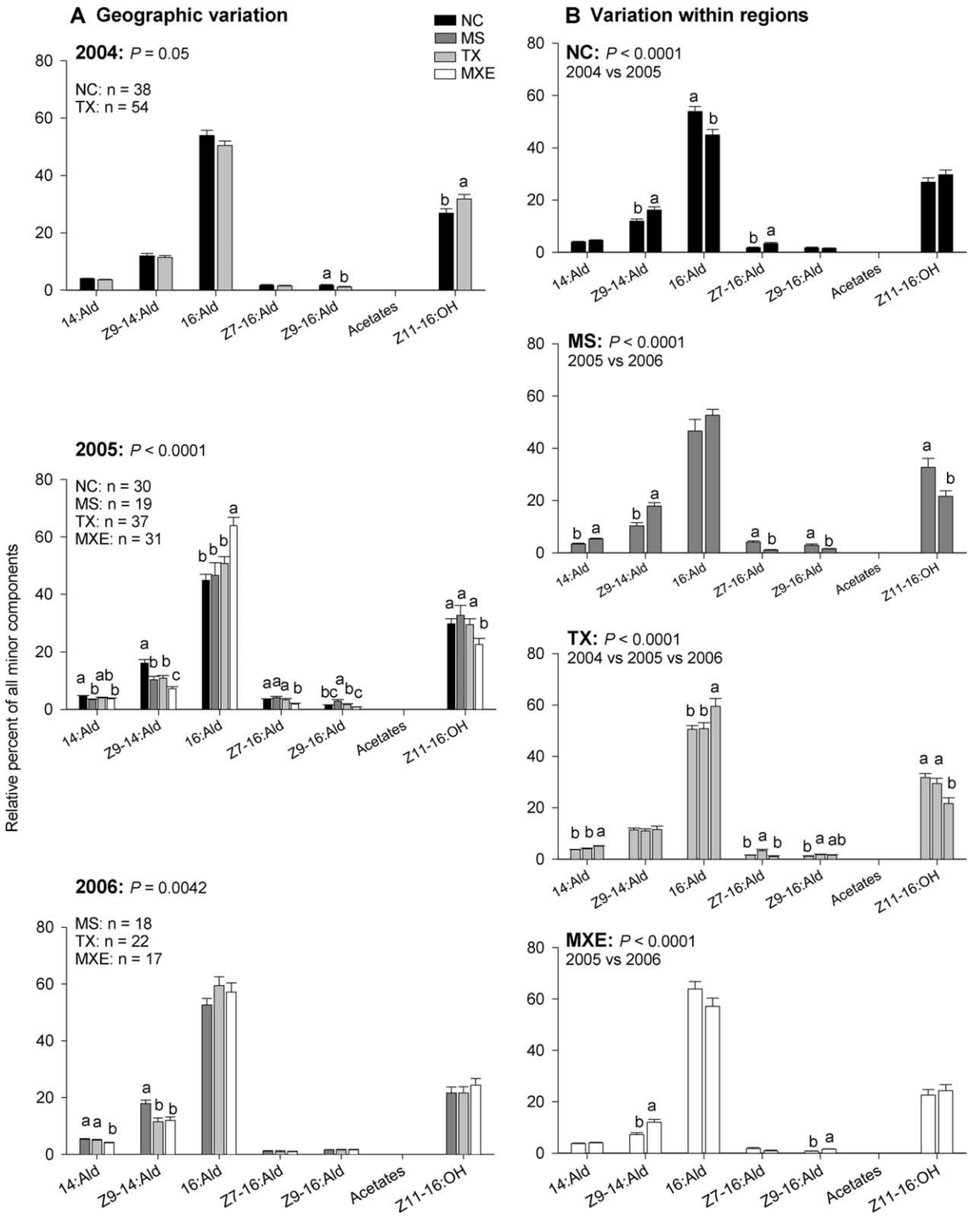

Figure 1. **(A) Geographic variation and (B) temporal variation within regions in *Heliothis virescens* pheromone gland composition. A and B are based on the same data. Overall differences for each graph were tested with a MANOVA using GLM in SAS 9.1, after which the means were separated using LSD. Comparisons are per pheromone component in each graph, and different letters for the same compound indicate significant differences. Acetates include the three compounds detailed in Fig. 2, which exist in *Heliothis subflexa* but not in *H. virescens*. MS, Mississippi; NC, North Carolina; TX, Texas; MXE, Eastern Mexico.**

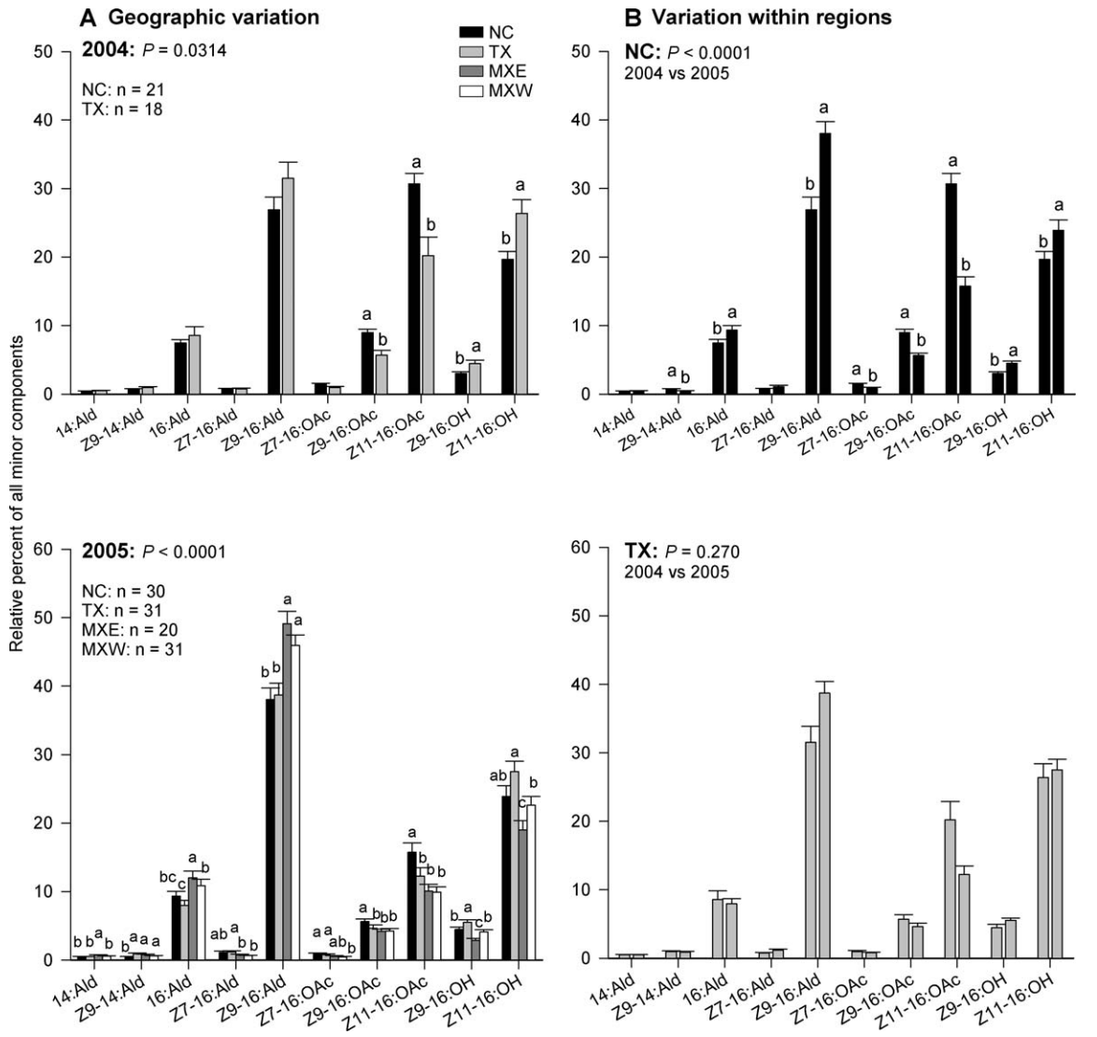

Figure 2. **(A) Geographic variation and (B) temporal variation within regions in *Heliothis subflexa* pheromone gland composition. A and B are based on the same data. Overall differences for each graph were tested with a MANOVA using GLM in SAS 9.1, after which the means were separated using LSD. Comparisons are per pheromone component in each graph, and different letters for the same compound indicate significant differences. NC, North Carolina; TX, Texas; MXE, Eastern Mexico; MXW, Western Mexico.**

Hs-females from North Carolina contained significantly less Z9–14:Ald (Fig. 2B), which does not appear to be important in the sexual communication of Hs (Groot et al. 2007). Also, the three acetates were significantly lower in 2005 than in 2004. In contrast, Hs-NC females tested in 2005 contained significantly more of their critical secondary pheromone component Z9–16:Ald, as well as 16:Ald and the 16-carbon alcohols, than females tested in 2004.

VARIATION IN MALE RESPONSE

Positive assortative attraction in the field

In 2005, 73 Hv females (34 Hv-NC and 39 Hv-TX) that were collected as eggs or larvae were used as lures in traps deployed in North Carolina fields. Of these, 22 females (10 Hv-NC and 12 Hv-TX) attracted a total of 83 Hv males. Significantly more Hv males were caught in traps baited with Hv-NC than with Hv-TX females ($P = 0.0388$, Fig. 3A).

Considering Hs, 51 out of 73 traps baited with Hs females that were collected as larvae from the field (27 Hs-NC females and 24 Hs-TX females) caught a total of 323 Hs males in North Carolina fields. Significantly more Hs males were caught in traps deployed with Hs-NC females than with Hs-TX females ($P = 0.0284$, Fig. 3B). These results indicate that both Hv and Hs males from North Carolina are assortatively attracted to conspecific females from their own region. It is important to reiterate that all females were collected as eggs or young larvae in the field and were reared to the adult stage in the same laboratory under identical temperature and photoperiod conditions.

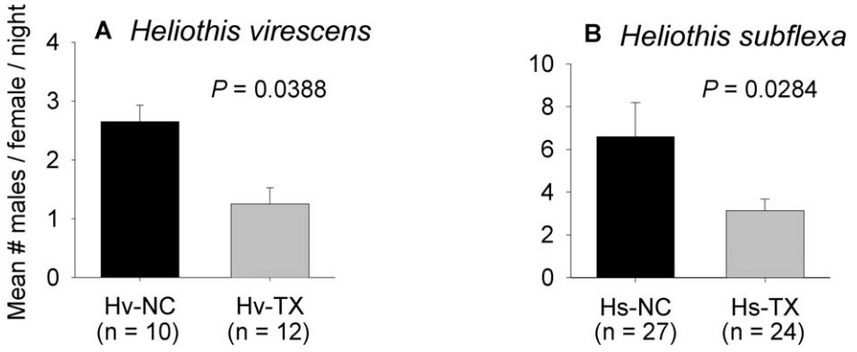

Figure 3. Differential attraction in 2005 of (A) *Heliothis virescens* (Hv) males to Hv females that were collected from North Carolina (NC) as eggs or from Texas (TX) as larvae, and (B) *Heliothis subflexa* (Hs) males to Hs females that were collected from NC or TX as larvae.

Variation in Hv male attraction in North Carolina

In 2005, a total of 51 Hv males were caught in traps baited with 29 Hv-NC females whose pheromone gland composition was analyzed. In 2006, a total of 625 Hv males were caught in traps baited with 103 Hv-NC females whose pheromone gland composition was analyzed. In 2005, we found a significant positive correlation between the number of Hv males captured and the relative amount of the critical secondary sex pheromone component of Hv, Z9–14:Ald ($P = 0.042$, Fig. 4, black circles). In the same year, this component was also produced in the highest amounts in Hv-NC females (see Fig. 1A). In contrast, in 2006 there was no correlation between Z9–14:Ald and the number of Hv males captured (Fig. 4, gray triangles). This difference in correlation between years could be partly due to the fact that the females used as lures in both years significantly differed in their relative amount of Z9–14:Ald ($P = 0.0049$), Hv females in 2005 containing 15.23% ± 1.19% (mean ± SEM) relative to all minor components, whereas those used in 2006 contained 9.61% ± 0.66%. However, Fig. 4 also shows that the relative percent of Z9–14:Ald present in the females extensively overlapped between the two years, so that the variation in male attraction between the two years could not be explained solely by the difference in live-female lures used. In addition, there was no correlation between the total amount of pheromone in the gland and either the relative amount of Z9–14:Ald in the glands or the number of Hv males captured (Fig. 4, right graphs).

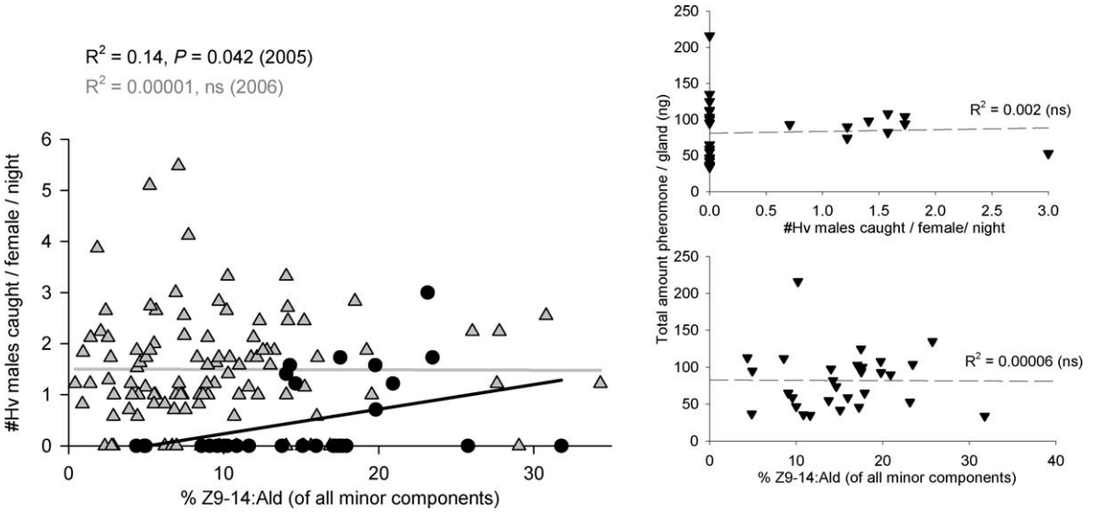

Figure 4. Variation in *Heliothis virescens* (Hv) male response to live-female lures in North Carolina. Mean number of Hv males caught per female lure per night in 2005 (circles) and 2006 (triangles) are plotted relative to two pheromone components. Black-filled symbols indicate significant correlations, and gray-filled symbols show the nonsignificant correlations. A significant positive correlation is shown in 2005, but not in 2006, between the number of Hv males caught and the relative amount of Z9–14:Ald. The graphs at right show correlations between 1) the relative amount of Z9–14:Ald and the total amount of pheromone extracted from the glands, and 2) the mean number of Hv males caught in the traps and the total amount of pheromone extracted from the glands.

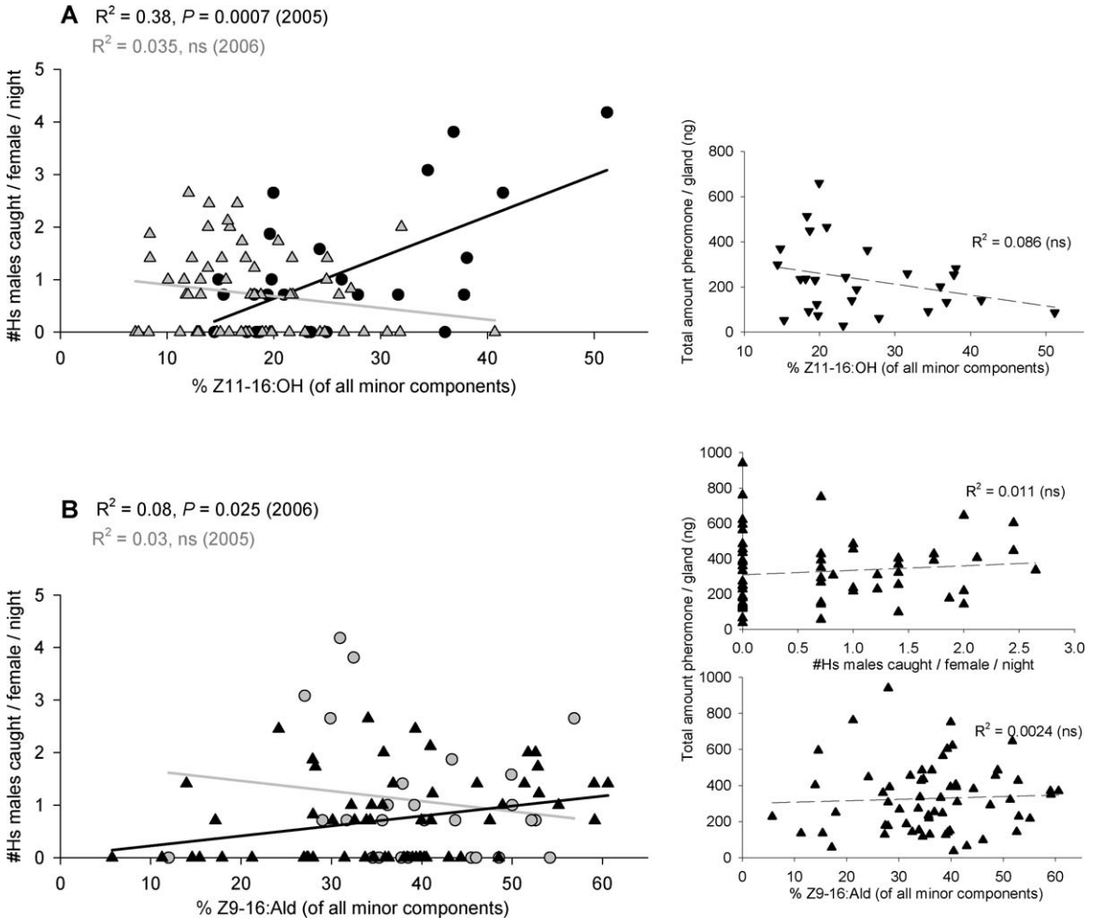

Figure 5. **Variation in *Heliothis subflexa* (Hs) male response to live-female lures in North Carolina. Mean number of Hs males caught per female lure per night in 2005 (circles) and 2006 (triangles) are plotted relative to three pheromone components. Black-filled symbols indicate significant correlations, and gray-filled symbols show the nonsignificant correlations. A. In 2005, a significant negative correlation between the number of Hs males caught and Z9–16:OAc. B. In 2005, a significant positive correlation between the number of Hs males caught and Z11–16:OH. C. In 2006, a significant positive correlation between the number of Hs males caught and the relative amount of Z9–16:Ald in the pheromone gland. The graphs at right show 1) correlations between the relative amount of the pheromone components that showed a correlation with the number of males caught, and the total amount of pheromone extracted from the glands (ng), and 2) the mean number of Hs males caught in the traps plotted against the total amount of pheromone extracted from the glands.**

Variation in Hs male attraction to females in North Carolina

In 2005, 27 Hs-NC females were used as live-female lures and their pheromone glands were analyzed; they caught a total of 140 Hs males. In 2006, 61 Hs-NC females caught a total of 102 Hs males. The mean number of Hs males caught per female in 2005 was significantly positively correlated with one of the Hs critical sex pheromone components, Z11–16:OH ($P = 0.0007$; Fig. 5A, black circles). However, in 2006, this relationship disappeared, and instead there was no correlation between this alcohol and the number of Hs males caught (Fig. 5A, gray triangles). The difference between years may be explained, at least in part, by a significant overall difference in the relative amount of Z11–16:OH ($P < 0.0001$) in the females used as lures in 2005 (32.92% $\pm$ 1.83%) and in 2006 (18.03% $\pm$ 0.86%). Thus, higher relative amounts of Z11–16:OH in 2005 appeared to attract relatively more males.

In 2006, the number of Hs males caught was significantly positively correlated with the relative amount of Z9–16:Ald, another critical secondary pheromone component of Hs ($P = 0.025$; Fig. 5B, black triangles). But no such correlation was found in 2005 (Fig. 5B, gray circles) and we found no significant difference in the relative amount of Z9–16:Ald in the pheromone glands of females tested in 2005 and in 2006 ($P = 0.203$). In addition, we did not find a correlation between the relative amount of Z9–16:Ald and the total amount of pheromone in the glands, or

between the total amount of pheromone and the number of males caught (Fig. 5B, right graphs).

In 2006, some Hs females attracted heterospecific males; in total 11 Hv males were caught by nine of the 218 Hs females used as lures (Table 3). These females produced relatively low amounts of acetate esters (6.15% ± 1.68% when calculated as percent of all pheromone components, following Groot et al. 2006) compared to Hs females that attracted only Hs males (9.35% ± 0.54% of the three acetate esters). This confirms our finding that cross-attraction of Hv males to Hs females may occur when Hs females produce relatively low amounts of acetates (Groot et al. 2006). The number of Hs males trapped was not correlated to any of the other pheromone compounds present in the female glands in both years.

Discussion

In this study we found significant variation in the premating pheromonal signals of two closely related and sympatric moth species, *Heliothis virescens* (Hv) and *H. subflexa* (Hs), not only between geographic regions but also within a region between years. The temporal variation was of a similar magnitude as the geographic variation. As far as we know, this is the first study reporting such temporal variation in moth chemical communication systems. The variation could be the result of natural selection, as has been generally hypothesized, or caused by stochastic events. In addition, we suggest a new hypothesis that may explain variation in premating pheromone signals: phenotypic plasticity might allow females to modulate pheromone gland composition based on their olfactory experience as larvae or early adults; males, likewise, may modulate their response to pheromones based on earlier experience. To distinguish among these hypotheses, variation in pheromone communication signals and responses, as well as the relative abundance of potentially interfering species, should be documented over multiple years in multiple regions. Because we only collected data in 2–3 consecutive years from four to five regions, and we could not characterize the entire semiochemical environment of the moth populations, our study should be viewed as a first exploration to assess whether the observed pattern in variation of female pheromone production and male response may be related to a complex optimization process that involves not only natural and sexual selection, but also olfactory experience.

To assess whether temporal variation in the pheromone signals may be due to phenotypic plasticity, we first consider the population densities and dynamics of the three species. Geographically, the relative population densities of Hv and Hs differ greatly across regions. Hv is a generalist on many important agricultural crops throughout the United States, and is widely distributed throughout North, Central, and South America, where its host plants are grown (e.g., Hartstack et al. 1979; Lopez et al. 1994; Chapin et al. 1997; Parajulee et al. 2004; Blanco et al. 2007). However, because the abundance of Hv is related to the intensity of agricultural production, it appears to be much less abundant in Mexico (Tafoya et al. 2002). On the other hand, Hs can only survive on plants in the genus *Physalis* (McElvare 1941; Sheck and Gould 1993; Bateman 2006), which grow in small patches along roadsides and margins of agricultural fields throughout the United States. In Mexico, however, Hs is highly abundant (Bautista 2006; M. Bateman, F. Gould and A. Groot, pers. obs.), because one of its host plants is the crop plant tomatillo (*Physalis philadelphica* Lam.), a major crop in Mexico since pre-Colombian times (Jenkins 1948).

Within regions, local population densities are affected by crop rotations, weather, and migration patterns of adult heliothines. Hv is known to be migratory (Stadelbacher 1981; Schneider et al. 1989; Han and Caprio 2004), but the migratory habits of Hs have not been described. Hz, like Hv, is a highly polyphagous and important agricultural pest in the United States as well as in Mexico (Laster 1972; Sparks et al. 1979; Hartstack et al. 1979; Lopez et al. 1994; Parajulee et al. 2004), so the geographic distribution of Hz broadly overlaps with the habitats of Hv and Hs, except where Hs is most abundant (e.g., Western Mexico). On some agricultural crops, local Hz populations can reach much higher levels than the other two heliothines (Parajulee et al. 2004). Importantly, Hz males appear to have the least fidelity for their species-specific pheromone blend, and are attracted in relatively large numbers not only to live Hv and Hs females (Table 3), but also to artificial Hv and Hs lures (see Table S1). Thus, although neither Hv nor Hs can successfully hybridize with Hz (Stadelbacher et al. 1983), when Hz populations are high, Hz males may physically harass Hv and Hs females, and interfere with pheromone emission and conspecific copulation attempts.

Our trapping experiments in 2004 (Groot et al. 2006), 2005, and 2006 to lure and trap local wild males in North Carolina to traps baited with live females showed large fluctuations in trap catch of Hv and Hs males between years (Table 3), which was also confirmed in synthetic lure experiments that we conducted in 2004 and 2005 (Table S1). Although pheromone trap data cannot be directly extrapolated to population densities, they offer a reliable measure of the relative abundance of heliothine species (e.g., Hartstack and Witz 1981; Lopez et al. 1994; Parajulee et al. 2004), and thus the chance of attracting con- or heterospecific males. We deployed a similar number of traps baited with Hv and Hs females each year, but four times as many Hv as Hs males were caught in 2004, half as many Hv males as Hs males were caught in 2005, and twice as many Hv males as Hs males were captured in 2006 in these traps (Table 3). Hence, the chance of attracting heterospecific Hv males was much higher for Hs females in 2004 and 2006 than in 2005. Interestingly, we never found the reverse pattern of Hs males being attracted to Hv females. In 2004 we

also caught a large number of Hz males, not only in traps baited with live Hv and Hs females (Table 3), but also in traps baited with synthetic Hv and Hs lures (Fig. S1). However, because we caught a similar number of Hz males in traps baited with Hs lure as in blank control traps, the Hz males caught in the Hs traps were likely not actively attracted to the Hs pheromone blend. In 2005, we caught only a few Hz males in any trap (Tables 3 and S1), indicating that Hz males were much less abundant than Hs males.

Even though our data do not permit a clear distinction between the "selection" and the "experience" hypothesis, subsequently we explore the variation we found in light of these hypotheses. If communication interference from Hs or Hz is in part responsible for variation in the Hv pheromone blend, we would expect Hv to contain higher levels of Z9–14:Ald when Hs and Hz males were relatively more abundant. Such a change within the same year would suggest that females modulate their pheromone blend based on olfactory experience, whereas production of more Z9–14:Ald in response to high Hs or Hz populations in the previous year would implicate selection across generations for females that optimized a blend to minimize interference (i.e., relatively more Z9–14:Ald). In North Carolina, Hv females contained significantly more Z9–14:Ald in 2005 than in 2004, which coincided with relatively greater abundance of Hs males in 2005 (Table 3). Hence, this correlation suggests that temporal variation in Z9–14:Ald may, at least in part, be attributable to female experience. Geographically, we found that females from North Carolina and Mississippi produced more of the critical secondary sex pheromone component Z9–14:Ald than females from Texas and MXE, at least in 2005 and 2006. In North Carolina, the variance in Hs population sizes, patchiness, movement, and abundance may be higher than in the other regions, because tomatillo, the host plant of Hs, has become a small but significant agricultural crop in North Carolina in recent years. Thus, the geographic variation could at least partly be due to selection.

For Hs we predicted an increase in Z9–16:Ald, a critical secondary sex pheromone component, when Hz populations are relatively high, because this component is also part of the Hz sex pheromone, but inhibits Hz attraction at high amounts (Table 3). The relative amount of this component was significantly higher in the two Mexican populations than in North Carolina and Texas in 2005. As described previously, the abundance of Hs in Mexico is likely to be higher than Hv or Hz, so there does not seem to be a correlation between the variation of Z9–16:Ald in Hs females and the chance of attracting heterospecific males, which suggests that this variation may be due to either stochastic events or to the presence of other moth species in Mexico that overlap this communication channel. Within North Carolina, the relative amount of Z9–16:Ald was significantly higher in 2005 than in 2004. Because the chance of catching Hz males was much higher in 2004 than in 2005 in North Carolina (see Tables 3 and S1), variation in this important component may be due to selection from the previous year.

For the pheromone components of Hs with a dual function (attraction of conspecific males as well as repellence of heterospecific males) we expected an increase with higher Hv and/or Hz populations to reduce heterospecific attraction. In 2005, Hs-NC females contained almost half as much Z11–16:OAc (15.74% $\pm$ 1.36%) as in 2004 (30.69% $\pm$ 1.53%). The dramatic decrease in Z11–16:OAc, which inhibits attraction of Hz and Hv males, coincided with lower trap catches of Hv and Hz males in the same year, suggesting that females might have adjusted the pheromone blend based on olfactory experience. Geographically, Hs-NC females produced significantly more of this acetate than females from other regions in both years (Fig. 2). In Western Mexico, where Hs is the dominant heliothine moth, the relative amount of Z11–16:OAc was $<$30% of that produced by North Carolina females. Hence, we also found a geographic correlation between the relative amount of this acetate and the chance of attracting Hv and Hz males. If the relative abundance of these species in different regions is more or less stable (i.e., if Hz and Hv are always relatively less abundant in Mexico than in North Carolina), then the geographic variation in pheromone blends may be due to selection. The alcohol Z11–16:OH is a critical sex pheromone component in Hs, and like Z11–16:OAc, it also inhibits the attraction of Hv and Hz. Yet, Z11–16:OH did not vary consistently with the relative abundance of Hv and Hz (see Fig. 2), probably because of its dual role in the pheromone gland. The alcohols serve as immediate precursors to their aldehyde and acetate derivatives (Tillman et al. 1999; Rafaeli 2002; Jurenka 2004), and higher amounts of aldehyde and acetate products usually coincide with lower amounts of the corresponding alcohols (Groot et al. 2005a; Sheck et al. 2006). Therefore, the metabolic pool of Z11–16:OH may distort correlations based purely on its behavioral function.

Similar to the female sex pheromone signals, the variation in male response to female sex pheromones in moths may also be affected by the presence and abundance of a) conspecifics, b) closely-related species that have similar pheromone components, c) other species that may generate background chemical "noise" and thus may influence the response thresholds to the native pheromone, and d) by abiotic factors that may affect pheromone release rates, adsorption to substrates, and dispersion in the atmosphere, as well as male flight capabilities (Raina 1988; McNeil and Deslile 1989; Dumont and McNeil 1992; Murlis et al. 2000). Our male attraction experiments were conducted mainly in September 2005 and August 2006, when the weather conditions were relatively comparable, although September 2005 was cooler and drier than August 2006 (Table 4). In both years, the male attraction experiments were conducted at the Clayton Central Crops Research Station, North Carolina, in fields with similar

Table 4. Weather conditions in Clayton, NC, when the experiments were conducted in 2004, 2005, and 2006 (from the weather station in Clayton).

Month	Year	Temperature in °C mean (max–min)	RH in % mean (max–min)	Rainfall in mm
August	2004	23.5 (28.6–19.2)	80 (96–56)	272.8
	2005	26.1 (31.8–21.5)	78 (96–51)	77.0
	2006	26.1 (31.7–21.3)	76 (95–49)	87.6
September	2004	21.4 (26.5–17.3)	82 (96–58)	46.5
	2005	23.7 (29.9–18.7)	72 (92–45)	33.0
	2006	20.6 (26.0–15.8)	80 (96–55)	87.4

vegetation types (*Physalis*, cotton, and soybean plants). Trap catches (mean number of males per trap per night) were generally low in female-baited traps, but this was also the case in traps baited with synthetic lures (see Table S1). For example, traps with synthetic Hv lures caught 0.017 Hv males/trap/night in 2004 and 0.13 in 2005, whereas traps baited with Hs lures caught 0.008 Hs males/trap/night in 2004 and 0.23 in 2005. Despite these low trap catches, we found a significant positive correlation in 2005 between the number of Hv males lured to calling females and the relative amount of Z9–14:Ald in female pheromone glands (Fig. 4). In 2006, when we caught many more Hv than Hs males, we did not find a correlation between Hv trap catch and the relative amount of Z9–14:Ald. Similarly, in Hs we found a significant positive correlation between the number of Hs males caught by live female lures and the relative amount of the critical secondary sex pheromone component Z9–16:Ald in pheromone glands in 2006. There was no such correlation in 2005, when Hs was relatively more abundant than Hv based on trap catches. Because these correlations coincided with a relatively higher abundance of the other species in the same years, these correlations could be due to experience-based modulation of male behavior. In contrast, the significant positive correlation between the number of Hs males caught and the relative amount of Z11–16:OH in Hs female glands in 2005 could be due to selection in response to the high abundance of Hv and Hz in the previous year. The dynamic nature of pheromone emission and male attraction suggest a coupling of male behavioral responses to the quality of the signals that females produce, in line with the behavioral coupling found in other studies (e.g., Clayton 1990; Houde and Endler 1990; Löfstedt et al. 1991; Zhu et al. 1997; Brodin and Haas 2006), as well as with the finding that male response may change with experience (Anderson et al. 2003; Andersson et al. 2007).

In the one cross-regional attraction experiment we conducted, North Carolina males of both Hv and Hs were preferentially attracted to conspecific local females from North Carolina over females that originated from Texas, which suggests assortative attraction of males to females from the same population. Limited gene flow between populations may select for variation in pheromone communication systems. Unfortunately, we were unable to conduct the reciprocal test in Texas, so this pilot experiment serves as a preliminary suggestion that positive assortative attraction may occur. Because the two sets of females were reared to adults in the lab under identical conditions and appeared to call at the same times during the night in the field (F. Gould, pers. obs), assortative attraction of males is likely not related to differences in calling times, as has been observed in other moth species (e.g., Cardé et al. 1975; Pashley et al. 1992).

In conclusion, the chemical premating signals of these two noctuid moths are more variable in time and space than expected based on the hypothesis that they are subject to strong stabilizing selection and empirical analyses of pheromone glands at a single point in time. There are numerous descriptive studies of geographic variation in pheromone blends, male responses, and attraction to artificial lures formulated to mimic females. To our knowledge, variation in chemical communication signals between years has not been described so far. In addition to selection and local stochastic events, the geographic and temporal variation of the premating signals may be affected by the habitat, which in our view includes not only abiotic factors, such as temperature, relative humidity, and day length, but also the presence and relative abundance of other species that may cause communication interactions and thus constitute the semiochemical environment. An effect of the habitat may arise if females vary their pheromone blend depending on local environmental conditions that they experienced as larvae or early adults—we refer to this as the "experience hypothesis." We are currently testing this hypothesis in the laboratory, and it should also be tested in the field. Variation due to experience is not heritable and therefore not due to selection. Hence, even though the finding of variation often leads to the conclusion that differences are due to selection which may lead to speciation, as in the Galapagos finches, we propose that not all variation may be subject to selection.

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Supporting Information

The following supporting information is available for this article:

Figure S1. Pheromone titers in the extracted pheromone glands of females on which Figure 1 and 2 in the manuscript are based.
Table S1. Trap catches in traps with synthetic lures.

Supporting Information may be found in the online version of this article.
(This link will take you to the article abstract).

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Supplementary Table 1. **Trap catches in traps with synthetic lures**

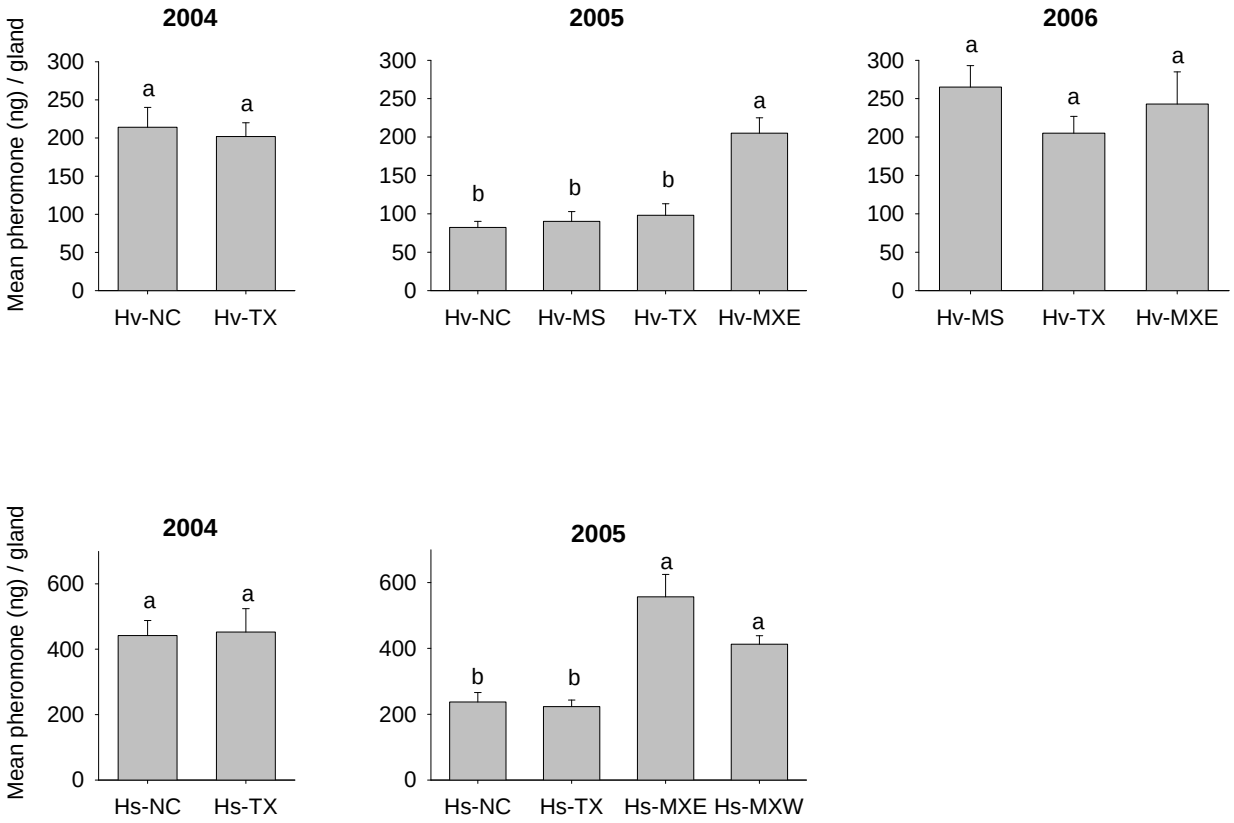
Year	***Heliothis virescens* (Hv) lure**			***Heliothis subflexa* (Hs) lure**			**Control lure**		
	Hv males	Hs males	Hz males	Hv males	Hs males	Hz males	Hv males	Hs males	Hz males
Aug. 2004 (28, 30[1])	**29**	**0**	**76**	**1**	**11**	**13**	**3**	**1**	**17**
Sep 2004 (21, 21[2])	**14**	**0**	**0**	**0**	**11**	**0**	**0**	**0**	**0**
Total 2004 (49, 51)	***43***	***0***	***76***	***1***	***22***	***13***	***3***	***1***	***17***
Jul 2005 (24, 20)	**65**	0	**2**	n.t.	n.t.	n.t.	0	0	0
Aug 2005	**190** (22, 24)	0	0	0	**223** (16, 30)	**1**	0	0	0
Sep 2005	**282** (20, 6)	0	**3** (20, 6)	0	**119** (24, 18)	0	n.t.	n.t.	n.t.
Total 2005	***437** (66, 50)*	*0*	***5** (44, 26)*	*0*	***442** (40, 48)*	***1** (16, 30)*	*0*	*0*	*0*

Hv lure: Complete Hv blend (7 components; see Groot et al. 2007); Hs lure: Complete Hs blend (7 components; see Groot et al. 2007); Control lure loaded with hexane only; Hz = *Helicoverpa zea*.

In parentheses the total number of traps baited with the lure, followed by the total number of nights that these traps were monitored. n.t. not tested.

[1]Each lure was deployed in 28 traps, and the experiment lasted for 30 nights in total (from 30 Jul to 31 Aug). New lures were deployed every 2 weeks, midway through the experiment. The experiment was designed following a complete randomized block design to minimize position effects.

[2]Each lure was deployed in 21 traps, and the experiment lasted for 18 nights in total (from 16 Sep to 7 Oct). New lures deployed midway through the experiment. All experiments were designed following a complete randomized block design to minimize position effects.



Supplementary Figure 1. **Pheromone titers in the extracted pheromone glands of females on which Figure 1 and 2 in the manuscript are based.**

Within each species an ANOVA was conducted using proc GLM in SAS (9.1) where year, region and their interaction were treated as fixed effects. There was no interaction between year and region in both species. The means were separated using a Tukey adjustment for multiple comparisons. Within each species, different letters above the bars indicate significant differences in total pheromone amount.