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Pheromonal Divergence Between Two Strains of *Spodoptera frugiperda*

Melanie Unbehend · Sabine Hänniger · Robert L. Meagher · David G. Heckel · Astrid T. Groot

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Abstract *Spodoptera frugiperda* consists of two genetically and behaviorally different strains, the corn- and the rice-strain, which seem to be in the process of sympatric speciation. We investigated the role of strain-specific sexual communication as a prezygotic mating barrier between both strains by analyzing strain-specific variation in female pheromone composition of laboratory and field strains, and also male attraction in wind tunnel and field experiments. Laboratory-reared and field-collected females from Florida exhibited strain-specific differences in their relative amount of (*Z*)-7-dodecenyl acetate (Z7-12:OAc) and (*Z*)-9-dodecenyl acetate (Z9-12:OAc). In wind tunnel assays, we did not find strain-specific attraction of males to females. However, in field experiments in Florida, we observed some differential attraction to synthetic pheromone blends. In a corn field, the corn-strain blend attracted more males of both strains than the rice-strain blend, but both blends were equally attractive in a grass field. Thus, habitat-specific volatiles seemed to influence male attraction to pheromones. In dose–response experiments, corn-strain males were more attracted to 2 % Z7-12:OAc than other doses tested, while rice-strain males were attracted to a broader range of Z7-12:OAc (2–10 %). The attraction of corn-strain males to the lowest dose of Z7-12:OAc corresponds to the production of this compound by females; corn-strain females produced significantly smaller amounts of Z7-12:OAc than rice-strain females. Although corn-strain individuals are more restricted in their production of and response to pheromones than rice-strain individuals, it seems that differences in sexual communication between corn- and rice-strain individuals are not strong enough to cause assortative mating.

Keywords Sexual communication · Male attraction · Fall armyworm · Corn- and rice-strain · Synthetic pheromone lures · Dose–response experiments · Lepidoptera · Noctuidae · Sympatric speciation

M. Unbehend (✉) · S. Hänniger · D. G. Heckel · A. T. Groot
Department of Entomology, MPICE, Hans-Knoell-Str. 8,
07745 Jena, Germany
e-mail: munbehend@ice.mpg.de

R. L. Meagher
USDA-ARS, CMAVE 1700 SW 23rd Dr.,
Gainesville, FL 32608, USA

A. T. Groot
Institute for Biodiversity and Ecosystem Dynamics,
University of Amsterdam, Science Park 904,
1098 XH Amsterdam, the Netherlands

Introduction

Many insect species produce sex pheromones that mediate sexual communication between males and females (Tamaki, 1985; Löfstedt and Kozlov, 1997). In Lepidoptera, females usually produce species-specific sex pheromones that exclusively attract conspecific males over long distances (Cardé and Baker, 1984; Tamaki, 1985; Cardé and Haynes, 2004). To find a suitable mating partner, males need to respond to the specific chemical signal that is emitted by a conspecific female (Löfstedt, 1993; Cardé and Haynes, 2004). Thus, changes in the pheromone signal of a female may result in reproductive isolation, which in turn can lead to speciation (Roelofs and Carde, 1974; Phelan, 1992; Baker, 2002; Smadja and Butlin, 2009). A model species to study the evolution of sexual communication is the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae), which consists of two strains, the Z- and the E-strain, that differ in their female-produced pheromone production and male response to pheromones (Klun and Cooperators, 1975; Smadja and Butlin, 2009; Lassance, 2010; Lassance et al., 2010; Wicker-Thomas, 2011). While Z-strain females produce 97:3 (*Z*)/(*E*)-11-tetradecenyl acetate (Z/E11-14:OAc)

(Klun et al., 1973), E-strain females emit 1:99 Z/E11-14:OAc (Kochansky et al., 1975). The production of different ratios Z/E11-14:OAc is based on a strain-specific allelic variation in a fatty-acyl reductase gene (pgFAR), which causes different substrate specificities of the enzyme and, thus, different female pheromones (Lassance et al., 2010). Males of both strains are specifically attracted to females of their own strain, although E-strain males have a broader response to pheromones than Z-strain males (Lassance, 2010). The two *O. nubilalis* strains seem to be sibling species, which mate assortatively, and exhibit low hybridization rates in the field due to strain-specific sexual communication (Lassance, 2010).

Similar to the European corn borer, the fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is an ideal model organism to study speciation because it also consists of two distinct strains, the corn- and the rice-strains (Pashley, 1986). Corn-strain individuals mainly occur in habitats that contain large grasses like corn and sorghum, while the rice-strain inhabits areas consist of small grasses like rice, bermuda grass, or turf grass (Pashley, 1986, 1989; Lu and Adang, 1996; Levy et al., 2002; Nagoshi et al., 2006, 2007; Machado et al., 2008). However, in most fields, both kinds of strains can be found in different proportions, and habitats containing only one strain are rare (Pashley, 1989; Meagher and Gallo-Meagher, 2003; Nagoshi et al., 2006, 2007). Although both strains are morphologically indistinguishable from each other, they exhibit several genetic differences in the mitochondrial cytochrome oxidase I (COI) and NADH dehydrogenase (ND1) genes (Pashley, 1989; Pashley and Ke, 1992; Lu and Adang, 1996; Levy et al., 2002; Meagher and Gallo-Meagher, 2003; Prowell et al., 2004; Nagoshi et al., 2006; Machado et al., 2008), esterase allozyme loci (Pashley, 1986), amplified fragment length polymorphisms (AFLP) loci (McMichael and Prowell, 1999; Busato et al., 2004; Prowell et al., 2004; Clark et al., 2007; Martinelli et al., 2007; Juárez et al., 2012), the copy number and organization of the fall armyworm rice-strain sequence (FR) (Lu et al., 1994; Nagoshi and Meagher, 2003), and in their triose phosphate isomerase (TPI) gene (Nagoshi, 2010). Furthermore, both strains differ in their timing of mating in the scotophase; corn-strain individuals call, mate, and oviposit approximately 3 hr earlier than rice-strain individuals (Pashley et al., 1992; Schöfl et al., 2009).

The pheromone composition of *S. frugiperda* females has been studied several times at different geographic regions (Tumlinson et al., 1986; Descoins et al., 1988; Batista-Pereira et al., 2006; Groot et al., 2008; Lima and McNeil, 2009). However, most studies have focused on the general composition of the female sex pheromone, irrespective of the female strain. The first pheromone component identified in *S. frugiperda* females was the major component, (*Z*)-9-tetradecenyl acetate (Z9-14:OAc) (Sekul and Sparks, 1967). Analyses of female pheromone glands and volatiles have shown that females from Florida emit ratios of 4.9:3.1:1.7:3.5:86.9 of dodecyl acetate (12:OAc), (*Z*)-7-dodecenyl acetate (Z7-12:OAc), 11-dodecenyl acetate (11–12:OAc), (*Z*)-11-hexadecenyl acetate (Z11-16:OAc), and Z9-14:OAc (Tumlinson et al., 1986). In addition to the major pheromone component, Z9-14:OAc, the critical secondary sex pheromone component, Z7-12:OAc, is important to attract *S. frugiperda* males in North and South America (Tumlinson et al., 1986; Andrade et al., 2000; Batista-Pereira et al., 2006). Because Z9-14:OAc and Z7-12:OAc are biologically active for male attraction they will be referred to as "pheromone components", according to the definition of Tamaki (1985). The importance of Z11-16:OAc, Z9-12:OAc and other minor compounds in attraction of males is not yet understood (Jones and Sparks, 1979; Tumlinson et al., 1986; Andrade et al., 2000; Fleischer et al., 2005; Batista-Pereira et al., 2006), so these will be referred to as "pheromone compounds".

Two independent studies have investigated strain-specific differences in the pheromone composition of females (Groot et al., 2008; Lima and McNeil, 2009). Each study found that females of both strains produce strain-specific relative amounts of different pheromone compounds (Groot et al., 2008; Lima and McNeil, 2009). However, the strain-specific pheromone variation differed between the two studies. We (Groot et al., 2008) found that corn-strain females from Florida exhibited significantly higher relative amounts of Z11-16:OAc, and lower relative amounts of Z7-12:OAc and (*Z*)-9-dodecenyl acetate (Z9-12:OAc) than rice-strain females. In contrast, Lima and McNeil (2009) found that corn-strain females from Louisiana produced significantly larger relative amounts of Z9-14:OAc as well as lower relative amounts of Z7-12:OAc and Z11-16:OAc compared to rice-strain females. The differing results of these studies suggest that geographic variation might influence the strain-specific pheromone composition of *S. frugiperda* females.

Considering all genetic, as well as behavioral (e.g., host plant choice, timing of reproduction, female pheromone) strain-specific differences, it seems that the two strains of *S. frugiperda* are in the process of sympatric speciation (Groot et al., 2010). Sympatric speciation requires the evolution of reproductive isolation mechanisms to reduce recombination between groups of individuals, as well as the coexistence of newly formed groups within the same area (Coyne and Orr, 2004). Strain-specific pheromone differences of corn- and rice-strain females could act as a reproductive isolation barrier if males show differential attraction to the different pheromone blends. The aim of our study was to examine the importance of sexual communication as a prezygotic mating barrier between the two strains of *S. frugiperda*. We determined a) whether lab- and field-collected corn- and rice-strain females differed in their

pheromone composition, and b) the biological relevance of strain-specific female sex pheromone differences on male mate choice in wind tunnel assays and pheromone attraction experiments in the field.

Methods and Materials

Spodoptera frugiperda Populations We conducted experiments with two different populations of each host-strain from Florida. The so-called laboratory populations, i.e., corn-strain (JSC3) and rice-strain (OnaR), originated from 100 to 200 larvae collected by RLM in Florida. JSC3 individuals were collected from corn plants near Homestead, Miami-Dade County in 2004, and OnaR larvae were collected from pasture grasses at the Range Cattle Research and Education Center, Ona, Hardee County in 2003. Populations were reared on artificial pinto bean diet for 2–3 yr in a mass culture at USDA, Gainesville, Florida, after which specimens of both strains were sent to the MPICE in 2006 to establish a colony. All individuals were screened for strain-specific cytochrome oxidase subunit I (COI) markers to confirm strain-identity, and reared for another 3 yr on artificial pinto bean diet. Since these two populations have been reared up to 6 yr under laboratory conditions, they will be referred to as the laboratory populations in all experiments.

The so-called field population of both strains descended from around 300 larval specimens collected in 2010 in Florida. Corn-strain larvae (FLC) were collected from a corn field at the Everglades Research and Education Center, Belle Glade, Palm Beach County (+26° 40′ 7.20″, −80° 37′ 57.63″), and rice-strain individuals (FLR) from a grass field at the Graham Farm in Moore Haven, Glades County (+26° 53′ 3.04″, −81° 7′ 21.17″). All larvae were shipped to the MPICE, and reared until adulthood on artificial pinto bean diet. Adults were screened for strain-specific COI markers to establish strain-specific colonies. Experiments with these populations were conducted after the colony was established (2nd laboratory generation); these populations will be referred to as field populations in all experiments. All insects were reared in climate chambers on a reversed light:dark (L:D) cycle, and a 14:10 L:D photoperiod at 26 °C and 70 % RH. Adults were fed with a 10 % honey-water solution, and random single-pair-matings were performed to avoid inbreeding and maintain both populations. Although we collected all insects from different locations in Florida, we do not assume genetic differences between populations because *S. frugiperda* is a highly migratory species (Sparks, 1979), that overwinters in Florida (Luginbill, 1928), suggesting high gene flow among populations. Furthermore, genetic analyses of corn-strain haplotypes in different habitats indicated a genetically homogenous corn-strain population in Florida (Nagoshi and Meagher, 2008).

Pheromone Extractions To determine strain-specific differences in the pheromone composition, and consistency of these differences between laboratory and field populations, pheromone extractions of the field population were compared to the pheromone extractions done previously and reported in Groot et al. (2008). Pheromone extractions of the field populations were performed in summer 2010 with newly collected corn-strain (FLC, 2nd generation) and rice-strain (FLR, 2nd generation) field populations from Florida. Pheromone glands of 2–4 d-old corn-strain and rice-strain virgin females were extracted during the scotophase according to strain-specific female calling times (corn-strain: 2–4 h, rice-strain: 5–7 h). Pheromone glands were excised from the female abdomen and placed singly into a glass vial containing 50 μl hexane and 125 ng pentadecane as internal standard. After extraction for 30 min, the gland was removed from the vial and the extract was stored at −20 °C until gas chromatographic analysis (see below).

Chemical Analysis Gas chromatography (GC) was performed using an HP7890 GC with a 7683 automatic injector, which injected 2–4 μl of each sample into a splitless inlet attached to a high resolution polar capillary column (DB-WAXetr (extended temperature range); 30 m× 0.25 mm×0.5 μm), using a flame-ionization detector (FID) at 250 °C. The GC was programmed from 60 °C with a 2 min hold to 180 °C at 30 °C/min, 230 °C at 5 °C/min, and finally, to 245 °C at 20 °C/min with a 15 min hold. Pheromone extracts of females were reduced from 50 μl to 2 μl under a gentle stream of nitrogen. The reduced 2 μl extract and 2 μl octane were transferred into a 50 μl vial within a crimp capped glass vial and injected into the GC. An internal standard containing four pheromone compounds of *S. frugiperda* (Z9-14:OAc, Z11-16:OAc, Z7-12:OAc, Z9-12:OAc) was injected into the GC each day before the first samples were analyzed to confirm retention times.

Preparation of Lures Synthetic pheromone lures were prepared to test attraction of *S. frugiperda* males. The four pheromone compounds identified from *S. frugiperda* females (Z9-14:OAc, Z11-16:OAc, Z7-12:OAc, Z9-12:OAc) were purchased from Pherobank (Wageningen, the Netherlands) to prepare lures (Table 1). Each pheromone lure consisted of a red rubber septum (Thomas Scientific, Swedesboro, NJ, USA) that was loaded with 100 μl of hexane containing 300 μg of the major component Z9-14:OAc (100 %) plus different amounts of minor compounds (0–18 %) relative to 300 μg Z9-14:OAc (Table 1). Before use, rubber septa were soaked in hexane overnight and air dried for 1 d. Pheromone solutions for the four different

Table 1 Field experiments to test the attraction of *Spodoptera frugiperda* males

Experiment	A) Strain-specific blends	B) Z7-12:OAc dose-response[a]	C) Z11-16:OAc dose-response[b]	D) Z9-12:OAc dose-response[b]
Pheromone blends	Corn-strain blend: 2 % Z7-12:OAc	0 % Z7-12:OAc	0 % Z11-16:OAc	0 % Z9-12:OAc
	13 % Z11-16:OAc 1 % Z9-12:OAc	2 % Z7-12:OAc	8 % Z11-16:OAc	1 % Z9-12:OAc
	Rice-strain blend: 4 % Z7-12:OAc	4 % Z7-12:OAc	13 % Z11-16:OAc	2 % Z9-12:OAc
	8 % Z11-16:OAc 2 % Z9-12:OAc	10 % Z7-12:OAc	18 % Z11-16:OAc	4 % Z9-12:OAc
	Hexane			
Field	Corn field, Belle Glade, FL		Corn field, Hague, FL	
	Grass field, Moore Haven, FL		Grass field, Moore Haven, FL	Peanut/grass field, Williston, FL

Other pheromone concentrations were as follows: 18 %=54 μg, 13 %=39 μg, 10 %=30 μg, 8 %=24 μg, 4 %=12 μg, 1 %=3 μg

[a] All septa contained 300 μg Z9-14:OAc, which was set to 100 %

[b] All septa contained 300 μg (100 %) Z9-14:OAc and 6 μg (2 %) Z7-12:OAc

experiments were prepared according to Table 1. To test the quality and quantity of the synthetic pheromone blends, 2 μl of each solution were analyzed by GC; the relative percentages of all lure compounds were confirmed by peak area integration, and lures were stored at −20 °C until use. Heath et al. (1986) showed that release rates of C12-C14 acetates (Z9-14:OAc, Z7-12:OAc, Z9-12:OAc) are similar to loading percentages of these compounds on rubber septa; however, the results of Tumlinson et al. (1990) suggest that the release rates of Z11-16:OAc from our lures might have been lower than the loaded percentages.

Wind Tunnel Experiments To assess strain-specific attraction of *S. frugiperda* males in the wind tunnel, experiments were performed in November 2009 in the laboratory of Prof. Manfred Ayasse at the Institute of Experimental Ecology, University of Ulm, Germany. Strain-specific attraction of *S. frugiperda* males was tested in a wind tunnel (200×75×75 cm) at 23 °C, 30 cm/s airflow, and 23 % RH. To adapt males to the low humidity, we placed all males, which were located in round plastic tubes covered with gauze, for about 1 h in the wind tunnel before the experiments started. Attraction of males was tested with choice experiments because in nature both kinds of strains can occur within one habitat and, thus, females might be located close to each other during calling. Choice experiments were conducted with the laboratory corn-strain (JS3C, 38th generation) and rice-strain (OnaR, 49th generation). Single 2-5-d-old, virgin males and females were placed in round plastic tubes (9.5 cm, 3.5 cm diam) that were closed with gauze at both ends. One plastic tube containing a male was mounted on a stand 30 cm high, and placed downwind in the middle of the wind tunnel. After the gauze was removed, each male was able to fly upwind, and given a choice between corn-strain and rice-strain females; three females of each strain were housed separately in round plastic tubes (9.5 cm, 3.5 cm diam) on stands above each other at 30 cm, 45 cm, and 60 cm height. We used 3 females to increase the chance that at least 1 of the females would call. The stands holding females of each strain were positioned upwind 26 cm apart. We examined the response of males on 5 consecutive nights; 18 males per night (9 corn-strain males and 9 rice-strain males) were tested repeatedly, so that every male was tested up to five times per night. After testing males one night, they were excluded from the experiment, and another subset of 18 males was tested in the following night. All males that were completely inactive for 4 min were excluded from a trial. Each active male was allowed 5 min to start upwind flight before exclusion from a trial. Active males that started upwind flight were observed until they contacted the source (i.e., one of the female tubes), and displayed courtship behavior. Various male behaviors (e.g., activity status, presence/absence zigzag flight, source contact, courtship) were recorded for 2–9 h within scotophase.

Field Experiments To assess strain-specific attraction of *S. frugiperda* males in the field, four different male trapping experiments were performed using: (A) strain-specific blends, (B) a range of Z7-12:OAc dosages, (C) a range of Z11-16:OAc dosages, and (D) a range of Z9-12:OAc dosages (Table 1). Plastic green-yellow-white Unitraps (Pherobank, Wageningen) were baited with synthetic pheromone lures, and, attached to a bamboo stick 1–2 m above the ground; traps were at least 15 m apart, as well as from field borders. All traps contained a Vaportape II insecticide strip (Hercon Environmental, Emigsville, PA, USA) to kill

males after they were trapped. These males were stored at -20 °C for strain-identification at a later stage (see below). All experiments were conducted using a complete randomized block design with 3 biological replicates per field. Traps were rotated and emptied every 2–3 d. Field experiments were conducted at the following sites in Florida: 1) a corn field at the Everglades Research and Education Center in Belle Glade (+26° 40′ 7.20″, −80° 37′ 57.63″) (experiments A and B); 2) a grass field at the Graham Farm in Moore Haven (+26° 53′ 3.04″, −81° 7′ 21.17″) (experiments A, B and C); 3) a corn field in Hague (+29° 47′ 7.40″, −82° 25′ 3.66″) (experiments C and D); and 4) in a peanut/pasture field in Williston (+29° 20′ 28.72″, −82° 34′ 18.88″) (experiment D).

DNA Extractions To determine the strain identity of all trapped males, one third of the thorax of each trapped male was homogenized in 500 µl TES buffer (100 mM tris(hydroxymethyl) aminomethane hydrochloride pH 8, 10 M ethylenediaminetetraacetic acid, 2 % sodium dodecyl sulfate), and 2.5 µl proteinase K and incubated at 55 °C overnight. Cetyltrimethylammonium bromide (80 µl, 10 % CTAB) and 170 µl 5 M sodium chloride were added to each sample followed by an incubation time of 10 min at 65 °C. After addition of 750 µl chloroform-isoamyl alcohol (24:1) and 30 min incubation on ice, the sample was centrifuged for 10 min at 10,000 rpm at 4 °C. Approximately 650 µl of the upper phase, together with 650 µl 100 % isopropanol, were transferred into a new tube, and incubated on ice for 1 h. The mixture was centrifuged for 45 min at 13,000 rpm at 4 °C, and the resulting DNA pellet was washed with 500 µl 70 % ethanol, and centrifuged for 10 min at 13,000 rpm at 4 °C. The extracted DNA was dissolved in 50 µl TES buffer, and stored at 4 °C until PCR amplification. All chemicals and buffers used for DNA extractions were purchased from Carl Roth GmbH & Co. (Karlsruhe, Germany).

Strain Identification To determine the strain-identity of each individual, strain-specific polymorphisms at the mitochondrial cytochrome oxidase I (COI) gene, as described by Nagoshi et al. (2006), were used. PCR amplifications were conducted using 1 µl DNA, 11.92 µl dH_2O, 2 µl 10x Taq buffer, 3 µl 10 mM primer mix, 2 µl 2 mM dNTPs, and 0.08 µl Taq polymerase (Metabion, Martinsried, Germany). CO1-58 (5′-GGAATTTGAGCAGGAATAGTAGG-3′) was used as forward primer, and JM77 (5′-ATCACCTCCWCCTGCAGGATC-3′) as reverse primer (Nagoshi et al., 2006). The thermo cycler was programmed for 2 min incubation time at 94 °C, followed by 35 cycles of 45 sec at 94 °C, 45 sec at 56 °C, 60 sec at 72 °C, and a final elongation at 72 °C for 10 min. The generated amplification products were further digested for 2 h at 37 °C with MspI and SacI (New England Biolabs, Ipswich, MA, USA). For this digestion, 4 µl PCR product were mixed with 0.6 µl NEB buffer 4, 1 µl H_2O and 0.4 µl MspI (MspI digest) as well as 0.6 µl NEB buffer 1, 0.06 µl 100x BSA, 0.94 µl H_2O, and 0.4 µl SacI (SacI digest). Each digest was mixed with 3 µl loading dye, and 4.5 µl of this mix were loaded on a 1 % agarose gel, and run at 110 V for 45 min. MspI digestion proved corn-strain identity, whereas SacI digestion detected rice-strain individuals.

Statistical and Graphical Analysis Statistical analysis was performed with R 2.11.1 (R Development Core Team 2007). Female pheromone data were log transformed to stabilize the variance, and analyzed using a multivariate analysis of variance (MANOVA) and a generalized linear model (GLM). A graphical illustration of the female pheromone production was generated with SigmaPlot 8.0 (Fig. 1). The attraction experiments in the wind tunnel were analyzed using a Pearson's *Chi-square* test and a GLM. The attraction experiments in the field were analyzed with a GLM using a Poisson distribution. If a treatment caught no moths, it was removed from the analysis. The quasi-Poisson distribution was used whenever the residual deviance of the data was larger than the residual degrees of freedom (over-dispersion). Graphical illustrations of the wind tunnel and field experiments were made with Microsoft Office Excel 2007.

Results

Strain-Specific Variation in the Pheromone Blend In this study, we compared the pheromone composition of a field population with previous data from our laboratory population (Groot et al., 2008). Corn- and rice-strain females of the

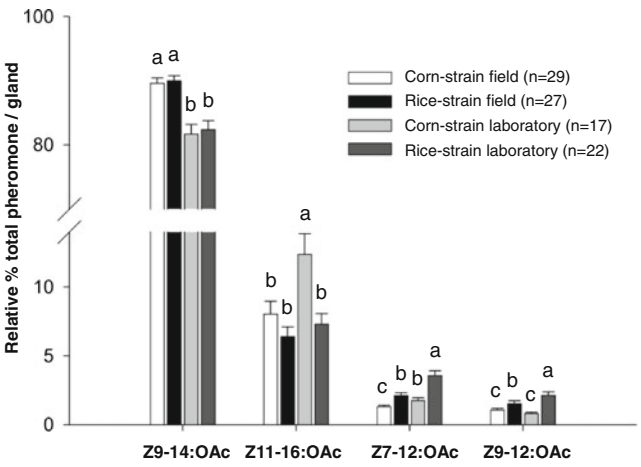

Fig. 1 Pheromone composition of *Spodoptera frugiperda* corn-strain and rice-strain virgin females from laboratory and field populations originating from Florida. The sum of all components adds to 100 %. Different letters above the bars indicate significant differences. Pheromone data of laboratory populations refer to Groot et al., 2008

laboratory, and field populations from Florida showed consistent strain-specific differences in their amount of Z7-12:OAc and Z9-12:OAc (Fig. 1). Rice-strain females of both the laboratory and field populations produced significantly higher relative amounts of Z7-12:OAc and Z9-12:OAc compared to corn-strain females of both populations (Fig. 1). As for Z11-16:OAc, laboratory corn-strain females exhibited significantly higher relative amounts of Z11-16:OAc compared to laboratory rice-strain females (Fig. 1). Such a difference was not found in the field populations (Fig. 1). The relative amount of the major sex pheromone component Z9-14:OAc was not significantly different between corn- and rice-strain females in either population (P=0.918, Fig. 1).

Population-specific Variation in the Pheromone Blend In addition to strain-specific pheromone differences, we also found differences between laboratory and field corn-strain females, as well as between laboratory and field rice-strain females for all four pheromone components (P<0.001 for Z9-14:OAc and Z7-12:OAc, P=0.009 for Z11-16:OAc, P=0.043 for Z9-12:OAc, Fig. 1). Corn-strain females of the field population produced lower relative amounts of Z7-12:OAc than corn-strain females of the laboratory population (Fig. 1). Similarly, field rice-strain females produced lower relative amounts of Z7-12:OAc than laboratory rice-strain females (Fig. 1). Corn-strain females of field and laboratory populations exhibited similar relative amounts of Z9-12:OAc, whereas rice-strain field females had significantly less Z9-12:OAc than laboratory rice-strain females (Fig. 1). Field and laboratory rice-strain females produced similar relative amounts of Z11-16:OAc, while corn-strain laboratory females contained higher relative Z11-16:OAc amounts than corn-strain field females (Fig. 1). The relative amount of the major component Z9-14:OAc was significantly lower in laboratory corn-and rice-strain females than in field corn- and rice-strain females (Fig. 1).

Male Attraction to Females in the Wind Tunnel Corn- as well as rice-strain males showed no strain-specific attraction in wind tunnel choice assays (*Chi-square test*: P=0.421, Fig. 2a). No significant strain-, choice- or strain x choice-effect was observed (Fig. 2a). Although males were flown multiple times per night, we could not detect any effect of flight experience on the male choice. Of all tested corn- and rice-strain males, 29–44 % were inactive and did not respond to any of the six presented females upwind (Fig. 2a). Most of the inactive males showed no response during the whole night (i.e., in all trials of one experiment), and generally males did not become inactive when tested multiples times per experiment. All active males that reached a source did fly within the odor plume, and did not reach a female just by chance. None of the tested males showed only zigzag flight behavior without afterwards contacting a tube

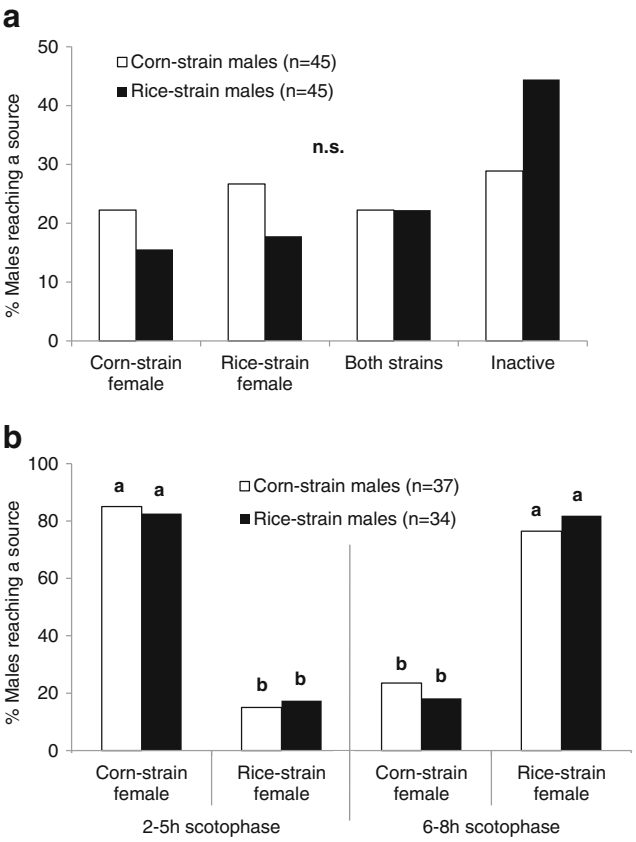

Fig. 2 Attraction of *Spodoptera frugiperda* corn-strain and rice-strain males to calling females in a wind tunnel. **a** Data represent the sum of all males that showed source contact, and male calling within five experiments performed on five consecutive nights. *n.s.* not significant. **b** Data represent the sum of all active males that showed source contact and male calling within five experiments in the early scotophase (2–5 h), and four experiments at the end of the night (6–8 h). *Different letters* above the bars indicate significant differences

containing females. Active males of both strains were attracted to strain-specific females (18–22 %), to females of the other strain (16–27 %), as well as females of both strains (22 %, Fig. 2a). We observed that most males responded to every female that was currently calling, irrespective of the female strain. Around 80 % of the active males of both strains were attracted to calling corn-strain females at the beginning of the scotophase, and to calling rice-strain females at the end of the scotophase (Fig. 2b).

Male Attraction to Synthetic Lures in the Field In the field, the strain-specific blends tested in experiment (A) revealed differential attraction to the corn-and rice-strain blend between habitats, but equal attraction to both blends within habitats (Fig. 3). Males of both strains were significantly more attracted towards the synthetic corn-strain lure than to the synthetic rice-strain lure in the corn field (Fig. 3a).

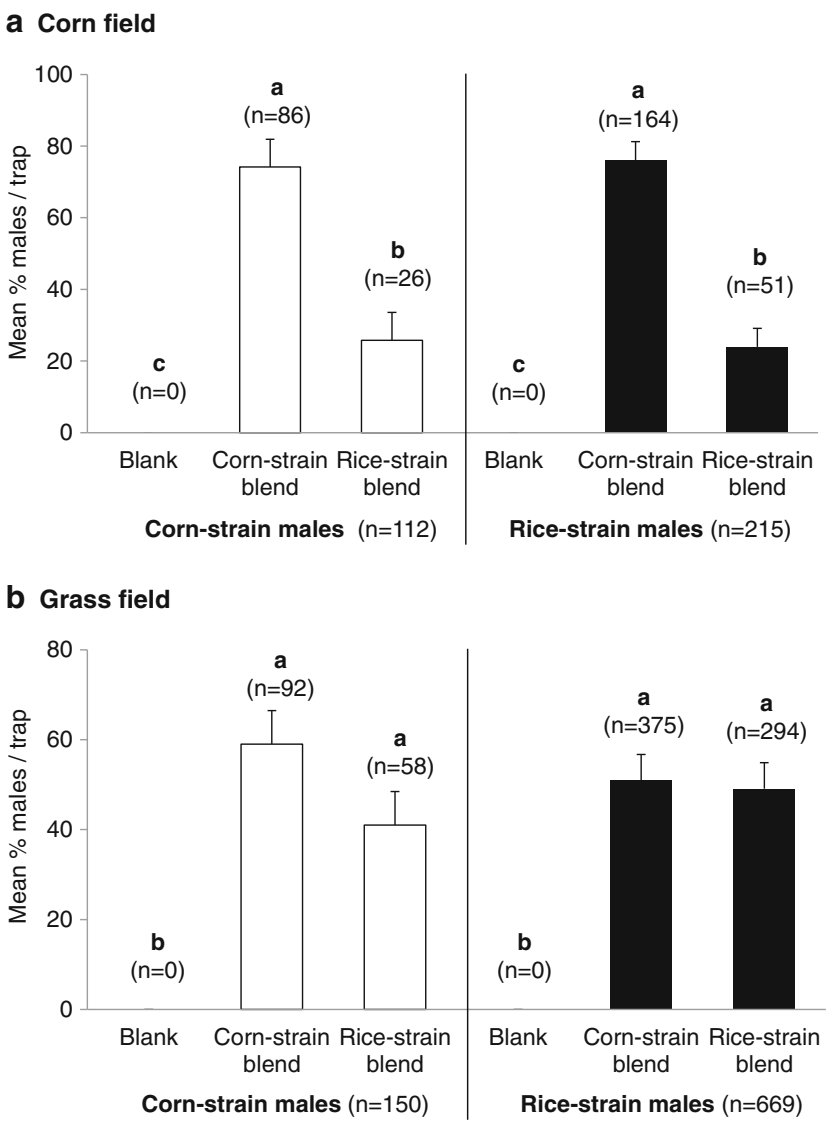

Fig. 3 Attraction of *Spodoptera. frugiperda* corn-strain and rice-strain males to strain-specific synthetic pheromone lures in a corn field (**a**) and in a grass field (**b**) in Florida. Different letters above the bars indicate significant differences. *n* = sample size

However, both strains were equally attracted towards corn- and rice-strain lures within the grass field (Fig. 3b).

Experiment (B), the dose–response experiment to Z7-12:OAc, evidenced strain-specific responses of corn- and rice-strain males within both kinds of habitats (Fig. 4). Corn-strain males in corn and grass habitats were significantly more attracted to 2 % Z7-12:OAc than to traps baited with 4 % or 10 % Z7-12:OAc (Fig. 4). Rice-strain males were attracted equally to traps with lures containing 2 % and 4 % Z7-12:OAc and were even attracted to 10 % of this component within both fields (Fig. 4). Males of both strains were attracted only towards lures containing Z9-14:OAc when Z7-12:OAc was added (Fig. 4).

The male response towards different doses of Z11-16:OAc (experiment C) was equal between both strains and both corn and grass habitats. Corn-strain males were similarly attracted to binary blends (100 % Z9-14:OAc and 2 % Z7-12:OAc; $n_{corn\ field}$=153, $n_{grass\ field}$=28) as to three-component blends containing 8 % Z11-16:OAc ($n_{corn\ field}$=220, $n_{grass\ field}$=51), 13 % Z11-16:OAc ($n_{corn\ field}$=188, $n_{grass\ field}$=40), or 18 % Z11-16:OAc ($n_{corn\ field}$=162, $n_{grass\ field}$=30). Although not statistically significant, corn-strain males seemed to be more attracted to the three-component blend containing 8 % Z11-16:OAc in both fields. Similar to the response of corn-strain males, rice-strain males did not differentiate between binary blends ($n_{corn\ field}$=202, $n_{grass\ field}$=276) and three-component blends containing different doses of Z11-16:OAc (8 %: $n_{corn\ field}$=194, $n_{grass\ field}$=331; 13 %: $n_{corn\ field}$=198, $n_{grass\ field}$=360; or 18 %: $n_{corn\ field}$=161, $n_{grass\ field}$=252).

In both corn and grass habitats, the addition of 1, 2, or 4 % of Z9-12:OAc to the binary blend (experiment D) did not significantly increase trap catches compared to the binary blend. Corn-strain males responded similarly to binary blends ($n_{corn\ field}$=96, $n_{grass\ field}$=54) as to three-component blends containing either 1 % Z9-12:OAc ($n_{corn\ field}$=139, $n_{grass\ field}$=60), 2 % Z9-12:OAc ($n_{corn\ field}$=111, $n_{grass\ field}$=

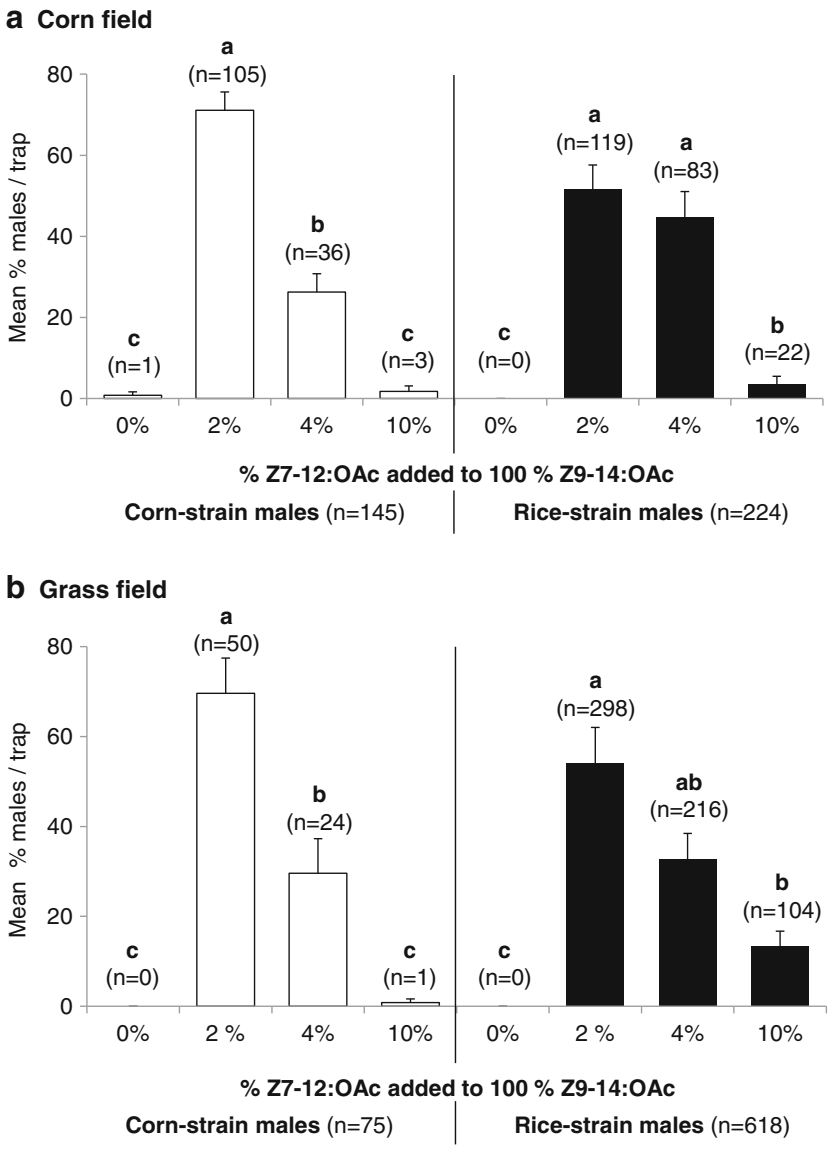

Fig. 4 Attraction of *Spodoptera frugiperda* corn-strain and rice-strain males towards different doses (0 %, 2 %, 4 %, 10 %) of Z7-12:OAc added to 100 % Z9-14:OAc in a corn field (**a**), and in a grass field (**b**), in Florida. Different letters above the bars indicate significant differences. *n*=sample size

59) or 4 % Z9-12:OAc ($n_{corn\ field}$=111, $n_{grass\ field}$=70). Like the corn-strain, rice-strain males were equally attracted to binary blends ($n_{corn\ field}$=84, $n_{grass\ field}$=207) as to three-component blends containing different doses of Z9-12:OAc (1 %: $n_{corn\ field}$=116, $n_{grass\ field}$=196; 2 %: $n_{corn\ field}$=73, $n_{grass\ field}$=187; or 4 %: $n_{corn\ field}$=72, $n_{grass\ field}$=243). Within the corn field, males of both strains showed a slight, but not significant, increase in attraction when 1 % Z9-12:OAc was added to the binary blend.

Discussion

In this study, we assessed the importance of sex pheromone differences between the two strains of *S. frugiperda* for differential male attraction, in order to estimate the role of sexual communication as a prezygotic mating barrier between both strains. We found: a) consistent pheromone variation between corn- and rice-strain females; but also b) significant pheromone variation between laboratory and field populations within the strains; c) no differential attraction of males in wind tunnel experiments; and d) some differential attraction of males to synthetic lures in the field. Although experiments were conducted with insect colonies that have been reared many years under laboratory conditions, we do not assume that laboratory breeding influenced their reproductive behavior because the same colonies were used by Schöfl et al. (2009) who found similar strain-specific timing differences in the reproduction as found previously (Pashley et al., 1992).

a) *Consistent Pheromone Variation between Strains*. Our finding that rice-strain females collected from the field contain higher relative amounts of Z7-12:OAc and Z9 12:OAc than corn-strain females from the field

confirms our previous results when we analyzed laboratory populations also originating from Florida (Groot et al., 2008). Since our field collections were from 2009, and the laboratory populations originated from field-collected larvae in 2003, this indicates that the strain-specific pheromone differences of *S. frugiperda* females are not an artifact that may have developed during laboratory rearing. Nevertheless, our findings contrast with those of Lima and McNeil (2009), who found that corn-strain females exhibited larger relative amounts of Z9-14:Ac, as well as lower relative amounts of Z7-12:OAc and Z11-16:OAc, compared to rice-strain females (Lima and McNeil, 2009). Most likely, the different findings are due to the fact that females from different geographic regions were used. We extracted laboratory and field females originating from Florida,while Lima and McNeil (2009) used females from Louisiana.

The pheromone differences of females from Florida and Louisiana could be related to different corn-strain specific mitochondrial COI haplotype profiles existing in the Florida and Louisiana populations (Nagoshi et al., 2008). The different haplotype profiles reflect the migration of corn-strain individuals through North America in two migration routes: an Eastern route from Florida northwards to Georgia and along the Atlantic coast, and a Western route from Texas northeastwards to Louisiana, Mississippi, Alabama, and into the Ohio Valley to the northeast (Nagoshi and Meagher, 2008; Nagoshi et al., 2008). If haplotype profile and migration differences influence female pheromone composition, then pheromones of females from regions of the Eastern migration route should be similar to each other but different from pheromones of females from the Western migration route and vice versa. To disentangle geographic from strain-specific variation, further pheromone studies from different geographic regions will be necessary.

The critical secondary sex pheromone component, Z7-12:OAc, showed similar strain-specific variation between corn and rice-strain females from Florida and Louisiana (Groot et al., 2008; Lima and McNeil, 2009). In different geographic regions, *S. frugiperda* males are attracted to the binary blends containing Z7-12:OAc and Z9-14:OAc (Andrade et al., 2000; Fleischer et al., 2005; Batista-Pereira et al., 2006). This consistent attraction of males, together with the geographically independent strain-specific variation in Z7-12:OAc in females, indicates that the critical component Z7-12:OAc is under stabilizing selection. In contrast, variation in Z11-16:OAc and Z9-12:OAc, both in the female glands (Groot et al., 2008; Lima and McNeil, 2009) and in the male response (see part d), indicates that Z11-16:OAc and Z9-12:OAc are under much less stabilizing selection.

b) *Pheromone Variation within Strains*. The fact that we found population-specific pheromone differences between our laboratory and field females implies that either differences were present before individuals were bred in laboratory, or that differences developed in the course of laboratory rearing. We do not assume population-specific pheromone differences *per se* based on the same origin of both populations in Florida. Laboratory rearing may influence the pheromone composition of *S. frugiperda* females to some degree, although strain-specific pheromone variation is preserved. The higher probability of inbreeding, genetic drift, founder effects, and bottlenecks, as well as the loss of long-range mate search and interspecific interactions, could result in a reduction of selection pressures acting on laboratory bred populations, which could cause an alteration of the pheromone composition of laboratory bred females (Miller and Roelofs, 1980; Haynes and Hunt, 1990). Examples of such changes have been found in *Argyrotaenia velutinana* (Miller and Roelofs, 1980), *Agrotis segetum* (Löfstedt et al., 1985), and *Trichoplusia ni* (Haynes and Hunt, 1990). Long lasting laboratory pure-strain matings of *S. frugiperda* likewise could have changed the pheromone composition in the females in our case, resulting in an increase of all minor components in at least one of both laboratory strains compared to field females (see Fig. 1). Based on a proposed pheromone biosynthesis pathway of *S. frugiperda*, a single-gene mutation in a fatty acyl reductase (FAR) would be sufficient to reduce the amount of the major pheromone component Z9-14:OAc, which would in turn lead to an increase in the amount of the minor compounds Z11-16:OAc, Z7-12:OAc, and Z9-12:OAc (Groot et al., 2008).

c) *No Differential Attraction of Males in Wind Tunnel Experiments*. Wind tunnel experiments showed that males of both strains were attracted mainly to corn-strain females at the beginning of the night and to rice-strain females at the end of the night, which corresponds to the strain-specific female calling times of *S. frugiperda* (Pashley et al., 1992; Schöfl et al., 2009). Although we found no strain-specific male attraction to females of their own strain in wind tunnel choice assays, Lima and McNeil (2009) reported an experiment that showed that *S. frugiperda* males of both strains exhibited "different responses to an array of concentrations and blends in the wind tunnel" (McNeil et al. unpublished data). These data imply that *S. frugiperda* males are able to show differential responses to pheromone blends in the wind tunnel and, thus, other factors might have influenced the male response in our

experiments. It is known that lepidopteran males show differential attraction behavior in the wind tunnel depending on host plant volatiles (Landolt et al., 1994; Deng et al., 2004; Yang et al., 2004), their age (Rojas, 1999), the pheromone dosage of the source and the ambient temperature (Charlton et al., 1993), chemical noise and wind turbulences (Liu and Haynes, 1993), as well as the wind speed, flight altitude, and ground pattern (Foster and Howard, 1999). We observed that *S. frugiperda* males were highly sensitive to slight changes in the wind tunnel parameters (e.g., temperature, wind speed), and stopped their response to any stimulus when environmental conditions were inadequate. Due to the construction of the wind tunnel, it was not possible to obtain higher percentages than 23 % relative humidity in the wind tunnel, which might explain why more than one third of all males were inactive and did not respond to any of the presented females. Most likely, males may have shown differential attraction in the wind tunnel if we had optimal environmental conditions and shifted the calling times of the females, so that corn- and rice-strain females would have called simultaneously.

d) *Some Differential Attraction of Males in the Field.* In a corn field, we found that corn- and rice-strain males preferred the synthetic corn-strain blend over the synthetic rice-strain blend. Such a preference was not found when the same blends were tested in a grass field. The presence of response to strain-specific lures could be explained by synergistic effects of specific corn field volatiles. For many lepidopteran species, it has been shown that the presence of host plant volatiles can synergize the male orientation towards female sex pheromones (Landolt et al., 1994; Landolt and Phillips, 1997; Ochieng et al., 2002; Deng et al., 2004; Reddy and Guerrero, 2004; Yang et al., 2004). Although synergistic plant volatile effects have not been described for *S. frugiperda,* adult moths can perceive at least 16 different host plant volatiles, and males show greater EAG responses to plant odors than females (Malo et al., 2004). Thus, host plant volatiles may enhance the attraction of both strains towards the corn-strain blend in a corn field.

Plant semiochemicals and non-host green leaf volatiles also can have an inhibitory effect on insect behavior by repelling them from certain hosts, thus providing proper host-selection (Reddy and Guerrero, 2004). In *Spodoptera littoralis*, a closely related species, plant terpenes can antagonize the pheromone signal in a reversible way and are able to reduce the firing response of pheromone receptor neurons that respond to the major pheromone component (*Z*)-9-(*E*)-11-tetradecadienyl acetate (Party et al., 2009). If plant volatiles are able to modulate pheromone perception in *S. frugiperda*, grass volatiles could reduce the ability of males to quantify doses of pheromone and differentiate between blends, which could explain why both strains did not differentiate between the synthetic corn- and rice-strain blend in a grass field.

The fact that we found no strain-specific attraction to the four-component blends suggests either that males of both strains have a similar response range and are not differentiated in this respect, or that the blends that we tested were not strain-specific enough. Even though we and Lima and McNeil (2009) found that Z7-12:OAc is present in significantly lower amounts in corn-strain females than in rice-strain females, variation in the other two compounds Z9-12:OAc and Z11-16:OAc is not consistent between the strains and is variable within the strains. This may have confounded a possible strain-specificity of our so-called corn-strain and rice-strain pheromone blend. That such a confounding factor may have occurred seems to be confirmed by our dose–response experiment, varying the dose of the critical pheromone component Z7-12:OAc. Corn-strain males were significantly more attracted when 2 % of Z7-12:OAc was added to the major component compared to 4 % and 10 %, while rice-strain males showed a much wider response range, from 2 % to 10 % of Z7-12:OAc. These differences in response are in accordance with the strain-specific female pheromone production, as corn-strain females produce smaller relative amounts of Z7-12:OAc than rice-strain females. These results suggest that *S. frugiperda* males from Florida are adapted to the strain-specific Z7-12:OAc differences in the females. The fact that corn-strain males differentiated between 2 % and 4 % Z7-12:OAc in our dose–response experiments shows that males were able to detect minor differences of 2 % between the tested synthetic lures. This in turn suggests that males similarly detected the differences between our strain-specific corn- and rice strain blends that also differed in their amount of Z7-12:OAc by 2 %. Furthermore, we found that no males of either strain were attracted when Z7-12:OAc was absent (0 %), which confirmed previous findings of Tumlinson et al. (1986) that this secondary component is necessary for male attraction to the major pheromone component, Z9-14:OAc.

Similar to previous field experiments conducted in Florida and Brazil (Tumlinson et al., 1986; Batista-Pereira et al., 2006), our Z11-16:OAc dose–response experiments showed that the addition of Z11-16:OAc to binary blends containing Z9-14:OAc and Z7-12:OAc did not increase capture rates compared to binary blends. This male response is in accordance with the female pheromone production, because Floridian field

females of both strains do not differ in their relative amount of Z11-16:OAc. Nevertheless, the amount of Z11-16:OAc in female pheromone glands (Groot et al., 2008; Lima and McNeil, 2009), as well as male attraction to this compound, differs between different geographic regions (Tumlinson et al., 1986; Andrade et al., 2000; Fleischer et al., 2005; Batista-Pereira et al., 2006). Field trapping experiments in Costa Rica showed that addition of Z11-16:OAc did marginally increase capture rates of binary blends (Andrade et al., 2000), and even doubled the attraction of males in Pennsylvania when Z11-16:OAc, together with Z9-12:OAc, was added to the binary blend (Fleischer et al., 2005). In contrast, EAG studies of laboratory *S. frugiperda* males from Mexico showed that males respond electrophysiologically to Z9-14:OAc, Z7-12:OAc, and Z9-12:OAc, but not to Z11-16:OAc (Malo et al., 2004).

We found no strain-specific attraction of males towards different doses of Z9-12:OAc, which is in contrast to the strain-specific Z9-12:OAc differences that we found between Floridian corn- and rice-strain females. However, males of both strains showed similar attraction in both fields to binary blends (Z9-14:OAc, Z7-12:OAc), but differential attraction between fields to four-component blends containing Z9-12:OAc. This differential attraction may be due to the addition of Z9-12:OAc. Furthermore, field experiments in Florida and Costa Rica showed that traps baited only with Z9-12:OAc were attractive for *S. frugiperda* males (Jones and Sparks, 1979; Andrade et al., 2000), and addition of Z9-12:OAc and Z11-16:OAc to binary blends doubled the attraction of males in Pennsylvania (Fleischer et al., 2005). If the amount/presence of Z9-12:OAc and Z11-16:OAc is unimportant for male attraction, results of the test of strain-specific blends should be similar to results of our Z7-12:OAc dose–response experiment where we tested 2 % and 4 % Z7-12:OAc, because strain-specific blends differed in their amount of Z7-12:OAc (corn-strain blend: 2 %, rice-strain blend: 4 %), Z9-12:OAc and Z11-16:OAc. However, when testing the strain-specific blend we found differences between habitats, while the Z7-12:OAc dose response experiment showed similar results between habitats. Thus, we cannot exclude the biological relevance of Z9-12:OAc or Z11-16:OAc for male attraction and/or synergistic effects of these compounds in combination with other pheromone components or plant volatiles, which could influence male attraction in the field.

In summary, overall, we found some consistent strain-specific differences in the sexual communication system of *S. frugiperda*. Laboratory and field females showed strain-specific pheromone differences in their relative amount of Z7-12:OAc and Z9-12:OAc. Although males were not attracted to females of their own strain in wind tunnel assays, which was most likely due to differential calling times of the females, we observed some differential attraction of males in the field. In a corn field, both corn- and rice-strain males were more attracted to our synthetic corn-strain blend than our synthetic rice-strain blend, while these blends were similarly attractive in a grass field. Furthermore, males of both strains showed strain-specific responses towards the critical component Z7-12:OAc. While corn-strain males were mainly attracted to 2 % Z7-12:OAc, rice-strain males were attracted to 2 % up to 10 % of this component. Together, these data suggest that strain-specific differences in sexual communication alone are marginal and probably not sufficient to cause assortative attraction.

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