Friedrich Schiller University Jena

Department of Biology and Pharmacy

Differences in pheromone composition between the two strains of the fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

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Melanie Marr

(born on 23th of January 1985 in Rudolstadt, Germany)

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Max Planck Institute (MPI) for Chemical Ecology
Department of Entomology (Director: Prof. David G. Heckel)
Beutenberg Campus
Hans-Knöll-Straße 8
D-07745 Jena, Germany

1st Supervisors: Dr. Astrid T. Groot, Dr. Gerhard Schöfl (MPI)
2nd Supervisors: Prof. Dr Stefan Halle, PD Dr. Günter Köhler (University Jena)

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1. Introduction

1.1. The fall armyworm

The fall armyworm *Spodoptera frugiperda* (J. E. Smith) is a major agricultural pest of crops throughout the Western Hemisphere (Pashley 1986). The fall armyworm can be found on more than 80 plant species and it is called the "pest of grasses" (Pashley 1988a). This nocturnal moth (Lepidoptera: Noctuidae) consists of two different stains, a so-called "corn" strain that primarily feeds on corn and a "rice" strain, which feeds on rice and various grasses (Pashley 1986). The two strains are morphologically indistinguishable from each other but they differ genetically at five allozyme loci (Pashley 1986), their mitochondrial DNA (Pashley 1989) and their nuclear DNA (Lu et al. 1992). Specifically, the two strains have different restriction sites in their cytochrome oxidase I (COI) gene (Meagher and Gallo-Meagher 2003; Nagoshi et al. 2006a) and ND1 gene (Pashley 1989), in the copy number and organisation of the FR (Fall armyworm Rice strain; Lu et al. 1994) sequence (Nagoshi and Meagher 2003a), and they can be distinguished by a number of AFLP loci (McMichael and Prowell 1999). Although their taxonomic status is still unclear, the rice and the corn strain exhibit too many genetic differences to be only biotypes of a single species (Pashley 1989).

Behaviourally, the strains exhibit different mating and calling times. While the corn strain is active in the first two-thirds of the night (average calling time is 2.5 hours in scotophase), the rice strain is mostly active in the last part of the night (average calling time is 6.5 hours in scotophase) (Pashley et al. 1992; Schöfl et al. submitted 2008). Other reproductive isolation mechanisms between the two strains of this species are different seasonality abundances (Pashley et al. 1992) and behavioural differences in oviposition preferences (Whitford et al. 1988). Furthermore, developmental differences (e.g. weight gain, developmental time) on different plants and artificial diet have been found between the two strains (Pashley 1988b; Whitford et al. 1988; Quisenberry and Whitford 1988; Pashley et al. 1995; Meagher at al. 2004).

1.2. Sex pheromones

1.2.1. The role of sex pheromones in moth species

Pheromones, which are produced by many insects, are crucial for the chemical communication system of males and females in a patchy environment (Tamaki 1985; Löfstedt and Kozlov 1997; Raina 1997). Within lepidopteran species, sex pheromones are

produced by the female to attract the conspecific sex for mating over long distances (Raina and Menn 1987; Löfstedt and Kozlov 1997; Raina 1997). Sex pheromones are essential for mating success, because moths in general are short-lived and need to find right mating partners while they are in the adult stage (Raina 1997). Nocturnal moth species release their pheromones in the scotophase and mate during the night, probably to avoid the higher risk of predation during the day (Raina 1997). Female moths exhibit a typical calling behaviour when emitting pheromones: they extrude their pheromone gland and expose the gland surface to the air to release a specific pheromone blend (Tamaki 1985; Raina and Menn 1987; Löfstedt and Kozlov 1997). The male moth follows the source of the pheromone release to find the conspecific female to mate with (Raina and Menn 1987; Mafra-Neto and Cardé 1994; Löfstedt and Kozlov 1997). When a male moth comes across a species-specific female, it courts in front of the female with rapid wing-fluttering and releases male-specific pheromones through its scent brushes or hairpencils (Tamaki 1985; Birch et al. 1990). Virgin females produce high amounts of pheromone to attract conspecific males, whereas the pheromone titer of mated females can dramatically decrease after mating (Raina 1993; Rafaeli 2005). This post-mating decline in the pheromone titer is probably due to factors (e.g. peptides) within the male sperm or the spermatophore that are transferred to the female during mating (Raina 1993; Kingan et al. 1995).

1.2.2. Biochemistry of sex pheromones in moths

Most sex pheromones that are released by female moths are unsaturated derivatives of fatty acids that exhibit an even numbered 12-18 carbon backbone and have an oxygen-containing functional group (e.g. alcohol, aldehyde or acetate ester) (Jurenka 2003; Jurenka 2004). In many moth species, the biosynthesis of pheromone components is based on products of the fatty acid synthesis, which are even numbered carbon chains (Jurenka 2004). In general, these products are palmitic acid (16 carbons) and stearic acid (18 carbons), which can be modified via a number of enzymes to build up the pheromone components (Raina 1993; Tillman et al. 1999; Jurenka 2004). Key enzymes of the pheromone production are desaturases, which introduce double bonds into saturated and monounsaturated carbon chains, as well as ß-oxidation enzymes, which are responsible for limited chain shortening by two carbons (Raina 1993; Jurenka 2004). After the production of an appropriate pheromone precursor that exhibits the right chain length and double bond position, the carbonyl carbon is converted into an oxygen-containing functional group

(Jurenka 2004). The modification of the functional groups is controlled by oxidases, reductases and acetyltransferases (Jurenka 2004).

The biological activity of lepidopteran pheromone components is affected by the position of the double bond and the chain length of the component (Tamaki 1985; Jurenka 2004). The presence of functional groups like alcohols, aldehydes and acetate esters increases the number of possibly different pheromone blends within the species-specific mate location system of moths (Jurenka 2004). The specificity of a pheromone blend to attract a certain species is influenced by the structural composition as well as the ratio between the different components (Tamaki 1985; Jurenka 2004). The relative amount of a single component in the pheromone blend of a female is not necessarily positively correlated with the amount of male response (e.g. the biological activity of the component) because minor compounds, which can be found only in small amounts within the pheromone blend, can be highly effective attractants for males (Sekul and Sparks 1976; Tumlinson et al. 1986).

1.2.3. Pheromone biosynthesis activating neuropeptide (PBAN)

In the brain of several moth species, a peptide has been identified, which induces pheromone biosynthesis in females during the scotophase (Raina and Klun 1984; Ando et al. 1988; Raina et al. 1989; Fabriás et al. 1994; Rafaeli and Jurenka 2003). This peptide, called pheromone biosynthesis activating neuropeptide (PBAN), consists of 33 amino acids and has an amidated carboxy-terminus (Riana et al. 1989; Kitamura et al. 1990; Rafaeli and Jurenka 2003; Jurenka 2004). The trophic hormone PBAN is produced in a circadian rhythm by three groups of neurons in the subesophageal ganglion of the female and is released through the corpora cardiaca into the hemolymph to induce the pheromone biosynthesis in the gland (Raina and Klun 1984; Raina and Menn 1987; Raina et al. 1989; Raina 1993; Rafaeli and Jurenka 2003; Rafaeli 2005). PBAN binds to a specific G-protein coupled PBAN-receptor in the membrane of the pheromone gland cells and activates the cAMP production or the influx of extracellular calcium (Rafaeli and Jurenka 2003; Jurenka 2004; Rafaeli 2005). Ca^{2+}-calmodulin complexes and cAMP act as second messengers that activate kinases and phosphatases to trigger the pheromone biosynthesis (Rafaeli and Jurenka 2003; Rafaeli 2005). Most of the female moths produce their pheromones *de novo* every night (Tamaki 1985; McNeil and Delisle 1989; Raina 1993) as response to circadian PBAN stimulation (Rafaeli 2005). Several studies suggest that PBAN does not regulate a particular enzyme within the pheromone biosynthetic pathway, but can affect different

enzymes, whereas it stimulates reductases or steps in, or prior to, the fatty acid synthesis in most moth species (Rafaeli and Jurenka 2003; Jurenka 2004; Rafaeli 2005; Tsfadia et al. 2007).

The injection of synthetic PBAN into female moths can be used to determine their pheromone composition in the photophase (Raina et al. 1989; Fabriás et al. 1994; Groot et al. 2005). Additionally, PBAN-injection into mated females can activate the pheromone production again (Rafaeli and Jurenka 2003). For some moth species it was shown that PBAN-injection can be applied to specify the females' native pheromone phenotype independent of mating status and age of the female and PBAN can help to reduce temporal and nocturnal variation in the pheromone titer of female moths (Groot et al. 2005).

1.3. Pheromone composition of *Spodoptera frugiperda*

The pheromone composition of *Spodotera frugiperda* females has been studied by several authors, at different geographic regions where the fall armyworm naturally occurs (Table 1). Not all components that can be found in the female gland may be pheromone components to attract conspecific males. Some components could be only by-products of the pheromone biosynthesis pathway to which males do not respond (Jurenka 2004), or they can be found in the gland but not in the emitted volatiles of the female (Tumlinson et al. 1986). A pheromone compound, which is biologically active in the attraction of conspecific males, is called "pheromone component", whereas all inactive chemical agents are called "compounds" (Tamaki 1985). To approve the biological activity of a pheromone compound, it is necessary to examine the physiological and behavioural male response for this compound with the help of electro-antennogramm (EAG) recordings and field lures.

The first pheromone component that has been found in *Spodoptera frugiperda* females was (Z)-9-tetradecenyl acetate (Z9-14:OAc) (Sekul and Sparks 1967). This is the major component of the sex pheromone of the fall armyworm females, as the blend consists of 70-85% of Z9-14:OAc. Evaluations of field lures in Florida indicated that a combination of the major component Z9-14:OAc and the minor component (Z)-7-dodecenyl acetate (Z7-12:OAc) was required for the biological activity of the fall armyworm pheromone lure and Z7-12:OAc was a necessary pheromone component, without which fall armyworm males were not attracted to the blend (Tumlinson et al. 1986). The major pheromone component Z9-14:OAc and the minor component Z7-12:OAc could be identified in fall armyworm populations from Brazil, French Guyana, Florida and Costa Rica, and males responded to both components every time (Table 1).

Other field studies of fall armyworm lures in Florida showed that a binary blend of the major component Z9-14:OAc and the minor component (Z)-9-dodecenyl acetate (Z9-12:OAc) attracted two to three times more males than Z9-14:OAc alone (Jones and Sparks 1979). The presence of the minor component Z9-12:OAc was not confirmed in fall armyworm females from Brazil, but this component was found in *Spodoptera frugiperda* populations from Florida and French Guyana (Table 1). The biological activity of Z9-12:OAc was confirmed in some field studies in Florida, but not in Costa Rica and Brazil (Table 1).

The second most abundant compound in the female blend of the fall armyworm is (Z)-11-hexadecenyl acetate (Z11-16:OAc), which was found in females from Brazil, French Guyana and Florida, whereas Z11-16:OAc differently affected males in the field (Table 1). In Mexico (Malo et al. 2001), synthetic lures containing Z11-16:OAc attracted fall armyworm males, which was not the case in Brazil (Batista-Pereira et al. 2006) and Costa Rica (Andrade et al. 2000). Different tests of pheromone lures in Florida showed that Z11-16:OAc in combination with Z9-14:OAc and Z7-12:OAc attracted most fall armyworm males in the field (Mitchell et al. 1985; Tumlinson et al. 1986; Meagher and Mitchell 1998). All commercial available fall armyworm lures contain at least Z9-14:OAc and Z7-12:OAc, whereas the amount and presence of Z11-16:OAc differs between different types of pheromone lures (Malo et al. 2001).

Two other minor compounds, dodecenyl acetate (12:OAc) and tetradecenyl acetate (14:OAc), were identified in females from Brazil and French Guyana, but not in females from Florida (Table 1). Field studies conducted so far did not indicate that 12:OAc or 14:OAc are necessary to attract fall armyworm males (Table 1). In addition to the acetate ester pheromone compounds, two aldehyde compounds have been identified in the pheromone glands of females from Florida, but only one of them (Z11-16:Ald) was biologically active in the field (Tumlinson et al. 1986). Another minor pheromone compound, namely (E)-7-dodecenyl acetate (E7-12:OAc), was detected only in fall armyworm females in Brazil and most males of the brazilian population were attracted to lures containing E7-12:OAc (Batista-Pereira et al. 2006). All other studies on the pheromone composition of fall armyworm females could not show that aldehydes or E7-12:OAc are biologically active pheromone components in this species (www.pherobase.com).

Table 1. Reported sex pheromone composition and male response of *Spodoptera frugiperda*. Data adapted from Groot et al. (2008) and www.pherobase.com. All numbers are percentage values. The total percent of all compounds add to 100%. ([1]Batista-Pereira et al. 2006; [2]Descoins et al. 1988; [3]Tumlinson et al. 1986; [4]Andrade et al. 2000; [5]Meagher and Mitchell 1998; [6]Mitchell et al. 1985)

Pheromone component	Sex pheromones within the female gland			Composition of pheromone lures that attracted most males				
	Brazil[1*]	French Guyana[2*]	Florida USA[3*]	Costa Rica[4*]	Florida USA[5*]	Florida USA[6*]	Florida USA[3]	Brazil[1]
Z9-14:OAc	82.8	73.6	69	99.6	80.3	81.61	79	98
Z7-12:OAc	0.8	1.1	4	0.4	0.5	0.45	5	1
12:OAc	0.6	0.43						
Z9-12:OAc		0.5	2			0.25	2	
14:OAc	1.5	0.53						
Z10-14:OAc	0.3							
Z11-14:OAc		1.2						
16:OAc		0.21						
Z11-16:OAc	12.9	16.6	9		19.2	17.69	10	
E7-12:OAc	1.2							1
E9-14:OAc		3.6						
Z11-16:Ald			3				3	
Z9-14:Ald			13					

16 Years ago, scientists thought that "pheromone chemistry may play a small role (if any) in strain separation" (Pashley et al. 1992), because so many pre-mating isolation mechanisms (e.g. host choice, mating and calling times, seasonal abundance) have been found between the corn and the rice strain. Using live females as lures, relatively more corn than rice males were attracted to corn females and relatively more rice than corn males were attracted to rice females (Pashley et al. 1992). In this thesis I examined the pheromone composition of both strains, to approve or negate the hypothesis that pheromone composition is another trait that distinguishes the two strains of *Spodoptera frugiperda*. The results are discussed to assess to what extent possible pheromone differences could act as pre-mating isolation mechanisms to avert interbreeding between corn and rice strain individuals.

Since no studies conducted so far describe the effect of PBAN on the pheromone production of *Spodoptera frugiperda* females, this study investigated to what extent the injection of synthetic PBAN influences the pheromone composition of corn and rice strain females in order to assess which enzymes are may be stimulated by PBAN-injection into

fall armyworm females. The results are discussed to estimate whether or not PBAN can be used to determine the native pheromone phenotype of *Spodoptera frugiperda* females.

Most surveys concerning the chemical communication system of *Spodoptera frugiperda* identified different compounds in the pheromone blend of females and showed that geographically different male responses to pheromone compounds exist (Table 1). Unfortunately, none of these studies described which strain of the fall armyworm was used for the determination of the females' pheromone composition and the male-response tests with pheromone lures. Hence, it is not clear whether the diverse results from different studies are due to geographic variation or strain-specific differences in the pheromone composition of the fall armyworm. In this study I determined the pheromone composition of fall armyworm corn and rice strain females to assess whether pheromonal differences between both strains exist. Additionally, to assess the heritability of possibly strain-specific pheromonal differences, corn and rice strain individuals were hybridized and their pheromone blends were compared with pure corn and rice strains. Since this study is based on data of laboratory-reared individuals, I conducted a further experiment with a recently collected *Spodoptera frugiperda* population from the field to assess the influence of laboratory rearing on the pheromone composition of the females. This study is the first attempt to characterize strain-specific pheromone differences of the fall armyworm, which could at least partly explain evolutionary forces that led to the differentiation of the two sympatric *Spodoptera frugiperda* strains. More specific knowledge of possible pheromonal differences between both fall armyworm strains could help to design strain-specific pheromone lures that could facilitate pest management strategies and trapping of strain-specific males for further experimental studies. The main results of this research are published in Groot et al. (2008) (see appendix).

2. Methods

2.1. *Spodoptera* strains

Laboratory Population: The corn and rice strain individuals were obtained in 2006 from laboratory colonies of Rob Meagher at USDA-ARS in Gainesville. The corn strain colony (JS3C) originated from over 100 larvae collected from corn plants near Homestead in Miami-Dade Co. in 2004 (Oct-Nov). The rice strain colony (OnaR) descended from over 200 larvae collected from pasture grasses from the Range Cattle Research and Education Centre, Ona, Hardee Co. in 2003 (May-Oct). The JS3C strain and the OnaR strain were reared on an artificial pinto bean diet for 10 and 21 generations in a mass culture at USDA Florida. In July 2006, individuals of these colonies were sent to our laboratory and screened for the strain-specific COI marker. The offspring of these individuals was randomly paired in single pairs to minimize inbreeding and to maintain genetic variation. This single pair mating design was performed for every generation to raise our own laboratory colony, which was reared another 15 generations on artificial pinto bean diet.

Field Population: *Spodoptera frugiperda* individuals were collected from a sweet corn field at Miami-Dade County (N 25°28'000" W80°24'234") in March 2008. The 120 collected individuals were screened for the strain-specific COI marker. Single pair matings were performed and the offspring was reared on artificial pinto bean diet.

The *Spodoptera frugiperda* individuals used for the experiment were placed in an early larval stage in environmental chambers at 26 ± 1 °C, 60 ± 10 % RH, with a 14:10 L:D photoperiod (dark cycle 11 a.m. – 21 p.m.). The larvae were reared up to adult eclosion individually in plastic cups comprising pinto bean diet. Emerged females were checked daily, fed with a 10% honey-water solution and left in the environmental chamber until the extraction of the pheromone gland. In all experiments two to four days old virgins were used for gland preparations.

2.2. Gland preparations

Gland preparations under “natural” conditions were carried out three to six hours into scotophase and the extracted females were not injected with synthetic PBAN. According to the strain-specific mating and calling times corn strain individuals were extracted three to four hours into scotophase and rice strain individuals five to six hours into scotophase (under “natural” conditions).

Gland preparations of both strains under "artificial" conditions (with synthetic PBAN injection) were done one to two hours before the beginning of the scotophase. Under "artificial" conditions virgin females were injected with synthetic HezPBAN (Pheromone Biosynthesis Activating Neuropeptide from *Heliothis zea*, Peninsula Laboratories, San Carlos, CA) to induce pheromone synthesis during the photophase. Three µl PBAN stock solution (200 pmol/µl in 50% methanol and 1N HCl) was diluted in 157 µl saline to obtain a dilution of 3.75 pmol PBAN/ul. Two µl of this solution (i.e. 7.5 pmol) was injected between the 8^{th} and 9^{th} abdominal segment of a female with a 10 µl syringe (31 gauge needle, Hamilton, Reno, NV), because pheromone glands of nearly all moth species are located between these two segments (Tamaki 1985). One to two hours after the PBAN injection, the lateral-posterior abdominal section of the female was gently squeezed until protrusion of the pheromone gland, which was excised from the abdomen. Glands from females under "natural" conditions were excised in the same way. In both cases the gland was placed into a glass vial containing 50µl hexane and 40 ng pentadecane as internal standard for half an hour. After the gland was removed from the vial, the gland extract was stored at -20°C until analysis via gas chromatograph (GC).

The first three experiments were conducted with individuals of our laboratory *Spodoptera frugiperda* populations, whereas the fourth experiment was carried out with females of the field population.

In the first experiment, to detect differences in the overall pheromone composition between the two strains, glands of corn and rice strain virgins were extracted at the same day under "natural" conditions.

In a second experiment, to assess the effect of PBAN on the amount of pheromone produced by the females, I compared gland extractions of both strains under "natural" conditions with extractions under "artificial" conditions. Therefore, glands of corn and rice strain females without PBAN injection were extracted at the same day as the glands of corn and rice females that were injected with 7.5 pmol PBAN. A PBAN dose-response experiment (females were injected with 1 pmol, 7.5 pmol or 20 pmol PBAN) was conducted only with rice strain females because few corn strain individuals were available during the time of this experiment. All rice strain females were injected with different doses of PBAN at the same time.

In the third experiment I determined the pheromonal differences between pure strain and hybrid females to assess the mode of inheritance of the pheromone compounds.

For this purpose, corn and rice strain virgins, as well as CxR hybrids (offspring of a corn mother and a rice father) and RxC hybrids (offspring of a rice mother and a corn father), were injected with 7.5 pmol PBAN and extracted at the same time. The mode of inheritance of all pheromone compounds was assessed by comparing the relative amount of each compound in the pure strain glands with the relative amount of the compounds in the hybrid glands.

The rearing of *Spodoptera frugiperda* individuals under constant laboratory conditions (simulating a "stable" environment) may influence the pheromone composition of the females. Therefore, in a fourth experiment pheromone glands of the offspring of recently collected corn strain females were extracted at the same time under "artificial" conditions (e.g. with 7.5 pmol PBAN-injection) and compared with the laboratory-reared strains. Gland preparations were carried out with the offspring (1st generation) of seven corn strain families from the field.

2.3. Chemical analysis of the pheromone composition

The volume of the gland extracts was reduced from 50 µl to two µl under a gentle stream of nitrogen. The two µl gland extract was placed in a 50 µl glass insert within a crimp-capped vial containing two µl octane to prevent evaporation of the sample. The whole extract was injected into a splitless inlet of a HP7890 gas chromatograph (GC) coupled with a high resolution polar capillary column (DB-WAXetr [extended temperature range]; 30m x 0.25mm x 0.5µm) and a flame-ionization detector (FID). The column temperature was set to 60°C for 2 min, afterwards risen to 180°C by 30°C/min and finally risen to 230°C by 5°C/min to elute all pheromone components of the gland. Afterwards, the column temperature was heated up to 245°C at 20°C/min and held for 15 min to clean the column for the next sample. The FID detector temperature was held at 250°C.

Many compounds have been identified from the glands of the fall armyworm females (Table 1). I tried to identify the following components within the female gland: 12:OAc, Z7-12:OAc, Z9-12:OAc, 14:OAc, Z9-14:OAc, Z11-14:OAc, 16:OAc, Z11-16:OAc and E7-12:OAc, as well as some alcohol and aldehyde analogs of these components. A synthetic component blend (components bought from Pherobank, Wageningen) was injected into the GC, every day before the samples were analyzed. To determine the pheromone composition of females, the retention time of every compound in the gland was compared to the retention time of synthetic components. After identification of the

females' pheromone compounds, a subset of samples was checked by GC-MS to approve the GC results. Gland extracts of ten females were injected into a HP6890 GC coupled to Massspec MS002 (Micromass, Manchester, UK) with electron ionization (EI) at 70eV, using a 30m x 0.25mm x 0.25µm DB-Wax column. The temperature was programmed in the same way as the HP7890 GC. The resulting mass spectra were analyzed with the help of a spectral database (Wiley MS library v 7) and compared to the compounds that were found in the females by GC analysis.

The ratios of the identified compounds were calculated with regard to 40 ng internal standard. I converted the amounts of all identified pheromone compounds to relative percentages of the total amount of all pheromone compounds in the gland. Hence, the sum of all identified pheromone compounds in the gland summed to 100%.

2.4. Statistical analysis

The statistical analysis was performed with the computer program SAS, version 9.1 (SAS Institute, 2002-2003). For all four experiments a multivariate analysis of variance (MANOVA in SAS) was performed to examine differences in the pheromone composition between corn and rice strains females under "natural" and "artificial" conditions, between PBAN injected pure strains and hybrids (CxR and RxC), and between field collected individuals under "natural" and "artificial" conditions. The means of all pheromone compounds were separated using LSMEANS with a Turkey adjustment for multiple comparisons.

Phenotypic correlations of each pheromone compound were conducted to detect potential differences in the enzymatic biosynthetic pathway of the pheromone production between the two strains of the fall armyworm. Therefore, a Pearson's correlation matrix (PROC CORR in SAS) was generated, using the percentages of every component relative to the total amount of pheromone per gland. The Pearson product-moment correlation coefficient specifies whether the individual variation in the mean proportions of the pheromone compounds is positively or negatively correlated, or uncorrelated. The phenotypic associations among pheromone compounds were performed to determine differences between laboratory corn and rice strain individuals under "natural" as well as "artificial" conditions and between laboratory CxR and RxC hybrid females under "artificial" conditions. The results of the phenotypic correlations were used to detect possible enzymatic differences in the pheromone biosynthesis of both *Spodoptera frugiperda*

strains. A theoretical biosynthetic pathway of the pheromone biosynthesis in the fall armyworm was constructed via evaluation of phenotypic correlation patterns.

To investigate the broad sense heritability (H^2) of each component in the pheromone blend, a linear mixed model analysis (PROC MIXED in SAS) was carried out with the seven corn strain families of the field population. The pheromone phenotype of each female is determined by a genetic component and an environmental component (P= G x E). The broad sense heritability analysis was used to detect how strong the phenotype of a pheromone component is influenced by environmental and genetic factors. Heritability values close to one evidence high genetic effects whereas values close to zero evidence high environmental effects. The broad sense heritability H^2 is the quotient of the between family variance and the total variance (between-family variance + within-family variance). The extracted females of each corn strain family were sisters, so the between-family variance in the numerator was multiplied by two to get a full-sib design approach.

3. Results

3.1. Pheromone composition of the fall armyworm

Seven pheromone compounds were identified in the glands of all fall armyworm females: the two major components Z9-14:OAc and Z11-16:OAc, and five minor compounds, namely 12:OAc, Z7-12:OAc, Z9-12:OAc, 14:OAc, Z11-14:OAc (Figure 1). The pheromone blends of the laboratory corn and rice strain females comprised only compounds with acetate esters as functional group. I could not detect any aldehyde or alcohol compounds within the pheromone blend of both strains. Furthermore, 16:OAc could not be detected in any fall armyworm blend, as well as E7-12:OAc, which was also not found via GC-MS.

3.2. Between-strain differences in pheromone composition

3.2.1. Gland preparations under "natural" conditions

When comparing corn and rice strain females that were extracted under "natural" conditions within the scotophase, significant differences were found in the relative amount of four of the seven compounds (Figure 1a). Corn strain females had a significantly higher relative amount of the second major component Z11-16:OAc compared to rice strain individuals (Figure 1a). The glands of rice strain females contained a significantly higher relative amount of the minor compounds 12:OAc, Z7-12:OAc and Z9-12:OAc than the glands of corn strain females (Figure 1a). There were no significant differences between the two strains of the fall armyworm in the relative amount of the major sex pheromone component Z9-14:OAc, and the two minor compounds 14:OAc and Z11-14:OAc (Figure 1a).

3.2.2. Gland preparations under "artificial" conditions

When corn and rice strain females were extracted under "artificial" conditions, the relative amount of each pheromone compound was also significantly different between the two strains. The two strains exhibited significant differences in the relative amount of three of the seven identified compounds (Figure 1b). Rice strain females contained a significantly higher relative amount of Z9-14:OAc and Z9-12:OAc than corn strain individuals, whereas the relative amount of Z11-16:OAc was significantly higher in corn strain females than in rice strain females (Figure 1b). The relative amounts of 12:OAc, Z7-12OAc and 14:OAc were not significantly different between the two strains (Figure 1b).

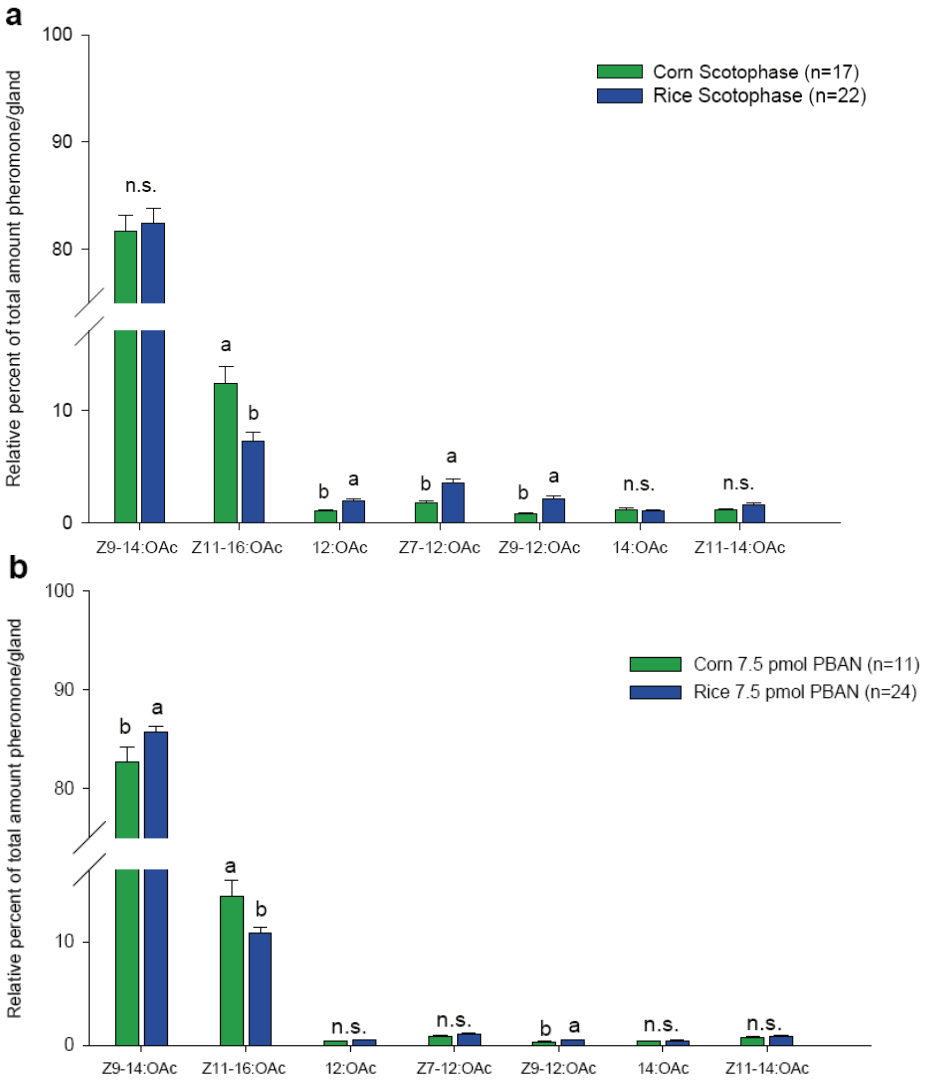

Figure 1. Between-strain differences in pheromone composition of the *Spodoptera frugiperda*. **A.** Pheromone composition of corn and rice strain females that were extracted under "natural" conditions into scotophase. Wilk`s lambda: P=0.0002. **B.** Pheromone composition of corn and rice strain females that were injected with 7.5 pmol PBAN. Wilk`s lambda: P=0.0042. The total percent of all compounds add to 100%. N.s.: not significant. Different letters above the bars indicate significant differences.

3.2.3. Comparison of pheromone composition under "natural" and "artificial" conditions

I detected significant differences between the two strains in the relative amount of four of the seven compounds under "natural" conditions and in the relative amount of three compounds under "artificial" conditions (Figure 1). The relative amount of the major component Z9-14:OAc was significantly different between the two strains under "artificial" conditions, but not under "natural" conditions (Figure 1). Furthermore, rice strain females under "natural" conditions contained significantly more 12:OAc and Z7-12:OAc than corn strain females, but not under "artificial" conditions (Figure 1). Z11-16:OAc was present in significantly higher relative amounts and Z9-12:OAc in

significantly lower relative amounts in corn strain than in rice strain females under both conditions (Figure 1). The relative amounts of 14:OAc and Z11-14:OAc were not different between the two strains under "natural" and "artificial" conditions (Figure 1).

3.3. Effect of PBAN on virgin pheromone production

When corn and rice strain females were injected with 7.5 pmol PBAN, significant differences were found in the relative amount of several pheromone compounds compared to non-PBAN-injected females (Figure 2).

Corn strain females that were injected with 7.5 pmol PBAN exhibited no significant differences in the relative amount of the two major components Z9-14:OAc and Z11-16:OAc compared to "natural" conditions without PBAN injection (Figure 2a). However, when corn strain females were extracted into photophase under "artificial" conditions, the relative amount of all five minor compounds (namely 12:OAc, Z7-12:OAc, Z9-12:OAc, 14:OAc, Z11-14:OAc) was significantly lower than under "natural" conditions (Figure 2a).

When rice strain females were extracted under "natural" conditions, the relative amount of the major component Z9-14:OAc was not significantly different compared to "artificial" extractions with 7.5 pmol PBAN injection (Figure 2b). The glands of rice strain females that were injected with 7.5 pmol PBAN contained a significantly higher relative amount of the second major component Z11-16:OAc as well as a significantly lower relative amount of all five minor compounds (Figure 2b).

3.4. Dose-dependent effect of PBAN

When rice strain females were injected with different doses of PBAN (1, 7.5 or 20 pmol), the pheromone composition was similar between the differently injected females (Figure 2b). Thus, different doses of PBAN had no significant effect on the pheromone composition of the rice strain females (Figure 2b).

The relative amount of the major component Z9-14:OAc was not significantly different between rice strain females that were injected with different doses of PBAN, except when females were injected with 20 pmol PBAN (Figure 2b). For all other pheromone compounds, the injection of 1 pmol PBAN gave similar results as the injection of 7.5 or 20 pmol PBAN (Figure 2b).

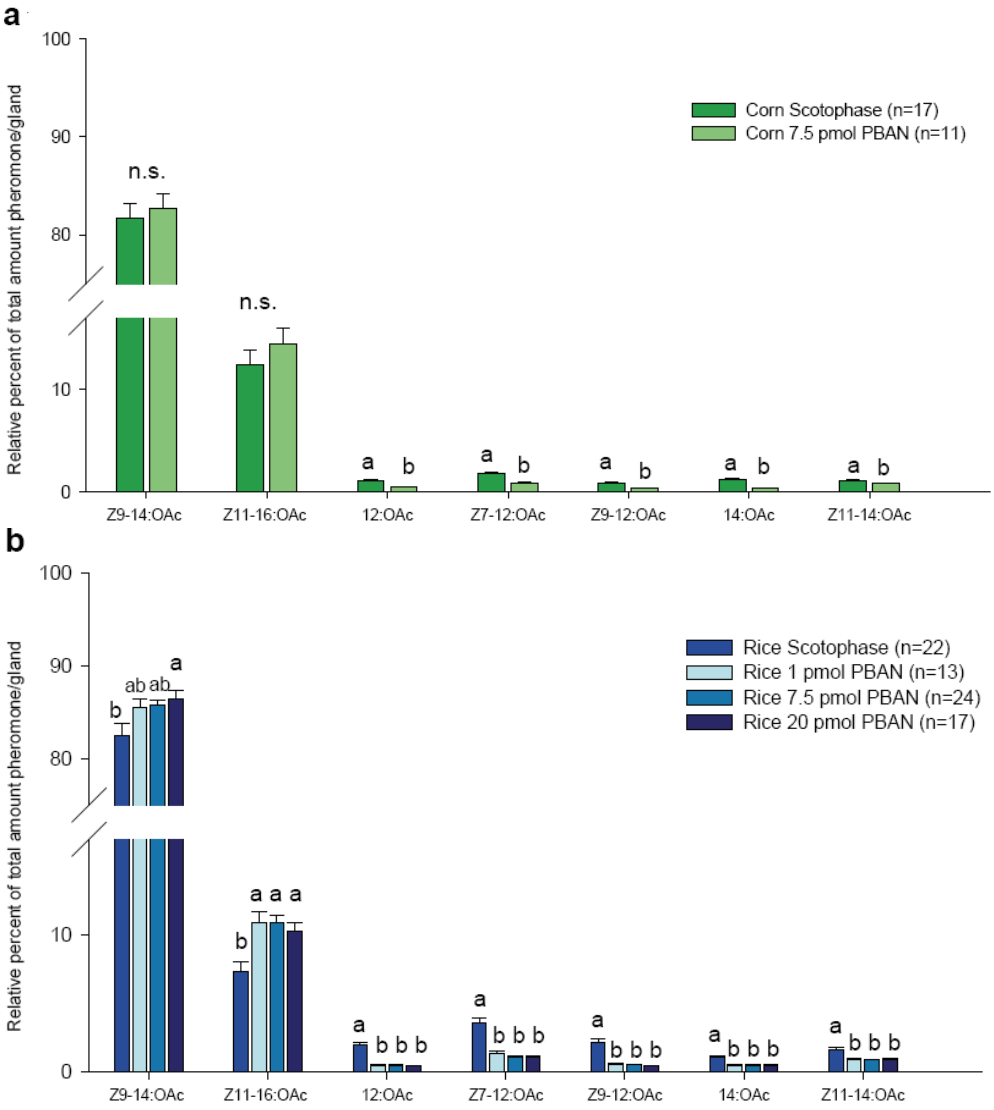

Figure 2. Effect of PBAN on the pheromone composition of corn and rice strain females. Females were either extracted under "natural" conditions in the scotophase or under "artificial" conditions with PBAN injection. **A.** Glands of corn strain females. Wilk`s lambda: P=0.0009. **B.** Glands of rice strain females. Wilk`s lambda: P<0.0001. The total percent of all compounds add to 100%. N.s.: not significant. Different letters above the bars indicate significant differences.

3.5. Mode of inheritance of pheromone components

3.5.1 Analysis of the seven-component blend

The RxC hybrid females contained significantly more of the major component Z9-14:OAc compared to the pure strains and the CxR hybrids (Figure 3a). The relative amount of the second major component Z11-16:OAc was significantly higher in corn strain and CxR hybrid individuals than in rice strain and RxC hybrid females (Figure 3a). Since the CxR hybrid females exhibited a similar relative amount of Z11-16:OAc as corn strain individuals and the RxC hybrids had a similar relative amount of this component as the rice strain females, this component seems to be maternally inherited. The three minor

compounds Z9-12:OAc, 14:OAc and Z11-14:OAc were significantly less present in corn strain females and all hybrids (CxR and RxC) compared to rice strain females, which suggests a corn-dominant inheritance of these minor compounds (Figure 3a). Rice strain females contained a significantly higher relative amount of Z7-12:OAc than corn strain individuals or hybrids, whereas both hybrid strains exhibited a significantly lower relative amount of this compound compared to the pure strains (Figure 3a). Therefore, the mode of inheritance of Z7-12:OAc is ambiguous. 12:OAc was significantly more present in rice strain females and RxC hybrids than in corn strain females, while CxR hybrids exhibited a similar relative amount of 12:OAc as the pure strains and RxC hybrids (Figure 3a). Thus, the relative amount of 12:OAc within the hybrid strains did not reveal a clear pattern of inheritance either.

3.5.2 Analysis of the four-component blend

Maybe not all of the seven compounds we have identified from the fall armyworm glands are active pheromone components that attract conspecific males. Field studies conducted so far showed that at least four components (Z9-14:OAc; Z11-16:OAc; Z9-12:OAc and Z7-12:OAc) have an effect on the attraction of fall armyworm males (Table 1). The possible role of the other compounds that we have found in *Spodoptera frugiperda* glands (12:OAc; 14:OAc and Z11-14:OAc) still needs to be investigated. Therefore, the original seven-component blend was transformed into a four-component blend, which consists of the four male attracting pheromone components Z9-14:OAc; Z11-16:OAc; Z9-12:OAc and Z7-12:OAc. For that reason, the compounds 12:OAc; 14:OAc and Z11-14:OAc were omitted and the relative percentages of the other four components were recalculated to set the relative amount of the whole blend again to 100%.

When the analysis was based on a four component blend, overall significant differences were found in the relative amount of each pheromone component for corn and rice strain females and for both hybrid strains (Figure 3b). Corn strain females and CxR hybrids contained significantly less of the major component Z9-14:OAc than the rice strain females and RxC hybrids (Figure 3b). However, Z11-16:OAc was found in significantly higher relative amounts in the glands of corn strain individuals and CxR hybrids compared to rice strain and RxC hybrid ones (Figure 3b). The ratio of these two major components was similar between corn strain individuals and CxR hybrids and between rice strain females and RxC hybrids, so that a maternal inheritance can be concluded. The relative amount of Z7-12:OAc was significantly higher in both pure strains than in both hybrid

strains, so that the mode of inheritance of Z7-12:OAc within the hybrid strains did not reveal any clear pattern (Figure 3b). Z9-12:OAc was present in significantly lower relative amounts in the corn strain and both hybrid strains than in the rice strain (Figure 3b). The relative amount of Z9-12:OAc was similar between the corn strain and both hybrid strains, which indicates a corn-dominant inheritance of this component.

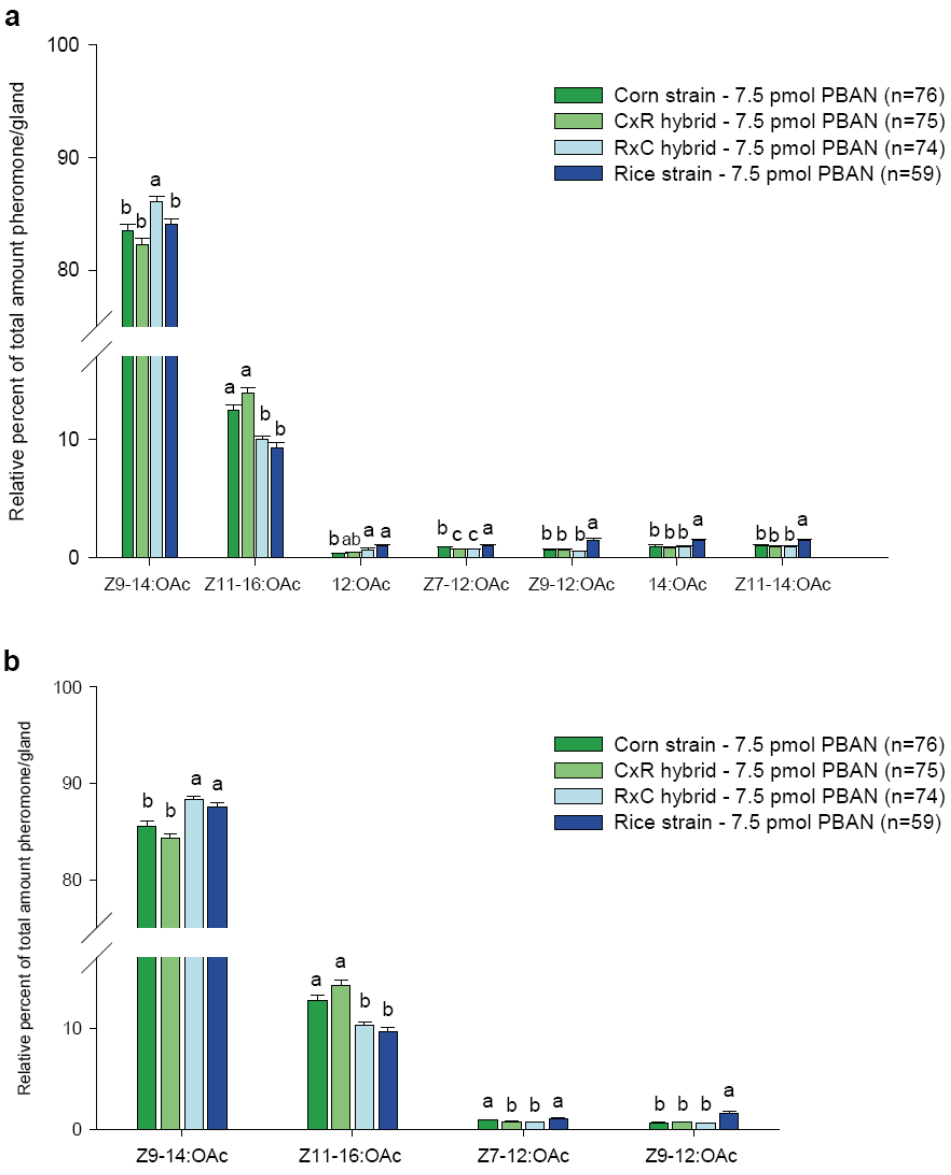

Figure 3. Mode of inheritance of the pheromone components of *Spodoptera frugiperda*. Comparison between the glands of pure corn and rice strain females, CxR hybrids and RxC hybrids. Females were extracted under "artificial" conditions into photophase (with 7.5 pmol PBAN injection). **A.** Seven-component blend. Wilk's lambda: P<0.0001. **B.** Four-component blend. Wilk's lambda: P<0.0001. The total percent of all compounds add to 100%. Different letters above the bars indicate significant differences.

3.6. Phenotypic correlations

The pheromone compounds of *Spodoptera frugiperda* females are not produced independently from each other, because they are linked due to their biosynthesis production pathway (see discussion, Figure 5 and 6). The phenotypic correlation matrices showed significantly different patterns of correlations which may reveal properties of the compounds' biochemical pathways (Tables 2-4, adapted from Groot et al. 2008).

3.6.1. Corn and rice strain correlations under "natural" conditions

When corn and rice strain females were extracted into scotophase, a significantly strong negative correlation was detected between the two major components Z9-14:OAc and Z11-16:OAc (Table 2). Hence, when more Z9-14:OAc is produced in the gland, less of the second major component Z11-16:OAc is produced and vice versa. Furthermore, the correlation analysis of the rice strain females showed significantly negative correlations between the major component Z9-14:OAc and all five minor compounds, whereas corn strain females exhibited only one significantly negative correlation between Z9-14:OAc and 14:OAc (Table 2). There were no significant correlations between the second major component Z11-16:OAc and all minor compounds within both strains (Table 2). A number of significantly positive correlations were detected between the minor compounds in the corn and rice strain glands (Table 2). A significantly strong positive correlation was found between Z7-12:OAc and Z9-12:OAc and between Z9-12:OAc and Z11-14:OAc within both strains (Table 2). Hence, when more of one of these minor compounds is produced, the amount of the correlated minor compound is increased too.

The correlation matrix of corn strain females showed further significantly positive correlations between 12:OAc and the three minor compounds Z7-12:OAc, Z9-12:OAc and Z11-14:OAc and between Z7-12:OAc and Z11-14:OAc (Table 2a).

A significantly strong positive correlation was detected between 12:OAc and the two minor compounds Z9-12:OAc and 14:OAc in rice strain females (Table 2b). Furthermore, significantly positive correlations were found between 14:OAc and the two compounds Z7-12:OAc and Z9-12:OAc and between Z11-14:OAc and both Z7-12:OAc and 14:OAc within the rice strain correlation matrix (Table 2b).

Table 2. Pearson's correlation coefficients of the pheromone compounds in glands of corn and rice strain females extracted in the scotophase under "natural" conditions.

A. Glands of corn strain females.

Corn strain (n=17)	Z9-14:OAc	Z11-16:OAc	12:OAc	Z7-12:OAc	Z9-12:OAc	14:OAc
Z11-16:OAc	-0.94****	—				
12:OAc	0.16	-0.44	—			
Z7-12:OAc	0.11	-0.38	0.58*	—		
Z9-12:OAc	-0.06	-0.25	0.63**	0.90****	—	
14:OAc	-0.51*	0.32	0.48	-0.005	0.17	—
Z11-14:OAc	0.006	-0.28	0.56*	0.70**	0.84****	0.13

B. Glands of rice strain females.

Rice strain (n=22)	Z9-14:OAc	Z11-16:OAc	12:OAc	Z7-12:OAc	Z9-12:OAc	14:OAc
Z11-16:OAc	-0.75****	—				
12:OAc	-0.52*	0.046	—			
Z7-12:OAc	-0.71***	0.22	0.39	—		
Z9-12:OAc	-0.75****	0.15	0.74****	0.77****	—	
14:OAc	-0.72***	0.41	0.67***	0.48*	0.60**	—
Z11-14:OAc	-0.70***	0.27	0.39	0.59*	0.80****	0.44*

Tables adapted from Groot et al. (2008). The sum of all components is set to 100%. Significant interactions are shown by asterisks. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, **** indicates $P < 0.0001$.

3.6.2. Corn and rice strain correlations under "artificial" conditions

When corn and rice strain females were injected with 7.5 pmol PBAN, significantly negative correlations were detected between the major component Z9-14:OAc and almost every other compounds except of Z7-12:OAc (Table 3). A significantly negative correlation was found between the second major component Z11-16:OAc and Z7-12:OAc within corn and rice strain glands and between Z11-16:OAc and Z9-12:OAc within the rice strain (Table 3). The correlation matrix of corn strain females showed significantly strong positive correlations between 14:OAc and Z11-14:OAc and between Z9-12:OAc and both 14:OAc and Z11-14:OAc (Table 3a). Furthermore, a significantly positive correlation was detected between 12:OAc and both Z9-12:OAc and 14:OAc as well as a significantly negative correlation between Z7-12:OAc and Z11-14OAc within the glands of corn strain females (Table 3a). The correlation analysis of the rice strain females showed significantly positive correlations between all of the five minor compounds 12:OAc, Z7-12:OAc, Z9-12:OAc, 14:OAc, Z11-14:OAc (Table 3b).

Table 3. Pearson's correlation coefficients of the pheromone compounds in glands of corn and rice strain females extracted in the photophase under "artificial" conditions (7.5 pmol PBAN injection).

A. Glands of corn strain females.

Corn strain (n=76)	Z9-14:OAc	Z11-16:OAc	12:OAc	Z7-12:OAc	Z9-12:OAc	14:OAc
Z11-16:OAc	-0.86****	—				
12:OAc	-0.27*	0.07	—			
Z7-12:OAc	0.27*	-0.37**	0.07	—		
Z9-12:OAc	-0.29*	-0.19	0.39***	0.03	—	
14:OAc	-0.44****	-0.05	0.34**	0.02	0.82****	—
Z11-14:OAc	-0.53****	0.15	0.05	-0.24*	0.63****	0.71****

B. Glands of rice strain females.

Rice strain (n=59)	Z9-14:OAc	Z11-16:OAc	12:OAc	Z7-12:OAc	Z9-12:OAc	14:OAc
Z11-16:OAc	-0.61****	—				
12:OAc	-0.59****	-0.03	—			
Z7-12:OAc	-0.31*	-0.40**	0.46***	—		
Z9-12:OAc	-0.53****	-0.28*	0.69****	0.84****	—	
14:OAc	-0.67****	0.03	0.45***	0.38**	0.58****	—
Z11-14:OAc	-0.57****	0.09	0.26*	0.28*	0.32*	0.73****

Tables adapted from Groot et al. (2008). The sum of all components is set to 100%. Significant interactions are shown by asterisks. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, **** indicates $P < 0.0001$.

3.6.3. CxR and RxC hybrid correlations under "artificial" conditions

When CxR and RxC hybrids were injected with 7.5 pmol PBAN, significantly negative correlations were detected between the major component Z9-14:OAc and the four compounds Z11-16:OAc, 12:OAc, 14:OAc and Z11-14:OAc in both hybrid crosses (Table 4). Furthermore, a significantly positive correlation was found between Z9-14:OAc and Z7-12:OAc within CxR hybrid glands as well as a significantly strong negative correlation between Z9-14:OAc and Z9-12:OAc in the gland of RxC hybrids (Table 4). The second major component Z11-16:OAc was not correlated with any other compound within RxC glands, but Z11-16:OAc was significantly positive correlated with both 14:OAc and Z11-14:OAc and significantly negative correlated with Z7-12:OAc in the glands of CxR hybrids (Table 4). All minor compounds of the CxR hybrids were nearly exclusive significantly positively correlated with each other (Table 4a). The correlation matrix of the RxC hybrids showed significantly positive correlations between all five minor compounds 12:OAc, Z7-12:OAc, Z9-12:OAc, 14:OAc, Z11-14:OAc (Table 4b).

Table 4. Pearson's correlation coefficients of the pheromone compounds in glands of CxR and RxC hybrid females extracted in the photophase under "artificial" conditions (7.5 pmol PBAN injection).

A. Glands of CxR hybrids.

CxR hybrid (n=75)	Z9-14:OAc	Z11-16:OAc	12:OAc	Z7-12:OAc	Z9-12:OAc	14:OAc
Z11-16:OAc	-0.96****	—				
12:OAc	-0.36**	0.18	—			
Z7-12:OAc	0.23*	-0.43***	0.43***	—		
Z9-12:OAc	-0.15	-0.08	0.37**	0.35**	—	
14:OAc	-0.51****	0.31**	0.48****	0.20	0.41***	—
Z11-14:OAc	-0.50****	0.35**	0.23*	0.05	0.28*	0.48****

B. Glands of RxC hybrids.

RxC hybrid (n=74)	Z9-14:OAc	Z11-16:OAc	12:OAc	Z7-12:OAc	Z9-12:OAc	14:OAc
Z11-16:OAc	-0.68****	—				
12:OAc	-0.79****	0.15	—			
Z7-12:OAc	-0.22	-0.22	0.36**	—		
Z9-12:OAc	-0.67****	0.10	0.69****	0.48****	—	
14:OAc	-0.78****	0.12	0.93***	0.35**	0.80****	—
Z11-14:OAc	-0.40***	0.01	0.31**	0.23*	0.34**	0.39***

Tables adapted from Groot et al. (2008). The sum of all components is set to 100%. Significant interactions are shown by asterisks. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, **** indicates $P < 0.0001$.

3.7. Pheromone composition of the field population

3.7.1. Gland preparations under "artificial" conditions

The analysis of the seven-component blend of the corn strain field population showed a high variation in the pheromone composition between the different corn strain families (Figure 4). When comparing the pheromone composition of the PBAN-injected corn strain families from the field, significantly different relative amounts of most pheromone compounds were identified between the glands of every single corn strain family (Figure 4; Table 5). This high between-family variation was not detected within single families, what demonstrated a strong heritability effect of the pheromone components in the fall armyworm. The blends of the corn strain field individuals showed also significant differences to the blends of laboratory-reared corn and rice strain females (Figure 4; Table 5). The major component Z9-14:OAc was present at significantly higher relative amounts within the field corn families 10, 11 and 24 compared to the field families 07, 14, 21 and the laboratory corn strain (Table 5). However, the relative amount of Z11-16:OAc was

higher within individuals that contained less Z9-14:OAc (Figure 4). The glands of the field families 07, 14 and 21 exhibited a significantly lower relative amount of Z7-12:OAc than the field families 10, 11, 24 and both laboratory strains (Table 5). The relative amount of Z9-12:OAc was similar between six of the seven corn strain families from the field and the laboratory corn strain, whereas the field family 25 and the laboratory rice strain exhibited significantly higher relative amounts of this component than all other fall armyworm females (Figure 4; Table 5). Further significant differences in the relative amount of the minor compounds 12:OAc, 14:OAc and Z11-14:OAc were present between the different corn strain field families and both laboratory strains (Table 5).

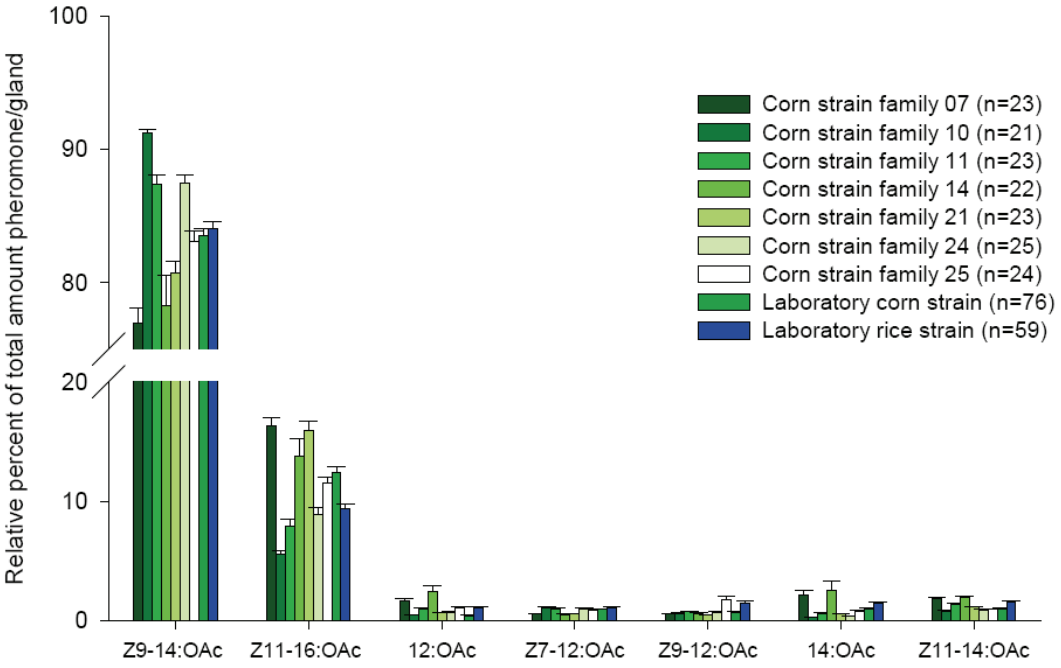

Figure 4. Pheromone composition of corn strain families of the field population and laboratory-reared *Spodoptera frugiperda* corn and rice strain individuals. Individuals were extracted under "artificial" conditions into photophase (7.5 pmol PBAN injection). Wilk's lambda: $P<0.0001$. The total percent of all compounds add to 100%. Significant differences between the pheromone composition of different *S. frugiperda* groups (corn strain field population, laboratory corn and rice strain) are shown in Table 5.

Table 5. Statistical differences in the amount of each pheromone component between field and laboratory *Spodoptera frugiperda* corn and rice strain females. Different letters for the amount of a single pheromone compound (rows) indicate significant differences between different families and/or strains.

Pheromone compounds	Corn strain families from the field population							Laboratory strains	
	Fam. 7	Fam. 10	Fam. 11	Fam. 14	Fam. 21	Fam. 24	Fam. 25	corn	rice
Z9-14:OAc	e	a	abc	e	de	ab	cd	d	bd
Z11-16:OAc	a	e	de	ab	a	cde	bc	b	cd
12:OAc	ab	cd	bc	a	cd	cd	bc	ce	bd
Z7-12:OAc	bc	a	a	c	bc	a	ab	a	a
Z9-12:OAc	b	b	b	b	b	b	a	b	a
14:OAc	a	c	bc	a	bc	c	bc	bc	ab
Z11-14:OAc	ab	d	bd	a	cd	d	d	d	abc

3.7.2 Broad sense heritability analysis

Because of the high within-strain and between-family variance of the corn strain field population, a broad sense heritability analysis was performed to assess if this variance is due environmental or genetic effects. The linear mixed model analysis showed that the between-family and within-family variance of the two major compounds Z9-14:OAc and Z11-16:OAc was much higher than the variance of the two minor components Z7-12:OAc and Z9-12:OAc (Table 6). The broad sense heritability value H^2 of each pheromone component indicated that the phenotype of each pheromone component was almost exclusive determined by genetic effects, because for every pheromone component the heritability value H^2 was close to one (Table 6). The heritability value H^2 of the second major pheromone component Z11-16:OAc was larger than one, even though normally $0 \leq H^2 \leq 1$. Since H^2 is only an estimated value, a H^2 value greater than one is possible due to stochastic effects. The results of this analysis showed again the strong heritability of each pheromone component in the fall armyworm corn strain individuals from the field.

Table 6. Broad sense heritability H^2 of the four pheromone components Z9-14:OAc, Z11-16:OAc, Z7-12:OAc and Z9-12:OAc of different corn strain families from the field population. H^2 = 2 * between-family variance/ [between-family variance + within-family variance].

Pheromone Component	Between-family variance	Within-family variance	Heritability H^2
Z9-14:OAc	16.753	17.396	0.981
Z11-16:OAc	18.984	17.286	1.047
Z7-12:OAc	0.054	0.084	0.779
Z9-12:OAc	0.206	0.250	0.904

4. Discussion

4.1. Pheromone differences between laboratory fall armyworm strains

The corn and rice strain females of the laboratory-reared *Spodoptera frugiperda* population significantly differed in the relative amounts of the compounds present in their pheromone gland. Corn strain females contained a significantly higher relative amount of the second major component Z11-16:OAc than rice strain females, whereas most of the minor components were more abundant in the glands of rice strain females. These differences were found even though individuals were reared for over 25 generations under constant laboratory conditions in environmental chambers and fed with the same artificial diet. Hence, there must be a strong genetic component that determines the pheromone composition of the two fall armyworm strains. Consequently, I can approve my initial hypothesis that pheromone composition is another trait that distinguishes the two strains of *Spodoptera frugiperda*, in addition to strain-specific differences for example in their host plant choice or timing of reproduction behaviour. To what extent these strain-specific pheromonal differences influence the premating isolation of both strains in the field still needs to be tested.

4.2. Mode of inheritance of pheromone differences

The pheromone composition of CxR and RxC hybrid females reveals the genetic basis (i.e. the mode of inheritance) of the strain-specific pheromone differences. Within lepidopteran species, males are the homogametic sex (ZZ) and females are heterogametic (ZW). The relative amount of the second major component Z11-16:OAc was maternally inherited. Comparing only the four "male attracting" components, the major component Z9-14:OAc was also maternally inherited. The genetic basis of both major components could be found either on the mitochondrial DNA or the differences may be due to a cytoplasmic effect, because both are always maternally inherited. Since no genes have been found on the W-chromosome so far, it is unlikely that pheromone-specific genes are located there. The three minor compounds Z9-12:OAc, 14:OAc and Z11-14:OAc showed a corn-dominant inheritance pattern, which suggest that their production is affected by a different set of genes than the production of Z9-14:OAc and Z11-16:OAc. These genes are likely to be found on the autosomes. The essential secondary component Z7-12:OAc, without which *Spodoptera frugiperda* males are not attracted (Tumlinson et al. 1986), revealed no clear pattern of inheritance. This component seems to be suppressed in the hybrids, because both hybrid strain contained less relative amounts of Z7-12:OAc than the pure strains. I assume

that the suppression of this crucial sex pheromone within both hybrid strains could act as pre-mating isolation mechanism between the two strains. EAG-recordings of *Spodoptera frugiperda* male antennae showed that the dose of a single pheromone component had a significant effect on the amplitude of the EAG-response (Malo et al. 2004). If *Spodoptera frugiperda* males exhibit a species-specific threshold for the detection of Z7-12:OAc, they could possibly be unable to find females which emit amounts of Z7-12:OAc smaller than the threshold-level (e.g. hybrid females). Hence, the probability to be attracted to a female that emits undetectable amounts of a crucial sex pheromone (e.g. Z7-12:OAc) would decrease. In further investigations it will be necessary to assess whether such pheromone detection thresholds exist in *Spodoptera frugiperda* males and to what extent differences between the two strains are detectable.

4.3. Biosynthetic pathway of pheromone production

4.3.1. Biosynthesis of major pheromone components

The general strong negative correlation between the two major components Z9-14:OAc and Z11-16:OAc suggests that their production is linked via a same precursor within the biosynthetic pathway. In *Spodoptera littoralis*, the biosynthesis of Z9-14:OAc includes the desaturation of palmitic acid (16:Acid) to Z11-16:Acid, the chain shortening of Z11-16:Acid to Z9-14:Acid and a final reduction and acetylation of Z9-14:Acid to Z9-14:OAc (Fabriás et al. 1994). Although Z9-14:OAc, but not Z11-16:OAc is a pheromone compound of *Spodoptera littoralis* (www.pherobase.com), we can assume a similar biosynthetic pathway of Z9-14:OAc between *S. littoralis* and *S. frugiperda*. In the fall armyworm, 16:Acid could be desaturated by a Δ11-desaturase to Z11-16:Acid, the precursor of both Z9-14:OAc and Z11-16:OAc (Groot et al. 2008; Figure 5). A chain shortening of Z11-16:Acid to Z9-14:Acid and further reduction and acetylation could form the major pheromone component Z9-14:OAc, whereas reduction and acetylation of Z11-16:Acid could produce the second major pheromone component Z11-16:OAc (Groot et al. 2008; Figure 5). Similar pathways concerning the biosynthesis of Z9-14:OAc and Z11-16:OAc have been found in other lepidopteran species (Roelofs and Bjostad 1984; Tillman et al. 1999; Jurenka 2003). The hypothetical biosynthetic pathway could explain the reciprocal ratio (strong negative correlation) of the two major components in the fall armyworm, because both components share the same precursor Z11-16:Acid (Figure 5). When more Z11-16:Acid is reduced and acetylated to Z11-16:OAc, less Z11-16:Acid is

available for chain shortening to Z9-14:Acid and thus, the amount of Z9-14:OAc would decrease while the amount of Z11-16:OAc increases.

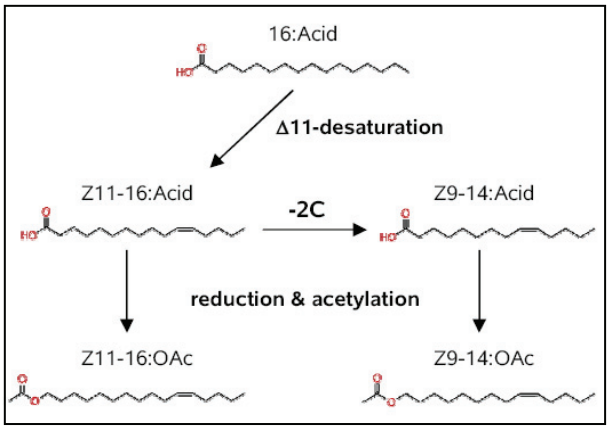

Figure 5. Hypothetical biosynthesis of the major pheromone components Z9-14:OAc and Z11-16:OAc of *Spodoptera frugiperda* females (based on Groot et al. 2008). -2C: chain shortening via ß-oxidation.

4.3.2. Biosynthesis of minor pheromone compounds

In most females, especially within PBAN-injected rice strain and RxC hybrid individuals, significantly positive correlations were present among the five minor compounds 12:OAc, Z7-12:OAc, Z9-12:OAc, 14:OAc and Z11-14:OAc, which suggests that they share the same precursors palmitic acid (16:Acid) and myristic acid (14:Acid) (Groot et. al 2008). Pheromone biosynthesis studies of several moth species propose that Δ11-desaturases are important for the biosynthesis of Z7-12:OAc, Z9-12:OAc, Z9-14:OAc and Z11-14:OAc (Roelofs and Bjostad 1984; Tillman et al. 1999; Jurenka 2003). Jurenka (2003) suggests that the biosynthetic pathways of Z7-12:OAc and Z9-12:OAc in females moths could also be controlled by Δ9-desaturases. Thus, a hypothetical biosynthetic pathway of the minor pheromone compounds of *Spodoptera frugiperda* was created based on the possible activity of Δ9- and Δ11-desaturases (Groot et al. 2008; Figure 6).

Δ9-desaturation of 14:Acid to Z9-14:Acid and further chain-shortening to Z7-12:Acid could lead to Z7-12:OAc, whereas Z7-12:OAc could also be formed via Δ11-desaturation of 16:Acid to Z11-16:Acid and additional chain shortening to Z9-14:Acid, the precursor of Z7-12:Acid (Groot et al. 2008; Figure 6). 14:Acid could be desaturated by a Δ11-desaturase to form Z11-14:Acid, which could be reduced and acetylated to Z11-14:OAc or transformed to Z9-12:Acid, the precursor of Z9-12:OAc (Groot et al. 2008; Figure 6). Z9-12:OAc could also be produced via chain shortening of 14:Acid to 12:Acid and further Δ9-desaturation of 12:Acid to Z9-12:Acid (Groot et al.

2008; Figure 6). The minor compounds 12:OAc and 14:OAc could be directly formed via reduction and acetylation of their precursors 12:Acid and 14:Acid (Groot et al. 2008; Figure 6).

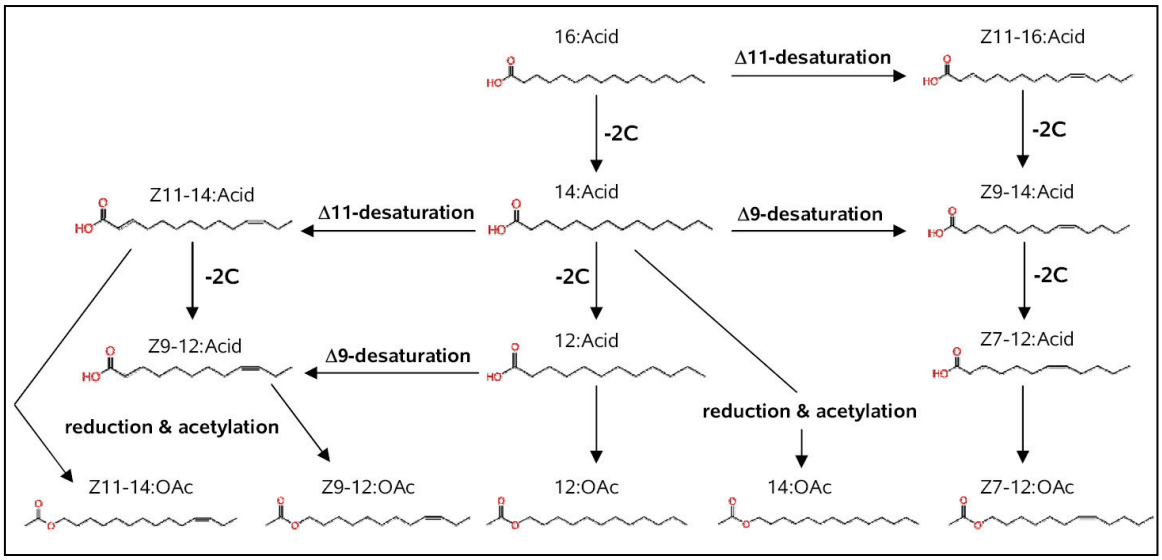

Figure 6. Hypothetical biosynthesis of the minor pheromone components Z11-14:OAc, Z9-12:OAc, 12:OAc, 14:OAc and Z7-12:OAc of *Spodoptera frugiperda* females (based on Groot et al. 2008). -2C: chain shortening via ß-oxidation.

4.3.1. Strain-specific differences of the pheromone biosynthetic pathway

Candidate genes that may explain the strain-specific sex pheromone differences of *Spodoptera frugiperda* could be found by investigating likely enzymatic differences in the pheromone biosynthetic pathway. Genetic studies concerning the chemosensory speciation in insects suggest that desaturase enzymes play a decisive role in the sexual isolation processes mediated by pheromone differentiation (Knipple et al. 2002; Roelofs et al. 2002; Roelofs and Rooney 2003; Smadja and Butlin 2008). The hypothetical pathway of the pheromone production in *Spodoptera frugiperda* implies that strain-specific desaturase differences could explain the genetic basis of the pheromone differences between corn and rice strain females. One difference between the two strains was the significantly higher relative amount of the second major component Z11-16:OAc within corn strain females, what could be explained by a rice strain-specific reduced activity of the Δ11-desaturase that converts 16:Acid to Z11-16:Acid (Groot et al. 2008). The amount of 16:Acid available for chain-shortening to 14:Acid would increase in rice strain females, when less 16:Acid can be converted into Z11-16:OAc. Since 14:CoA could be an important precursor for the production of minor compounds, a higher relative amount of 14:CoA in rice strain females would also increase the relative amount of the minor compounds. Significantly higher

relative amounts of most of the minor compounds were indeed found in the glands of rice strain females compared to corn strain individuals, which approves the hypothetical pathway and possible strain-specific Δ11-desaturase activities.

Smadja and Butlin (2008) suggest that evolutionary shifts in the pheromone blend of insects can be mediated via inactivation of desaturase enzymes or activation of non-functional desaturases. The evolution of different sex pheromones among closely related *Ostrinia* species (Lepidoptera: Pyralidae) seems to be affected by the activation of non-functional desaturase transcripts (Roelofs et al. 2002). Genetic studies evidenced that the same three desaturase transcripts (of a Δ9-, a Δ11- and a Δ14-desaturase) are present in the pheromone glands of *Ostrinia nubilalis* and *O. furnacalis* females, but in each species only one of the three transcripts (the "functional transcript") is translated into a protein which affects the biosynthesis of their pheromone components (Roelofs and Rooney 2003). In *Ostrinia nubilalis* the "functional transcript" encodes a Δ11-desaturase, whereas *O. furnacalis* females translate the "functional transcript" of a Δ14-desaturase, which is a non-functional transcript in *O. nubilalis*, into a functional enzyme (Roelofs et al. 2002; Roelofs and Rooney 2003). Since both *Ostrinia* species use different desaturases for their pheromone biosynthesis, they exhibit significant differences in their pheromone composition (Roelofs et al. 2002). Genetic data evidence that *Ostrinia furnacalis* populations derived from *O. nubulalis* populations a million years ago and the underlying speciation process could be initiated by mutational shifts in the pheromone composition of *O. furnacalis* females and the concomitant response of some rare *O. nubulalis* males to the new pheromone blend (Roelofs and Rooney 2003).

The activation of such non-functional desaturase transcripts could also play a role in *Spodoptera frugiperda*, especially with regard to the possibly strain-specific biosynthesis of male-attracting pheromone components. Phenotypic correlation analyses of Z7-12:OAc indicate that this crucial sex pheromone component could be produced by the precursor 14:Acid in rice strain females, whereas 16:Acid seems to be the precursor of Z7-12:OAc in corn strain females (Groot et al. 2008). This would mean that the production of Z7-12:OAc could be controlled by a Δ9-desaturase in rice strain females and by a Δ11-desaturase in corn strain females (Groot et al. 2008). Alternatively, a rice strain-specific Δ7-desaturase could be involved in the production of Z7-12:OAc (Groot et al. 2008), but since Δ7-desaturases have not been identified in moths so far, this suggestion is unlikely. These results suggest that corn and rice strain females of *Spodoptera frugiperda* could have different types of desaturases or exhibit similar desaturase enzymes

with different substrate-preferences, which could explain the differences in their pheromone composition. Further investigation will be necessary to assess whether or not the two strains of the fall armyworm exhibit different desaturases, enzyme-activities or -specificities (e.g. stereo- or region-specificity) that could explain the strain-specific pheromonal differences. Finding the genetic basis of possible strain-specific desaturase genes or transcripts will help to gain insight into the evolutionary process of pheromone divergence between the two strains of *Spodoptera frugiperda*.

4.4. Effect of PBAN on pheromone composition

The pheromone compositions of PBAN- and non-PBAN-injected corn and rice strain females showed that more strain-specific differences were present when females were extracted under "natural" conditions compared to "artificial" extractions during the day. Even though PBAN-injection reduced the level of pheromonal differences between both strains, significantly strain-specific differences were still detectable. These results suggest that PBAN-injection can not be used to determine a similar pheromone phenotype as in native fall armyworm females. Since recent studies proposed that PBAN-injected female moths can be extracted independently of their age or mating status at every time of the day (Raina et al. 1989; Fabriás et al. 1994; Rafaeli and Jurenka 2003; Groot et al. 2005), PBAN-injection can be a useful tool to extract a higher number of females than would be possible under "natural" conditions. The preparation of many individuals is necessary to detect pheromone differences between different groups of females (e.g. pure strains vs. hybrids or laboratory vs. field individuals) and to find the genetic basis of sex pheromones in *Spodoptera frugiperda*.

In *Spodoptera littoralis* it was shown that PBAN stimulates the activity of the reductase that converts acyl-CoA to intermediate alcohol precursors of the pheromone compounds (Martinez et al. 1990; Fabriàs et al. 1994), whereas studies of *Spodoptera exigua* suggest that PBAN activates steps of the fatty acid biosynthesis (Jurenka 1997). The injection of PBAN into fall armyworm females resulted in a significant decrease of all minor compounds in both strains and a significant increase of Z11-16:OAc, at least in rice strain females (an increase of Z11-16:OAc was also present in corn strain females, but this was not significant compared to the relative amount of Z11-16:OAc in non-PBAN-injected corn strain females). Since significant differences between PBAN-injected and non-PBAN-injected corn and rice strain females could not be identified for all pheromone compounds

(e.g. Z9-14:OAc) and PBAN injection seemed to increase as well as decrease the amounts of different pheromone compounds, it is unlikely that PBAN affects a step of the fatty acid synthesis. Hence, we can assume a similar effect of PBAN on the pheromone production in the species *Spodoptera littoralis* and *S. frugiperda* (i.e. the stimulation the activity of reductase). Groot et al. (2008) suggest that PBAN activates the fatty acyl reductase (FAR) that coverts Z11-16:CoA to Z11-16:OH in the fall armyworm. This would mean that PBAN injection results in a higher amount of the intermediate alcohol Z11-16:OH and consequently increases the amount of the second major component Z11-16:OAc (Groot et al. 2008). This could explain the result that in PBAN-injected corn and rice strain females higher relative amounts of Z11-16:OAc were present than in females extracted under "natural" conditions (i.e. without PBAN injection). Alternatively, it could be possible that two different kinds of the Δ11-desaturase are active in *Spodoptera frugiperda* females (Groot et al. 2008). PBAN could specifically activate the Δ11-desaturase that converts 16:OAc to Z11-16:OAc, so that more Z11-16:OAc would be produced (Groot et al. 2008). Further investigations with labelled pheromone precursors and intermediates as well as inhibition-studies with enzyme inhibitors (e.g. herbicides) could be helpful to assess the rate-limiting steps for PBAN in species like *Spodoptera frugiperda* (Raina 1993; Rafaeli 2005; Tsfadia et al. 2007).

4.5. Pheromone composition of the corn strain field population

The corn strain field population showed a huge variation in the pheromone composition between the seven corn strain families. The pheromone compositions of these corn strain families ranged from being similar to the laboratory-reared corn or rice strain females to everything beyond, between or under this similarity. Thus, one can not deduce from the pheromone composition whether or not the extracted female belonged to the corn or the rice strain. Unfortunately, I could not get any rice strain females from the field to make a complete pheromonal comparison between corn and rice strain field individuals. Hence, I do not know whether rice strain individuals from the field exhibit a similarly high between-family variation in their pheromone composition as the corn strain field females of this study.

If we assume that rice strain females exhibit a lower between-family variation in their pheromone composition as corn strain females, one could explain how pheromone differences may act as pre-mating isolation mechanism in spite of the high between-family variance of corn strain females. Laboratory hybridisation studies of *Spodoptera frugiperda*

showed that backcrosses between RxC hybrid females (offspring of a rice mother and a corn father) and pure corn or rice strain males failed, whereas CxR hybrid backcrosses produced fertile clutches (Schöfl et al. unpublished data). Thus, negative fitness consequences would arise if rice strain females mate with corn strain males due to infertility of RxC hybrids. Whitford et al. (1988) also found that RxC hybrids exhibit lower mating- and hatch-rates and produce fewer numbers of eggs than CxR hybrids. These findings indicate that it may be costly for rice females to attract corn males, which would mean a strong selection on rice females to attract rice males and not corn males. In corn females such a cost does not seem to occur, because CxR hybrids are as fertile as the parental strains (Schöfl et al. unpublished data). Hence, variations in the pheromone composition of corn strain females could arise because hybridisation due to low strain-specificity of the pheromone blend would have no negative fitness consequences.

In contrast to the findings of Schöfl et al. (unpublished data) and Whitford et al. (1988) are population genetic studies of both strains, which indicate that natural hybridisation mostly occurs between rice strain females and corn strain males (RxC) compared to the reciprocal cross (CxR) (Nagoshi and Meagher 2003b; Prowell et al. 2004; Nagoshi et al. 2006b). An unidirectional mating incompatibility between laboratory-reared corn strain females and rice strain males and between F_1 hybrid females (CxR and RxC) mated with both pure strain males was also found by Pashley and Martin (1987). Other laboratory studies evidenced no incompatibilities of inter-strain matings (Quisenberry 1991). Hence, hybridisation compatibility and mating behaviour of the fall armyworm seems to be an inconsistent trait (Prowell et al. 2004). Since we do not know to what extent intra-strain matings may have selective (dis)advantages, further field studies are necessary to draw conclusions from possible strain-specific selection differences, which could affect strain-specific pheromone differences.

The high between-family and comparatively low within-family variance of the pheromone blend of the corn strain field population showed that the pheromone composition in the *Spodoptera frugiperda* corn females was highly heritable (broad sense heritability $H^2 \sim 1$). Hence, the pheromone phenotype of these corn strain females was basically determined by genetic rather than environmental effects, at least in a narrow sense. Since geographical differences in the pheromone composition of fall armyworm females exist (Tumlinson et al. 1986; Descoins et al. 1988; Batista-Pereira et al. 2006), we can not exclude the possibility of environmental effects that influence the pheromone phenotype of *Spodoptera*

frugiperda females. The high between-family variance of corn strain females could be influenced by environmental effects, if for example the parents of these individuals had their origin at different geographic regions. Since the parents of our corn strain population were collected from the same sweet corn field in Florida at the same time in spring, and fall armyworm individuals are present throughout the year in Florida and usually overwinter there (Pashley 1988a), it seems to be unlikely that the pheromonal differences between the corn strain families are due to migration events of corn strain females from different geographic regions. Nevertheless, I do not know whether the corn strain females are genetically different or not and thus, I can not exclude that the pheromonal differences are due to environmental effects. Since the individuals were stored after the gland preparation in a -80°C freezer, further investigations concerning their genetic background can be done to explain how possible genetic effects could influence the phenotype of their pheromone composition. Furthermore, general studies are necessary to assess to what extent geographic variation in the sex pheromone composition, which has been described for *Spodoptera frugiperda* by several authors, is affected by environmental or genetic effects.

Despite the fact that all corn strain families exhibit significantly different pheromone compositions, a higher number of significantly between-family differences was detected in the relative amounts of the two major components Z9-14:OAc and Z11-16:OAc compared to the two minor components Z7-12:OAc and Z9-12:OAc. This could be explained by different selection pressures affecting the major and minor components due to possibly different strain-specificities and range-distance activities of the pheromone components. Roelofs and Cardé (1977) proposed that the single pheromone components of a females' sex pheromone could have different functions (e.g. range-distance activities) and thus would evoke different behavioural responses in lepidopteran males. They distinguished between long-range pheromone components, which induce males to fly upwind, and close range components, which are not essential for the upwind flight behaviour, but which (in combination with long-range components) elicit mating-specific response in the males (e.g. landing, wing-fluttering, releasing of male pheromones, copulation behaviour) (Roelofs and Cardé 1977). Field evaluations of *Spodoptera litura* suggested that the major pheromone component (Z,E)-9,11-14:OAc seemed to act as long-range signal up to 100 meters, whereas the minor component (Z,E)-9,12-14:OAc, together with the major component, seemed to operate as a close range signal up to few meters (Nakamura 1980).

The pheromone components of fall armyworm females could also exhibit different range-distance activities, whereas the two major components Z9-14:OAc and Z11-16:OAc could act as long-distance signals to attract males over great distances, and the two minor components Z7-12:OAc and Z9-12:OAc, in combination with the major components, could act as close-range signals that influence males close to the female to start with the mating procedure. Laboratory studies approve this hypothesis, because it was shown that fall armyworm males exhibit a lower threshold response for Z9-14:OAc than for Z9-12:OAc (Hirai and Mitchell 1981) and thus, males can detect Z9-14:OAc over greater distances than Z9-12:OAc.

If the different components are important at different distances, it could be possible that the emitted ratio of the two major components Z9-14:OAc and Z11-16:OAc is less strain-specific than the ratio of the minor components Z7-12:OAc and Z9-12:OAc. This could lead to different selection forces that act on the genotype and phenotype of the major and minor pheromone components of *Spodoptera frugiperda*. If males are attracted to a wide range of different ratios of the major components Z9-14:OAc and Z11-16:OAc, the relative amounts of these components could be under weak selection, which would explain the high between-family variance. The ratio of the two major components could be more important for the attraction of species-specific *Spodoptera frugiperda* males than for strain-specific corn and rice males, because pheromone components within lepidopteran species are often used by more than one species (Cardé and Haynes 2004; www.pherobase.com). The pheromone components that we have identified in the glands of *Spodoptera frugiperda* females are common in other species of the genus *Spodoptera*, for example *S. praefica* emits Z9-14:OAc, Z11-16:OAc, Z7-12:OAc and Z9-12:OAc (Landolt et al. 2003); *S. descoinsi*, *S.exempta* and *S. latifascia* release Z9-14:OAc, Z11-16:OAc and Z11-14:OAc from their gland (www.pherobase.com); *S. exigua* and *S. eridania* both use Z9-14:OAc and Z11-16:OAc (Tumlinson et al. 1990; Mitchell and Tumlinson 1994) and *S. androgea*, *S. cilium*, *S. depravata* and *S. evanida* exhibit Z9-14:OAc in their blend (www.pherobase.com). Thus, the release of a fall-armyworm-specific ratio of Z9-14:OAc and Z11-16:OAc could act as pre-selective mate choice mechanism for males, to distinguish *Spodoptera frugiperda* from other lepidopteran females. If the major pheromone components of fall armyworm females attract males of both strains over long distances, the minor pheromone components Z7-12:OAc and Z9-12:OAc could be necessary for the strain-specific attraction of males and the ratio of the minor components could be under strong selection, which would explain the low between-family variance.

The relative amount of Z9-12:OAc in the female gland seemed to be strain-specific, because the laboratory-reared rice strain females exhibited significantly higher relative amounts of this component than the corn strain females. Also six of the seven corn strain families of the field population exhibited similar relative amounts of Z9-12:OAc as the corn strain laboratory population, whereas one family contained a similar relative amount of this component as the laboratory rice strain. Whether or not this "rice strain like relative amount" of the one corn strain family is due to genetic differences still needs to be tested. The strain-specific amount of Z9-12:OAc in the pheromone blend of a female in combination with a detection-threshold of Z7-12:OAc within males (see discussion part 4.2.) could act as strain-specific mate choice mechanism that facilitate the reproductive isolation between the two strains of the fall armyworm. Further detailed investigations need to be done to analyze whether or not the major components Z9-14:OAc and Z11-16:OAc act as long-distance signals to attract species-specific males and the minor components Z7-12:OAc and Z9-12:OAc act as close-range signals to attract strain-specific males of *Spodoptera frugiperda*.

4.6. Further perspectives

Now that I have shown that the two strains of the fall armyworm exhibit differences in their pheromone composition, strain-specific field lures need to be tested. If the ratios of the pheromone compounds that I found in the glands are directly linked with the pheromone ratios that are emitted by the female, lures with a higher amount of the second major component Z11-16:OAc should be more attractive to corn than to rice strain males. Such studies could be helpful for agriculture management, since the fall armyworm is a serious pest of several crops in the United States (Pashley 1986). In addition to the test of strain-specific lures in field experiments, the effect of environmental conditions on the pheromone composition needs to be investigated. The effect of temperate or tropical environmental conditions on the pheromone composition could be analyzed with the help of Genotype x Environment (GxE) experiments. Furthermore, electro-antennogramm (EAG) recordings are necessary to detect strain-specific differences in the male response of *Spodoptera frugiperda*. By phenotyping and genotyping all four backcross families (C x [CxR], C x [RxC], R x [CxR], R x [RxC]), the genetic basis of the pheromone composition and the effect of hybridization in the fall armyworm can be determined. Additionally, candidate genes (Δ9-, Δ11-desaturases, FAR) that could explain the strain-specific sex pheromone differences should be further investigated.

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6. Summary

The two host strains of *Spodoptera frugiperda* exhibited significant differences in the pheromone composition of the females, at least when they were reared under constant laboratory conditions. To what extent these strain-specific pheromonal differences influence the premating isolation of both strains in the field still needs to be tested. It seems that a strong genetic component determines the pheromone composition of the two strains. In both strains the major components of the sex pheromone seemed to be maternally inherited, whereas most of the minor components showed a corn-dominant inheritance pattern. Strain-specific differences in Δ-desaturases or a fatty acid reductase could explain the pheromonal differences between corn and rice strain females. The injection of synthetic PBAN into fall armyworm females caused pheromonal differences compared to non-PBAN-injected females, but significant strain-specific differences were still detectable in PBAN-injected females. The analysis of a corn strain field population showed results that were not comparable to the laboratory population. Further studies will be necessary to understand the possible influence of pheromonal differences on the speciation process of the two sympatric species of *Spodoptera frugiperda*.

Die beiden *Spodoptera frugiperda* Wirtsstämme zeigten signifikante Unterschiede in der Pheromon-Komposition der Weibchen, zumindest wenn die Individuen unter konstanten Laborbedingungen gezüchtet wurden. In welchem Ausmaß diese wirtsspezifischen Pheromonunterschiede die Paarungs-Isolation der beiden Wirtsstämmen im Feld beeinflusst, muss noch untersucht werden. Es scheint, dass die Pheromon-Komposition von beiden Stämmen stark genetisch beeinflusst ist. In beiden Stämmen scheinen die Hauptpheromonkomponenten mütterlich vererbt zu sein, während die meisten Nebenpheromonkomponenten corn-dominant vererbt werden. Wirtsspezifische Unterschiede in Δ-Desaturasen oder Fettsäure-Reduktasen könnten die Pheromon-unterschiede zwischen beiden Wirtsstämmen erklären. Die Injektion von PBAN in *Spodoptera frugiperda* Weibchen führte zu Pheromonunterschieden im Vergleich zu nicht-injizierten Weibchen, wobei jedoch wirtsspezifische Unterschiede in der Pheromon-Komposition auch in PBAN-injizierten Weibchen erkennbar waren. Die Analyse eines corn-Wirtsstammes einer Feld-Population zeigte keine vergleichbaren Resultate zu der Labor-Population. Weitere Untersuchungen sind notwendig um zu verstehen, wie Pheromonunterschiede den Artbildungsprozess der zwei sympatrischen *Spodoptera frugiperda* Arten beeinflussen kann.

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8. Appendix

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Research

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Host strain specific sex pheromone variation in *Spodoptera frugiperda*

Astrid T Groot*[1], Melanie Marr[1], Gerhard Schöfl[1], Sybille Lorenz[2], Ales Svatos[2] and David G Heckel[1]

Address: [1]Max Planck Institute for Chemical Ecology, Dept. Entomology, Hans-Knöll Strasse 8, 07745 Jena, Germany and [2]Max Planck Institute for Chemical Ecology, Research group Mass spectrometry, Hans-Knöll Strasse 8, 07745 Jena, Germany

Email: Astrid T Groot* - agroot@ice.mpg.de; Melanie Marr - mmarr@ice.mpg.de; Gerhard Schöfl - gschoefl@ice.mpg.de; Sybille Lorenz - lorenz@ice.mpg.de; Ales Svatos - svatos@ice.mpg.de; David G Heckel - heckel@ice.mpg.de

* Corresponding author

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Abstract

Background: The fall armyworm *Spodoptera frugiperda* (Lepidoptera; Noctuidae) consists of two distinct strains with different host plant preferences for corn and rice. To assess whether pheromonal-mediated behavioral isolation accompanies the habitat isolation on different host plants, we compared the sex pheromone composition among females of the two strains. Pheromone glands were extracted with or without injection of pheromone biosynthesis activating neuropeptide (PBAN). To assess the mode of inheritance of this variation, we also analyzed the pheromone composition of F_1 hybrid females.

Results: Relative to intra-strain variation, the pheromone composition of the two strains differed significantly. Corn strain females contained significantly more of the second most abundant pheromone compound Z11-16:Ac (m), and significantly less of most other compounds, than rice strain females. When females were injected with PBAN before their glands were extracted, the differences between the strains were less pronounced but still statistically significant. The pheromone composition of hybrid females showed a maternal inheritance of the major component Z9-14:Ac (M) as well as of Z11-16:Ac (m). Most other compounds showed an inheritance indicating genetic dominance of the corn strain. The within-strain phenotypic correlations among the various components were consistent with their hypothesized biosynthetic pathway, and between-strain differences in the correlation structure suggested candidate genes that may explain the pheromone differences between the two strains. These include Δ9- and Δ11 desaturases, and possibly also a Δ7-desaturase, although the latter has not been identified in insects so far.

Conclusion: The two host strains of *S. frugiperda* produce systematically differing female sex pheromone blends. Previously-documented geographic variation in the sexual communication of this species did not take strain identity into account, and thus may be partly explained by different strain occurrence in different regions. The finding of pheromone differences reinforces the possibility of incipient reproductive isolation among these strains, previously shown to differ in the timing of nocturnal mating activity and host plant use. Finding the genetic basis of the pheromone differences, as well as these other biological traits, will help to elucidate the role of premating isolation in the continuing differentiation of these two strains that may eventually lead to speciation.

Background

In night-flying moths, highly specific, long distance, pheromonal communication ensures that males and females can find each other and mate. Females produce a species-specific sex pheromone in a specialized gland at the tip of their abdomen, to which males of the same species are attracted [1,2]. Moth pheromones usually consist of a blend of two or more components of even-numbered C_{10}–C_{18} straight-chain, unsaturated derivatives of fatty acids, with the carbonyl carbon modified to form an oxygen-containing functional group (alcohol, aldehyde, or acetate ester) [3,4]. The species-specificity of each blend is determined by the particular combination of the components, as well as their relative ratios (e.g., [5-7]). Although there are thousands of moth species with unique pheromone blends (e.g., [8]), the evolutionary processes that resulted in this diversity of sexual communication signals are still poorly understood (e.g., [9-11]). To gain insight into the evolution of premating isolation between species it is essential to quantify the level and possible causes of variation in the premating signals within species on which selection may operate.

The fall armyworm (FAW, *Spodoptera frugiperda* J. E. Smith) offers an ideal opportunity for investigating intraspecific variation in sex pheromone communication, because two sympatrically occurring strains have already been recognized [12,13]. One feeds predominantly on corn (the corn strain, C) and the other on rice and various pasture grasses (the rice strain, R). These strains can be identified by several molecular markers, e.g. allele frequency differences at three allozyme markers [12,14], several strain specific DNA sequence variants in the mitochondrial COI gene [15,16] and ND1 gene [17], AFLP markers [18,19], and the FR repetitive nuclear DNA sequence extensively present in R and mostly absent from C [14,20]. Co-occurrence of typically strain-specific markers in the same individuals has provided evidence of some naturally-occurring hybridization in the field [18].

Given that natural hybridization can occur, factors that might limit it and thus maintain the genetic integrity of two different strains are of interest. The two strains differ in the timing of their mating activity; female calling (emission of pheromone) and mating occurs early in the night for C and during the last half of the night for R [21,22]. When using live females as lures in pheromone traps and typing a subset of males caught in these traps, significantly more C males were caught in traps with C females, and R females attracted more R males [21]. Under laboratory conditions, we have found that both strains mate assortatively to some degree as well (G. Schöfl, A. Dill, A.T. Groot, unpubl. res.), which would inhibit hybridization and enhance divergence between the strains.

The female sex pheromone composition and male attraction in the field have been studied in several regions within the North and South American range of *S. frugiperda* (see Table 1). The female pheromone glands were found to contain Z9-14:Ac as the major compound (to which we will refer as M), comprising up to 83%, as well as the second most abundant compound Z11-16:Ac (m), and a number of compounds present in low amounts, such as Z9-12:Ac and Z7-12:Ac (see Table 1) [23-25]. In addition, Brazilian *S. frugiperda* females produce E7-12:Ac [25], a compound not found in other populations so far.

Table 1: Means and Coefficients of variation (CV) of each compound when 7 compounds and when 4 compounds of the sex pheromone gland of *Spodoptera frugiperda* are considered.

	Corn – Sc[1] (n = 17)		Rice – Sc[1] (n = 22)		Corn[2] (n = 76)		C × R[2] (n = 75		R × C[2] (n = 74)		Rice[2] (n = 59)	
7 Compounds	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV
Z11-16:Ac (m)	12.4	50	7.3	50	12.5	33	13.9	33	10.0	24	9.3	33
Z9-14:Ac (M)	81.8	7	82.4	8	83.5	5.5	82.3	6	86.1	5	84.0	5
14:Ac (a)	1.1	65	1.1	59	1.0	117	0.9	52	0.9	113	1.5	62
Z11-14:Ac (b)	1.1	35	1.6	55	1.0	69	1.0	40	1.0	61	1.5	41
12:Ac (c)	1.1	42	2.0	44	0.4	53	0.5	51	0.7	180	1.0	81
Z9-12:Ac (d)	0.8	52	2.1	55	0.7	89	0.7	77	0.6	70	1.5	86
Z7-12:Ac (e)	1.8	48	3.6	47	0.9	45	0.8	54	0.7	37	1.1	51
4 Compounds												
Z11-16:Ac (m)	12.8	50	7.7	51	12.8	33	14.3	33	10.3	25	9.7	33
Z9-14:Ac (M)	84.6	7	86.3	6	85.6	5	84.2	5	88.3	3	87.6	4
Z9-12:Ac (d)	0.8	53	2.3	58	0.7	97	0.7	78	0.6	77	1.6	89
Z7-12:Ac (e)	1.8	49	3.8	48	1.0	45	0.8	54	0.8	39	1.1	53

[1]Glands extracted in the scotophase; [2]Glands extracted from PBAN-injected females

Not all compounds that are found in the sex pheromone glands of females are "pheromone components" which are by definition important in the attraction of conspecific males (e.g. [6]; some may be unavoidable by-products of the pheromone biosynthetic pathways [4]. The components that have been found to be attractive for *S. frugiperda* males include the major component Z9-14:Ac (M), as well as one of the components present in only low amounts, Z7-12:Ac (to which we refer as e). This is the crucial secondary pheromone component of *S. frugiperda*, because blends without it are not attractive for males of this species [23-29]. The addition of other components has given different results in different regions, which suggests that the sexual communication of this species varies geographically [25], similar to what has been found in several other noctuid moth species (e.g., [30-32]. Specifically, when Z11-16:Ac (m) was added to the blend, significantly more males were attracted in Mexico and Costa Rica [26,27], but not in Florida [23]. In Pennsylvania, adding Z11-16:Ac (m) as well as Z9-12:Ac (d) to the two-component blend attracted twice as many males as the two-component blend [29]. By contrast, in Brazil the addition of Z11-16:Ac to the two-component blend did not increase attraction of *S. frugiperda* males, but the addition of E7-12:Ac, that was found uniquely in glands of Brazilian females, did [25].

Surprisingly, none of the above-described studies mention whether corn or rice strain females were analyzed or whether corn or rice strain males were attracted to the different blends. This is despite the documentation of both strains in North America [12,13,15,21] as well as in Brazil [33]. Because of the lack of distinction between the two strains in previous pheromone studies, the variation found in the different regions could be at least partly due to sampling of two different strains in the different areas.

Another possible source of variation found in the pheromone composition is the time of day at which glands are extracted. Female moths usually produce pheromone *de novo* every night [34], and in many species the timing of pheromone synthesis and release is controlled by Pheromone Biosynthesis Activating Neuropeptide (PBAN) [35]. Extraction of glands after dark (i.e. in the scotophase) yields those compounds that have accumulated there in response to natural PBAN produced in the suboesophagal ganglion and released from the corpus cardiacum into the hemolymph [36,37]. Experimental injection of commercially available PBAN induces pheromone production within 2–3 hours, independent of the time of day or the physiological state (mating status and age) of the female [35,38-41]. This thus can exclude the time of day as a possible source of variation, which is important in the two strains of *S. frugiperda*, because they differ in their time of mating activity and thus likely also the time of pheromone production at night. Previously, we found that injecting females with PBAN can be a simple and convenient method of determining a female's native pheromone phenotype [42]. However, PBAN can act at different stages in the pheromone biosynthetic pathway in different species, reviewed by [4,41]. Therefore, it is important to assess the effect of PBAN injections on the pheromone composition in the two strains.

The aim of our study was to determine whether the pheromone composition in the glands of corn strain females differs from that of rice strain females. We analyzed the pheromone composition of F_1 hybrid females as well, to assess the mode of inheritance of pheromone variation. We found signficant differences in pheromone composition between corn and rice strain females. Although PBAN reduced the variation in both strains, the pheromone composition remained significantly different. The relative amounts of the two most abundant components, Z9-14:Ac (M) and Z11-16:Ac (m), were maternally inherited, while three minor compounds were inherited in a manner indicating genetic dominance of alleles from the corn strain. Linking these differences to the phenotypic correlations between the pheromone compounds, as well as to a hypothetical scheme of the biosynthetic pathway of the pheromone of this species, suggests that differential activity of a Δ11-, Δ9-, and/or possibly also a Δ7-desaturase may underly the pheromone variation between the two strains.

Results

Between-strain pheromone variation

Comparing the gland content between corn and rice strain females that had been extracted under natural conditions in the scotophase, we found a significant overall difference in the pheromone composition as determined by the relative amounts of most of the minor compounds (Fig. 1; see Table 1 for Coefficients of Variation). Corn strain females contained significantly more Z11-16:Ac (m) and significantly less 12:Ac (c), Z9-12:Ac (d), and Z7-12:Ac (e) than rice strain females. The relative amount of the major component Z9-14:Ac (M) was the same in the two strains, as well as the relative amount of 14:Ac (a) and Z11-14:Ac (b).

Effect of PBAN on pheromone composition

In both strains, glands of females injected with PBAN differed significantly in their pheromone composition from glands of females that were extracted in the scotophase (Fig. 2a and 2b). In both strains, significantly more Z11-16:Ac (m) and significantly less of all other minor compounds (a-e) were found in glands of PBAN-injected females. There were no differences between the glands of rice strain females injected with different amounts of PBAN (1, 7.5 or 20 pmol) (Fig. 2b). Despite the increase

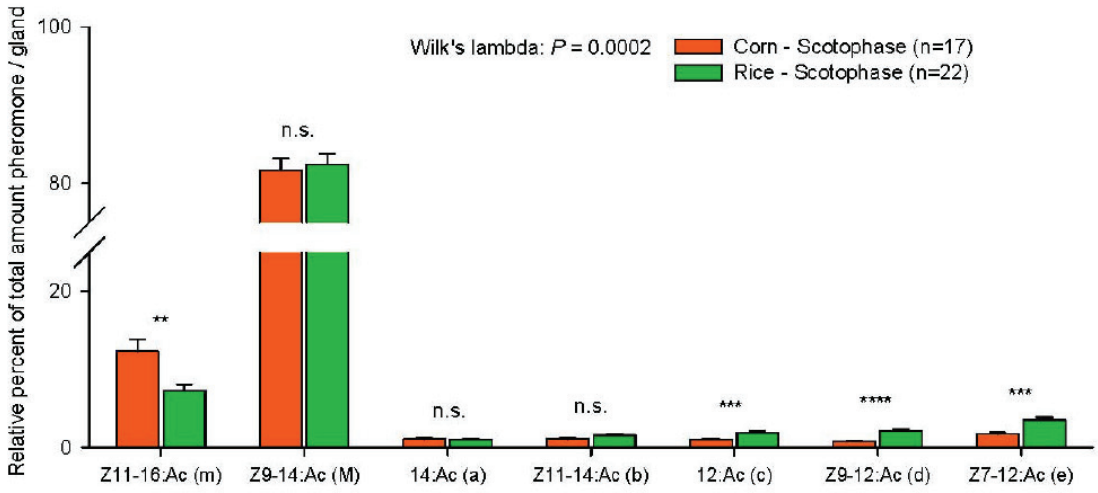

Figure 1
Between-strain comparisons of the pheromone composition when glands were extracted from females under natural conditions, i.e. 4–6 h into scotophase. The total percent of all depicted compounds add to 100%. N.s: not significant, * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, **** indicates $P < 0.0001$.

of Z11-16:Ac (m) and a reduction of most compounds in both strains, the overall pheromone composition still differed significantly between the two strains after injection with 7.5 pmol PBAN (Fig. 2c). Specifically, there was still significantly more Z11-16:Ac (m) and significantly less Z9-12:Ac (d) in corn strain than in rice strain females, although differences in the relative amounts of 12:Ac (c) and Z7-12:Ac (e) were no longer significant.

Mode of inheritance of pheromone variation

The hybrid CxR females (offspring of C females and R males) contained similar amounts of the major component Z9-14:Ac (M) as the corn and the rice strain females, while the reciprocal cross RxC females contained significantly more of this component than the other three groups (Fig. 3; see Table 1 for Coefficients of Variation). CxR females contained similar relative amounts of Z11-16:Ac (m) as corn strain females, while RxC females contained similar relative amounts of Z11-16:Ac (m) as rice strain females. This suggests a maternal inheritance of the relative amount of this component. All other compounds were present in similar amounts in both CxR females and RxC females. For 14:Ac (a), Z11-14:Ac (b), and Z9-12:Ac (d), the relative amounts in the hybrids were similar to corn strain females and significantly lower than rice strain females, suggesting genetic dominance of alleles from the corn strain in the production of these compounds in the hybrids. The remaining two compounds 12:Ac (c) and Z7-12:Ac (e) showed a different pattern: RxC females contained significantly more 12:Ac than corn strain females, and both hybrids contained significantly less Z7-12:Ac than the two parental strains.

Not all compounds that we identified from the female glands may be pheromone components functioning to attract males. From the field studies conducted so far, at least the four components Z9-14:Ac (M), Z11-16:Ac (m), Z9-12:Ac (d), and Z7-12:Ac (e) have been shown to affect the attraction of *S. frugiperda* males in one or more populations (see Table 2). The possible attractive role of the other compounds remains to be investigated. When we omitted these other compounds and based our analysis only on the four components that are known to affect the attraction of conspecific males (i.e. the total amount of these four components was set to 100%, after which the relative percentages of each of the four components were recalculated), we also found a significant overall difference in pheromone composition between the corn and the rice strain (see Fig. 4). Specifically, corn strain females contained significantly more Z11-16:Ac (m) and significantly less Z9-12:Ac (d) than rice strain females in all comparisons. The major component Z9-14:Ac (M) was only significantly different between corn and rice strain females when injected with 7.5 pmol PBAN (Fig. 4b). The hybrid females contained similar relative amounts of Z9-14:Ac (M) and Z11-16:Ac (m) as their mothers, indicating a maternal inheritance of the relative amount these main components. Hybrid females of both types contained sim-

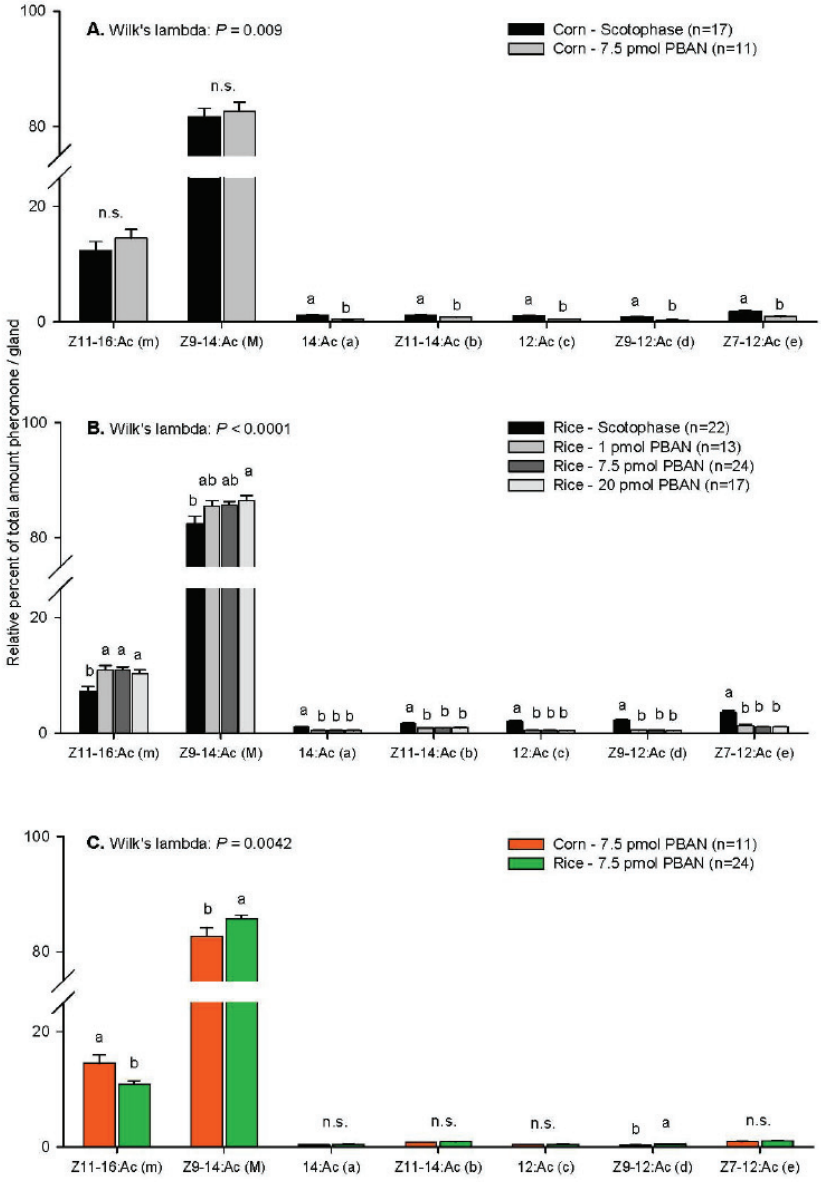

Figure 2
Effect of PBAN on the pheromone composition in the females of the two strains. Glands that were extracted from females in the scotophase were compared to glands that were extracted from females that had been injected with PBAN. A. Glands from corn strain females. B. Glands from rice strain females. C. Comparsion between corn and rice strain glands that were extracted from females that had been injected with 7.5 pmol PBAN. In each graph the total percent of all depicted compounds add to 100%. Different letters above the bars of one component indicate significant differences. N.s: not significant.

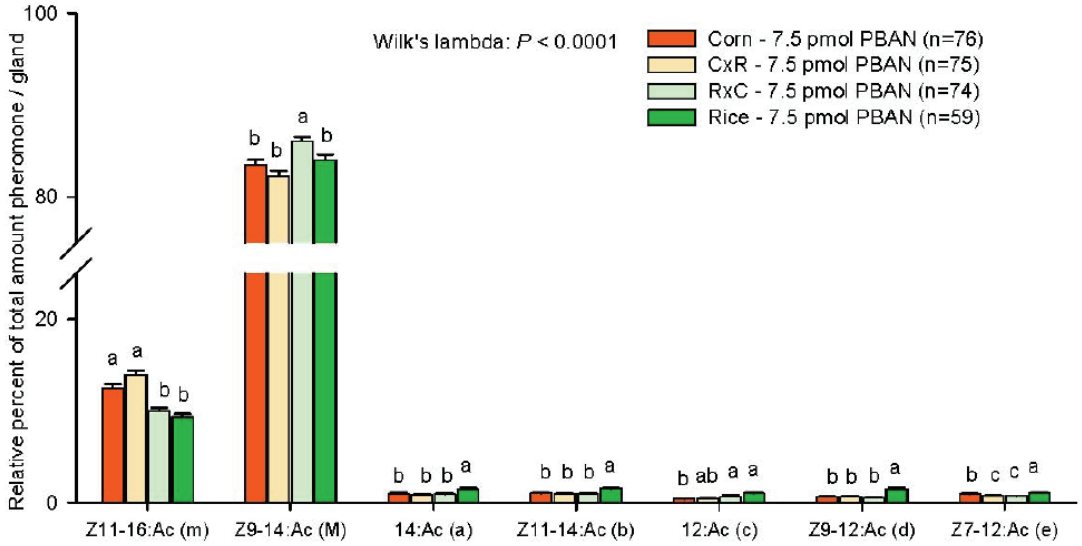

Figure 3
Mode of inheritance of the pheromone composition. Gland extracts from offspring of corn females mated with rice males (CxR) and offspring of rice females mated with corn males (RxC) were compared with gland extracts from corn and rice strain females. All females have been injected with 7.5 pmol PBAN before gland extractions. The total percent of all depicted compounds add to 100%. Different letters above the bars of one component indicate significant differences. N.s: not significant.

ilar amounts of Z9-12:Ac (d) as corn strain females, similar to what was found when including all pheromone compounds (Fig. 3). The critical secondary sex pheromone component Z7-12:Ac (e) was significantly higher in scotophase-extracted rice strain females than in corn strain females, but did not differ between PBAN-injected corn and rice strain females. This compound was significantly lower in hybrid females.

Phenotypic correlations

The previous comparisons have been based on mean proportions of pheromone gland compounds of different groups of females. The proportions within a particular group of females vary slightly from individual to individual around these mean values. The phenotypic correlation matrix describes whether this variation in different components is positively or negatively correlated, or uncorrelated (see Additional files 1, 2, 3). Because the compounds are not produced independently of one another, but are connected due to their biochemical pathways (see Fig. 5), the pattern of correlations may reveal properties of those pathways. A pattern seen within all groups of females tested consisted of strong negative correlations between the major component Z9-14:Ac (M) and both Z11-16:Ac (m) and 14:Ac (a) (orange cells in Additional files 1, 2, 3). Thus, for all groups of females, when more Z9-14:Ac is produced, less Z11-16:Ac and 14:Ac are produced and vice versa. Also, in all groups of females the minor compounds b-e were mostly positively correlated with each other (green cells in Additional files 1, 2, 3). Another pattern seen within all groups of females, except in corn females whose glands were extracted in the scotophase (Additional file 1a), is the strong positive correlations between 14:Ac (a) and the other minor compounds (b-e), and the strong negative correlations between M and b-e (yellow cells in Additional files 1, 2, 3). The most striking difference between PBAN-injected corn females and CxR hybrid females on the one hand, and the other groups of females on the other hand, is the positive correlation between the major component M and the critical secondary sex pheromone component e in the first two groups (blue cell in Additional file 2a and 3a). In scotophase-extracted corn females and in RxC females this correlation is absent, while in the other groups of female this correlation is negative.

Discussion

The relative amounts of the compounds present in the sex pheromone glands of our laboratory-reared corn and rice strain of *S. frugiperda* were significantly different. Rice

Table 2: Reported sex pheromone components of *S. frugiperda*

	Female sex pheromone					Synthetic lures that attracted most males					
	Fl, USA[1]	French Guyana[2]	Brazil[3]	**Corn***	**Rice***	PA, USA[4]	Fl, ** USA[1,5]	Mexico[6]	Costa Rica[7]	French Guyana[2]	Brazil[3]
Z11-16:Ac (m)	9	16.66	12.9	**12.4**	**7.3**	17.69		10.3		15.5	
Z9-14:Ac (M)	69	73.75	82.8	**81.7**	**82.4**	81.61	99.42	77.8	99.4	83	98
14:Ac (a)		0.53		**1.1**	**1.1**						
Z11-14:Ac (b)		1.2	1.5	**1.1**	**1.6**						
12:Ac (c)		0.43	0.6	**1.1**	**1.9**						
Z9-12:Ac (d)	2	0.5	trace	**0.8**	**2.1**	0.25				0.5	
Z7-12:Ac (e)	4	1.12	0.8	**1.8**	**3.6**	0.45	0.58	11.9	0.6	1	1
E7-12:Ac			1.2								1
Z9-14:Al	13	3.59									
Z10-14:Ac			0.3								
16:Ac		0.21									
Z11-16:Al	3										

All numbers in one column add to 100%. Female sex pheromone refers to gland extracts. Tumlinson et al. (1986) also analyzed the volatile blend emitted from the glands and found 2.6% Z11-16:Ac, 90.1% Z9-14:Ac, 1.2% Z11-14:Ac, 1.9% 12:Ac, 2.2% Z9-12:Ac, 0.21% 16:Ac and 3.2% Z7-12:Ac. Note that the Z9-14:Al which was found in the gland extracts, was not found in the emitted volatiles. Compounds in bold are the compounds that we could identify in the gland extracts studied here; all of these were found in both strains. Letters behind these compounds are given the facilitate the reading. *Means of the relative amounts that we found in glands extracted from females in the scotophase. ** In FL the same 4-component blend as used by Fleischer et al. (2005) was tested and attracted similar amounts of males as the 2-component blend.[1][23]; [2][24]; [3][25]; [4][29]; [5][28]; [6][27]; [7][26].

strain females contained a significantly lower relative amount of Z11-16:Ac (m) and a correspondingly higher amount of most other minor compounds than corn strain females. Connecting these differences to the hypothetical biosynthetic pathway of these compounds (Fig. 5), the differences between the strains could be explained by a reduced activity of the Δ11 desaturase converting 16:CoA to Z11-16:CoA in the rice strain. This would increase the relative amount of 16:CoA available for chain-shortening to 14:CoA, the precursor of the minor compounds (c-e) that were found in higher relative amounts in the rice than in the corn strain.

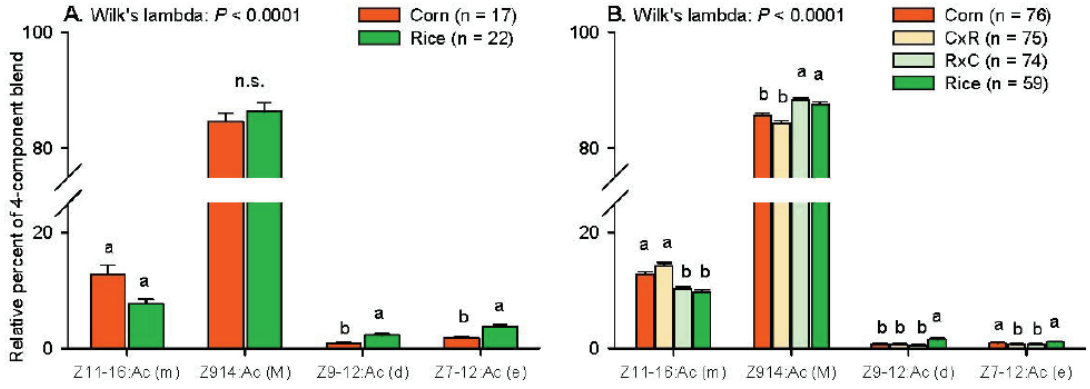

Figure 4
Between-strain comparisons of the pheromone composition between corn and rice strain females, including only the four compounds that have been shown to affect conspecific male attraction. The total percent of the four compounds add to 100%. Different letters above the bars of one component indicate significant differences. N.s: not significant.

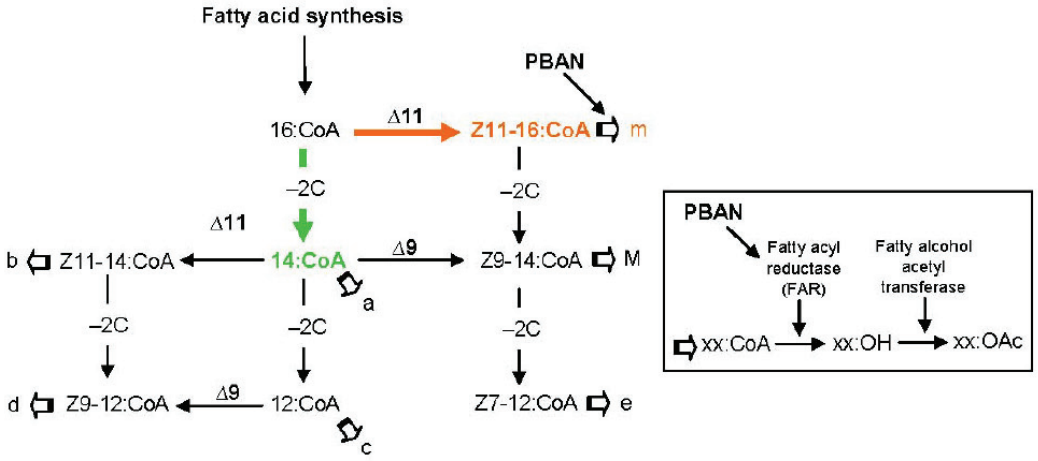

Figure 5
Proposed pathways of the biosynthesis of the pheromone components in *S. frugiperda* (based on biosynthetic pathways described for other moth species by Jurenka, 2003). The interplay of desaturation and chain shortening of 16-, and 14-carbon acyl-CoA derivatives produce mono-unsaturated acyl-CoA precursors that are then reduced and acetylated to produce acetate esters. The number that follows Δ indicates the position of the double bond introduced by the desaturase into the acyl-CoA. -2C indicates chain-shortening by two carbons through β-oxidation. The order of desaturation and chain shortening results in different compounds. The open arrows stand for reduction and acetylation. The letters behind the open arrows stand for the pheromone compounds mentioned in all Tables and Figures.

The biosynthetic scheme can also account for much of the observed correlation structure within groups of females. It is important to note that since M is the dominant compound, always accounting for more than 80% of the total, modest variations in its production would have proportionally greater effects on the relative amounts of the minor compounds. Also, an increase in the formation of one product would entail a decrease in the formation of the other. The universal and strong negative correlations between the major component Z9-14:Ac (M) and both Z11-16:Ac (m) and 14:Ac (a) are consistent with the assumption that Z11-16:CoA is the precursor for both M and m, and that 14:CoA is the precursor of M and a. Another pattern common to many of the groups is the positive correlation among the four minor compounds b-e, that all share the same common precursor 14:CoA. If the amount of 14:CoA varies between females within a group, then a larger amount could result in a concomitant increase of all products. The significant positive correlation between the major component Z9-14:Ac (M) and the critical secondary sex pheromone component Z7-12:Ac (e), found only in PBAN-injected corn and CxR females, may be explained by an increased conversion of 16:CoA to Z11-16:CoA coupled to a limited availability of fatty acyl reductase (FAR), so that an excess of Z11-16:CoA is chain-shortened (through β-oxidation) to Z9-14:CoA and Z7-12:CoA. Alternatively, PBAN may enhance β-oxidation in the corn strain.

The finding that injection of PBAN changed the ratio of the pheromone compounds in both the corn and the rice strain suggests that PBAN does not primarily stimulate the synthesis of fatty acid hormone precursors, as in most moth species [43-46], but instead primarily stimulates a step later in the biosynthetic pathway, as in *Spodoptera littoralis* [47,48]. In general, the precursors of moth pheromones are derived from the fatty acid synthesis, which are stearic acid (18:CoA) and palmitic acid (16:CoA). These acids can then be desaturated through Δ11- and Δ9-desaturase, and reduced through β-oxidation to shorter chain length fatty acids (e.g. 14:CoA, 12:CoA), after which they are reduced and acetylated to form the acetate esters [49]. In *S. littoralis*, PBAN acts on the reduction step of the fatty acids to form the intermediate alcohols [47,48], which is directly followed by the next enzymatic reaction (acetylation) to form the end products [41]. It seems likely that a similar mode of action of PBAN occurs in *S. frugiperda*: after the formation of Z11-16:CoA, PBAN may activate a fatty acyl reductase (FAR), so that Z11-16:OH is formed, which is then converted to Z11-16:Ac (m) (see Fig. 5). Alternatively, PBAN may specifically activate the Δ11-desaturase that converts the 16:CoA to Z11-16:CoA. This

may be a different Δ11-desaturase than the one converting 14:CoA to Z11-14:CoA, similar to the differential substrate preference found for Δ9-desaturases [50]. This would explain the significant increase of Z11-16:Ac (m) in glands of PBAN injected females compared to glands of females that were extracted under natural conditions in the scotophase in both strains (see Fig. 2a and 2b). Despite this effect on the pheromone composition in both strains, decreasing the differences between the two strains, significant differences between the strains were still detectable.

The pheromone composition in hybrid females reveals the mode of inheritance of the pheromone differences between the two strains. The relative amounts of Z11-16:Ac (m) and probably also 12:Ac (c) in the pheromone glands are maternally inherited. In addition, the major component Z9-14:Ac (M) shows a maternal inheritance as well when only the four components are compared that have been shown to affect the attraction of *S. frugiperda* males (see Fig. 4). The other compounds, 14:Ac (a), Z11-14:Ac (b), and Z9-12:Ac (d), are inherited differently, in a corn-dominant way, suggesting that the production of these compounds are affected by a different set of genes. The production of the essential secondary component Z7-12:Ac (e), without which *S. frugiperda* males are not attracted [23], seems to be suppressed in the hybrid females, as both contained less than either parent. We are currently conducting crosses and backcrosses to identify the genetic basis of all pheromone differences between these two strains.

Candidate genes that may possibly underlie the sex pheromone differences between corn and rice strain females can be found by linking these differences to likely enzymatic differences in the biosynthetic pathway (Fig. 5), as well as by comparing the phenotypic correlations between the pheromone compounds. This is a novel approach that we developed recently [51] to generate a list of candidate genes that may explain pheromone differences between species. The most obvious difference in pheromone composition between the two strains of *S. frugiperda*, i.e. a higher relative amount of Z11-16:Ac (m) in corn than in rice strain females, suggests that a Δ11-desaturase, converting the 16:CoA to Z11-16:CoA, is more active in corn than in rice strain females. In addition, the strong positive phenotypic correlation in the rice strain (and RxC females) between 14:Ac (a) and 12:Ac (c) with Z7-12:Ac (e) suggests a coupling that could be explained by a Δ7-desaturase. This coupling is absent in corn females, at least when injected with PBAN. Thus, a Δ7-desaturase, if present, could be restricted to the rice strain. However, so far Δ7-desaturases have not been identified in insects. An alternative explanation is that Z7-12:Ac in rice-strain females is produced via a conversion of 14:CoA by Δ9-desaturase to Z9-14:CoA, after which Z9-14:CoA is β-oxidized to Z7-12:Ac (e). In corn females (and CxR females), the positive correlation beween Z9-14:Ac (M) and Z7-12:Ac (e), and not between M and 14:Ac, suggests that Z7-12:Ac (e) in this strain is produced via Δ11-desaturation of 16:CoA, which is subsequently β-oxidized to Z9-14:Ac (M) and Z7-12:Ac (e), as mentioned above. Thus, both Δ11-desaturase and Δ9-desaturases, and possibly Δ7-desaturase, are candidate genes that may be differentially active between the two strains. Desaturases have been identified from pheromone glands of many moth species, e.g., [52]. In the genus *Spodoptera*, Δ9- and Δ11-desaturases have been characterized in *S. exigua*, *S. littura* [52] and *S. littoralis* [53]. These identifications will facilitate the assessment of whether and which of these genes vary between the two strains.

Now that we have found significant differences in the pheromone composition between the two strains, the next steps are to evaluate a) whether different ratios of the pheromone blend are differentially attractive to corn and rice strain males, and b) how the between-strain variation is related to the geographic variation in sexual communication that has been found in the past. Strain-specific lures will probably be more effective to assess the population distributions of the two strains than the commercial lures that have been used so far, e.g., [27,54-56]. If the ratios found in the glands are an indication of the ratios emitted by the females, a blend with a larger relative amount of Z11-16:Ac is likely to be more attractive to corn than to rice strain males. If Z11-16:Ac is less important in the attraction of rice strain males, this might explain why the addition of this compound to the two-component blend did attract more males in Mexico [27] and Costa Rica [26], but not in Florida [23] or Brazil [25]: the latter experiments may have been conducted in areas or periods when mostly rice-strain males were present.

Conclusion

The two strains of *S. frugiperda* are not only differentiated in their host use and their timing of sexual activities at night, but also in their sex pheromone composition. We found significant differences in the pheromone blend between the two strains when we considered the seven compounds that were identified from the pheromone gland of this species (see Table 1), and when we only considered the four pheromone components that have been shown to affect the attraction of *S. frugiperda* males. Even when females were injected with PBAN, which reduced the among-strain differences, the pheromone composition still significantly differed between the two strains. The pheromone composition of the hybrid females suggests a maternal inheritance of the relative amount of Z11-16:Ac (m) and a genetic dominance of alleles from the corn strain in the production of 14:Ac (a), Z11-14:Ac (b),

and Z9-12:Ac (d). The traits that differentiate the two host strains of this species (host use and differential timing of sexual activities at night) are most likely not independent of each other. For example, host plants have been found to directly or indirectly affect the sexual communication in moths [57-59]. Finding the genetic basis of both sexual (i.e. behavioral) and host plant (i.e. habitat) differentiation in this species will potentially give insight into the interaction between behavioral isolation and habitat isolation, and their role in speciation [60].

Methods

Insects

FAW corn and rice strains were obtained in 2006 from lab-reared colonies of R. Meagher at USDA-ARS in Gainesville, Fl. The corn strain was established from > 100 larvae collected from corn plants near Homestead in Miami-Dade Co., Fl, throughout October and November 2004, and was called JS3C. The rice strain colony originated from > 200 larvae collected from pasture grasses from the Range Cattle Research and Education Centre, Ona, Hardee Co., Fl, between May 2003 and October 2003, and was named OnaR. Both colonies were reared in mass culture for 10 and 21 generations, respectively, on a pinto bean-based artificial diet at USDA Florida. In July 2006, a subset of these colonies were transferred to our laboratory. Upon receiving the colonies, 48 JS3C and 56 OnaR individuals were screened for the strain-specific COI marker. All but three JS3C individuals had the RFLP marker that is associated with the corn strain, and all OnaR individuals had the RFLP marker that is associated with the rice strain [15,16]. Offspring of the three ambiguous JS3C individuals were not included in subsequent rearing. Individuals used for our experiments had been reared for another 15 generations in our laboratory, in environmental chambers at 26 ± 1°C, 60 ± 10% RH, and a 14:10 L:D photoperiod, in a single pair mating protocol that is specifically designed to maintain the genetic variation and to avoid selection of any females. In short, in every generation an equal number of offspring is chosen from each of 30 single pair matings. The offspring from these matings are randomly paired, again in single pairs, in the next generation to maximize effective population size and avoid possible shifts in allele frequencies. Adults used for the pheromone experiments were placed as larvae in a reversed L:D chamber, where lights were off from 11.00 – 21.00 h. Pupae were kept individually in plastic cups and checked daily for emergences. Emerged females were given a honey-water solution and left in the same rearing chamber until their glands were extracted. Pheromone glands were extracted from 2–3 day old virgin females.

Gland extractions

Two groups of glands were extracted, one to assess the between-strain variation and mode of inheritance of strain differences (Group I), and one to assess the effect of PBAN (Group II). Of group I, glands were extracted from corn and rice strain females, as well as from hybrid female offspring from crosses with corn females and rice males, referred to as CxR, and from crosses with rice females and corn males, referred to as RxC. To minimize the effect of one specific cross, glands were extracted from offspring of 9–10 crosses. All females were injected with Hez-PBAN (Peninsula Laboratories, San Carlos, CA) ca. 1–2 h before the scotophase. A stock solution of *Hez*-PBAN (200 pmol/μl in 50% methanol and 1 N HCl) was diluted in saline to 3.75 pmol/ul within 1 hr of injection. Females were injected with 2 μl of this dilution, using a 10 μl syringe (Hamilton, Reno, NV) with a 31 gauge needle that was inserted ventrally between the 8th and the 9th abdominal segments. All females were injected at similar times, 1–2 h before the scotophase, and glands were dissected 2–3 hours later. Of group II, glands were extracted either from females 4–6 h into scotophase, or from females that were injected with PBAN ca. 1–2 h before the scotophase. To assess whether possible changes in the pheromone composition was dependent on the dose of PBAN injected, rice females were injected with 2 ul of PBAN of either 0.5, 3.75 or 10 pmol/ul. Because of a limited number of corn females available at the time of this experiment, corn females were injected only with 2 ul of 3.75 pmol/ul (the second dilution). Two to three hours after PBAN injection, the glands were dissected from the females.

All pheromone glands were dissected and placed in conical vials containing 50 μl hexane and 40 ng pentadecane as internal standard. After 30–40 min, the glands were removed and the extracts were stored at -20°C until analysis. The hexane extract 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 (i.e. 3–4 μl) of extract was injected into a splitless inlet of a HP7890 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 (FID), programmed from 60°C with a 2 min hold, to 180°C at 30°C/min, then to 230°C at 5°C/min, during which all the pheromone components eluted. The column was then heated to 245°C at 20°C/min and held at this temperature for 15 min to clean the column before the next analysis. The FID detector was held at 250°C.

Chemical analysis

Of all the compounds that have been previously identified from glands of *S. frugiperda* (see Table 1), we identified the following from the pheromone glands: Z9-14:Ac (M), Z11-16:Ac (m), 14:Ac (a), Z11-14:Ac (b), 12:Ac (c), Z9-12:Ac (d), and Z7-12:Ac (e). The letters behind the compound are given to facilitate the reading. All of these

compounds were found in both strains. These compounds were first identified by injecting synthetic compounds into the GC described above to compare their retention times with the retention times of the peaks present in the gland extracts. In addition, the alcohol and some of the aldehyde analogs of these compounds (i.e., Z9-14:Ald, Z11-16:Ald, Z9-12:OH and Z7-12:Ac) were injected as well to make sure that these compounds have a different retention time in our settings and GC program. All synthetic compounds were bought from Pherobank, Wageningen. After the first identification, the chemical identities of all peaks in the gland extracts were checked by GC-MS. A subset of extracts was injected into an HP6890 GC coupled to Masspec MS002 (Micromass, Manchester, UK) with electron ionization (EI) at 70 eV, and separated using a 30 m × 0.25 mm × 0.25 μm DB-Wax column, with the same temperature program as described above. The recorded mass spectra were compared to those of known standards injected in the same manner and using spectral database (Wiley MS library v 7). The gland extracts were additionally screened for a possible detection of E7-12:Ac. This compound was not found in the 10 extracts examined.

The amount of each pheromone compound was calculated relative to the 40 ng internal standard. Every day, before and after each GC sequence, we injected authentic standards of the pheromone compounds mentioned above 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. Because there is high variance among female moths in total gland pheromone content, even within treatments, most researchers analyze differences between the amount of each component after converting amounts to percentages relative to the most abundant compound (i.e., the "major" component) (e.g. [8,23]. Therefore, we compared the pheromone composition between different groups of females by converting all amounts to relative percentages of the total amount of all pheromone components in the glands.

Statistical analysis

To compare the pheromone compositions between the two strains, a multivariate analysis of variance (MANOVA) was conducted, using SAS, version 9.1 (SAS Institute, 2002–2003), with all the females of a group that were a) extracted during the scotophase, or b) injected with 7.5 pmol PBAN. In addition, to assess the effect of PBAN on the pheromone composition within each strain, a separate MANOVA was conducted within each species. To determine the mode of inheritance of the difference in pheromone composition between corn and rice strain females, another MANOVA was conducted, comparing the pheromone composition between the corn strain females, the rice strain females, the CxR hybrid females, and the RxC hybrid females. In every test, the means of all pheromone compounds were separated using least-squares means (LSMEANS), with a Tukey adjustment for multiple comparisons.

Phenotypic correlations

To assess possible enzymatic differences between the two strains in the biosynthetic pathway of the FAW pheromone, we analyzed phenotypic associations among pheromone components in the two strains by generating a Pearson's correlation matrix (PROC CORR in SAS). For this correlation matrix we used the percentages of each compound relative to the total amount of all the pheromone compounds that we distinguished. Separate correlation matrices were constructed for the pheromone composition found in glands of a) corn or rice females from which glands were extracted under natural conditions, i.e. in the scotophase, b) corn or rice females that were injected with 7.5 pmol PBAN, which glands were extracted at the same time as the hybrid females, and c) hybrid CxR or RxC females that were injected with 7.5 pmol PBAN as well. Since such associations can give insight into biosynthetic pathways of the pheromone components, we also constructed a (hypothetical) scheme of this pathway.

Abbreviations

The chemical compounds are abbreviated following the standard shorthand notation for pheromone molecules [61]. For example, (*Z*)-11-hexadecen-1-yl acetate is abbreviated Z11-16:Ac. The corresponding aldehyde is abbreviated Z11-16:Al. PBAN: Pheromone Biosynthesis Activating Neuropeptide.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ATG designed the experiments. MM conducted the experiments. ATG, MM and GS analyzed the data. SL and AS carried out the chemical analyzes and identifications. ATG and DGH interpreted the data and drafted the manuscript. MM, GS, SL and AS critically revised the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1

Pearson's correlation coefficients of pheromone compounds in glands of A) corn strain females, and B) rice strain females, extracted in scotophase. The tables show positive and negative phenotypic correlations between all pheromone compounds. The colors of the cells coincide with the colors in the proposed biosynthetic pathway of the compounds in Figure 5.

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Additional file 2

Pearson's correlation coefficients of pheromone compounds in glands of A) PBAN-injected corn strain females, and B) PBAN-injected rice strain females. The tables show positive and negative phenotypic correlations between all pheromone compounds. The colors of the cells coincide with the colors in the proposed biosynthetic pathway of the compounds in Figure 5, while the blue cells indicate a strikingly different correlation from that found in other females.

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Additional file 3

Pearson's correlation coefficients of the pheromone compounds in glands of A) CxR hybrid females, and B) RxC hybrid females. The tables show positive and negative phenotypic correlations between all pheromone compounds. The colors of the cells coincide with the colors in the proposed biosynthetic pathway of the compounds in Figure 5, while the blue cell indicates a strikingly different correlation from that found in other females.

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Additional file 1

A. Corn (n = 17)	Z11-16:Ac (**m**)	Z9-14:Ac (**M**)	14:Ac (**a**)	Z11-14:Ac (**b**)	12:Ac (**c**)	Z9-12:Ac (**d**)
Z9-14:Ac (**M**)	**-0.94******	—				
14:Ac (**a**)	0.32	**-0.51***	—			
Z11-14:Ac (**b**)	-0.28	0.006	0.13	—		
12:Ac (**c**)	-0.44	0.16	0.48	**0.56***	—	
Z9-12:Ac (**d**)	-0.25	-0.06	0.17	**0.84******	**0.63****	—
Z7-12:Ac (**e**)	-0.38	0.11	-0.005	**0.70****	**0.58***	**0.90******

B. Rice (n = 22)	Z11-16:Ac (**m**)	Z9-14:Ac (**M**)	14:Ac (**a**)	Z11-14:Ac (**b**)	12:Ac (**c**)	Z9-12:Ac (**d**)
Z9-14:Ac (**M**)	**-0.75******	—				
14:Ac (**a**)	0.41	**-0.72*****	—			
Z11-14:Ac (**b**)	0.27	**-0.70*****	**0.44***	—		
12:Ac (**c**)	0.046	**-0.52***	**0.67*****	0.39	—	
Z9-12:Ac (**d**)	0.15	**-0.75******	**0.60****	**0.80******	**0.74******	—
Z7-12:Ac (**e**)	0.22	**-0.71*****	**0.48***	**0.59***	0.39	**0.77******

The sum of all components is set to 100%. Significant interactions are shown in bold.

* indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, **** indicates $P < 0.0001$

Additional file 2

A. Corn (n = 76)	Z11-16:Ac (**m**)	Z9-14:Ac (**M**)	14:Ac (**a**)	Z11-14:Ac (**b**)	12:Ac (**c**)	Z9-12:Ac (**d**)
Z9-14:Ac (**M**)	**-0.86******	—				
14:Ac (**a**)	-0.05	**-0.44******	—			
Z11-14:Ac (**b**)	0.15	**-0.53******	**0.71******	—		
12:Ac (**c**)	0.07	**-0.27***	**0.34****	0.05	—	
Z9-12:Ac (**d**)	-0.19	**-0.29***	**0.82******	**0.63******	**0.39*****	—
Z7-12:Ac (**e**)	**-0.37****	**0.27***	0.02	**-0.24***	0.07	0.03

B. Rice (n = 59)	Z11-16:Ac (**m**)	Z9-14:Ac (**M**)	14:Ac (**a**)	Z11-14:Ac (**b**)	12:Ac (**c**)	Z9-12:Ac (**d**)
Z9-14:Ac (**M**)	**-0.61******	—				
14:Ac (**a**)	0.03	**-0.67******	—			
Z11-14:Ac (**b**)	0.09	**-0.57******	**0.73******	—		
12:Ac (**c**)	-0.03	**-0.59******	**0.45*****	**0.26***	—	
Z9-12:Ac (**d**)	**-0.28***	**-0.53******	**0.58******	**0.32***	**0.69******	—
Z7-12:Ac (**e**)	**-0.40****	**-0.31***	**0.38****	**0.28***	**0.46*****	**0.84******

Females were injected with 7.5 pmol PBAN, at the same time as the hybrid females (Group 1). The sum of all components is set to 100%. Significant interactions are shown in bold.

* indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, **** indicates $P < 0.0001$

Additional file 3

A. CxR hybrids (n = 75)	Z11-16:Ac (**m**)	Z9-14:Ac (**M**)	14:Ac (**a**)	Z11-14:Ac (**b**)	12:Ac (**c**)	Z9-12:Ac (**d**)
Z9-14:Ac (**M**)	**-0.96******	—				
14:Ac (**a**)	**0.31****	**-0.51******	—			
Z11-14:Ac (**b**)	**0.35****	**-0.50******	**0.48******	—		
12:Ac (**c**)	0.18	**-0.36****	**0.48******	**0.23***	—	
Z9-12:Ac (**d**)	-0.08	-0.15	**0.41*****	**0.28***	**0.37****	—
Z7-12:Ac (**e**)	**-0.43*****	**0.23***	0.20	0.05	**0.43*****	**0.35****

B. RxC hybrids (n = 74)	Z11-16:Ac (**m**)	Z9-14:Ac (**M**)	14:Ac (**a**)	Z11-14:Ac (**b**)	12:Ac (**c**)	Z9-12:Ac (**d**)
Z9-14:Ac (**M**)	**-0.68******	—				
14:Ac (**a**)	0.12	**-0.78******	—			
Z11-14:Ac (**b**)	0.01	**-0.40*****	**0.39*****	—		
12:Ac (**c**)	0.15	**-0.79******	**0.93*****	**0.31****	—	
Z9-12:Ac (**d**)	0.10	**-0.67******	**0.80******	**0.34****	**0.69******	—
Z7-12:Ac (**e**)	-0.22	-0.22	**0.35****	**0.23***	**0.36****	**0.48******

Females were injected with 7.5 pmol PBAN.

The sum of all components is set to 100%. Significant interactions are shown in bold.

* indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, **** indicates $P < 0.0001$

9. Statutory declaration

I hereby declare that the thesis has been written by myself without any external unauthorised help and that it has not been previously presented to any university for evaluation. The only sources used were the ones referred to. All parts which have been adopted either literally or in a general manner from these sources have been referred to accordingly. Any parts, words or ideas, which are based on other sources, have been acknowledged as such.

Jena, 19.02.2009 ……....

Melanie Marr