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**Variation in sexual communication and its role in divergence of two host strains of the noctuid moth *Spodoptera frugiperda***

Unbehend, M.

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# Chapter 3

## Geographic variation in sexual attraction of *Spodoptera frugiperda* corn- and rice-strain males

Melanie Unbehend<sup>1</sup>, Sabine Hänniger<sup>1</sup>, Gissella M. Vásquez<sup>2</sup>,  
María Laura Juárez<sup>3</sup>, Dominic Reisig<sup>4</sup>, Jeremy N. McNeil<sup>5</sup>,  
Robert L. Meagher<sup>6</sup>, David A. Jenkins<sup>7</sup>, David G. Heckel<sup>1</sup> and  
Astrid T. Groot<sup>1,8</sup>

<sup>1</sup>Department of Entomology, Max Planck Institute for Chemical Ecology (MPICE), Hans-Knoell-Str. 8, 07745 Jena, Germany

<sup>2</sup>Department of Entomology, North Carolina State University, Raleigh, 27695 North Carolina

<sup>3</sup>Sección Zoología Agrícola, Estación Experimental Agroindustrial Obispo Colombres, Las Talitas (T4104AUD), Tucumán, Argentina

<sup>4</sup>North Carolina State University, Vernon James Research & Extension Center, 207 Research Station Road, Plymouth, 27962 North Carolina

<sup>5</sup>University of Western Ontario, Department of Biology, Biological & Geological Sciences Building, London, Ontario, N6A 5B7 Canada

<sup>6</sup>USDA-ARS, CMAVE, 1700 SW 23rd Dr., Gainesville, 32608 Florida

<sup>7</sup>Tropical Agriculture Research Station, 2200 Pedro A. Campos Ave., Suite 201, Mayaguez, 00680 Puerto Rico

<sup>8</sup>Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, the Netherlands

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**Abstract.** The corn- and rice-strains of *Spodoptera frugiperda* exhibit several genetic and behavioral differences and appear to be undergoing ecological speciation in sympatry. Previous studies reported conflicting results when investigating male attraction to pheromone lures in different regions, but this could have been due to inter-strain and/or geographic differences. Therefore, we investigated whether corn- and rice-strain males differed in their response to two synthetic pheromone blends in different regions in North America, the Caribbean and South America. These two blends mirrored differences we had previously documented between females of both strains and trapped males were classified by a strain-specific mitochondrial DNA marker. We found that corn-strain males preferred Blend 1, i.e. 100% (Z)-9-tetradecenyl acetate (Z9-14:OAc), 13% (Z)-11-hexadecenyl acetate (Z11-16:OAc), 2% (Z)-7-dodecenyl acetate (Z7-12:OAc), 1% (Z)-9-dodecenyl acetate (Z9-12:OAc), over Blend 2 (100% Z9-14:OAc, 8% Z11-16:OAc, 4% Z7-12:OAc, 2% Z9-12:OAc) in South America (Peru, Argentina) and half of the Puerto Rico and Florida field sites, but were equally attracted to both blends in Canada, North Carolina, and the other half of the Puerto Rico and Florida field sites. Furthermore, corn-strain males showed geographic variation in response to different doses of Z7-12:OAc. In contrast, rice-strain males showed almost no geographic variation in response to different pheromone blends. Addition of the minor compound (*E*)-7-dodecenyl acetate to Z9-14:OAc was attractive to males in North Carolina, but not in Peru. Overall, our results suggest that corn-strain males are more restricted in their response to pheromone blends than rice-strain males and this specificity shows some geographic variation.

**Key Words.** Sexual communication, Fall armyworm, Lepidoptera, Noctuidae, Synthetic pheromone lures, Field experiments, Dose-response experiments.

## **Introduction**

Geographic variation in the sexual communication signals of animals is a widespread phenomenon, being reported in frogs (Ryan et al., 1996; Bernal et al., 2005; Pröhl et al., 2006; Jang et al., 2011), birds (Mundinger, 1982; Slabbekoorn and Smith, 2002; Podos and Warren, 2007), fish (Gonzalez-Zuarth et al., 2011) and insects (Ackerman, 1989; Miller et al., 1997; Zhu et al., 2009). This variation can be the result of isolation by distance, with a positive correlation between genetic dissimilarity and geographic distance (Balaban, 1988; MacDougall-Shackleton and MacDougall-Shackleton, 2001; Lampert et al., 2003; Bernal et al., 2005), but this is not always the case (Tilley et al., 1990; Seppä and Laurila, 1999; Leblois et al., 2000; Kaefer et al., 2012). Furthermore, mating signals can be influenced by environmental factors such as temperature (Delisle and Royer, 1994; Roeser-Mueller et al., 2010; Olvido et al., 2010; Green et al., 2012), humidity (Kumar and Saxena, 1986, Royer and McNeil, 1991; 1993), photoperiod length (Delisle and McNeil, 1987; Gemeno and Haynes, 2001), host plant volatiles (Landolt and Phillips, 1997; Reddy and Guerrero, 2004) or interspecific olfactory cues (Groot et al., 2010a) that vary geographically.

Geographic variation in sexual communication systems has been reported in several lepidopteran species (Tòth et al., 1992; McElfresh and Millar, 1999; Wu et al., 1999; Gemeno et al., 2000; Kawazu et al., 2005; Groot et al., 2009) and is of interest because changes in the sex pheromone signal and/or response to sex pheromones could result in reproductive isolation and subsequently may lead to speciation (Roelofs and Cardé, 1974; Phelan, 1992; Baker, 2002; Smadja and Butlin, 2009). Understanding the level and extent of intra-specific geographic variation in pheromone-mediated mating will help to identify the factors responsible for the geographic differences and provide insight in the evolution of reproductive isolation between populations.

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is an excellent model system to study the evolution of reproductive isolation, as there are two genetically and behaviorally distinct strains, the corn- and rice-strain, occurring sympatrically throughout North- and South America (Pashley, 1986). Both strains appear to be undergoing ecological speciation in sympatry and reveal several possible prezygotic isolation barriers (Groot et al., 2010b). These include differential host plant choice (Pashley, 1986; Pashley, 1989; Lu and Adang, 1996; Levy et al., 2002; Nagoshi et al., 2006; Machado et al., 2008), strain-specific mating times in the scotophase (Pashley et al., 1992; Schöfl et al., 2009), as well as differences in the female sex pheromone composition (Groot et al., 2008; Lima and McNeil, 2009; Unbehend et al., 2013). The sex pheromone of *S. frugiperda* was identified by Tumlinson et al. (1986) to consist of (*Z*)-9-tetradecenyl acetate (Z9-14:OAc) as the major sex pheromone component, and (*Z*)-7-dodecenyl acetate (Z7-12:OAc) as critical secondary sex pheromone component. A number of other minor compounds like Z11-16:OAc and Z9-12:OAc have also been identified from the gland (Tumlinson et al., 1986; Groot et al., 2008; Lima and McNeil, 2009), but with unclear behavioral function so far (Unbehend et al., 2013). Analysis of sex pheromone gland extracts from females collected in Florida showed that corn-strain females contained significantly lower relative amounts of Z7-12:OAc and (*Z*)-9-dodecenyl acetate (Z9-12:OAc) than rice-strain females (Groot et al., 2008; Unbehend et al., 2013). However, different male trapping experiments conducted in Louisiana and Florida showed no consistent attraction of males to females of their own strain (Pashley et al., 1992; Meagher and Nagoshi, 2013; Unbehend et al., 2013), which suggests that differences in the female pheromone are not sufficient to cause assortative mating in the field.

Nevertheless, there is evidence that there are geographic differences in the female sex pheromone blend (Tumlinson et al., 1986; Batista-Pereira et al., 2006; Groot et

al., 2008; Lima and McNeil, 2009), as well as in male response (Jones and Sparks, 1979; Tumlinson et al., 1986; Mitchell et al., 1985; Andrade et al., 2000; Fleischer et al., 2005; Batista-Pereira et al., 2006; Unbehend et al., 2013). For example, while females from Brazil (Batista-Pereira et al., 2006) produce (*E*)-7-dodecenyl acetate (E7-12:OAc), those from Florida, Louisiana or French Guyana do not (Descoins et al., 1988; Groot et al., 2008; Lima and McNeil, 2009). In addition, studies on females originating from Florida and Louisiana provide evidence of geographic variation in the production of sex pheromone by females of both strains (Groot et al., 2008; Lima and McNeil, 2009; Unbehend et al., 2013). Numerous studies have shown that male trap catch varies with the pheromone blend used, for example, while the minor compound (*Z*)-11-hexadecenyl acetate (Z11-16:OAc) did not affect male attraction in Florida and Brazil (Tumlinson et al., 1986; Batista-Pereira et al., 2006; Unbehend et al., 2013), it may be necessary in Costa Rica and Pennsylvania (Andrade et al., 2000; Fleischer et al., 2005). However, most of these studies did not determine the strain identity of the males captured. Consequently, the variation in male attraction observed in these studies could either be due to strain-specific and/or due to geographic differences.

To disentangle strain-specific variation from geographic variation in male response, we investigated the response of corn- and rice-strain males to different synthetic pheromone blends in six different countries in North America, the Caribbean and South America. To determine whether males exhibit strain-specific geographic differences in their attraction, we tested (A) two synthetic 4-component blends (Blend 1 and 2) in different fields in Canada, North Carolina, Florida, Puerto Rico, Peru and Argentina; (B) different doses of Z7-12:OAc in Florida, Puerto Rico and Peru; (C) different doses of Z11-16:OAc in Florida and Peru; and (D) different doses of E7-12:OAc and Z7-12:OAc in Peru and North Carolina.

## **Methods and Materials**

### *Male Trapping Experiments*

To test whether a certain synthetic pheromone blend is equally attractive for corn and rice-strain males in different geographic regions, four different trapping experiments were conducted in six different regions in North America, the Caribbean and South America (Table 1). For experiment (A), we prepared two synthetic 4-component blends (Blend 1 and 2) based on strain-specific pheromone differences found in laboratory females from Florida by Groot et al. (2008). Blend 1 and 2 consisted of 100% Z9-14:OAc with different percentages of Z11-16:OAc, Z7-12:OAc and Z9-12:OAc (see Table 2), as described by Unbehend et al. (2013). Both blends were tested in Canada, the United States (North Carolina and Florida), Puerto Rico, Peru and Argentina (Table 1).

To evaluate the relative importance of Z7-12:OAc for male attraction in Florida, Puerto Rico and Peru (experiment B), different percentages of Z7-12:OAc (0%, 2%, 4%, 10%) were added to the major pheromone component Z9-14:OAc alone, without Z11-16:OAc or Z9-12:OAc (Table 2). The used percentages were chosen to examine whether Z7-12:OAc is necessary for male attraction in all regions and fields (0%, lures baited only with Z9-14:OAc), to test whether males can distinguish between 2% and 4% Z7-12:OAc, and to investigate a possible repellent effect of high dosages of Z7-12:OAc (10%).

To assess whether Z11-16:OAc would affect male attraction, we conducted experiment (C), in which different amounts of Z11-16:OAc (0%, 8%, 13%, 18%) were added to a “minimal blend”, consisting of 100% Z9-14:OAc and 2% Z7-12:OAc (Table 2). The minimal blend (0% Z11-16:OAc) was used as control, 8% and 13% Z11-16:OAc reflect the percentages found in rice- and corn-strain females from Florida, respectively (Groot et al., 2008). To test possible repellent effects,

18% was used as the highest concentration. Experiment C was conducted in Florida and Peru.

**Table 1** *Spodoptera frugiperda* trapping experiments conducted in North America, the Caribbean and South America

COUNTRY	LOCATION & COORDINATES	FIELD	EXPERIMENT <sup>1</sup>	EXPERIMENTER	DATE	
<b>Canada</b>	Ontario	+43° 4' 26.08", -81° 20' 21.81"	Corn	A	JMN	Sep. 2011
<b>North Carolina</b>	Plymouth	+35°50' 46.19", -76° 39' 46.24" +35°51' 01.93", -76° 39' 11.28" +35°51' 48.80", -76° 39' 33.61"	Soybean Cotton; Grass Soybean; Corn	A, D	DR	Sep. 2011
<b>Florida<sup>2</sup></b>	Belle Glade	+26° 40' 7.20", -80° 37' 57.63"	Corn A	A, B	MU, SH	April- May 2010
	Hague	+29° 47' 7.40", -82° 25' 3.66"	Corn B	C	RLM	Sept. 2011
	Moore Haven	+26° 53' 3.04", -81° 7' 21.17"	Grass	A, B, C	MU, SH	April- May 2010
<b>Puerto Rico</b>	Santa Isabel	+17° 59' 0.93", -66° 23' 29.88"	Corn A	A	MU,SH, ATG,DAJ	April 2010
		+17°57' 30.65", -66° 23' 32.43"	Corn B	A, B		
<b>Peru</b>	Lima	-12° 4' 51.56", -76° 57' 9.14"	Corn	A, B, C, D	GV	May- July 2011
<b>Argentina</b>	El Molino	-27° 20' 11.1", -65° 41' 25.8"	Corn	A	MLJ	Dec. 2010- Jan. 2011

<sup>1</sup> Experiments: A) Test of strain-specific blends, B) Z7-12:OAc dose-response, C) Z11-16:OAc dose-response, D) Importance of E7-12:OAc

<sup>2</sup> Data adapted from Unbehend et al. 2013.



To test the importance of the isomers Z7-12:OAc and E7-12:OAc in North and South America (experiment D), we added different doses of E7-12:OAc and Z7-12:OAc (0%, 1%, 2%) to 100% Z9-14:OAc (Table 2). The minimal blend (2% Z7-12:OAc + 100% Z9-14:OAc) was used as control, and as equivalent we prepared an E-blend with 2% E7-12:OAc and 100% Z9-14:OAc. To investigate a possible interaction effect of both isomers together, 1% as well as 2% of E- and Z7-12:OAc were added to 100% Z9-14:OAc. The fourth experiment (Exp. D) was carried out in North Carolina and Peru.

We were not able to conduct all four experiments in all countries, due to technical limitations (i.e. limited time availability of collaborators, limited access to infested field sites and variability of moth population densities). All data from trapping experiments in Florida were published previously (Unbehend et al., 2013) and were included in this study for comparison. In all experiments, the synthetic pheromone lures were placed in plastic green-yellow-white Unitraps (Pherobank, Wageningen, the Netherlands), which contained a Vaportape II insecticide strip (Hercon Environmental, Emigsville, PA, USA) to kill the males captured. At each site, traps were hung just above the crop canopy (1-2 m above the ground depending on crop phenology), spaced 15m apart and at least 15 m from the edge of the field using a complete randomized block design. There were three replicates per treatment per field (n=3), except for experiments conducted in North Carolina, where each replicate was conducted in a different field (Table 1). Traps were rotated and emptied three or four times, depending on the number of treatments (Exp. A: n=3; Exp. B-D: n=4), and traps were rotated every 1-6 days, depending on the population density in the field. The males captured were stored at -20°C until strain-identification in the laboratory (see below).

*Preparation of Pheromone Lures*

All pheromone compounds used to prepare lures were bought from Pherobank (Wageningen, the Netherlands), and had a purity of  $\geq 99\%$ . Red rubber septa (Thomas Scientific, Swedesboro, NJ, USA) were soaked in hexane for 24 hours and air dried before they were loaded with 100  $\mu\text{l}$  hexane containing 300  $\mu\text{g}$  of the major pheromone component Z9-14:OAc plus different amounts, relative to 300  $\mu\text{g}$  Z9-14:OAc, of the minor compounds Z11-16:OAc, Z7-12:OAc, Z9-12:OAc, and E7-12:OAc (Table 2). All prepared lures were stored in glass vials at  $-20^\circ\text{C}$  until used 1-3 months later in the field. Each lure was only used once within one experiment (for  $\sim 1-3$  weeks), and we did not observe a decrease in lure-effectiveness at the end of an experiment. The release rates of Z9-14:OAc, Z7-12:OAc, E7-12:OAc, and Z9-12:OAc were probably similar to the amounts loaded on the septum (Heath et al., 1986), although Z11-16:OAc might have been released in lower amounts than the loaded percentages (Tumlinson et al., 1990).

**Table 2** Composition of pheromone lures to test the attraction of *Spodoptera frugiperda* males in the field

Experiment and Lures <sup>1</sup>		Z9-14:OAc	Z11-16:OAc	Z7-12:OAc	E7-12:OAc	Z9-12:OAc
<b>A</b>	Blend 1	100%	13%	2%	-	1%
	Blend 2	100%	8%	4%	-	2%
	Blank (Hexane)	-	-	-	-	-
<b>B</b>	0%		-	-	-	-
	2%	Z7-12:OAc	100%	-	2%	-
	4%		-	4%	-	
	10%		-	10%	-	
10%	-		-	-		
<b>C</b>	0%		-	-	-	-
	8%	Z11-16:OAc	100%	8%	-	-
	13%		13%	-	-	
	18%		18%	-	-	
18%	-		-	-		
<b>D</b>	2%	Z7-12:OAc		-	2%	-
	2%	E7-12:OAc	100%	-	-	2%
	1+1%	Z/E7-12:OAc	-	1%	1%	-
	2+2%	Z/E7-12:OAc	-	2%	2%	-

<sup>1</sup>Compound concentrations were as follows: 100% = 300  $\mu\text{g}$ , 18% = 54  $\mu\text{g}$ , 13% = 39  $\mu\text{g}$ , 10% = 30  $\mu\text{g}$ , 8% = 24  $\mu\text{g}$ , 4% = 12  $\mu\text{g}$ , 2% = 6  $\mu\text{g}$ , 1% = 3  $\mu\text{g}$ .

### *Chemical Analysis*

The purity and composition of the prepared pheromone solutions were verified by gas chromatography (GC) analysis, using a HP7890 gas chromatograph with a 7683 automatic injector. A 2  $\mu$ l aliquot of each pheromone solution used for the preparation of the pheromone lures (see Table 2) was injected into a splitless inlet attached to a polar capillary column (DB-WAXetr; 30m  $\times$  0.25mm  $\times$  0.5 $\mu$ m) and a flame-ionization detector (FID). The GC program ran from 60°C, with a 2 min hold, to 180°C at 30°C/min, 230°C at 5°C/min and to 245°C at 20°C/min, followed by a 15 min hold at 245°C to clean the column for the next sample. The FID detector was held at 250°C.

### *Strain Identification*

The strain identity of all trapped males was determined via two strain-specific markers, i.e. MSPI- and SACI-digest of the mitochondrial COI gene, which are known to be diagnostic for strain-identification of the two fall armyworm strains in North and South-America (Meagher and Gallo-Meagher, 2003; Nagoshi et al., 2006; Nagoshi et al., 2007). DNA of all males captured was extracted as described by Unbehend et al. (2013) using CTAB (Cetyltrimethyl-ammonium bromide) and isopropanol for DNA precipitation. The extracted DNA was tested at MPICE for strain-specific polymorphisms at the mitochondrial COI gene by amplification and strain-specific digestion (Nagoshi et al., 2006; Unbehend et al., 2013). The amplified part of the COI gene was digested with MSPI as well as SACI and analyzed electrophoretically on a 1% agarose gel (Unbehend et al., 2013). MSPI digestion detected corn-strain individuals, whereas SACI digestion proved rice-strain identity (Nagoshi et al., 2006).

### *Statistical Analysis*

Data of each field site of one experiment (Exp. A – D) were singly analyzed with a generalized linear model (*GLM*) with a Poisson distribution, or a quasi-Poisson distribution if the residual deviance of the data was larger than the residual degrees of freedom (over-dispersion), using the R software 2.11.1 (R Development Core Team, 2007). To assess whether there was any effect of geographic location, field crop, and/or any strain-specific effect that influenced male attraction, data of experiment A and B were additionally analyzed with a multivariate analysis of variance (*MANOVA*). Treatments that did not catch any moths within any rotation in any of the three biological replicates per field were excluded from the statistical analysis. Whenever a certain blend attracted one or more males, zero values were included in the analysis. Graphical illustrations were made with Microsoft Office Excel 2007. In all graphs, we averaged the number of males of all rotations of one treatment, calculated one percentage value for each of the 3 biological replicates, and plotted the mean percentage of males per trap (i.e. sum of all biological replicates divided by 3). In the statistical analysis, only raw data (no means) were used.

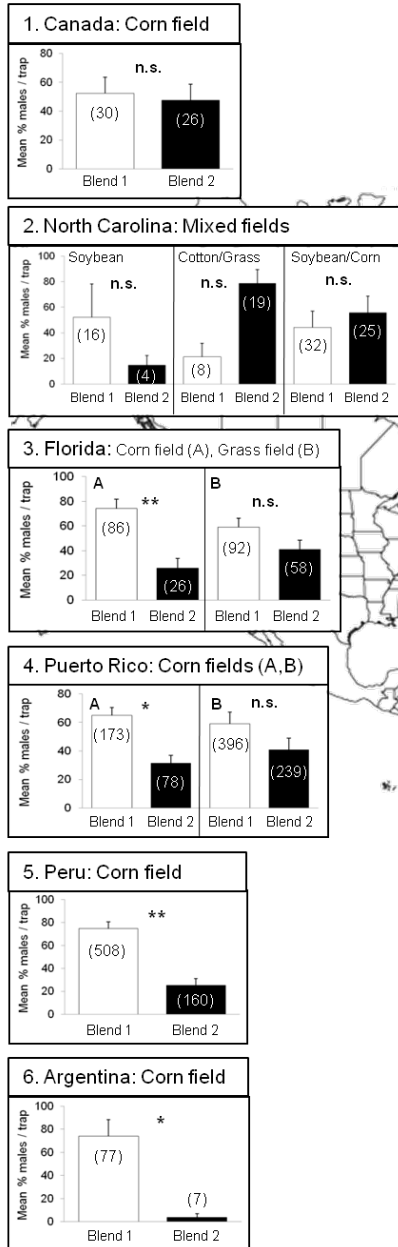
### **Results**

Overall, the field tests showed that *S. frugiperda* males of both strains exhibited some geographic variation in their attraction to two different synthetic 4-component-blends (Blend 1 and Blend 2) in North America, the Caribbean and South America (Fig.1). We found a significant effect of the geographic region, the field crop as well as an interaction effect between geographic region x strain (*MANOVA*:  $P < 0.001$ ; Fig. 1). Corn-strain males showed a significantly higher attraction to Blend 1 (100% Z9-14:OAc, 13% Z11-16:OAc, 2% Z7-12:OAc, 1% Z9-12:OAc) than to Blend 2 (100% Z9-14:OAc, 8% Z11-16:OAc, 4% Z7-12:OAc,

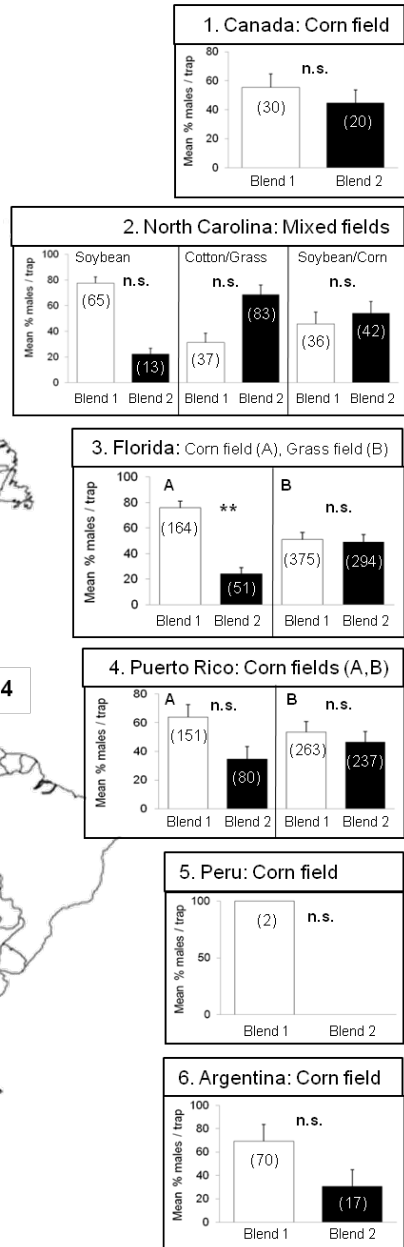
2% Z9-12:OAc) in corn fields in Florida, Puerto Rico (field A), Peru and Argentina, but did not show a preference for any of the two blends in corn fields in Canada and Puerto Rico (field B), the mixed habitats in North Carolina or a grass field in Florida (Fig.1). Rice-strain males were equally attracted to Blend 1 and Blend 2 in all cases, with only one exception in a corn field in Florida, where Blend 1 was more attractive than Blend 2 (Fig. 1). Both strains only differentiated between the two blends when they were tested in a corn field, where males of both strains were more attracted to Blend 1 than to Blend 2 (Fig. 1). Control traps baited with hexane were usually empty in all fields (data not shown), but caught males in Argentina (n=2) and in Puerto Rico (n=19 in field A, n=2 in field B). Interestingly, 18 out of the 19 males found in control traps in corn field A in Puerto Rico were caught during the first trap rotation at a time where male density was extremely high (over 50% of all males caught in this experiment were caught at the date of the first rotation).

The Z7-12:OAc dose-response experiments, where 0%, 2%, 4% or 10% of Z7-12:OAc was added to 100% Z9-14:OAc, showed a significant effect of geographic region and strain, as well as an interaction effect between geographic region x strain, and field crop x strain (*MANOVA*:  $P < 0.001$ ; Fig. 2). Interestingly, corn-strain males exhibited a greater difference in their response to Z7-12:OAc than rice-strain males (Fig. 2). The highest number of corn-strain males was captured with lures containing 2% Z7-12:OAc and was significantly different from all other ratios at three field sites in Florida and Puerto Rico, while in Peru lures with 2% and 4% Z7-12:OAc were equally attractive. Furthermore, lures containing no Z7-12:OAc, considered an essential pheromone component, attracted 37 corn-strain males in Puerto Rico (Fig. 2A).

**Corn-strain males caught**



**Rice-strain males caught**



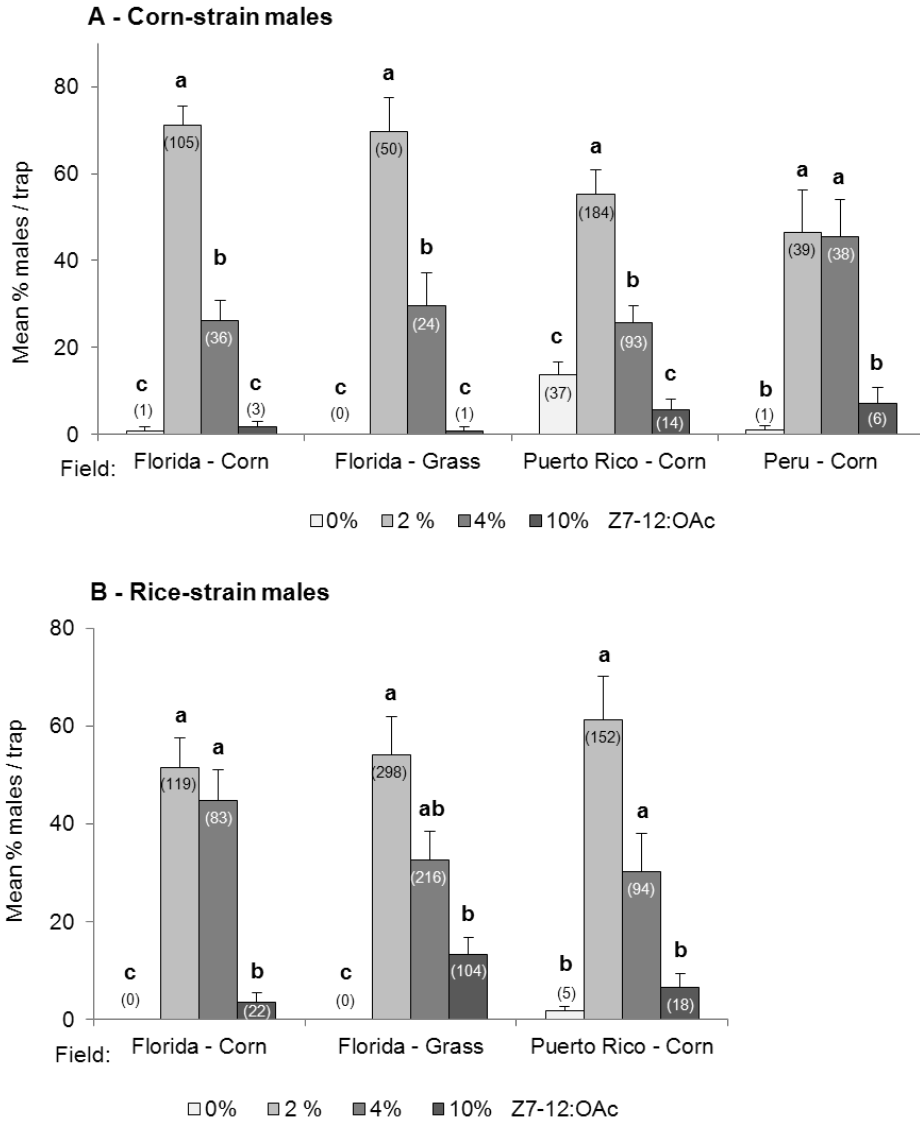
**Figure 1.** Attraction of *S. frugiperda* corn- and rice-strain males to two 4-component blends (Blend 1 and 2 in Table 2) in North America, the Caribbean and

South America. Bars show the mean percentage of males caught per trap and per biological replicate. There were three biological replicates per field (n=3), except for all fields in North Carolina (n=1) and for rice-strain males in Peru (n=1), where only one replicate caught males. The Standard errors in all fields in North Carolina show the variation between rotations (n=3), while all other error bars show the variation between biological replicates (n=3). Numbers in brackets represent the total number of males caught, \*=  $P < 0.05$ , \*\*=  $P < 0.01$ , n.s.=not significant. Data from Florida are adapted from Unbehend et al. (2013)

In contrast to corn-strain males, rice-strain males showed a similar response to different concentrations of Z7-12:OAc in all regions tested, being equally attracted to blends containing 2% or 4% Z7-12:OAc as well as showing some level of response to lures with 10% Z7-12:OAc (Fig. 2B). No data could be gathered in Peru, as only corn-strain males were found in this field (Fig. 2A).

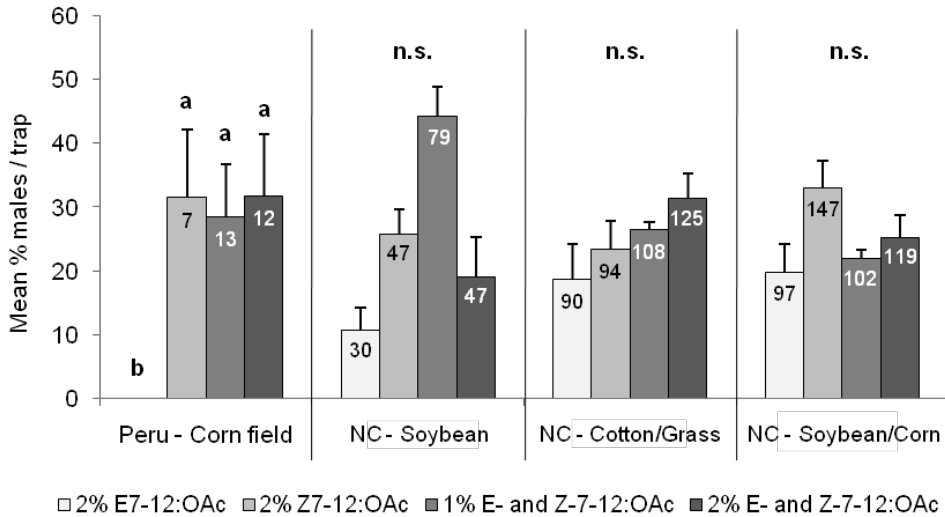
Testing different doses of Z11-16:OAc (0%, 8%, 13%, 18%), added to the minimal blend (i.e. 100% Z9-14:OAc and 2% Z7-12:OAc), revealed that corn-strain males from Peru were equally attracted to binary blends with and without Z11-16:OAc. More precisely, corn-strain males were similarly attracted to binary blends (n=44), as to three-component blends containing 8% Z11-16:OAc (n =26), 13% Z11-16:OAc (n= 29), or 18% Z11-16:OAc (n = 22). This result was similar to previous observations of corn- and rice-strain males in Florida (Unbehend et al. 2013). As no rice-strain males were caught in the corn field in Peru, we could not investigate the response of rice-strain males to different doses of Z11-16:OAc.

Testing different doses (0%, 1%, 2%) of E7-12:OAc and Z7-12:OAc, added to 100% Z9-14:OAc, showed that *S. frugiperda* males from Peru were not attracted to traps baited only with 2% E7-12:OAc added to Z9-14:OAc, but were equally attracted to all other blends tested (Fig. 3).



**Figure 2.** Attraction of *S. frugiperda* corn-strain (A) and rice-strain (B) males to different doses of Z7-12:OAc added to 100% Z9-14:OAc in different fields. Different letters above the bars indicate significant differences. Error bars show the variation between biological replicates (n=3). Numbers in brackets represent the total number of males caught. Data from Florida are adapted from Unbehend et al. (2013)





**Figure 3.** Attraction of *S. frugiperda* males to different doses of E7-12:OAc and Z7-12:OAc added to 100% Z9-14:OAc in different fields. The standard errors in all fields in North Carolina (NC) show the variation between rotations (n=3), while error bars in Peru show the variation between biological replicates (n=3). Numbers in the bars represent the total number of males caught. Different letters above the bars indicate significant differences. n.s.= not significant

In North Carolina, *S. frugiperda* males were equally attracted to synthetic blends to which E7-12:OAc, Z7-12:OAc or E- and Z-7-12:OAc was added (Fig. 3). Unfortunately, we were not able to identify the strain-type of any of the trapped males because of DNA degradation of the samples.

## Discussion

We investigated the variation in attraction of *S. frugiperda* corn- and rice-strain males in North America, the Caribbean and South America, and found a) some geographic variation in corn-strain male attraction to synthetic 4-component blends and to different doses of Z7-12:OAc, b) almost no geographic variation in rice-

strain male attraction to different synthetic blends, c) no variation in male attraction to the minor compound Z11-16:OAc, and d) some evidence of geographic variation in response to E7-12:OAc. Taken together, our results indicate that corn-strain males are more specific and restricted in their response to pheromone blends than rice-strain males and this specificity shows some geographic variation.

### *Geographic Variation in Corn-Strain Male Responses*

Testing two different 4-component blends revealed that corn-strain males were equally attracted to both blends in Canada and North Carolina, but preferred Blend 1 over Blend 2 in South America, i.e. in Argentina and Peru. Interestingly, Blend 1 mimics the pheromone composition of corn-strain females in Florida that we previously found (Groot et al. 2008). However, corn-strain males in Florida and Puerto Rico showed a preference for Blend 1 in one of the two fields tested at each site, but were equally attracted to Blend 1 and 2 in the other (Fig. 1).

Differential male attraction between fields could be caused by habitat-specific volatile differences. For example, the two corn fields in Puerto Rico, which were only 4 km apart, were planted with different corn varieties that were in different phenological states during the trapping period and were differentially treated with insecticides. This could result in different background odor profiles, which in turn may have influenced the attraction of corn-strain males to the two different 4-component blends used in the first experiment. Due to the fact that both strains show host plant preferences (Pashley, 1986; Nagoshi et al., 2006; Machado et al., 2008), it seems likely that males exhibit differential responses to female sex pheromones in different habitats, emitting host or non-host volatiles.

Previously, it was shown that corn-strain males varied in their attraction to sex pheromone blends in different fields with different host plants in north and south

Florida (Meagher and Nagoshi, 2013; Unbehend et al., 2013). On the one hand, host plant volatiles may facilitate male responsiveness, as electroantennogram studies showed that *S. frugiperda* moths can detect at least 16 plant volatiles, and male EAG responses are higher than those of females (Malo et al., 2004). However, addition of plant volatiles to the female sex pheromone did not enhance trap catches of *S. frugiperda* males in different field studies (Meagher, 2001; Malo et al., 2002). On the other hand, the differences may be due to antagonistic effects of plant volatiles on the response of *S. frugiperda* males towards pheromone blends. Party et al. (2009) showed that plant terpenes are able to antagonize the pheromone-evoked signal in *Spodoptera littoralis* (Boisduval), a closely related species. Clearly, additional research is needed to determine to what extent host- or non-host plant volatiles either synergize or antagonize the response of corn- and rice-strain males to pheromone blends.

The differential attraction of corn-strain males to pheromone blends in different regions could also be explained by genetic differences between *S. frugiperda* populations from North America, the Caribbean and South America. Population genetic analyses of *S. frugiperda* samples collected throughout the Western Hemisphere reported mainly the absence of isolation by distance between populations from different regions (Clark et al., 2007; Martinelli et al., 2007; Belay et al., 2012), which indicates no geographically restricted gene flow probably due to the high migratory ability of *S. frugiperda* (Luginbill, 1928; Sparks, 1979). However, these analyses did not take into account strain-specific differences and in several cases the strain-type of captured individuals was unknown (Clark et al., 2007; Belay et al., 2012). Genetic studies on populations from Arkansas and Florida showed significant genetic variation among populations, both within and between the two strains (Lewter et al., 2006). Furthermore, corn-strain individuals exhibit different mitochondrial haplotype profiles between populations from a)

Florida, Puerto Rico, Georgia and b) Texas, Brazil, Mississippi, Alabama, Louisiana (Nagoshi et al., 2007; Nagoshi et al., 2008; Nagoshi and Meagher, 2008; Nagoshi et al., 2010). Thus, genetic differences could play a role in differential attraction of corn-strain males to synthetic blends in different regions.

Besides different host plant volatiles or genetic differences, the responses of corn-strain males could have been influenced by geographically varying environmental factors like temperature or humidity. In a previous wind tunnel study, we observed that males of both strains were highly sensitive to changes in temperature or humidity and stopped their response to calling females whenever humidity or temperature was low (Unbehend et al., 2013). Thus, it is possible that males may respond differently to pheromone blends in regions with dry and cold climate compared to (sub)tropical climate zones.

The male response could also be influenced by intra-specific competition, if males would respond differently to a sex pheromone in the presence of many competing males. *Spodoptera frugiperda* is a highly migratory species that can have continuous generations in tropical and subtropical areas, and can migrate into temperate regions during summer (Luginbill, 1928; Sparks, 1979). When we compared the total number of males caught per region and field in the first experiment, we found that the lowest number of males was caught in the most northern (Canada) and most southern region (Argentina). These low population densities in the temperate climate regions suggest that besides temperature and humidity differences, population structures in a field are different between different climate zones. Possibly, the response of males is density-dependent: if there are many competing males, male choice may become less specific or occur in a shorter time frame, in order to mate as soon as possible before a competing male arrives.

In addition to variation in response to two synthetic 4-component blends, corn-strain males also exhibited significant geographic differences in their attraction to different doses of Z7-12:OAc. In Florida and Puerto Rico, corn-strain males were more attracted to the 2% dose than to other doses tested, but were equally attracted to 2% and 4% Z7-12:OAc in Peru (Fig. 2). Furthermore, some corn-strain males from Puerto Rico were attracted to Z9-14:OAc alone, even though Z7-12:OAc has been considered an essential secondary component, without which males are not attracted (Tumlinson et al., 1986; Unbehend et al., 2013). The fact that the response of corn-strain males to Z7-12:OAc significantly varied between regions suggests that females may also vary in their relative amount of Z7-12:OAc across different regions. Although previous data indicated that the production of Z7-12:OAc is under strong stabilizing selection (Unbehend et al., 2013), in light of the data presented here, selection pressures may be different in different regions.

#### *Geographic Variation in Rice-Strain Male Responses*

In general, rice-strain males were equally attracted to Blend 1 and Blend 2 in different fields in North America, the Caribbean and South America (Fig. 1). In addition, rice-strain males from Florida and Puerto Rico were similarly attracted to 2% and 4% Z7-12:OAc and were also found in traps baited with 10% of this component (Fig. 2). Thus, rice-strain males showed a broader response spectrum to different sex pheromone blends than corn-strain males. If the pheromone response spectrum of males is determined by environmental factors like temperature and humidity, and/or by intra-specific competition, it could be possible that rice-strain males are more resistant to climate changes and/or competition, which would explain why we observed almost no geographic differences in the response of rice-strain males.

However, we did find some variation in the response of rice-strain males, and another study showed that the attraction of rice-strain males to corn- and rice-strain females varied depending on field location and season in Florida (Meagher and Nagoshi, 2013). If geographic variation in the female pheromone composition exists, the composition of our synthetic pheromone blends might not be typical for all regions we tested. Rice-strain males may have shown differential attraction in the field if we would have used region-specific female pheromone blends as baits.

#### *Male Attraction to the Minor Compound Z11-16:OAc*

Testing the importance of Z11-16:OAc for male attraction showed that corn-strain males from Peru were equally attracted to blends with and without different doses of Z11-16:OAc, similar to the response of corn- and rice-strain males in Florida (Unbehend et al., 2013). These data suggest that Z11-16:OAc is not an essential component for *S. frugiperda* male attraction, which is supported by the observation that *S. frugiperda* males from Mexico did not respond electrophysiologically to Z11-16:OAc (Malo et al., 2004). Although the release ratio of Z11-16:OAc might have been lower than the loaded percentages (Exp. A , C), this should not have any influence on the male response because all data suggest that Z11-16:OAc does not seem to be necessary for male attraction, and does not decrease male attraction (Tumlinson et al., 1986; Andrade et al., 2000; Fleischer et al., 2005; Unbehend et al., 2013).

#### *Geographic Variation in Male Attraction to E7-12:OAc*

The E-isomer of the critical secondary sex pheromone component Z7-12:OAc has been found in *S. frugiperda* females from Brazil, and males from this region responded electrophysiologically to E7-12:OAc and exhibited a higher attraction to binary blends (Z9-14:OAc and Z7-12:OAc) when E7-12:OAc was added (Batista-

Pereira et al., 2006). On the other hand, E7-12:OAc was not detected in pheromone gland extracts of females from Florida, Louisiana or French Guyana (Tumlinson et al., 1986; Descoins et al., 1988; Groot et al., 2008; Lima and McNeil, 2009), suggesting geographic variation in the presence of this compound in the female pheromone blend. In our trapping experiments, we found that males from Peru were not attracted to traps baited only with E7-12:OAc and Z9-14:OAc, but were similarly attracted to all other blends that contained Z7-12:OAc (Fig. 3). Thus, males from Peru appear to distinguish between both isomers and need Z7-12:OAc, but not E7-12:OAc, for attraction. This result contrasts our findings in North Carolina, where such a differentiation did not occur. However, while the *S. frugiperda* males captured in Peru were corn-strain individuals, those caught in North Carolina could not be strain-typed, but probably belonged to both strains. Hence, we currently cannot exclude the possibility that corn- and rice-strain males show differential strain-specific attraction to E- and Z-7-12:OAc. Different isomers of a pheromone component are usually critical for attraction of males and can even lead to speciation, as shown in the two pheromone strains of *Ostrinia nubilalis* (Hübner) which differ in their production and response to (Z)- and (E)-11-tetradecenyl acetate (Lassance, 2010). Taken together, geographic variation in response to E7-12:OAc seems to exist, but additional experiments are required to evaluate the importance of E7-12:OAc for both strains in different regions.

### *Conclusions*

We found some geographic variation in attraction of *S. frugiperda* corn-strain males to two synthetic 4- component blends and different doses of Z7-12:OAc in North America, the Caribbean and South America. Rice-strain males showed almost no geographic variation in their attraction to different synthetic pheromone blends. One aspect that merits further attention is the possibility that habitat-specific volatiles influence the male response to pheromone blends in different

fields. Males were equally attracted to different doses of Z11-16:OAc, but appeared to exhibit region-specific differences in their attraction to E7-12:OAc. Overall, the data show geographic variation in the response of *S. frugiperda* males to pheromone blends. If this variation coincides with geographic variation in female pheromone composition, then geographic differentiation between populations could occur.

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